Desert ants use olfactory scenes for navigation

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Desert ants, *Cataglyphis fortis*, forage for dead arthropods in the Tunisian salt pans. Both the unpredictable food distribution and the high surface temperatures might account for the fact that the ants do not use any pheromone trails. However, *Cataglyphis* has been shown to still use olfactory cues for navigation. For instance, the ants locate sparsely distributed food or pinpoint their inconspicuous nest entrance by following odour plumes. In this study we found that, as well as using odours to pinpoint a target, the ants might use environmental odours as olfactory landmarks when following habitual routes. When analysing odours collected at 100 positions in the desert, we found spatially distinct gradients of a range of different environmental odorants. Furthermore we confirm that individual foragers followed forager-specific routes when leaving the nest. Therefore these ants could potentially learn such olfactory landscape features along their stable routes. We, hence, asked whether ants could learn and use olfactory cues for route guidance. We trained ants to visit a stable feeder and presented them with a sequence of four different odours along the way. Homing ants that had already passed the odour alley on their way back were displaced to a remote test field and released at the starting point of an identical alley. Control ants that experienced the alley only during the test situation focused their search on the release point. Ants that had experienced the odours during training, however, biased their nest search towards the odour alley and performed straight walking segments along the alley. Hence, we found that ants learnt the olfactory cues along their homeward route and used these cues in the absence of other navigational information. Hence, desert ants seem to be able to use odour information to follow routes.

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Desert ants are expert navigators and their study has led to the unravelling of many mechanisms of navigation (for reviews see e.g. Wehner, 2003, 2009). While foraging, these ants have to travel through a hostile desert environment in which an unpredictable food distribution and high surface temperatures force the ants to forage individually rather than orienting along pheromone trails. The initial navigational mechanism for a forager is path integration (Muller & Wehner, 1988; Ronacher, 2008; Wehner & Srinivasan, 2003). Through the use of a skylight compass (Wehner & Muller, 2006) and some kind of odometer (Wittlinger, Wehner, & Wolf, 2006) ants can keep track of the direction and distance in which they have travelled such that they are continuously informed about their position relative to the nest. In addition to this strategy ants can use learnt information from visual cues to pinpoint a place of interest or to follow a habitual route (Wehner, Cheng, & Cruse, 2014; Zeil, 2012). Learning of visual cues for route guidance

allows ants to develop idiosyncratic routes and robustly travel back and forth between the nest and plentiful feeding sites (Collett, 2010; Collett, Dillmann, Giger, & Wehner, 1992; Graham, Fauria, & Collett, 2003; Kohler & Wehner, 2005; Mangan & Webb, 2012; Sommer, von Beeren, & Wehner, 2008; Wehner, Michel, & Antonsen, 1996; Wystrach, Schwarz, Schultheiss, Beugnon, & Cheng, 2011). Furthermore, it has been shown that ants can take advantage of other sensory cues when available (Buehlmann, Hansson, & Knaden, 2012a; Kleineidam, Ruchty, Casero-Montes, & Roces, 2007; Seidl & Wehner, 2006). One of these modalities is olfaction (Steck, 2012; Wolf, Wittlinger, & Bolek, 2012). For instance, *Cataglyphis fortis* ants are capable of using an odour to accurately localize the inconspicuous nest entrance after experiencing it during a training phase (Steck, Hansson, & Knaden, 2009). Moreover, they are even able to learn the nest position relative to a complex array of multiple odorants around the entrance (Steck, Knaden, & Hansson, 2010). These findings led to the question whether olfaction also plays a role in a more natural setting when the ants are foraging within the salt pan. *Cataglyphis fortis* ants use olfaction to detect and locate dead arthropods (Buehlmann, Graham, Hansson, & Knaden, 2014) and to augment other

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navigational systems to pinpoint their nest by following a nest-produced odour plume (Buehlmann, Hansson, & Knaden, 2012b).

Here we investigated whether the desert environment provides odour features that might be useful for route navigation. Furthermore, we asked whether *C. fortis* ants can use olfactory information as a route-defining cue.

METHODS

Chemical Environment

To study the ants' chemical environment we collected odour samples in their natural foraging area in the Tunisian salt pan. The salt pan near the village of Menzel Chaker (34°96′N, 10°41′E) is mainly devoid of vegetation and is a rather homogeneous habitat, although the flat ground has some structure, i.e. the salt crust can be interrupted by clefts, sandy areas or small pieces of wood or halophytic plants. A 100 m × 100 m grid was established using strings fixed with nails (mesh width, 10 m) and 100 odour samples were taken at the intersection points by using poly-dimethylsiloxane (PDMS) tubes. Pieces of PDMS analytical tube (length: 4 mm; inner (outer) diameter: 1.5 (2.3) mm; RCT Reichelt Chemietechnik GmbH &Co, Heidelberg, Germany) were cleaned overnight in the laboratory with 4:1 acetonitrile/methanol, pre-conditioned at 230 °C for 4 h under nitrogen flow in a tube conditioner (Gerstel) and stored in clean vials before use. In the field, PDMS tubes were placed at the sample sites by inserting them slightly into the ground. Five PDMS tubes were used per sample site. Cleaned glass petri dishes were used to cover the sampling sites in order to facilitate the relocation of the analytical tubes. To decrease the direct sun radiation, horizontal aluminium plates (250 × 250 × 0.5 mm) were installed 10 cm above each sampling site. Odours were collected for 84 h. Analytical tubes placed into cleaned glass petri dishes on top of the desert ground served as blanks. After collection the analytical tubes were stored in the freezer (−80 °C) for around 3 months until measurement.

Analytical tubes were analysed using a GERSTEL thermode-sorption unit (TDU) coupled to a GERSTEL cooled injection system (CIS 4) on an Agilent GC (gas chromatography) 7890 A connected to a mass selective detector (MSD) 5975 C. The MS (mass spectrometry) operated in electron ionization mode (70 eV) with scans from 33 to 450 atomic mass units. Chemical compounds were separated on a 30 m × 250 μm HP-5 MS ultra inert column with a 0.25 μm film coating (Agilent Technologies; 19091S-433UI). Helium was used as carrier gas (constant flow 1 ml/min). The TDU temperature was increased from 30 °C to 210 °C at a rate of 30 °C/min and was held at 210 °C for 10 min. The thermodesorbed compounds were trapped in the cooled injection system (CIS 4) at −50 °C. The GC run started by heating the injection system from −50 °C to 220 °C at a rate of 12 °C/min and kept the end temperature for 5 min. The GC oven was set to 40 °C for 2 min and, a temperature ramp of 10 °C/min followed. The end temperature of 260 °C was held for 5 min. In each measurement two pieces of analytical tubes were used. Data were processed with MSD ChemStation Data Analysis Application (Agilent Technologies) and DataTrans.

Compounds were identified by comparison of mass spectra and KI values with those available in the NIST 2.0 mass spectra database (http://www.nist.gov/srd/nist1a.cfm). We do not intend to provide a complete description of the chromatograms but rather wanted to test, for a few selected substances, whether spatial gradients of environmental odours exist in the desert environment. Because of the huge number of peaks in the chromatograms (874 peaks with different retention times) we selected those peaks that reached a fit with NIST that was over 800 and additionally were found in at least six of the 100 sample sites. The relative quantity of a compound was calculated from the detected peak area normalized against the internal tube peak present throughout the measurements and the maximal value was set to 1. After selecting eight compounds by these rules we plotted the occurrence of these selected compounds for the 100 sample sites.

Individual Foraging Tracks

Foraging paths of individual *C. fortis* foragers from one colony were tracked with GPS. Ants were individually colour coded and several foraging paths were tracked by following the ants with a GPS measurement device (GARMIN eTrex 20 or 30). GPS co-ordinates were taken every second. Owing to high satellite coverage the GPS units indicated an expected error for absolute position of less than 2 m. However, by following a predefined route and comparing the GPS output we found that even small movements of less than 20 cm were correctly tracked. GPS data were transferred to a PC using EasyGPS (http://www.easygps.com) and paths were analysed in Matlab (Mathworks, Natick, MA, U.S.A.). To analyse whether individual ants take consistent paths through the salt pan, the overall heading direction for the path segments from 6 to 50 m from the nest was determined. Having 10 ants with only one path each allowed us to calculate the mean pairwise difference between these paths (between-ant comparisons). Then for each ant from another group of 10 ants we took the difference in trajectory direction of two paths (within-ant comparisons). We could then ascertain the proportion of ants whose heading direction difference was smaller than the mean pairwise difference in trajectory direction between ants. We tested the significance of this proportion with a sign test.

Olfactory Route Cues

To test whether ants are able to use olfactory information as part of their general route navigation we trained *C. fortis* foragers for at least 1 day to visit a feeding site containing biscuit crumbs that was located 10 m from their nest. The ants rapidly learnt to shuttle back and forth between the nest and the feeder. During training, ants experienced the following odours along the beeline of the route: (1) methyl salicylate, (2) decanal, (3) indole and (4) nonanal. As described in previous studies, these odours neither innately attract nor repel naïve ants, are learnt equally well and can be distinguished by the ants when applied in the current concentrations (Steck et al., 2009). Odours were presented in the sequence 11223344, with intervals of 1 m between each odour and 1.5 m gaps between the end of the odour array and nest and feeder. Odours were diluted in hexane (1:50) and 30 μl was pipetted onto a filter paper that was put in a 2 ml glass vial placed into the ground such that the opening was slightly lower than the ground level. The odours were renewed every 30 min which should guarantee that odour sources were never depleted (Steck et al., 2010). As the wind direction was mostly relatively stable, we were able to align the odour alley perpendicular to wind direction.

For tests, experienced ants returning from the feeder were captured just before they entered the nest and the search paths of these so-called zero-vector ants was recorded after releasing the ants at a distant test field either in the presence of the same (+/+ ants) or the reversed odour (+/+∗ ants) sequence. A 25 m × 25 m test field (mesh width, 1 m) was painted on the desert ground about 100 m from the training ground and the paths were recorded for 5 min on gridded paper. Wind direction was measured by using a small wind vane placed on the test field. As a control we recorded the search paths of ants that had been trained to the same feeder distance but had not experienced any odours along the route. These ants were later tested either without (−/− ants) or with (−/+ ants)

odours. Paths were digitized using GraphClick (www.arizona-software.ch/graphclick) and then analysed in Matlab.

The search paths were analysed regarding their symmetry along the nest-to-feeder direction and perpendicular to the nest-to-feeder direction. The sum of the total path lengths in either the upper and lower halves or the left and right halves of the test field and the difference in the total path lengths between the two halves were calculated. This difference divided by the total path length gives a value between -1 and $+1$ where a value of 0 means that paths were symmetrically distributed about an axis (either vertical or horizontal). A Wilcoxon signed-rank test was used to test the null hypothesis that the data come from a distribution whose median is zero (i.e. symmetrical search paths). For a more detailed analysis, the paths were divided into 0.5 m chunks and the ants' heading direction for each chunk was calculated. Chi-square tests were applied to test for a disproportionate frequency of headings parallel to the odour alley (H = homeward direction $\pm 45°$ and F = foodward direction $\pm 45°$) or perpendicular to the odour alley (L = left $\pm 45°$ and R = right $\pm 45°$) relative to an expected uniform distribution. Finally, for headings in both the homeward direction $\pm 45°$ and foodward direction $\pm 45°$ we determined segments of the paths where the ants kept walking in this direction for at least 10 m (i.e. 20 consecutive path chunks). Such a segment ended when the ants' direction changed to the L or R quadrant for more than 0.5 m. We tested the occurrence of these long, directed path segments from tests with the odour present (+/+, +/+∗ and $-/+$) against their frequency in the control test without odours ($-/-$) using the Fisher's exact test and a Bonferroni correction.
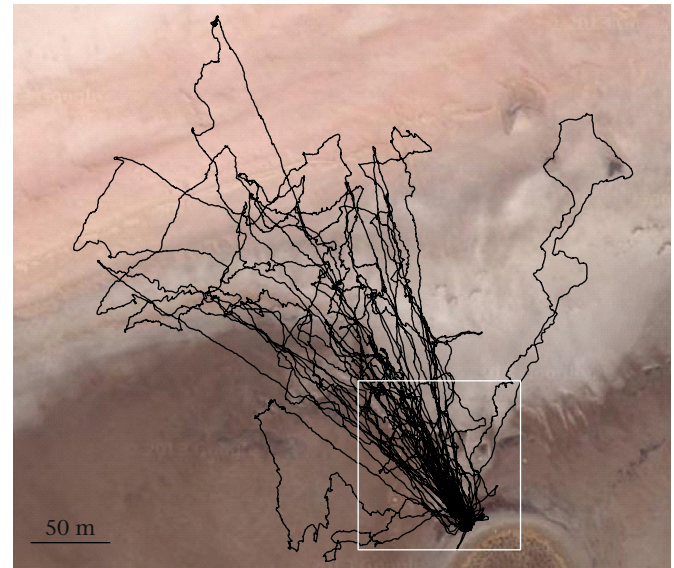
Ethical Note

Experiments were designed to minimize the impact on the tested desert ants.

RESULTS

The Desert Environment Provides Odour Information

For the eight environmental odours that we selected from the 100 samples, we found place-specific concentration gradients

Figure 2. Individual directions of foraging ants. Black lines: foraging paths of 20 ants. White square: the 100 m × 100 m odour sampling area (see Fig. 1).

(Fig. 1) within the ants' foraging area. We can thus say that the environment provides olfactory information that could potentially be used for route navigation.

Ants Take Consistent Paths Through the Uncluttered Terrain

To use place-specific odours for navigation requires that a navigating subject repeatedly passes a place such that it can form an association between place and place-specific odour. We therefore asked whether ants take consistent paths through the salt pan over a series of foraging runs. The analysis of multiple foraging journeys of individual ants revealed forager-specific initial heading directions (Fig. 2) with all 10 ants having a smaller difference in trajectory direction than the mean pairwise difference between ants (sign test: $P = 0.002$), which is consistent with former findings on sector fidelity of experienced *Cataglyphis* foragers (Schmid-
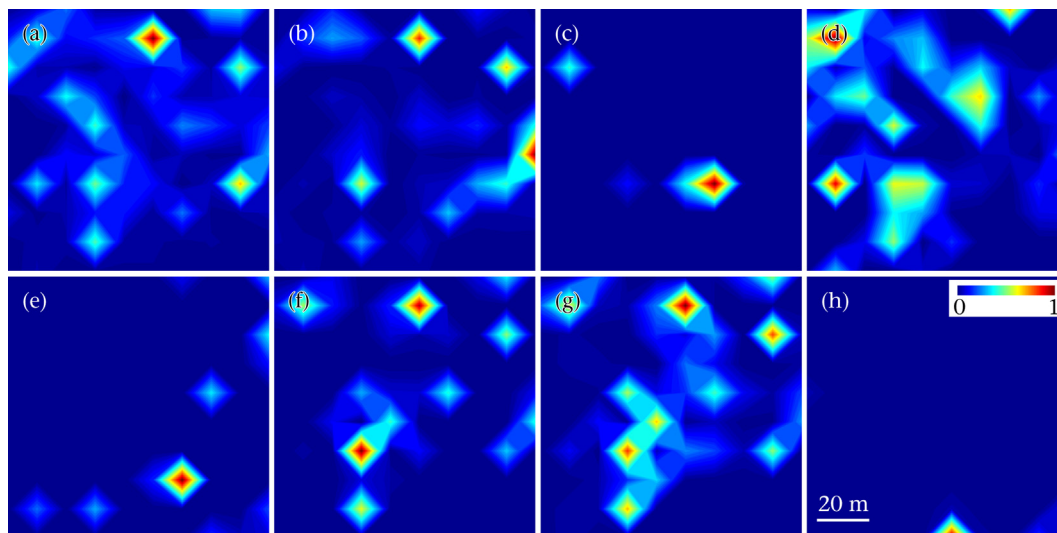
Figure 1. Heat map representation of relative odour gradients for eight odours identified in the salt pan habitat (sampling area: 100 m × 100 m; see white square in Fig. 2). (a) 6,10-dimethyl-2-undecanone; (b) 2,10-dimethyl-9-undecenal; (c) β-bisabolene; (d) tridecanal; (e) 3-ethyltoluene; (f) 2-dodecanone; (g) 2-undecanone; (h) 2-methyl-7-nonadecene. 0 gives low concentrations and 1 high concentrations (relative to internal standard).

Hempel, 1983; Wehner, Harkness, & Schmid-Hempel, 1983; Wehner, Meier, & Zollikofer, 2004). Our finding suggests that individuals do take consistent paths through the salt pan which is a prerequisite for the use of olfactory route information.

Ants Associate Directional Information with Odours

As the desert environment provides odour features (Fig. 1) and as ants take consistent paths through their environment (Fig. 2), we asked whether ants could learn and use odours for navigation. Ants that did not experience any odours during training and the test searched symmetrically around the point of release which is the nest position defined by the path integrator (−/− ants; Fig. 3d; Wilcoxon signed-rank test: about the horizontal axis: $W = 0$, $P = 0.63$; about the vertical axis: $W = 0$, $P = 0.39$; $N = 20$ ants). However, ants that had been trained with odours biased their search to that part of the test field where the odours were placed

(+/+ ants; Fig. 3a; Wilcoxon signed-rank test: $W = 1$, $P < 0.001$; $N = 29$ ants). At the same time ants' searches were symmetrically distributed about the vertical axis (Wilcoxon signed-rank test: $W = 0$, $P = 0.52$). To further investigate the effect of the odours we analysed the heading direction of entire search paths for each 0.5 m chunk. While +/+ ants mainly walked in homeward or foodward directions (Fig. 4a; chi-square test: $\chi^2_1 = 76.1$, $P < 0.001$), −/− ants did not show any directional preference (Fig. 4a; chi-square test: $\chi^2_1 = 0.1$, $P = 0.77$). Furthermore, +/+ ants exhibited significantly more long, straight 10 m path segments aligned with the nest to feeder direction (Fisher's exact test and Bonferroni correction: $P = 0.003$; Fig. 4b; $P = 0.64$; Fig. 4c). These straight segments were produced after ants had searched for a while. Ants had a median walking distance of 30.25 m before they exhibited the long 10 m segments (shortest distance 23 m; longest distance 54 m). As naïve ants that experienced the odours during the test for the first time (−/+) did not exhibit long, straight 10 m path segments (Fisher's
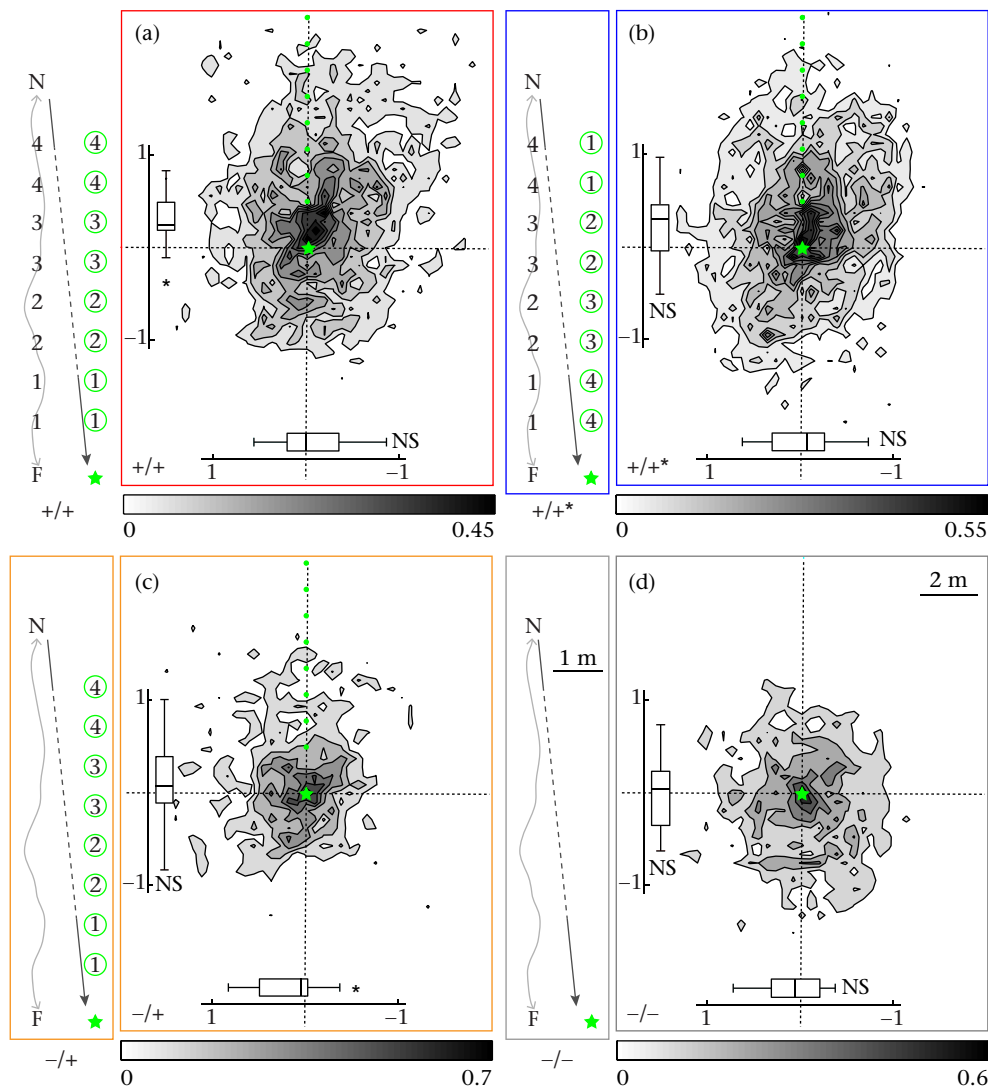
Figure 3. Routes taken by ants in four experimental conditions. Search densities in the four conditions are shown, with the experimental paradigms on the left for each condition (all to the same scale). Nest (N) to feeder (F) distance: 10 m; numbers: odour alley (for odour names see Methods section); arrow: displacement of trained homing ants from nest to remote test field; asterisk: point of release. Nest search was recorded for 5 min. Test conditions: (a) odour present during training and test (+/+, N = 29 ants); (b) sequence of odours reversed during test (+/+*, N = 25 ants); (c) odours only present during test (−/+, N = 20 ants); (d) no odour during training or test (−/−, N = 20 ants). Symmetry indices of the nest searches about horizontal and vertical axes are shown in box plots (median, 25th and 75th percentiles (edges of the boxes), whiskers (extreme values not considered as outliers); Wilcoxon signed-rank test: *P < 0.05; for statistics see Results). Numbers below the gradient bars depict the relative search densities (%) per square (0.3 m × 0.3 m) of the test ground. Vertical axis: nest-to-feeder direction. Based on the information collected with the wind vane, some paths were mirrored along the vertical axis such that, at the time of release, odour plumes were always pointing left.
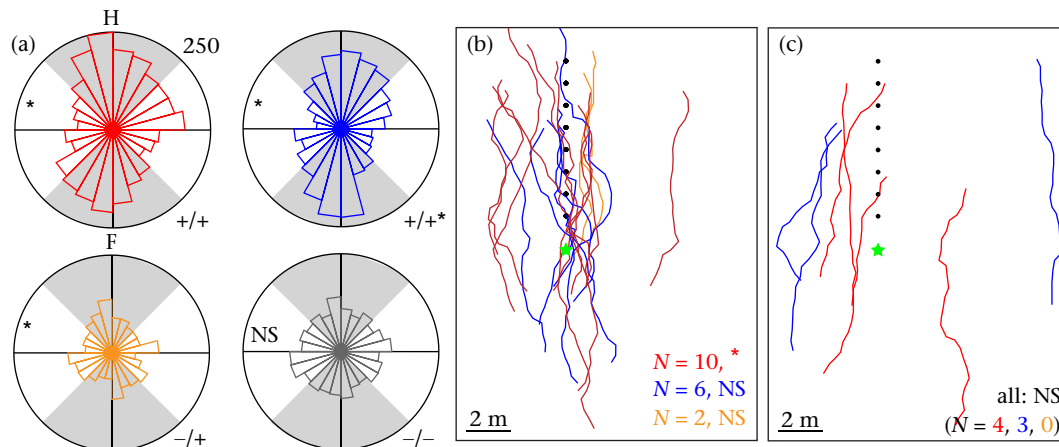
Figure 4. Length of ants' path segments in the homeward direction. (a) Directional distribution of all search paths broken into 0.5 m chunks (bin size: 15°; H: homeward direction; F: foodward direction). Distributions were tested for a disproportionate frequency of headings parallel (H ± 45° and F ± 45°: grey sectors) or perpendicular to the odour alley (right ± 45° and left ± 45°: white sectors) relative to an expected uniform distribution (chi-square test: *$P < 0.05$; for statistics see Results). Test conditions: +/+ (red), +/+∗ (blue), −/+ (orange), −/− (grey). For details see Fig. 3. Straight 10 m paths in homeward (feeder-to-nest, b) or foodward (nest-to-feeder, c) direction. Red: +/+ ants; blue: +/+∗ ants; orange: −/+ ants; asterisk: point of release; black circles: position of odour cues (for alley information see Fig. 3). Occurrence of these long, directed path segments from tests with the odour present (+/+, +/+∗ and −/+) were tested against their frequency in the control test without odours (−/−; b: $N = 0$ of 20 ants; c: $N = 1$ of 20 ants). Fisher's exact test and Bonferroni correction: *$P < 0.05$; for statistics see Results. Based on the information collected with the wind vane, some paths were mirrored along the vertical axis such that, at the time of release, odour plumes were always pointing left.

exact test and Bonferroni correction: $P = 0.49$; Fig. 4b; $P = 1$; Fig. 4c), the odours did not seem to innately guide the ants in the feeder to nest direction. However, we found a weak innate response of −/+ ants to the unknown odours when looking at the path distribution about the vertical axis (Fig. 3c; Wilcoxon signed-rank test: about the horizontal axis: $W = 0$, $P = 0.23$; about the vertical axis: $W = 1$, $P = 0.03$; $N = 20$ ants) and the preference for the nest to feeder direction (Fig. 4a; chi-square test: $\chi^2_1 = 5.0$, $P = 0.03$) that could be explained by ants being slightly attracted by the unfamiliar odours. We therefore conclude that the ants that were trained and tested with the same olfactory array later made use of olfactory route information.

Did the ants only learn the presence or also the sequence of the olfactory cues? When we trained ants to the same sequence of odours but tested them with a reversed sequence (+/+∗ ants) they also biased their search on the part of the test field containing the odours (Fig. 3b; Wilcoxon signed-rank test: about the horizontal axis: $W = 0$, $P = 0.05$; about the vertical axis: $W = 0$, $P = 0.88$; $N = 25$ ants), oriented themselves along the feeder to nest axis (Fig. 4a; chi-square test: $\chi^2_1 = 117.6$, $P < 0.001$) and exhibited straight 10 m path segments in the feeder to nest direction (Fisher's exact test and Bonferroni correction: $P = 0.03$; Fig. 4b; $P = 0.62$; Fig. 4c). However, this bias parallel to the olfactory array was less pronounced than for ants that were trained and tested with the same odour sequence (+/+). This difference is small, however and we are unable to draw a conclusion as to whether the ants learned the odour sequence.

DISCUSSION

In ants the use of olfactory cues for guidance is well documented regarding the utilization of pheromone trails laid by conspecifics (see e.g. Czaczkes, Grueter, & Francis, 2015; Steck, 2012 and references therein). However, what is less clear is whether ants might be able to learn and use environmental odour cues for route navigation. Our investigation addressed three questions related to the use of environmental odours. (1) Does the desert environment provide stable odour features that might provide route information? (2) In a flat, uncluttered terrain with little visual information, do ants take consistent paths that would enable them to learn such

information? (3) Can ants follow a route defined only by odour information?

The study of visually guided route navigation in ants has been recently augmented by quantitative approaches to understand the information in natural visual environments (see e.g. Philippides, Baddeley, Cheng, & Graham, 2011; Stuerzl & Zeil, 2007; Towne & Moscrip, 2008; Zeil, Hofmann, & Chahl, 2003). Here we took a similar approach to investigate the odour information available in the ants' salt pan habitat. We showed that over an area of 100 m × 100 m, environmental odours are present and that spatial concentration gradients exist (see Fig. 1). While at least one of the identified odours has already been described as a volatile from marine salts (Silva, Rocha, & Coimbra, 2009), we can only speculate about the origin of the other substances. The salt crust covering this habitat is occasionally interrupted by clefts, sandy areas or small pieces of wood or halophytic plants all of which might be odour sources. We did not intend to identify compounds that ant foragers actually use for navigation. We rather showed that there are potential environmental odours that could be used for navigation. Although we focused our analysis on eight odorants, the huge number of different compounds detected by our GC analysis (874 peaks in total) suggests that olfactory information provided by the habitat is highly complex.

Little is known about the occurrence of environmental odours over larger scales in the natural habitats of animals that navigate using olfaction (Budick & Dickinson, 2006; Carde & Willis, 2008; DeBose & Nevitt, 2008). Atmospheric trace gases have been sampled and analysed over hundreds of kilometres (Wallraff & Andreae, 2000) in order to relate spatial gradients in their ratios with findings regarding the homing behaviour of pigeons, *Columba livia*. Another example of an olfactory landscape has been described in the natural habitat of seabirds that travel for hundreds of kilometres over the otherwise rather featureless ocean (Nevitt, 1999; Nevitt, Veit, & Kareiva, 1995). In this case dimethyl sulphide, a compound indicating rich food areas, seems to be used by birds for navigation.

When looking at natural foraging paths of *C. fortis* foragers we found that foraging individuals repeatedly passed the same areas of the salt pan (see Fig. 2). This is consistent with other examples of sector fidelity in desert ants (Schmid-Hempel, 1983; Wehner et al.,

1983; Wehner et al., 2004). The foraging paths of individual ants were spread across 90°, i.e. ants headed preferentially towards the salt pan. We think these paths were not uniformly distributed as previously reported (Schmid-Hempel, 1983; Wehner et al., 1983; Wehner et al., 2004) because this colony is located at the edge of the salt pan which restricts the foraging area. Owing to the place-specific environmental odours and the idiosyncratic routes of the ants, we asked whether ants are capable of learning and using olfactory cues when repeatedly travelling along routes.

When we trained ants to a situation in which they always had to pass an alley of odours on their way between the nest and a stable feeder, the ants indeed learnt something about these odours. Such trained ants were biased along the odour alley when experiencing these odours at a remote test field (see Fig. 3). Furthermore, in the presence of familiar odours ants more often produced long path segments ($\geq$10 m) in the homeward direction (see Fig. 4).

How might ants use olfactory information to follow a route? In the better known case of visual route guidance there are two well-discussed mechanisms that might have analogues for olfactory guidance (for a review see Collett, Chittka, & Collett, 2013 and references therein). Alignment image matching allows ant foragers to walk along a habitual route (Graham & Cheng, 2009; Harris, Graham, & Collett, 2007; Wystrach, Beugnon, & Cheng, 2011) by aligning themselves such that the current panoramic view best matches views stored on previous trips along that route. Ants are also capable of associating visual cues along a route with so-called local vectors (Collett, Collett, Bisch, & Wehner, 1998). These memorized vectors (referenced to a celestial compass) can encode a path segment of a specific distance and direction from the familiar location (Collett & Collett, 2009) and would be recalled when an ant encounters a familiar location.

In terms of odour guidance the first of these visual strategies (alignment image matching) would be equivalent to setting the direction by trying to replicate the sensory experience learnt during training. If the prevailing wind is relatively stable then it might be possible to fix a course relative to a wind carrying familiar odours. Alternatively, olfactory-driven local vectors would involve the familiar odour triggering the recall of associated directions which are then set using a celestial compass. How these olfactory route cues are used and also how this interacts with wind direction and other cues needs to be studied in more detail.

It is already known that food odours can prime the recall of navigational memories. For instance, honeybees, *Apis mellifera*, fly back to a familiar feeding site when the scent that is associated with that location is blown into their hive (Reinhard, Srinivasan, Guez, & Zhang, 2004). Similarly, the black garden ant, *Lasius niger*, can associate an odour with the navigational memories required to reach the corresponding feeding site (Czaczkes, Schlosser, Heinze, & Witte, 2014). Our results suggest that ants may also be capable of associating odours experienced along routes (rather than just at a goal) with navigational instructions.

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