Current Biology

Homing Ants Get Confused When Nest Cues Are Also Route Cues

Highlights

- Ants search less accurately for the nest when nest cues also appear as route cues

- When part of a nest cue appears also as a route cue, ants focus on the unambiguous part

- These results indicate that desert ants have little sequential route knowledge

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In Brief

Homing ants localize the nest by help of visual and olfactory cues. Huber and Knaden show that the ants focus their nest search on those cues that they experience at the nest only and ignore cues that are omnipresent in the environment. Ants even focus on the unambiguous parts of compound cues when parts of these cues are less informative.

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Homing Ants Get Confused When Nest Cues Are Also Route Cues

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SUMMARY

The desert ant *Cataglyphis fortis* inhabits the salt pans of Tunisia. Individual ants leave the nest for foraging trips that can cover distances of more than 1,500 m [1]. Homing ants use path integration [2, 3], but they also rely on visual [4] and olfactory [5] nest-defining cues to locate the nest entrance. However, nest cues can become ambiguous when they are ubiquitous in the environment. Here we show how ants behave during the nest search when the same cues occur at the nest and along the route. Homing ants focused their search narrowly around a visual or olfactory cue that in training they had experienced only at the nest. However, when ants were trained to the same cue not only at the nest but also repeatedly along the foraging route, they later exhibited a less focused search around the cue. This uncertainty was eliminated when ants had a composite cue at the nest that consisted of two components, one unique to the nest and another that also occurred along the route. Here, the ants focused their search on that part of the binary blend that was presented only at the nest and ignored the other, ubiquitous component. Ants thus not only seem to be able to pinpoint their nest by following learned visual and olfactory cues, but also take into account which cues uniquely specify the nest and which, due to their ubiquity, are less informative and so less reliable.

RESULTS AND DISCUSSION

Here we asked whether an ant's search for its nest is more focused when a cue occurs only at the nest and nowhere else along the ant's route to the nest. In order to test this, we trained ants to forage within an aluminum channel (Figure 1A). This setup excluded external visual cues but enabled the ants to use celestial cues for path integration (PI) [2]. Foraging ants were trained to pick up cookie crumbs from a feeder located 10 m from the nest entrance. The inconspicuous nest entrance in the channel was labeled with two black cards that were fixed to the opposite

channel walls surrounding the entrance hole (unambiguous training in Figure 1A).

In a second training paradigm, we increased the difficulty of the ants' task by adding several identical pairs of black cards along the route. The ants now passed five additional cues on their way between nest and feeder (ambiguous training in Figure 1A). After at least 3 hr of training (i.e., approximately 30 training runs), homing ants were caught 3 m in front of the nest entrance and were—together with their biscuit crumb—transferred to a test channel (i.e., a remote parallel channel not connected to any nest or exit; Figure 1B). Under this situation, ants ran off most of their PI vector in the familiar surroundings and, after displacement to the test channel, ran off the remaining vector and started a well-described systematic search [5, 6] for the nest entrance (Figure 1B). Within the test channel, the ants experienced the nest cue (Figure 1). Ants were released 4 m in front of the nest cue to guarantee that any cue-centered search was not due to PI (which would have resulted in a focused search 1 m in front of the test cue). We tracked this nest search by recording the first six 180° turning points after the ant had passed the cue for the first time, as the distance between turning points provides information about the ant's confidence in a nest-defining cue [5]. For each experimental group, we tested at least 20 individuals, with each individual being tested only once.

Ants trained with the unambiguous nest cue exhibited a search that was notably more focused (Figure 2A, orange) than the search of ants that had been trained with the ambiguous nest cue (Figure 2A, red). We next tested whether this effect is restricted to visual cues only or whether it is a general effect that can be observed in other modalities, e.g., olfactory cues, as well. We trained ants in the same situation, but instead of visual cues, we presented an odor cue either at the nest only (Figure 1A, unambiguous training) or at the nest and five times along the foraging route (ambiguous training). We used a binary blend of decanal and methyl salicylate, i.e., common plant volatiles that are neither innately attractive nor repulsive to naive ants but can be learned and distinguished by *Cataglyphis fortis* [5]. Again, ants that during the training experienced the odor at the nest only (Figure 2B, orange) later strongly focused their search on this unambiguous nest cue. However, ants that were trained with an ambiguous nest cue (i.e., experienced the same odor at the nest and several times along the route) exhibited a less focused search (Figure 2B, red).

Could the different search patterns at unambiguous and ambiguous cues be due to artifacts from the experimental design? As

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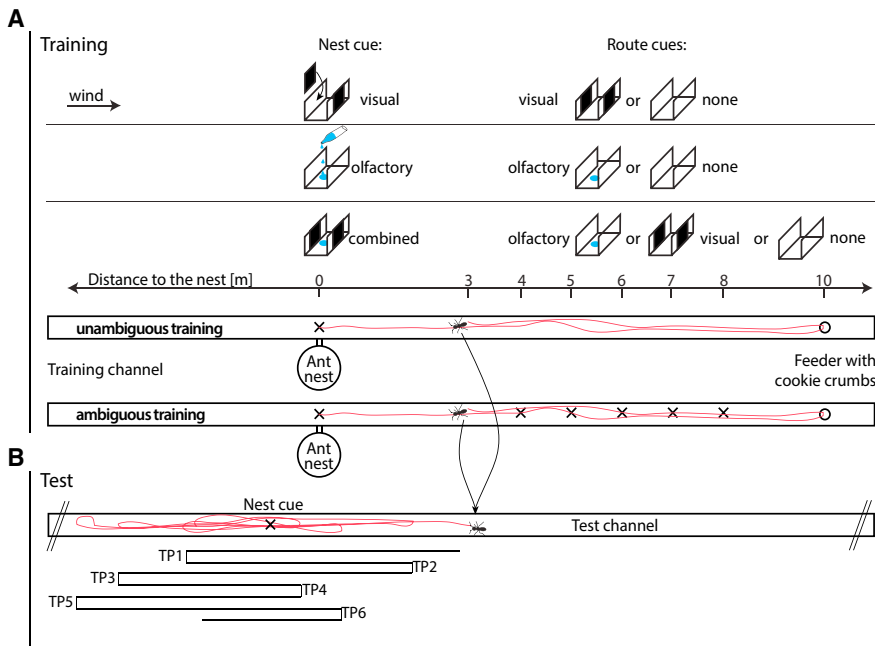
Figure 1. Experimental Design

(A) Aluminum training channel (length, 16 m; width, 7 cm; height, 7 cm) that is connected to a nest (surrounded by a circular barrier resulting in all ants leaving the nest and ending up in the channel system) and contains different combinations of nest and route cues (×, positions of cues at nest entrance only [unambiguous training] or at nest entrance *and* along the route [ambiguous training]) and a feeder (Petri dish filled with cookie crumbs).

(B) Aligned identical test channel to which an ant (caught during homing in training channel) is transferred for the analysis of its subsequent nest search. Red lines, training run and nest search; TP1–TP6, turning points during search that are used for analysis. Curved arrows depict the position from which the homing ant was displaced to the test channel. Please consider that in both training situations homing ants ran off most of their vector in familiar surroundings.

described, we allowed ants to start their last homing run in the training channel, capturing them 3 m before the nest entrance, and tested them after displacement in a test channel that contained only a single cue (Figure 1B). Ants trained with nest and route cues could potentially have become confused by the fact that in the test channel, only the nest cue was provided. Furthermore, ants could potentially depend on some working memory; i.e., after passing route cues, the ants could expect the nest cue to appear after a specific time. This could again have been affected by testing of ants that were captured after they had already passed the route cues.

We therefore trained additional groups of ants as before. However, this time, ants were captured at the feeder before they were released into the test channel (Figures S1A and S1B); i.e., ants performed their last complete homing run in the test channel, which this time contained—depending on the training situation—either the nest cue only or the nest and route cues. Although the ants experienced an identical set of cues in the training and test channels, and although any kind of potential working memory was not interrupted by the test procedure, ants still relied less on cues when they were ambiguous (Figure S1C). Finally, when the cues were put in a strong conflict with the path integrator (by displacing them 3 m along the test channel, so that the fictive nest as defined by the path integrator would be 3 m away from the tested nest cue), ants still focused their search on the unambiguous nest cue, but now completely ignored the ambiguous one (Figure S1D).

We conclude that the extra route cues disturb the ants' ability to specify precisely the position of the nest. A simple hypothesis as to why ants rate ambiguous and unambiguous nest cues differently would be that cues encountered along the route become associated with the motor command "carry on" and cues encountered at the nest become associated with "search here." In the ambiguous training, the cues would be associated with both "carry on" and "search here," thus leading ants to do both at each cue. Future experiments will reveal whether the ants are also able to rate single nest cues differently when these cues predict the nest position with more or less reliability (e.g., a visual cue that appears only during every second homing run or slightly changes its position relative to the nest entrance).

One can envision that ants might also experience composite cues that activate more than one information channel. A bush close to the nest definitely provides visual input, but it might also have associated odor cues. Having shown that ants exhibit a more focused search at unambiguous nest cues, we next asked what happens when the cue at the nest has two components, of which one labels the route and nest cues and the other occurs only at the nest (Figure 3). We have previously shown that ants trained with a blend of odors and later tested with the individual components of this blend exhibit a rather unfocused nest search (Figure S2A; see also [5]). Thus, we trained ants with a composite nest cue consisting of two components and route cues with a single component. When we tested them later with individual components of the mixture, the ants focused their search on the nest significantly more when the nest cue was labeled with the unique nest component than when it was the component shared with the route cues. Ants did so regardless of whether in training methyl salicylate or decanal was the unambiguous part of the nest cue (Figures 3A and 3B; Figure S2C). Interestingly, the same was true when the cue was bimodal. Ants learn to associate combined visual-olfactory cues with the nest entrance and do not respond (or at least respond significantly less) to the individual components (Figure S2B; see also [7]). When we trained ants to such a combined nest cue, in which either the olfactory or the visual part was ambiguous, the ants later responded to individual compounds but mainly to those that were unique to the nest cue (Figures 3C and 3D; Figure S2D).

We conclude that ants can adopt an appropriate behavioral response to cues of differing levels of ambiguity and that they even do so for individual components of uni- and bimodal compound cues.
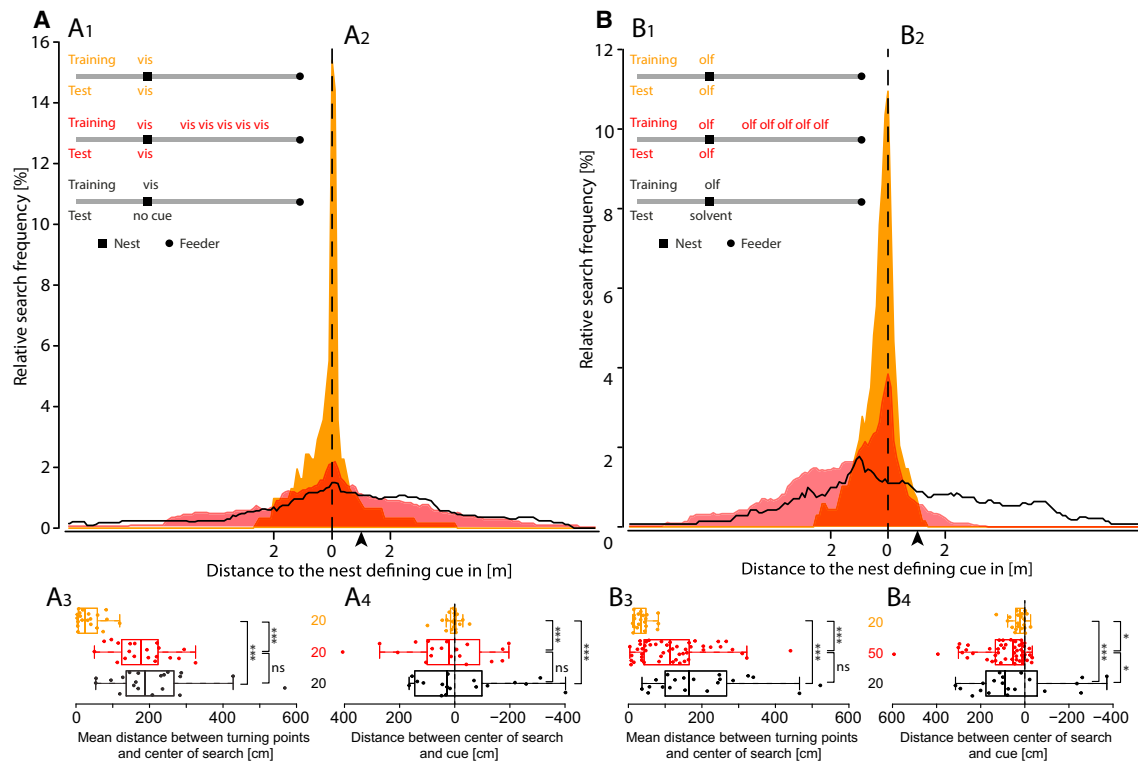
Figure 2. Ants Exhibit Focused Nest Searches at Unambiguous Cues Only

(A) Search patterns at a visual cue.

(B) Search patterns at an olfactory cue.

(A$_1$ and B$_1$) Training and test situations. vis, visual cue consisting of black cards (7 cm × 10 cm) placed on channel walls (see Figure 1A); olf, olfactory cues consisting of each 0.4 μL methyl salicylate and decanal diluted in 39.2 μL hexane and pipetted every 20 min in front of the nest entrance (see Figure 1A).

(A$_2$ and B$_2$) Search-density plots based on the first six turning points (see Figure 1B) after training with single nest cue (orange) or nest plus route cues (red). Black curves, control experiments with ants trained with nest cue and tested without any cues. Dashed line, position of nest cue; arrowhead, position of nest defined by path integrator. The feeder was positioned at 10 m.

(A$_3$ and B$_3$) Spread of search. Boxplots of the quantified search densities are shown. Each dot depicts the average distances between the first six turning points and the center of search (i.e., the mean of these six turning points) (see Figure 1B).

(A$_4$ and B$_4$) Accuracy of search. Each dot depicts the average distances between the center of search and the tested nest cue. Box, upper and lower quartile; line, median value of all tested ants; whiskers, values lying 1.5 times the length of the box from either end of the box.

Numbers beside boxplots depict sample sizes. **p < 0.01, ***p < 0.001 (Kruskal Wallis test with Dunn's post hoc test). Statistics for accuracy data were performed based on the distance (i.e., the absolute values) between the center and the cue. See also Figure S1.

To cope with the extremely harsh conditions of the Tunisian saltpans, *Cataglyphis fortis* has developed an efficient navigational toolkit that allows it to reduce the dangerous foraging time outside of the nest: the ants combine PI [2] with the use of local nest-defining cues [4, 5] and are able to associate cues of any given modality with the nest entrance [8]. The present study, however, shows that *Cataglyphis* can to some extent cope with a situation in which cues are ambiguous: nest-defining cues that are ubiquitous in the environment are ambiguous. Consequently, ants exhibit a strongly focused search at a cue that they experienced only at the nest but focus their nest search less on cues that they experienced at several places (Figure 2). The informative value of the nest cue is increased if it includes features of the same or a different sensory modality that is absent in route cues. A nest-defining bush might look similar to other bushes but might become unique if it is associated with a specific smell. Ants are known to learn such bimodal cues extremely quickly [7].

When ants are trained to a bimodal cue at the nest, it takes ~15 learning trials before the ant has learned that both modalities are closely linked. These ants in a test situation do not respond to the individual compounds anymore but only perform a focused nest search in the presence of both cues [7]. However, when we not only trained the ants to the combined nest cue but also presented one compound of the bimodal cue repeatedly along the route, this close link between both modalities was not formed. Instead, the ants focused their search at an individual component of a mixture so long as this was the unambiguous component one in the training situation (Figure 3). Obviously, cues along the route can influence the pattern learning at the nest, with the ants later focusing on those parts of the pattern that were unambiguous.

Traditionally, it was assumed that insects use different navigational cues like, e.g., compass cues and landmarks in a dominance hierarchy, with one system dominating the other in a context-specific manner [9, 10]. However, recent finding suggest
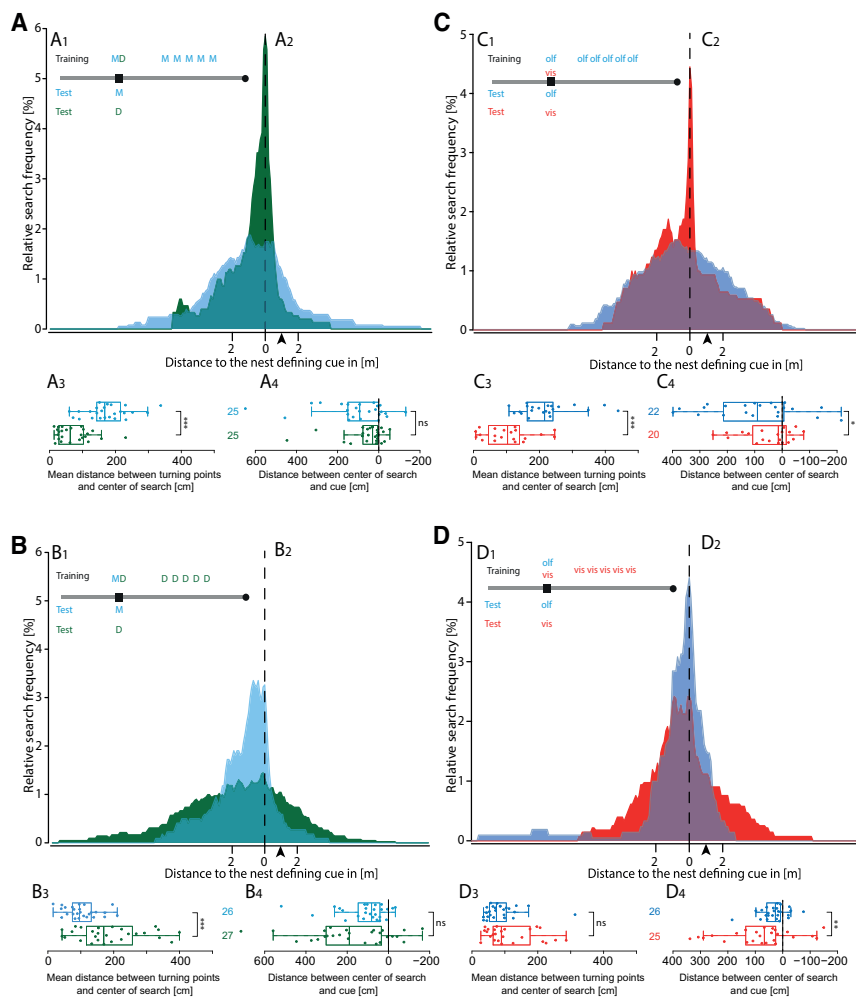
Figure 3. Ants Rate Predictability of the Individual Components of Compound Cues

(A and B) Search patterns of ants that were trained with a binary blend as nest cue plus route cues of a single odor.

(A1 and B1) Training and test situations. MD, M, and D indicate olfactory cues consisting of a binary blend (MD), or the individual components (methyl salicilate or decanal), respectively (for details on odor amounts, see Figure 2).

(A2) Search-density plots based on the first six turning points at decanal (D, green) and methyl salicilate (M, blue) when decanal was presented at nest only and methyl salicilate at nest *and* along the route.

(B2) Search density at decanal (D, green) and methyl salicilate (M, blue) when methyl salicilate was presented at nest only and decanal at nest *and* along the route.

(A3, A4, B3, and B4) Boxplots of the quantified search characteristics (for details, see Figure 2).

(C and D) Search patterns of ants that were trained with a bimodal cue but were tested with a single (visual or olfactory) compound only.

(C1 and D1) Training and test situations.

(C2) Search density at visual cue (red) and olfactory cue (decanal plus methyl salicilate, blue) when the visual cue was presented at nest only and the olfactory cue at nest *and* along the route.

(D2) Search density at visual cue (red) and olfactory cue (blue) when olfactory cue was presented at nest only and visual cue was presented at nest *and* along the route.

(C3, C4, D3, and D4) Boxplots of the quantified search characteristics (for details, see Figure 2).

Numbers beside the boxplots depict sample sizes. ***$p < 0.001$, *$p < 0.05$ (Mann-Whitney U test). See also Figure S1 for alternative paradigm. Arrowhead, position of nest defined by path integrator. See also Figure S2.

that systems operate simultaneously and that ants [10–12], as well as humans [13], rate the predictability of cues when they experience cue conflicts. This rating obviously does not necessarily need any second-order processing of cue predictability, as generally assumed in Bayesian theory, but can take into account a proxy that covaries with the cue's uncertainty [12]. When, e.g., PI and visual cues are put in conflict, ants show stronger weighting of the PI direction when the length of the PI vector increases [12]. As the direction predictability of the PI increases with its length, the stronger bias toward PI versus visual memory when ants are far away from the nest suggests optimal cue integration. Interestingly, although the directional precision increases with the length of the PI vector, the certainty of the ant regarding the nest position decreases due to accumulating errors. Again the ants take this reduced certainty into account by increasing the area they cover during the systematic nest search [14, 15].

In another study, it was shown that ants adjust their food-search strategy according to the food distribution [16]. Ants searching for protein-rich food (which usually is sparsely and randomly distributed) exhibit a wide search pattern, whereas ants searching for carbohydrates (which usually appear patched

and in a renewable and therefore predictable way) exhibit a focused search. An adaptive response to cues of differential predictability hence seems to be a general phenomenon in an ant's life.

However, our study for the first time shows that one and the same cue provokes different search behavior depending on the previous experience of the animal, whether this cue is ubiquitous or occurs only in a single site. The ant then adjusts its landmark-based nest search accordingly. The learning walks of ants [17] and the learning flights of wasps and bees [18] are behavioral signs that hymenoptera do have inbuilt strategies for emphasizing nests and feeding sites in their memories, and there is also behavioral evidence that ant routes are influenced more by visual cues close to the nest than by visual cues further away [19]. The question then arises, why aren't all the identical cues along the route treated like both nest and route cues? The likely answer is that in part they are, but that the ants' navigational strategies do result in some separation so that along the route ants "carry on" more than "search here" while the balance is tilted the other way at the nest. However, the strategies are insufficiently sophisticated to effect a clean distinction. A complete separation could in principle be made through counting

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cues along a route as suggested in [20] and [21] and/or by measuring distance intervals between cues. However, our results suggest that when nest and route cues become too similar, ants do not follow any of those strategies, but rather they ignore nest cues that they also have seen several times along the route. Ants have been shown to learn numerous visual cues along routes and to do so even with multiple routes [22]. This impressive ability to learn and store such an overwhelming amount of information might be partly explained by our finding that the ants rate the informative value of individual cues and mainly focus on the unambiguous ones.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
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- QUANTIFICATION AND STATISTICAL ANALYSIS

AUTHOR CONTRIBUTIONS

R.H. and M.K. designed the experiments, analyzed the data, and wrote the paper. R.H. conducted the experiments.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Methyl salicylate	SigmaAldrich	CAS 119-36-8
Decanal	SigmaAldrich	CAS 112-31-2
Deposited Data		
Analyzed data	This paper	N/A
Experimental Models: Organisms/Strains		
Cataglyphis fortis (Tunisian desert ant)	Tunisian Salt Pan	N/A
Software and Algorithms		
Statistical Software: GraphPad InStat	GraphPad	https://www.graphpad.com/scientific-software/instat/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Markus Knaden (mknaden@ice.mpg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The model of this study is the Tunisian desert ant *Cataglyphis fortis* inhabiting the open salt pans of Tunisia. The experiments were performed within the ants' habitat, i.e., a salt pan close to the village Menzel Chaker (Sebkhet Bou Jemel, 34°96'N, 10°41'E) in summers 2015 and 2017. All subjects of this study were female ants belonging to the worker caste.

METHOD DETAILS

In order to test the ants' use of visual and/or olfactory nest-defining cues, we connected the nest entrance via a tube (diameter 2cm, length, 20cm) from below with an aluminum channel (length, 16 m, width, 7cm, height, 7cm, open top to provide the ants with celestial cues) in which the ants were allowed to search for food. Ants were trained to a feeder (a Petri dish containing biscuit crumbs *ad libitum*) that was 10 m away from the inconspicuous exit of the channel to the nest entrance (Figure 1A).

Training with unambiguous nest cues (either a visual cue (two black cards (7cm x 10cm) at the opposite walls at the nest entrance), or an olfactory cue (each 0.4μL methyl salicylate and decanal diluted in 39.2μL hexane and pipetted every 20min in front of the nest entrance), or a bimodal cue (i.e., a combination of the visual and the olfactory cues): Ants arriving at the feeder for the first time, were marked with a time-specific color code in order to keep track of their training history.

Training with ambiguous nest cues: In a second training paradigm, in addition to the nest-defining cue, 5 route-defining cues were installed along the way from the nest to the feeder (in 4, 5, 6, 7, and 8 m distance from the nest entrance). These cues were either identical to the nest-defining cue, or consisted only of a part of it (olfactory cues: only one odor as route cues, while the nest was always marked with a blend of two odors; bimodal cues: only one modality (visual or olfactory), while the nest-defining cue was bimodal.

After at least 3 hr of training (i.e., approximately 30 training runs), homing ants were caught 3 m in front of the nest entrance and were – together with their biscuit crumb – transferred to a test-channel (i.e., a remote parallel, identical but longer (30 m) channel that was not connected to any nest or exit, Figure 1B). Under this situation, ants ran off most of their PI vector in the familiar surrounding and after displacement to the test channel ran off the remaining vector and started a well described systematic search [5, 6] for the nest entrance (Figure 1B). Ants were released 4 m downwind of the nest cue (curved arrows in Figure 1) to guarantee that any cue-centered search was not due to PI (which would have resulted in focused search 1 m downwind of the test cue). Using a measuring tape placed alongside the test channel, we tracked this nest search by recording the positions of the first six 180°-turning points after the ant had passed the cue for the first time (turning points were only considered, when the ant followed the new direction for at least 10 cm). By calculating the average of the first six turning points we identified the center of search. The distance between the turning points and this center informs us about the ant's confidence in that cue [5]. The distance between the center of search and the provided cue informs us about the accuracy of the nest search. Turning points that were performed before the ant had passed the cue were excluded from the analysis. For each experimental group we tested at least 20 individuals with each individual tested only once. Data were visualized by using a custom-written macro in Excel and R.

QUANTIFICATION AND STATISTICAL ANALYSIS

For each ant we analyzed the spread of the search by calculating the average distance of the first six turning points and the center of search (average of the six turning points), and the accuracy of the search by calculating the distance between the center of search and the nest-defining cue. For each analysis this resulted in at least 20 values per experimental group (as defined by the sample size). The values of different test groups were then compared by the Mann-Whitney U test (when only two groups were compared) or by Kruskal-Wallis with Dunn's posthoc test for multiple comparisons (when more than 2 groups were compared). All statistical calculations were performed in the GraphPad InStat. The resulting p values are provided in the figures and corresponding figure legends.