Smells like home:

Olfactory landmarks in desert ant orientation

Dissertation

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Introduction

Insect Olfaction - From Perception to Behaviour

The chemical sense is for many insects the most important sensory modality to exploit the environment with success, i.e. to survive and to reproduce. In this context, relevant chemical signals can be classified into environmentally-derived volatiles and pheromones which are produced by conspecifics. Environmentally-derived odours guide an insect to food sources and substrates for oviposition. Pheromones on the other hand are used for communication i.e. to find mating partners. Especially social insects, such as ants are known to use a multitude of pheromones to organize the life inside (i.e. queen pheromones) and outside (i.e. trail pheromones) their nests (Hoelldobler & Wilson, 1990). However, the species studied in the present thesis, the desert ant *Cataglyphis fortis*, contrary to most ants was thought to rely more or less exclusively on a vision-based orientation system during foraging runs instead of pheromone trails. I was amazed to find that *C. fortis* in addition is equipped with a sophisticated chemical sense of orientation.

This Chapter aims at giving a brief overview of the insects' olfactory system, from perception to behaviour.

Perception

To detect volatile chemical stimuli insects typically use a pair of head appendages, the antennae. Insect antennae come in many different shapes (Fig. 1), but always display a similar structure. The third antennal segment carries most of the olfactory sensilla, which all host between two and five olfactory sensory neurons (OSNs) and additional non-neuronal cells (Keil, 1999) (Fig.2). The dendritic segment of the OSN is embedded in the cuticular apparatus of the olfactory sensilla, a barrier which needs to be
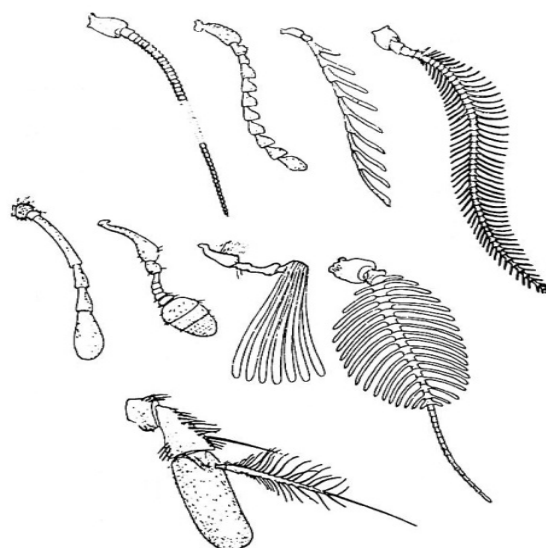
Fig. 1. A gallery of insect antennae. (After Eidmann & Kuehlhorn, 1970).
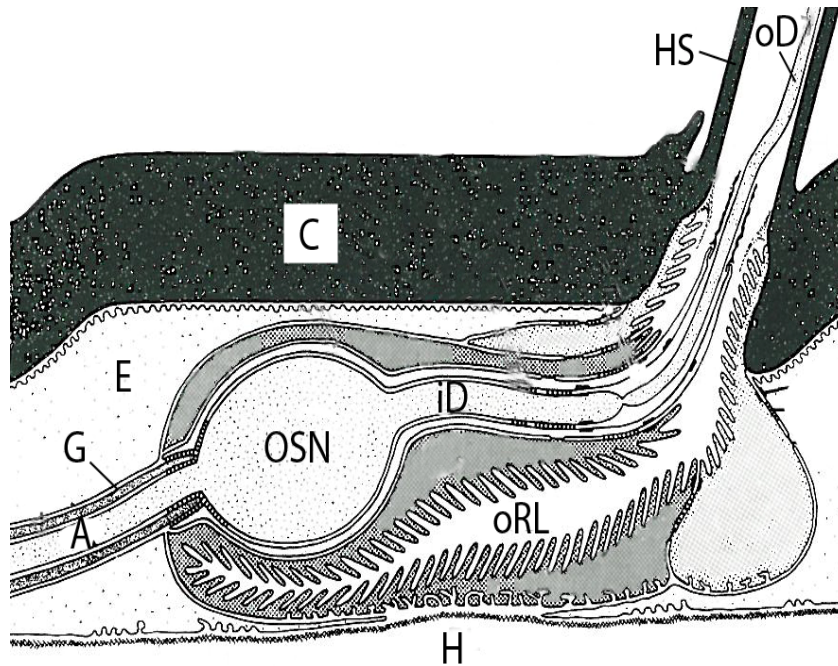
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Figure 2. Schematic drawing of an olfactory sensillum in longitudinal section. Only one sensory neuron (OSN) is shown with inner dendritic segment (iD) and the inner receptor-lymph space (iRL). The axon (A) is enveloped by a glial cell (G). The outer dendritic segment (oD) proceeds into the lumen of the hair shaft (HS) and is surrounded by the outer receptor-lymph space (oRL). C, cuticle; BL, basal lamina; E, epidermis; H, hemolymph. Non-neuronal cells are coloured in grey (After Keil & Steinbrecht, 1987).

overcome in order for the odorants to reach the nervous system. Pores present in the cuticle of the sensilla allow the volatile molecules to penetrate this barrier. Inside the pore the odorant molecules encounter the aqueous sensillum lymph, which fills the hollow sensillum hair. This lymph that surrounds the dendritic membrane represents the next barrier to be overcome by the mostly hydrophobic odorant molecules on their way to the dendrite and its ORs. Odorant-binding proteins (OBPs) and pheromone-binding proteins (PBP) are believed to transport the molecules from the inside of the sensillum wall across the lymph to the ORs (Vogt & Riddiford, 1981). Each OSN typically expresses one OR gene. In addition, a second OR-like gene is expressed in most OSNs (in *Drosophila* called Or83b (Larsson *et al.*, 2004)). The insect ORs belong either to the family of G-protein-coupled receptors (Buck & Axel, 1991) or are related to ionotropic receptors and appear as odorant-gated ion channels (Benton *et al.*, 2009). Some ORs are sharply tuned to particular compounds, whereas others are more broadly tuned and respond to multiple, structurally similar chemicals (Stensmyr *et al.*, 2003; Hallem & Carlson, 2006; Kreher *et al.*, 2008). Here at the OR level, the translation of the chemical information from the

outer world (i.e. the odorants) into an electrical signal (i.e.neuronal activity) takes place.

At the beginning of the signal transduction pathway the odorant binds to the odorant-specific part of the OR dimere. The binding of the odorant to the OR in turn activates Or83b. This activation most likely occurs via two pathways (Fig. 3). The activation of Or83b makes it permeable to ions, inducing an ion flow, i.e. an electrical signal. This electrical signal forms a slow receptor potential that travels down the dendrite to the cell body area. Here action potentials, spikes, are triggered.
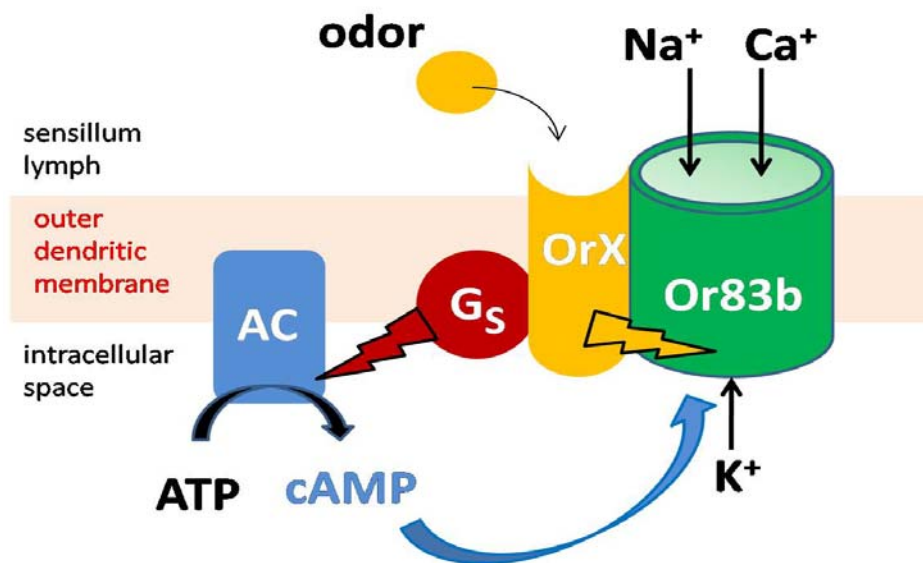

Figure 3. After binding of the odor molecule to the OR protein (OrX), a direct activation (yellow flash) causes Or83b to open and induces a fast cation flow (Na^+, Ca^{2+}, K^+) (ionotropic pathway). In a slower, metabotropic pathway the transduction cascade activates a G-protein (Gs) and an adenylyl cyclase (AC) (red flash), which in turn activates Or83b via cAMP (blue flash) (Sato *et al.*, 2008; Wicher *et al.*, 2008) (After Hansson *et al.*, 2010).

Processing

The electrical information in shape of action potentials is transmitted via the axon of the OSN and travels down the antennal nerve to the first odour-processing centre of the insect brain, the antennal lobe (AL). In most insects, the axons of antennal OSNs project to the ipsilateral AL. In flies, however, some primary afferents project bilaterally to both ALs, whereas others project exclusively to the ipsilateral AL (Strausfeld, 1976; Stocker, 1994). Unilateral afferent projections may be important for supplying and processing

laterality information (Stocker, 1994), e.g. in osmotropotaxis and stereo perception (see Manuscript II). In the ALs all axons of the OSNs expressing a given OR converge to form a spherical neuropil called glomerulus (e.g. Tolbert & Hildebrand, 1981). This glomerular unit consists of OSN axonal branches, projection neuron (PN) dendrites, and input from a network of local inhibitory interneurons (LN) (e.g. Tolbert & Hildebrand, 1981; Gao *et al.*, 2000; Vosshall *et al.*, 2000) (Fig. 3). In most systems studied so far glomeruli can be regarded as functional units in odour processing (e.g. Hildebrand & Shepherd, 1997; Hansson & Anton, 2000). The typical insect AL contains 50-100 glomeruli, while some ant species possess more than 400, which might reflect the elaborate chemical communication in the social ants (Goll, 1967; Zube & Roessler, 2008). As each glomerulus receives input from one type of OSN, the olfactory pathway is largely hardwired up to this level. However, at the next neural level the glomeruli are interconnected via LNs (Vosshall *et al.*, 2000) and PN dendrites (Strausfeld, 1976; Stocker *et al.*, 1990) (Fig. 4). This glomerular interconnectivity is responsible for the distribution of olfactory information across ensembles of principal neurons and thus for first-order processing in the AL.

Figure 4. Schematic illustration of the *Drosophila* olfactory system. The axons of neurons expressing the same type of OR converge in one glomerulus in the antennal lobe. Each colour (red, green and blue) denotes a specific type of OSN expressing a specific OR. Dashed purple lines outline glomeruli. In *Drosophila*, each excitatory PN sends dendrites into a single glomerulus (Stocker, 1994), where it receives extensive input from the innervating OSNs. The PN subsequently sends an axon to higher brain centres (purple). The inhibitory LNs (orange) in *Drosophila* interconnect multiple glomeruli. (After Bargmann, 2006; Keene & Waddell, 2007).

The possibility to record action potentials from interneurons extracellularly (Boeckh & Boeckh, 1979) and intracellularly (Matsumoto & Hildebrand, 1981), and later techniques for optical imaging of neural activity (Galizia *et al.*, 1997; Joerges *et al.*, 1997; Sachse & Galizia, 2002) have facilitated observation of information processing in the ALs. These and subsequent studies have revealed that chemical information, including odour identity, stimulus intensity, and odour blends, is coded over the glomerular array.
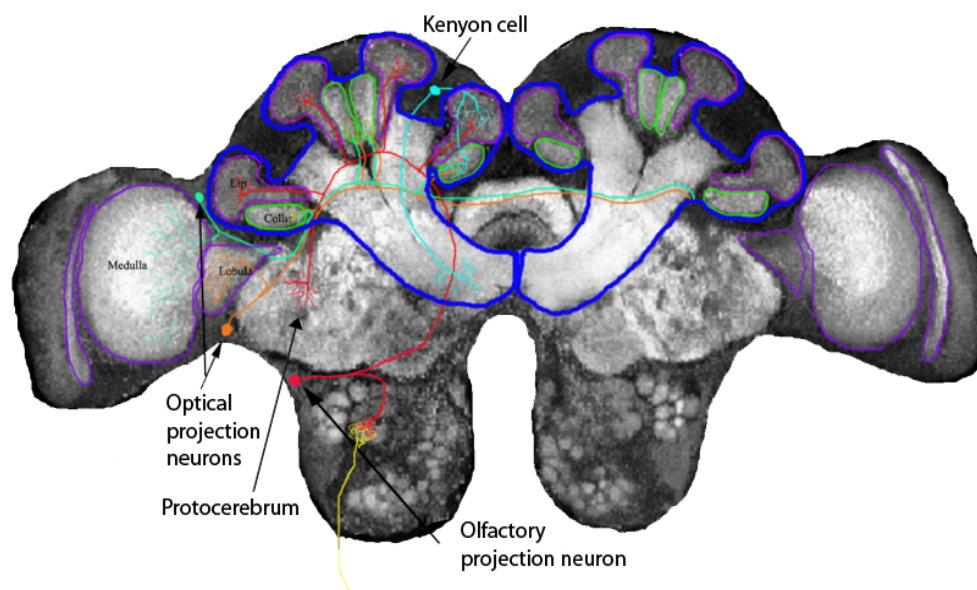

Figure 5. Whole-mount of a *Cataglyphis* brain with outlines of neuropils and neurons of interest. Mushroom bodies (blue), lip region (violet), collar region (green), optical lobes (purple). Optical projection neurons (orange or light green) and olfactory projection neuron (red). (After Seid & Wehner, 2008).

Via PNs olfactory information travels from the ALs to higher brain centres, i.e. the mushroom bodies and the lateral protocerebrum (Strausfeld, 1976) (Fig. 5) where further processing takes place. The insect mushroom bodies have been found to be involved in associative olfactory learning and memory (Menzel *et al.*, 1996; Zars *et al.*, 2000; Heisenberg, 2003). Especially *Hymenoptera* are famous for their impressive ability to learn and distinguish a multitude of chemical stimuli and these striking learning abilities are very likely reflected in the conspicuous morphology of the higher brain centres of these species. In *Hymenoptera*, especially in ants, the mushroom bodies are particularly well developed and may occupy up to 40% of the brain volume (Gronenberg & Hoelldobler, 1999).

Another characteristic of the hymenopteran mushroom bodies is the subdivision of the calyces into specific regions that receive input regarding different sensory modalities. Visual information feeds into the collar region, whereas olfactory information is projected to the lip region (Gronenberg, 1999; Gronenberg, 2001) (Fig. 5). Via the dendrites of kenyon cells these two regions are interconnect (Gronenberg, 2001; Gronenberg & Lopez-Riquelme, 2004; Strausfeld *et al.*, 2009) suggesting a close interplay of the modalities. Indeed, multimodal processing seems to be the rule rather than the exception. Enhanced crossmodal perception and learning was shown in a wide variety of *Hymenoptera* (Waeckers & Lewis, 1994; Rowe, 1999; Hebets & Papaj, 2005; Kulahci *et al.*, 2008).

Thus, odour-guided behaviour clearly does not depend only on direct olfactory input but to a large extent also on additional sensory input from other modalities and on previous experience, i.e. learning.

To allow studies of learning in insects a number of behavioural paradigms have been used. One classic and highly useful experimental paradigm for studying associative learning in harnessed honey bees takes advantage of the proboscis extension reflex (PER) (Bitterman *et al.*, 1983). Here, the reflexive extension of the bee's proboscis was conditioned with odour as the conditioned stimulus and sucrose solution as the unconditioned stimulus (Bitterman *et al.*, 1983). In my experimental paradigms I also made use of a stereotypic behaviour of *Cataglyphis fortis*; ant nest searching, in order to study associative olfactory and visual learning (Steck *et al.*, 2009a (Manuscript I)).

Behaviour

The study of insect olfaction not only aims at answering how the peripheral and the central olfactory systems work, but also how they are involved in eliciting odour-dependent behaviour, i.e. olfactory neuroethology.

Odour-guided behaviour in general implies a movement prompted by volatile chemicals in the surroundings. In kinesis an organism changes its speed or its rate of turning in reaction to a stimulus, whereas chemotaxis describes directed movements relative to an odour source (Fraenkel & Gunn, 1961). This motion could either guide the organism towards the odour source (attraction) or away from it (repulsion). In the rather rare case of still air the volatiles are distributed from an odour source by diffusion, building up a concentric concentration gradient around the source. In order to reach the location of the

highest concentration the animal simply has to follow the concentration gradient, either by comparing the input of both antennae (osmotropotaxis) (Borst & Heisenberg, 1982) or through space and over time (klinotaxis) (Fraenkel & Gunn, 1961).

However, air is hardly ever totally calm and the concentric diffusion gradient around an odour source is normally distorted into an odour plume that consists of discontinued odour filaments. Although the frequency of odour packages and the change of the concentration at the border of the odour package reveal information about the distance to the odour source (Murlis *et al.*, 1992), plume-tracking insects usually orient upwind in order to locate an odour source, i.e. they take the wind into account as the primary directional cue (anemotaxis) (Fig. 6).
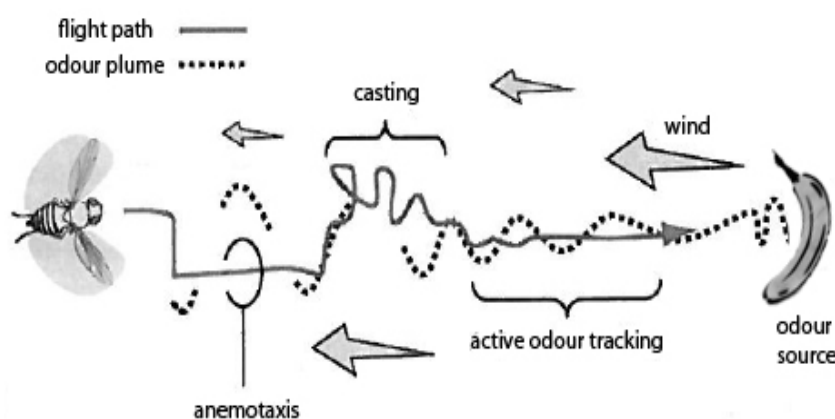

Figure 6. During flight, *Drosophila* tends to fly upwind (anemotaxis), cast in order to re-acquire a lost plume, and continue upwind flight after relocating the plume. (After Chow & Frye, 2009).

Typically, an odour package triggers the upwind flight of the insect. When contact with the odour plume is lost, the surge is followed by a behaviour referred to as "casting", in which upwind movement ceases and the animal flies to and fro in increasingly wider lateral zigzags across the wind line (Baker & Kuenen, 1982; Kuenen & Cardé, 1994; Cardé & Willis, 2008; Chow & Frye, 2009). Once the odour filament is relocated, the active odour tracking starts, where the animal tries to stay in the filament (Fig. 6). To accomplish this task, the insect benefits from paired sensory organs to measure the odour concentration simultaneously at two different points in space, as shown for *Drosophila* adults (Borst & Heisenberg, 1982) and larvae (Louis *et al.*, 2008), honey bees (Martin, 1965) and ants (Hangartner, 1967). Clearly, the majority of these studies focus on the navigational strategies that insects employ to find a distant odour source by navigating along its wind-borne plume.

Another perspective on olfactory orientation has the community involved in the investigation of animal navigation. Here, additionally to the strategy to pinpoint an odour

source itself, the source was shown be used as a landmark that guides the subject to a target positioned relative to the odour source. This kind of orientation has been discussed as one of the major tools used in pigeon homing and in the long distance migration of fish (DeBose & Nevitt, 2008). The present thesis aims at investigating this kind of olfactory orientation on a much smaller scale, taking advantage of the well-studied orientation system of the desert ant *Cataglyphis fortis*.

Cataglyphis fortis

The long-legged ants of the genus *Cataglyphis* typically inhabit steppes and deserts of the Old World (Wehner, 1983). The species used in the following studies is the Saharan desert ant *Cataglyphis fortis* FOREL 1902 (Wehner, 1983) (Fig. 7). It inhabits the hostile 'Chotts' (mostly dry, inland salt pans of the Sahel region) or coastal regions. This ant species is very tolerant of a high salt content in soil. Nests are subterraneous and have an inconspicuous entrance. As *C. fortis* feeds on other arthropods that have perished in the heat, workers tend to leave their nest during the hottest hours of the day. The ants possess a number of adaptations in order to avoid heat-induced damage. The most important adaptation is to minimise the time spent outside the nest. Therefore, *C. fortis* has remarkably long legs, enabling it to reach high locomotor speeds of up to $1ms^{-1}$ (Wehner,

Figure 7. Forager of *Cataglyphis fortis*. (Photo: M. Knaden)

1983). As prey is usually scarce, small and randomly distributed, *C. fortis* always makes single search runs and neither pheromone trails nor recruitment are used. After encountering a prey item, returning to the nest as fast as possible is absolutely essential for a forager's survival. A typical search run is illustrated in Figure 8. Search runs lead an ant up to 150 meters away from the nest and last for up to two hours, covering a total distance of more than one kilometre (Wehner, 1983). The animals return to the nest on a direct path instead of retracing the tortuous outbound journey (Fig. 8). This task is accomplished by using an egocentric system of reference known as path integration (Wehner, 1982). The animal's path integration system continually processes directional and distance information, producing a home vector. The home vector is defined as the vector pointing from the actual position of the ant to the nest entrance. The first component of the home vector, direction, is determined with the aid of a celestial compass (Wehner, 1982; Cheng *et al.*, 2006). The length of the home vector is

determined by a step integrator (Wittlinger *et al.*, 2006; Wittlinger *et al.*, 2007). However, the *C. fortis* step integrator seems to work only in combination with visual input (Sommer & Wehner, 2005). Thus, the ant's odometer apparently combines idiothetic (leg movements) and external (visual input) cues to estimate the distance covered (Steck *et al.*, 2009b).

Although this path integration system leads the ant back to the nest with relatively high accuracy, the inconspicuous nest entrance might still sometimes be missed. In such cases, where animals have completed their home vector without finding the nest entrance, the ants initiate a systematic search programme. The searching ant performs loops of increasing size in constantly changing directions, somewhat like the casting behaviour of odour-attracted, flying insects that have lost the odour plume. Search loops start and end at roughly the same
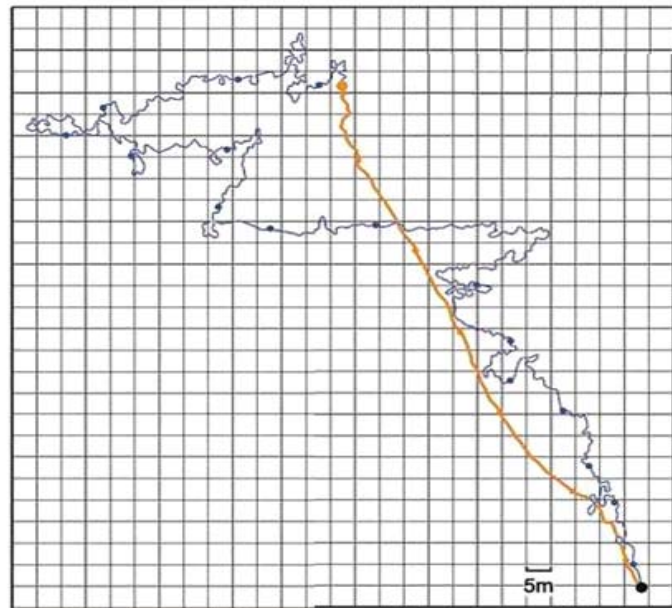

Figure 8. Outbound (blue) and homebound run (red) of an individually foraging worker of *Cataglyphis fortis*. Black circle, nest entrance; red circle, feeding site; small blue circles, time markers (every 60 sec). (After Wehner & Papi, 1992).

point, ensuring that the area where the nest entrance is supposed to be is searched most intensively (Wehner & Srinivasan, 1981; Mueller & Wehner, 1994). The accuracy of the path integrator decreases with increasing foraging distance. Simultaneously the search pattern broadens with increasing foraging distance (Merkle *et al.*, 2006). This means that the more secure an ant is concerning the position of its nest, the narrower is the search pattern displayed. The present studies take advantage of this well-studied and stereotyped search strategy of homing ants. When forced to accomplish homebound runs in an experimental channel paradigm, the animals perform one-dimensional searching movements forwards and backwards instead of two-dimensional loops. The position most often visited is where the nest entrance is expected to be. A nest search narrowly pacing around the position of interest results in a short median distance between turns and this position, whereas a broad search pattern is reflected in a long median distance. This is

comparable to studies concerning the accuracy of the path integrator with regard to the foraging distance (Merkle *et al.*, 2006). Similarly, in my experiments the narrowness of an ant's search reflects the search accuracy and thus, the ants' level of certainty as to the position of the nest (Steck et al., 2009a).

In addition to the egocentric path integration system described above, another system of orientation the ants can rely on is in action. After having travelled considerable distances, ants use visual landmarks to finally pinpoint the nest (Wehner *et al.*, 1996). Such geographic information is used in particular close to the nest entrance, that is, when they have 'reeled off' their home vector (Bisch-Knaden & Wehner, 2003; Wehner, 2003; Bregy *et al.*, 2008). This strategy ensures that ants, in spite of cumulative errors in the path integrator, find their way back to the nest. *C. fortis* lacks stereopsis and focusing mechanisms in its eyes. Therefore, information about the third dimension is gained only during motion, in the form of motion parallax. Experiments with cylindrical landmarks of different sizes and with different distances to the ants' goal revealed that size and distance are indeed confounded (Åkesson & Wehner, 2002). Hence, ants possess only a two-dimensional perception and memory of images, or photographic snapshots instead of three-dimensional representations of their surroundings.

C. fortis uses yet another strategy to compensate for navigational uncertainty. When, for example, approaching a familiar food source anemotactic and olfactory cues were shown to be employed (Wolf & Wehner, 2000). Instead of heading directly for the feeder, the ants steer slightly downwind, taking advantage of the wind that is typically blowing from a constant direction. Thus, animals can pick up odour filaments emitted by the food. They then reliably reach the food source by following this scent trail upwind (Wolf & Wehner, 2005). Thus, desert ants are able to take into account the direction of the wind as well as food odours and orientate according to them.

Hence, *Cataglyphis fortis* is a well-established model for navigation, and uses egocentric and geocentric cues for this purpose. However, the role of environmentally-derived odours so far was unknown. The main topic of the present thesis is the discovery of a sophisticated olfactory orientation system that complements the ants' navigational toolkit.

Aims and Questions

The desert ant *Cataglyphis fortis* so far was a model organism in which to study visual orientation. It is known to use path integration for large-scale navigation. In the vicinity of its home the ants in addition use visual landmarks to finally pinpoint the nest entrance. The present study now aims at investigating the olfactory navigational capabilities of *C. fortis*, and the following main questions were asked:

- Are there site-specific odours in the ants' habitat that could provide olfactory landmark information? (Manuscript I)

- Is *C. fortis* able to learn the association between an odour source and the nest entrance, i.e. do the ants use olfactory landmarks for homing? (Manuscript I)

- Can *C. fortis* locate its nest entrance relative to surrounding odour sources, i.e. use the olfactory scenery for homing? (Manuscript II)

- Do these ants perceive the complex olfactory surrounding employing a stereo sense of smell? (Manuscript II)

- How does *C. fortis* learn and memorize a bimodal nest-defining cue, i.e. a combined visual and olfactory landmark? (Manuscript III)

Overview of Manuscripts

Manuscript I

Smells like home:

Desert ants, *Cataglyphis fortis*, use olfactory landmarks to pinpoint the nest

Kathrin Steck, Bill S. Hansson & Markus Knaden

Frontiers in Zoology, 2009, 6:5

The results of the first manuscript suggest that the ants' habitat provides potential olfactory landmark information. Furthermore, we were able to show that *C. fortis* is able to learn the association between environmentally-derived odours and the nest entrance, and use this olfactory landmark information for homing.

K. Steck, M. Knaden and B. Hansson designed the experiment. K. Steck conducted the experiments and wrote the manuscript. M. Knaden and B. Hansson edited the manuscript.

Manuscript II

Do desert ants smell the scenery in stereo?

Kathrin Steck, Markus Knaden, and Bill S. Hansson

Animal Behaviour 2010, 79: 939-945.

The second manuscript describes the orientation behaviour of *C. fortis* in complex olfactory surroundings. I artificially created an olfactory landscape with place-specific blends, where the ants had to pinpoint the nest entrance relative to surrounding odour sources. The results suggest that ants achieve this by employing a stereo sense of smell.

K. Steck, M. Knaden and B. Hansson designed the experiment. K. Steck conducted the experiments and wrote the manuscript. M. Knaden and B. Hansson edited the manuscript.

Manuscript III

Memory goes crossmodal: visual and olfactory navigation in ants

Kathrin Steck, Bill S. Hansson and Markus Knaden
submitted in January 2010

In the third manuscript I compare visual and olfactory landmarks and investigate how unimodal and bimodal stimuli are learned and memorised. I found accelerated acquisition of a bimodal nest-defining cue compared to unimodal cues. Additionally, extended training with the bimodal landmark caused unimodal cues to no longer be recognised, suggesting fusion of the two unimodal cues to a bimodal unity.

K. Steck, M. Knaden and B. Hansson designed the experiment. K. Steck conducted the experiments and wrote the manuscript. M. Knaden and B. Hansson edited the manuscript.

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Smells like home: Desert ants, *Cataglyphis fortis*, use olfactory landmarks to pinpoint the nest

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Abstract

Background: *Cataglyphis fortis* ants forage individually for dead arthropods in the inhospitable salt-pans of Tunisia. Locating the inconspicuous nest after a foraging run of more than 100 meters demands a remarkable orientation capability. As a result of high temperatures and the unpredictable distribution of food, *Cataglyphis* ants do not lay pheromone trails. Instead, path integration is the fundamental system of long-distance navigation. This system constantly informs a foraging ant about its position relative to the nest. In addition, the ants rely on visual landmarks as geocentric navigational cues to finally pinpoint the nest entrance.

Results: Apart from the visual cues within the ants' habitat, we found potential olfactory landmark information with different odour blends coupled to various ground structures. Here we show that *Cataglyphis* ants can use olfactory information in order to locate their nest entrance. Ants were trained to associate their nest entrance with a single odour. In a test situation, they focused their nest search on the position of the training odour but not on the positions of non-training odours. When trained to a single odour, the ants were able to recognise this odour within a mixture of four odours.

Conclusion: The uniform salt-pans become less homogenous if one takes olfactory landmarks into account. As *Cataglyphis* ants associate environmental odours with the nest entrance they can be said to use olfactory landmarks in the vicinity of the nest for homing.

Background

As a result of its amazing navigational capabilities, the desert ant *Cataglyphis fortis* has become a model organism for studying orientation [1-4]. In search of food, individual ants depart on tortuous routes often leading them more than 100 m from the nest. Once they find a food item, the ants return directly to the inconspicuous nest entrance. The ability to navigate so precisely has so far been thought to result from two synergistic visual systems. For long-distance navigation, the ants perform path-integration, getting the direction of movement by a skylight compass [1,2] and the distance by a step integrator [5]. Owing to the egocentric nature of this kind of orientation, errors may accumulate during the forage run. Therefore, as soon as the path integrator has guided the ants to the vicinity of the nest, they shift their attention to visual landmarks in the immediate surroundings of the nest [3,4]. Hence, the large-eyed *Cataglyphis* has been deemed a typical vision-guided insect. The role of olfaction has so far been considered to be restricted mainly to nest mate recognition [6,7] and to the localization of food [8]. Food is usually distributed randomly as the ants forage for dead

arthropods [9]. Furthermore, route pheromones used by the ants would be rather short-lived given the hot ground – up to 60°C – of the salt-pan. Both the unpredictable food distribution and the high surface temperature might account for the fact that mass recruitment and orientation along odour trails have never been observed in members of the genus *Cataglyphis* [10]. Here we found location-specific blends in the salt-pans that differed in their composition of single-odour components. As the varying components showed electroantennogram (EAG) activity in *Cataglyphis*, they represent potential olfactory landmarks that the ants could use as cues for fine-scale navigation.

Furthermore, we show that *Cataglyphis* ants use environmental olfactory information for homing. We designed an experimental paradigm in which the ants associated a visually inconspicuous nest entrance with a given monomolecular odour. Not only did the ants learn to associate the nest with the training odour, but they were also able to distinguish this odour from a set of non-training odours. Hence, *Cataglyphis* ants are able to learn odours in the vicinity of the nest entrance and use these odours to pinpoint the nest during homing.

Results
Potential olfactory landmarks are present in the salt-pan habitat
Despite its homogenous appearance, the flat ground within the salt-pan habitat differs slightly in its soil structure. Covered by a continuous salt crust, the surface is occasionally interrupted by clefts or by pieces of wood and halophytic plants, signs of past periods of flooding (Figure 1A). In order to check whether these structures result in different habitat odours, we used gas chromatography to analyse headspace samples of continuous salt crust, cleft salt crust, wood and halophytic plants. The emitted volatiles for each sample were relatively constant over two consecutive days, whereas the chromatograms differed among the samples (Figure 1B). We identified five components (Figure 1C) that are known as common plant volatiles http://www.Pherobase.com and tested them for EAG activity (Figure 1C). All components generated antennal responses. In summary, the microhabitat blends were stable over time, differed between samples, and could be detected by the ants. Hence, they present potential olfactory landmarks.

Ants learn to associate the nest entrance with environmental odours in their surroundings
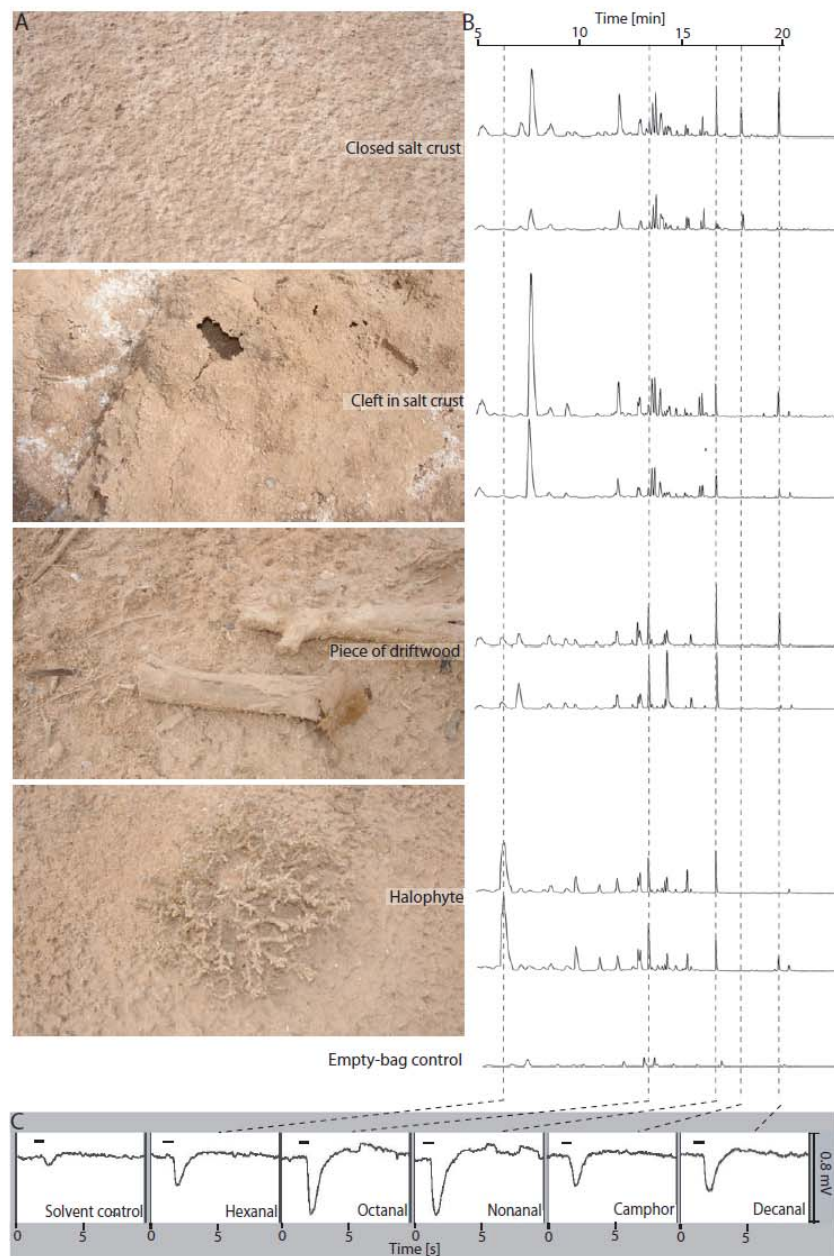Having shown that the habitat offers potential odour-landmark information, we checked whether the ants were able to use such information for navigation. Ants were trained to forage within an open linear channel to a feeder 8 m downwind from the nest entrance (Figure 2). At the

inconspicuous exit hole leading from the channel to the nest, we applied one of four mono-molecular odours (nonanal, decanal, methyl salicylate or indole) to the channel floor. Two of these odours were also present in the samples from the habitat (Figure 1), and all four showed electroantennographic activity but did not trigger any innate attraction in naïve ants (Additional file 1). Would the ants learn to associate the nest entrance with the given odour? Would they distinguish the training odour from other non-training odours? And would they be able to recognise the training odour against a background of an odour mixture? We captured homing ants at the nest entrance and released them in a test channel that was identical to the training channel in its dimensions and orientation but lacked a nest entrance. The release point was 1 m downwind of an odour stimulus that either
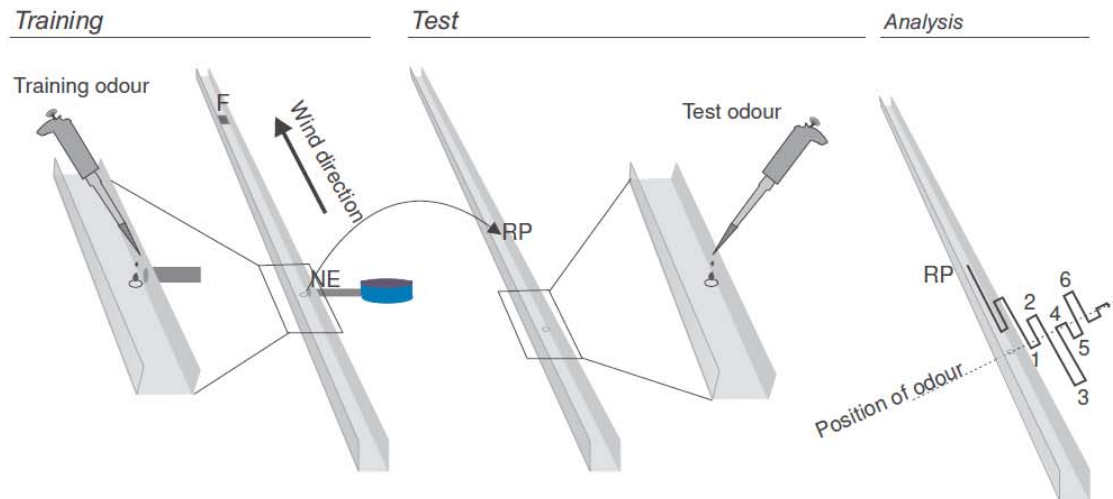
i) was identical to the training odour,

ii) consisted of another odour,

iii) consisted of a mixture of four odours including the training odour,

iv) consisted of a drop of the solvent hexane (solvent control).

In order to check whether the ants focused their search on the position of the applied odour, we recorded the turning points of the nest-searching ants in the test channel. We analysed six turning points after the ant had passed the odour for the first time for the median distance to the stimulus. The values of the differentially treated groups were tested for significant differences by Kruskal-Wallis analysis and a Dunn's post hoc test. When tested with the trained odour, the ants' search was well directed (Figure 3). However, when they were tested with non-trained odours the ants' search did not differ from that displayed when tested with the solvent control (Figure 3). Hence, the ants were able to associate each of the four odours with the nest entrance and were able to distinguish the learned odour from the non-training odours.

Furthermore, when ants were trained to indole and tested with a mixture of all four odours, their search accuracy again decreased. However, in this case they were still better-directed than they were when tested with the solvent control (Figure 4). Thus, the ants were less sure about the position of the nest when the trained odour was provided in a blend during the test. However, they were still able to recognise the learned odour against the background of three additional odours.

Figure 1

Olfactory landmarks in the *Cataglyphis* habitat. A. Sample locations. B. Location-specific gas chromatographic profiles collected on consecutive days are displayed next to the corresponding photo. Dashed lines depict identified components that were used for EAG recordings. C. EAG responses of *Cataglyphis* to the identified components. Horizontal bars indicate the stimulus duration.

Figure 2
Experimental paradigm. *Training*. Nest situated within blue border strip; channel width and height, 7 cm, length, 16 m; position of feeder (F), 8 m downwind from nest entrance (NE); Training odour, 20 µl of either indole, nonanal, decanal, methyl salicylate (each diluted 1:50 in hexane), or hexane as solvent control. Odours were reapplied every 20 min. *Test*. Capture site of ants at NE; point of release at RP; position of odour in test channel 1 m upwind of RP; ants were tested with training odour, non-training odour, or solvent control. *Analysis*. Schematic search run. Six turning points after the ant had passed the odour for the first time were analysed for their median distance to the stimulus.

Discussion

Desert ants, *Cataglyphis*, are known to rely on path integration [1,2,5] and visual landmarks [3,4] during homing. In the present study, we ask whether ants associate their nest entrance with environmental odours. By collecting and analysing volatiles from different positions within the salt-pan habitat of *C. fortis*, we show that the environment provides the ants with potential odour-landmark information, i.e. with different place-specific blends (Figure 1). The blend components that were identified induced EAG responses in these ants. Can the ants make use of such information? Yes, they can. Homing ants that were trained to the nest marked by an odour focused their nest search on this odour in a test situation, but they did not search at the solvent control (Figure 3, [11]). Hence, *Cataglyphis* ants can learn the association between the nest entrance and an environmental odour and use this information for homing.

The salt-pan habitat is not an odour-free space, interrupted by single odour peaks but, rather, is loaded with a variety of odours (Figure 1). Hence, when using olfactory information as landmarks, ants must be able to distinguish a learned odour from other odours. As the ability to discriminate among odours relates to the dissimilarity among molecules, bees frequently confuse odours that share functional groups or have similar chain lengths [12]. The most similar among the four odours tested in the present account were the aldehydes nonanal and decanal; they have the same functional group and chain lengths that differ by only one carbon atom, whereas methyl salicylate and indole have different chemical structures (Figure 3A). Even so, the ants were able to discriminate among all of them (Figure 3). Ants that were tested with a non-trained odour did not avoid this odour during the nest search as naïve ants seemed to do (Additional file 1). The training on one odour resulted in an odour-specific response by homing ants, which is one prerequisite for odour-landmark navigation.

The use of odour landmarks requires a further skill: the olfactory background of a stimulus might change dramatically when for example the wind direction changes and an odour source suddenly appears upwind of the landmark. In order to use an olfactory landmark, an ant must be able to identify the learned odour against a changing background of odours. *Cataglyphis* ants fulfilled this demand when trained on indole and tested with a blend of four odours including indole. Ants that were tested with the blend showed a less focused search than ants that
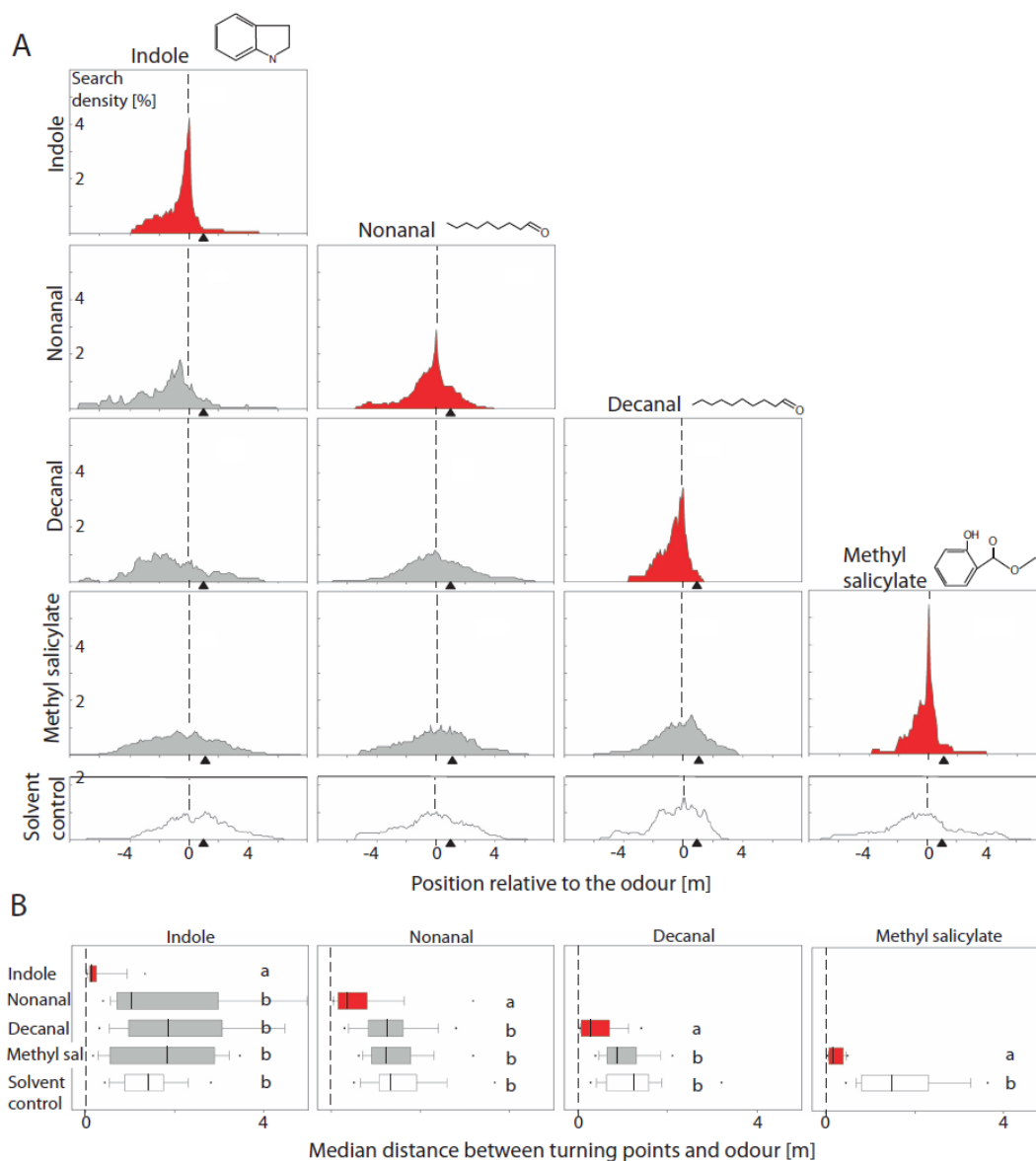
Figure 3
Discrimination among odours. A. Relative search densities of ants tested with the training odour (red plots), non-training odours (grey plots), or with the solvent as a control (white plots). Diagram columns, training odours; diagram rows, test odours; dashed line, position of odour; black arrow heads, point of release; sample size, 20 ants per plot. Search plots include the first six turning points after the ants had passed the odour for the first time. For details of graph construction see [11]. B. Median distances between the turning points and the position of odour (dashed line). Box plot: black line, median; box, inter-quartile range; whiskers, 10th and 90th percentiles, black dots, outliers. Within each plot, diagram letters indicate significant differences (p < 0.01, Dunn's post hoc test) between the groups.
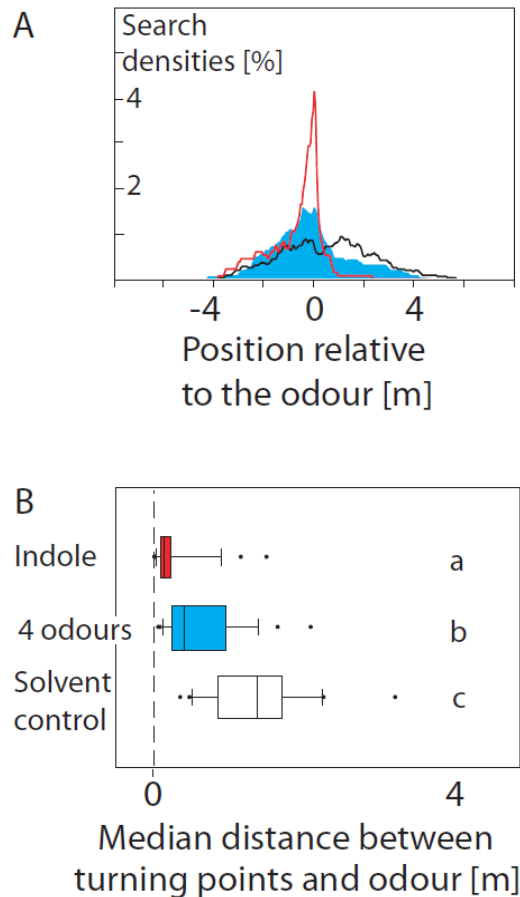
Figure 4
Recognition of a learned odour in a blend. A. Relative search densities of ants that were trained with indole and tested either with indole (red line), with the solvent (black line), or with a blend of indole, nonanal, decanal, and methyl salicylate (blue area). For details see Figure 3. B. Median distances between the turning points and the position of odour (dashed line). For details see Figure 3. Diagram letters indicate significant differences (p < 0.01, Dunn's post hoc test) between the groups.

were tested with the trained odour only, but still a more focused search than ant tested with the solvent control (Figure 4).

Hence, the ants can use olfactory information for homing. Trail following is the predominant means of orientation in a large number of ant species [13]. These trails always consist of ant-derived trail pheromones. Unlike orientation that is guided by pheromones, orientation that uses non-pheromonal chemical cues is less well investigated. Carpenter ants can be trained to search for food on specific species of trees. Trained ants decide for the right tree species even when tactile cues are experimentally excluded. Therefore, the ants seem to use tree-derived chemical cues [14]. Bees have been shown to associate different odours with different feeding places. Blowing a learned odour into the hive triggered the trained bees to visit the corresponding feeder, i.e. navigational memory can be evoked just by providing the learned odour [15]. Finally, it has been shown that around cities, spatial gradients of atmospheric volatiles exist [16] and seem to be used by pigeons to pinpoint their loft [17]. Unlike bees and pigeons, the desert ant *Cataglyphis* has so far been a model only for visually guided orientation.

We are amazed to discover that while keeping track of the path integrator and learning visual landmarks, these ants can also collect information about the olfactory world. In future experiments, we hope to clarify how visual and olfactory landmarks interact to provide accurate information regarding, for example, nest location.

Conclusion
The desert ant *Cataglyphis fortis* has been deemed a typical vision guided insect. Here we show that the ants' habitat exhibits location-specific blends that are stable over time and can be detected by the ants. Therefore, the environment provides potential olfactory landmarks. We also show that the ants can associate a specific place (the nest entrance) with an odour, can distinguish between learned and non-learned odours, and, finally, are able to recognize a learned odour in front of a complex blend. Hence, they can make use of the olfactory information offered by the environment to pinpoint their nest entrance.

Methods
Location-specific scents in the salt-pan habitat
We collected ground structures from the ants' habitat in a salt-pan close to Menzel Chaker, Tunisia, in order to analyse their odour composition. Samples of dead wood, plants, clefts and closed salt crust were brought to the laboratory within odourless oven bags. The air within each of the oven bags was exchanged for 3 litres of purified air. An empty bag served as control. The samples were put in an oven and kept at 45°C for 3 hours. The air within the bags was pumped over a thermal desorption filter containing Carbotrap B and Tenax [18]. The next day we again collected odours from the same samples under identical conditions. Headspace samples were analysed on an Agilent Technologies 7890A GC-MS system fitted with a GERSTEL Thermal Desorption Unit and equipped with an HP5ms column (30 m × 250 μm × 0.25 μm). Helium (1 ml/min

constant flow) was used as a carrier gas. Samples were heated to 40°C for 5 min. Temperature was then increased at a rate of 5°/min to 200°C. After another 20 min, the temperature was further increased at a rate of 30°/min to 280° for 10 min. Dominant peaks were identified using the NIST 2005 library and were confirmed by the injection of synthetic reference compounds.

EAG activity of identified components
We recorded EAG responses for 7 different components (5 identified habitat odours: hexanal, octanal, nonanal, camphor, decanal (Figure 1C); 2 non-habitat odours: indole, methyl salicylate [data not shown]). The basal end of the isolated antenna was inserted into a glass microelectrode filled with standard insect saline solution. The tip of the antenna was cut off and inserted into the reference electrode filled with the same saline solution. The antenna was placed in a humidified air stream (0.3 l/min, blown through a glass tube with 8 mm diameter with its end positioned about 1 cm from the antenna). We inserted a Pasteur pipette cartridge into a small hole in the tube, 10 cm from the outlet. The cartridge contained 2 µl of diluted odour (1:100 in hexane) dropped on a piece of filter paper. To deliver the odour stimulus, we puffed purified air (0.2 s at 0.1 l/min) through the odour cartridge, using a stimulus flow controller (SFC-2, Syntech®, The Netherlands). EAG signals from the antenna were amplified with a head-stage preamplifier (EAGPro, Syntech®, The Netherlands) and further processed with a PC-based signal processing system (EAGPro, Syntech®, The Netherlands).

The ants' ability to use olfactory landmarks
Ants were trained in an aluminium channel open at its top to a feeder 8 m downwind of the nest entrance (Figure 2). Each ant arriving at the feeder was individually marked by a two-colour code. At the nest entrance, we dropped 20 µl of a diluted mono-molecular odour (1:50 in hexane). The odour was reapplied every 20 min to ensure an olfactory cue at any time. Training experiments were performed with nonanal, decanal, methyl salicylate and indole. In addition to nonanal and decanal that were present in the ants' habitat, we used methyl salycilate and indole because they are also common plant volatiles with high boiling points, i.e. are easy to handle under hot desert conditions. These odours elicited electroantennographic activity in *Cataglyphis fortis* (nonanal and decanal, Figure 1C, data for methyl salicylate and indole not shown) but did not generate any innate attraction in naïve ants (Additional file 1). Returning ants (with an experience of at least 15 forage runs) were captured at the nest entrance and transferred, together with a food item to ensure homing motivation, to a remote parallel test channel. The release point was situated 1 m downwind of the odour stimulus. The ants were tested either with the training odour, with one of the non-training odours, or with a blend of four odours containing the training odour. In the solvent control, the ants that were trained to one of the four odours were tested with a pure solvent stimulus. We tested 20 ants in each test situation and every animal was tested only once. As a measure of the ants' search accuracy, we used the median distance of the first six turning points from the position of the odour after the ants had passed the odour for the first time (Figure 2C).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KS and MK designed and carried out the experiments and performed statistical analysis. All authors were involved in interpreting the results and writing the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Response of naïve ants to the study odours. The data provided give evidence that the odours used in this study are not innately attractive to Cataglyphis.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-9994-6-5-S1.doc]

Acknowledgements

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Supplemental Data (Manuscript I)

Response of naïve ants to the study odours.

Additional Fig 1 – Response of naïve ants to the study odours. A. Indole, B. Nonanal, C. Decanal, D. Methyl salicylate, E. Blend of the four components. Dashed line, position of odour; black arrowheads, point of release; sample size, 20 ants per plot. Search plots include the first 6 turning points after the ants had passed the odour for the first time.

Manuscript II

Do desert ants smell the scenery in stereo?

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Desert ants, *Cataglyphis fortis*, navigate individually in the inhospitable saltpans of Tunisia using path integration for long-distance navigation, and visual and olfactory landmarks for fine-scale orientation in the vicinity of the nest entrance. Here, we show in a field experiment that the ants are able to locate the nest entrance within a two-dimensional olfactory array. Ants were trained to forage in an open channel and to memorize the nest entrance relative to four odours that were applied at the corners of an invisible quadratic array. In a test situation, the ants pinpointed the fictive nest only when the odours were present at their learned positions. Our results suggest that the ants had learned the olfactory scenery around their nest. Furthermore, unilaterally antennectomized ants could not pinpoint the nest within a two-dimensional array. Hence, this kind of orientation depends on the simultaneous input of both antennae, that is, on a stereo sense of smell. Until now, insects and mammals, including humans, have only been known to use bilateral sensory input to follow a concentration gradient of an odour. Our evidence suggests that desert ants require a stereo sense of smell to make use of the olfactory scenery around their nest for homing.

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Animals use olfactory information for navigational means over a range of spatial scales. Seabirds pinpoint food-rich habitats over hundreds of kilometres based on plankton-derived olfactory cues (Nevitt et al. 1995), while pigeons, *Columba livia*, seem to use the olfactory landscape for homing (Wallraff 2004). Behavioural studies on insect olfaction have mainly focused on odour-evoked upwind flights to reach food or a potential mating partner (Murlis et al. 1992). In this study we investigated how the desert ant *Cataglyphis fortis* uses complex olfactory surroundings for homing. Because of its sophisticated visual navigational capabilities, *C. fortis* has become a model organism for studying orientation (Wehner 2003). In search of food, individual ants depart on tortuous routes that can take them more than 100 m from the nest. After finding a food item, an ant returns directly to the inconspicuous nest entrance. This precise navigational feat is accomplished with help from two synergistic systems, namely path integration for long-distance navigation (Wehner 2003) and landmark orientation for navigating in the vicinity of the nest (Wehner & Raeber 1979). To accomplish the latter, foragers leaving the nest are known to take a snapshot of their visual surroundings. Returning from the foraging trip, they pinpoint the nest by matching the actual image with the stored snapshot (Akesson & Wehner 2002). Recently, we found that the habitat also provides the ants with potential olfactory landmarks and that *Cataglyphis* is able to make use of such information for homing by associating environmentally derived odours with the nest entrance (Steck et al. 2009). In the present study, we asked whether the ants not only learn to associate the nest with a single odour but even remember the topography of odour sources in the vicinity of the nest. We designed an experimental paradigm according to which the ants had to memorize the position of the nest entrance within an array of four spatially separated odour sources. We tested whether the presence of all odours and their correct positions within the array were mandatory for precise homing and, finally, whether accurate orientation within the two-dimensional array required input from both antennae or only one. To sum up our questions: do the ants learn the olfactory surroundings of the nest and do they 'view' this olfactory scenery using a stereo sense of smell?

METHODS

In a field experiment in a Tunisian saltpan, we trained ants to forage within an open channel. The visually inconspicuous nest entrance was a 1 cm hole in the channel wall, situated within a complex olfactory array. Four odours were placed on the channel ground at the corners of a fictive square (side length: 7 cm). Methyl salicylate (M) and decanal (D) were placed at the channel wall adjacent to the nest entrance, with M upwind and D

downwind of the nest; nonanal (N) and indole (I) were placed opposite the nest entrance, with N upwind and I downwind of the nest (Fig. 1). We used 0.4 µl of each compound diluted in 20 µl hexane. These compounds are neither innately attractive nor repellent to naïve ants, can be learned equally well, and can be distinguished (Steck et al. 2009). As the ants do not antennate the ground while walking (see Supplementary Material), odours are perceived as volatiles via olfaction rather than via taste. Odours were reapplied every 20 min. A feeder, providing the ants with biscuit crumbs, was placed 8 m downwind from the nest entrance (Fig. 1).

To see what information this odour array provided the ants, we used a photoionization detector (Aurora Scientific Inc., Ontario, Canada, Model 200A) to visualize the distribution of a tracer volatile in the channel with wind blowing in a nest-to-feeder direction. The photoionization detector probes air and exposes the vapour sample to high-intensity ultraviolet light that ionizes the nonair chemical substances. Ions are then collected on positive and negative electrodes, creating a current proportional to the contaminant concentration. Under field conditions (wind blowing in the nest-to-feeder direction), we applied a tracer gas at a position in the channel corresponding to the position of one of the odours of the array and probed the air in the channel at each cross of a fictive 1 cm grid at a height corresponding to that of the antennae of walking ants. The concentration directly above the applied odour was set as 100% and the rest of the data were normalized. Owing to the symmetric application of the odour array, we could then extrapolate the distribution for all four odours. This technique does not provide any information about the absolute concentration of odours but describes how the relative concentrations change over several centimetres in the channel (Fig. 2a). The visualization of the volatiles revealed place-specific

blends. The composition and the concentrations of the volatiles changed over a distance as short as the span of an ant's antennae (Fig. 2b). Thus, when homing ants approached their nest, they came across a 'landscape' of position-specific blends of odours. Note that the nest entrance does not converge with the highest odour concentration.

Would the ants be able to learn the position of the nest entrance relative to the surrounding odour sources and make use of this olfactory scenery to locate the nest entrance? To find out, we captured, at the nest entrance, homing ants that had accomplished at least 15 training runs and released them individually to a remote test channel 1 m downwind of an olfactory array (Fig. 1) that was either identical to the training array or had the positions of the odour sources exchanged. We expected the latter test to show whether the ants had memorized the positions of the four odour sources or had simply learned that they first had to pass the blend of the downwind odours D and I and then search directly downwind of M and N. To see whether ants need both antennae to accomplish the task, we trained and tested unilaterally antennectomized ants either with the olfactory array or with a point odour source (a mixture of all four odours placed directly at the nest entrance). To assess the impact of unilateral antennectomy on search accuracy, we trained and tested another group of ants with intact antennae with the point odour source. To see which odours of the training array were necessary and sufficient for generating a focused nest search, the test array was reduced to two odours, located either adjacent to the nest entrance or opposite it. For further details see Figs 4 and 5 in the Results. Finally, we tested whether the concentration of odours affected the ants' search accuracy (see Appendix for Methods and Results). The nest searches of 30 homing ants in each of the different test arrays were analysed and each ant was tested only once. Channel
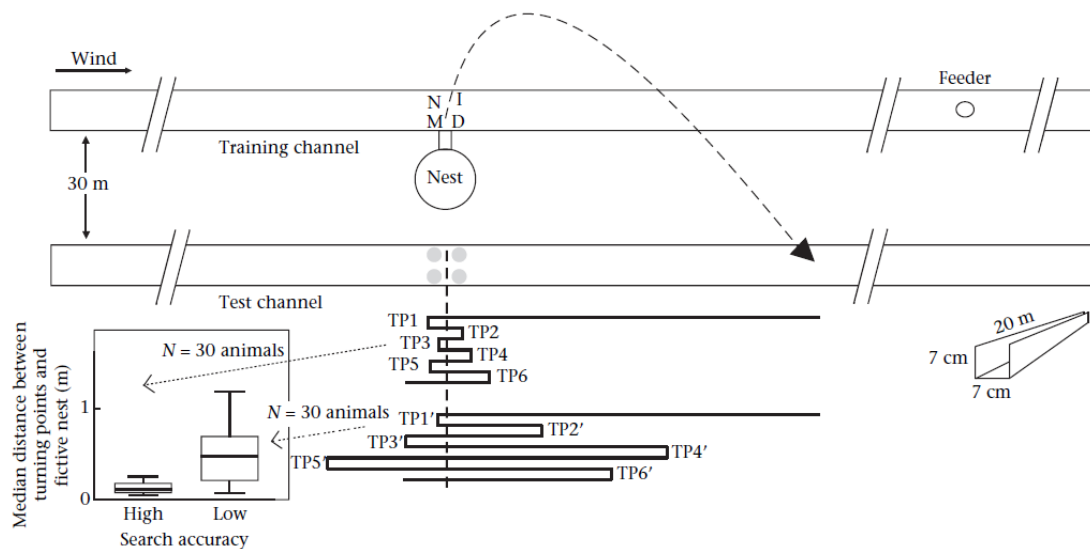

Figure 1. Experimental paradigm. The training channel consisted of 10 identical 2 m modules. The nest was connected to the training channel via a tube. The training array consisted of 0.4 µl of decanal (D), methyl salicylate (M), indole (I) and nonanal (N), each diluted 1:50 in hexane. A feeder was placed 8 m downwind from the nest entrance. The dashed arrow indicates transfer of individually marked homing ants (that had accomplished at least 15 foraging runs) from the nest entrance to a remote test channel. Odours were placed in the test array (grey circles) in the test channel 1 m upwind of the point of release. The various test arrays used are shown in Figs 4 and 5. Inset figure depicts the channel dimensions. Two schematic search runs (grey lines) are shown, one of a narrow and one of a broad nest search. For clarity, the runs are projected outside of the channel. Six turning points (TP1–TP6) after the ant had passed the test array for the first time were analysed for their median distance to the fictive nest (dashed line). Short median distances result in high search accuracy and long median distances in low search accuracy. The example box-and-whisker plots show the median, interquartile range and 10th and 90th percentiles.
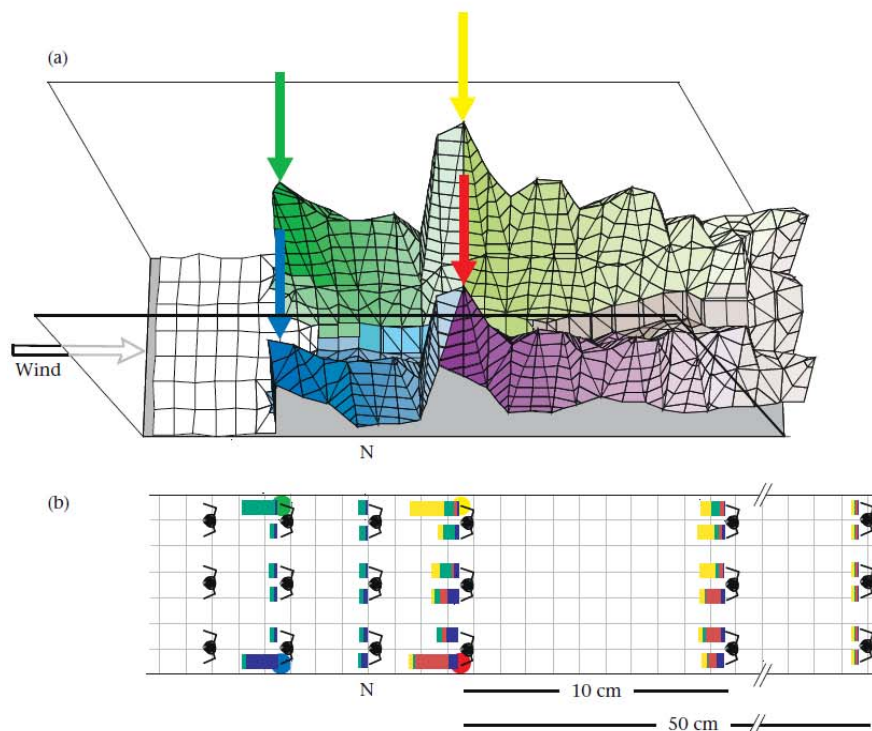
Figure 2. Odour distribution within the training channel. (a) Visualization of the odour distribution by the photoionization detector. Concentration of a tracer volatile was measured on each cross of a fictive 1 cm grid on the channel ground. Concentration measured directly above the odour source was set as 100% and the rest of the data were normalized. Data were extrapolated for each odour source of the array. Green: nonanal; blue: methyl salicylate; red: decanal; yellow: indole; N: position of nest entrance. Coloured arrows point to the position of the odour sources on the channel ground. (b) Relative concentrations of the four volatiles as detected by each antenna of ants at different positions within the channel. The size of the bars over the antennae depicts normalized odour concentration, from 100% (directly at odour source) to <5% (e.g. 50 cm downwind from odour source). Odours are colour coded as in (a). Circles depict the positions of odour sources.

modules with the test array were never used again either as no-odour modules or as other test array modules.

Experiments with visual landmarks have demonstrated that the search broadens when homing ants face a new landmark panorama and can no longer match the stored and the actual snapshots (Wehner & Raeber 1979). Hence, the breadth of the search reflects the ants' accuracy while pinpointing the nest. Therefore, we used as a measure of search accuracy the median distance between the first six turning points and the nest location in the array relative to the odours (called 'fictive nest', Figs 1, 3). We used a measuring tape placed alongside the test channel to track and record the positions of the ants' turning points. If the training and the test arrays appeared identical to the ants, a narrow search would have been likely, whereas any detected differences between training and test arrays, that is, any changes in the olfactory scenery, would have caused the ants' search pattern to become less precise and they would have broadened their search (Fig. 1).

RESULTS

Ants that were tested with the same array that was used in the training situation displayed a narrow search (Fig. 3) with short distances between turning points and the fictive nest (Fig. 4). When the odour pairs adjacent and opposite the nest were exchanged, the ants broadened the search (Fig. 3) and search accuracy significantly

decreased (Fig. 4); thus memorization of the blend sequences appears unlikely to have been the only characteristic guiding the ants home. Rather, the ants had also memorized an odour's location, that is, whether it was left or right during homing.

Compared to intact ants, unilaterally antennectomized ants displayed a significantly broader nest search (Fig. 3) and travelled five times longer between turning points and the fictive nest (Fig. 4). When ants were trained to associate the nest entrance with a point source landmark, antennectomized animals headed for the nest as accurately as the intact control ants (Fig. 4). Thus, unilateral antennectomy did not affect the ants' ability to orient by olfactory cues in general, but impeded the orientation within an olfactory array.

When the test array was reduced to two odours, either adjacent or opposite the nest entrance, the ants still searched for the fictive nest in the correct position but were less focused (Fig. 5). The downwind odours I and D alone were not accepted as a nest-defining cue and search accuracy was low (Fig. 5). When tested with only the two upwind odours M and N, ants showed the same search accuracy as they showed when tested with the full array control (Fig. 5). Therefore, the actual odour composition at the nest entrance seemed to be necessary and sufficient for pinpointing the entrance, while the presence of those odours perceived on the way to the nest was not crucial. However, when the downwind odours were present, they had to be at the correct position (Fig. 5). Thus,
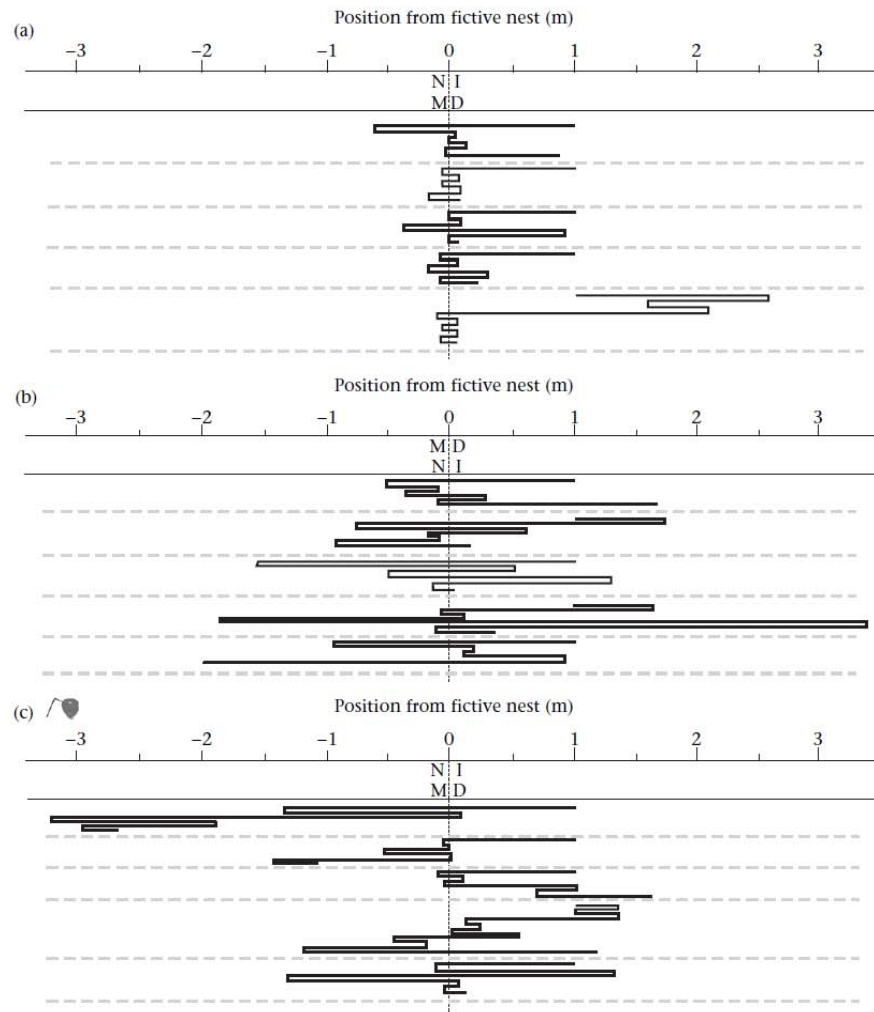
Figure 3. Example runs of intact ants tested with (a) the training array and (b) an exchanged array (with the positions of the odour sources exchanged) and (c) of unilaterally antennectomized ants tested with the training array. Compare with Fig. 4. Position of odour array, i.e. fictive nest: 0 m; point of release: 1 m. The first six turning points after the ant passed the nest for the first time were taken for analysis. For clarity, the runs are projected outside the channel on two dimensions. Horizontal dashed lines separate example runs.

ants had not simply learned mixtures of odours but, instead, had memorized the exact positions of the odour sources and the nest entrance relative to them.

Furthermore, the total amount of volatiles, that is, the amount of olfactory input, did not alter the ants' homing performance (Appendix Fig. A1).

DISCUSSION

In addition to their vision-guided orientation, *Cataglyphis* ants can learn to associate environmental odours with the nest entrance and use this knowledge to pinpoint the nest (Steck et al. 2009). The ability to pinpoint an odour source has been described for a wide range of insect species such as bark beetles, fruit flies, moths and mosquitoes (Murlis et al. 1992, and references therein). In these experiments, the animals simply had to follow a scent plume to find

the odour. Furthermore, when honeybees, *Apis mellifera*, were trained to associate an odour with a food source, blowing that odour into the hive triggered these bees to carry out foraging flights in the direction of the food source (Reinhard et al. 2004). The olfactory navigation hypothesis suggests that birds learn an olfactory landscape by associating smells perceived at the home with the directions from which they are carried by winds (Papi et al. 1971; Wallraff 2004 and references therein; for seabirds see De Bose & Nevitt 2008 and references therein). Apparently, wind is the crosslink embedding atmospheric blends into a directional reference scale provided by the birds' compass. A plausible explanation for how pigeons can home after long-distance displacement is given by a simulated navigation based on the olfactory landscape (Wallraff 2004). Stable olfactory landscapes that could provide sufficient navigational information have been shown for mid-scale ranges of about 300 km in diameter (Wallraff & Andreae 2000) and
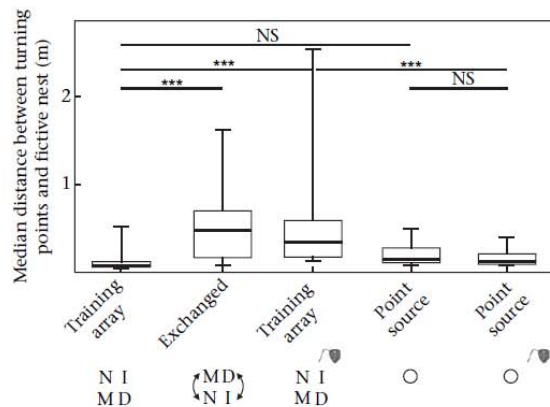
Figure 4. Median distances between turning points and the fictive nest of intact ants tested with the training array or an exchanged array (with the positions of the odour sources exchanged), unilaterally antennectomized ants trained and tested with the training array, intact ants trained and tested with a point odour source and unilaterally antennectomized ants trained and tested with a point odour source. Circle depicts point source landmark consisting of a mixture of all four odours. $N = 30$ ants per plot. Black line: median; box: interquartile range; whiskers: 10th and 90th percentiles. Statistical differences between the groups are shown for selected pairs (***$P < 0.001$, Kruskal–Wallis test with a Dunn's post hoc analysis).

for large-scale global ranges (Kettle et al. 1999). However, the difficulty of manipulating odours on such large scales complicates the in-depth investigation into how birds learn and use the olfactory landscape for navigational means. Our recent findings that *Cataglyphis* ants make use of environmental odours on a much smaller scale (Steck et al. 2009) enabled us to manipulate side-specific local blends and by doing so to decipher the navigational impact of the olfactory scenery.

Here we investigated whether ants were able to pinpoint the nest relative to surrounding odour sources. In short, did *Cataglyphis* ants memorize the topographic distribution of olfactory landmarks as if they were elements of an olfactory scenery? To answer this question, we trained ants in a situation where the nest entrance was situated within a two-dimensional array of four odours (Fig. 1). Therefore, the nest did not converge with the highest odour

concentration (Fig. 2). Ants facing the same situation during the test focused their search on the fictive nest, that is, they learned the nest position relative to the surrounding odour sources (Figs 3, 4).

What are the underlying mechanisms of orientation within complex olfactory surroundings? The following two hypotheses are possible.

(1) The ants simply memorize the sequence of changing blends while moving upwind, that is, they focus their search at the position where they no longer smell the blend of the downwind odours but still smell the blend of the upwind odours.

(2) The ants memorize the olfactory topography, that is, which odours are positioned on the right or the left side during the ants' home-bound runs.

To discriminate between these hypotheses, we tested trained ants with a manipulated array (Figs 3, 4). When the down- and upwind odour pairs appeared in the training order (i.e. the ants had to pass I and D first) but the sides of the odours within the pairs were exchanged, the animals were less confident about the nest location (Fig. 4). Therefore, hypothesis 1, the ants learn the sequences of blends only, appears unlikely. The homing ants moreover memorized an odour to be on their left or on their right side. A further strong indication that the ants learned the spatial distribution of the odour sources rather than just the sequence of blends is the finding that unilateral antennectomy affected the ants' ability to orient in the complex array (Figs 3, 4). Learning the sequence of blends would have been possible with only one antenna. To sum up, we demonstrated that homing ants make use of olfactory topography in the vicinity of the nest entrance.

Do the ants succeed in navigating in an olfactory landscape by using a stereo sense of smell? When the olfactory system of *Drosophila*, which usually receives bilateral input, is restricted to unilateral input, the animals can no longer pinpoint odour sources (Borst & Heisenberg 1982; Louis et al. 2007). Louis et al. (2007) argued that this is a result of a decreased signal-to-noise ratio rather than of a loss of stereo smell. In our study, the loss of orientation within the complex array of unilaterally antennectomized ants was not caused by a decreased sensory input, as the loss of one antenna did not compromise the ants' ability to locate a point odour source (Fig. 3). Thus the signal-to-noise-ratio hypothesis does not explain our results.

What morphological requirements could be needed to process bilateral sensory input simultaneously? Although in *Drosophila*

Figure 5. Median distances between turning points and the fictive nest of ants tested with two odours adjacent to the nest, opposite to the nest entrance, downwind of the nest, upwind of the nest, with the upwind odour pair at its training position but the downwind odours exchanged, and with the solvent (S) control. $N = 30$ ants per plot. Black line: median; box: interquartile range; whiskers: 10th and 90th percentiles. Statistical differences between the group of ants trained and tested with the olfactory array (see Fig. 4 left box) and the other groups are shown for selected pairs (***$P < 0.001$, Kruskal–Wallis test with a Dunn's post hoc analysis).

stimulation of one antenna results in ipsi- and contralateral activation in the antennal lobes (Stocker et al. 1990; Younossi-Hartenstein et al. 2002) fruit flies can discriminate an intensity gradient across the two antennae (Borst & Heisenberg 1982; Duistermars et al. 2009). In contrast to *Drosophila*, in hymenoptera odours activate the antennal lobes only ipsilaterally (Sandoz et al. 2002) which might even strengthen the animals' ability to process bidirectional input. Indeed, conditioning experiments with honeybees revealed antenna-specific odour learning, that is, the bees' olfactory system side-specifically processes the input from the left and the right antenna (Sandoz et al. 2002). In our experiments the visualization of the volatiles' distributions in the channel revealed considerable concentration differences over a distance as small as the span of an ant's antennae (Fig. 2b). Therefore it is very likely that the ants use the different input of both antennae, that is, a stereo sense of smell, to navigate in complex olfactory surroundings.

Stereo olfaction has been demonstrated for rats, *Rattus norvegicus*, and humans: rats can localize an odour source with a single sniff (Rajan et al. 2006) and humans are able to follow an odour trail (Porter et al. 2007). Both rats and humans perform less accurately when the bilateral olfactory input is reduced to a unilateral one. The use of a bilateral olfactory input has also been shown in insects. Honeybees (Martin 1965) and *Drosophila* (Borst & Heisenberg 1982; Duistermars et al. 2009) can localize an odour source most efficiently when they receive bilateral input from both antennae, and *Lasius fuliginosus* ants use bilateral olfactory input to follow a pheromone trail (Hangartner 1967). Our results that unilaterally antennectomized ants lose their ability to orient in an olfactory landscape, whereas they are still able to pinpoint a simple odour point source, can best be explained by the use of a stereo sense of smell.

At the beginning of the last century, Forel (1910) predicted that insects might also use bilateral antennal input to 'read' the topographical information conveyed by olfactory landmarks. This idea was later picked up by Von Frisch (1947). Our evidence suggests that homing *Cataglyphis* perceives the olfactory scenery around the nest in stereo and uses these olfactory landscapes for homing.

Acknowledgments

Supplementary Material

Supplementary material associated with this article is available, in the online version, at doi:10.1016/j.anbehav.2010.01.011.

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APPENDIX

Does the amount of volatiles affect the ants' homing accuracy?

During training and testing, we reapplied the odours every 20 min on the channel ground. Owing to the high environmental temperature and the high volatility of the odours, we expected the odour landmarks to change considerably within this 20 min time window. We collected air samples from the landmarks during four consecutive 5 min periods after odour application and analysed the samples in a gas chromatograph. The amount of each of the four volatiles detected in the first sample during minutes 0–5 was defined as 100%. The concentrations of volatiles in the following three samples were normalized. During the 20 min between the reapplications of the odours, their concentrations dropped by at least 80% (Fig. A1a). However, when ants were trained with an array that was 20 min old and were tested with a newly applied one, they still displayed an accurate search pattern (Fig. A1b). In addition, ants that were trained with a continuously renewed odour array and tested with one 20 min old again displayed a focused search (Fig. A1b). Hence, it was not the intensity of the landmarks, that is, the total amount of volatiles, but rather the mixture of odours that was crucial for the ants' search accuracy.

When the odour array was reduced by two odour sources as well as when the ants were antennectomized, the accuracy of the ants' nest search decreased. This effect was not due to the reduced amount of volatiles or the reduced number of receptors. If it had been, one would have expected an effect of the changing odour concentrations, too.

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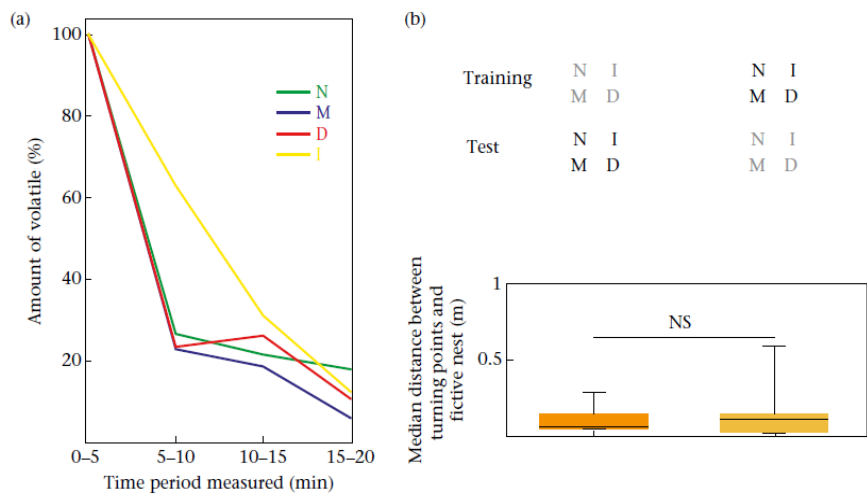**Figure A1.** (a) Normalized amounts of volatiles measured for time periods between reapplications of odour array. (b) Effect of changing volatile concentration between training and test runs on search accuracy. Grey caption depicts old arrays, i.e. with lower amounts of volatiles; black caption depicts freshly reapplied arrays, i.e. with higher amounts of volatiles. $N = 15$ ants per plot. For further details, see Fig. 4.

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Supplementary Material (Manuscript II)

Does the amount of volatiles affect the ants' homing accuracy?

During training and testing, we reapplied the odours every 20 minutes on the channel ground. Due to the high environmental temperature and the high volatility of the odours, we expected the odour landmarks to change considerably within this 20-min time window. We collected air samples from the landmarks during four consecutive 5-minute periods after odour application and analyzed the samples in a gas chromatograph. The amount of each of the four volatiles detected in the first sample during minutes 0-5 was defined as 100%. The concentrations of volatiles in the following three samples were normalized. During the 20 minutes between the reapplications of the odours, their concentrations dropped by at least 80 % (Supplementary Material Fig.1a). However, when ants were trained with a 20-minute-old array and were tested with a newly applied one, they still displayed an accurate search pattern (Supplementary Material Fig. 1b). Accordingly, ants that were trained with a continuously renewed odour array and tested with 20-minute-old one, again displayed a focused search (Supplementary Material Fig. 3c). Hence, not the intensity of the landmarks, i.e. the total amount of volatiles, but rather the mixture of odours was crucial for the ants' search accuracy.

When the odour array was reduced by two odour sources as well as when the ants were antennectomized, the accuracy of the ants' nest search decreased. Could this effect have been due to the reduced amount of volatiles or the reduced number of receptors? No, because if this had been the case, one would assume that the total amount of volatiles, i.e. the odour concentration, would influence the ants' performance as well.
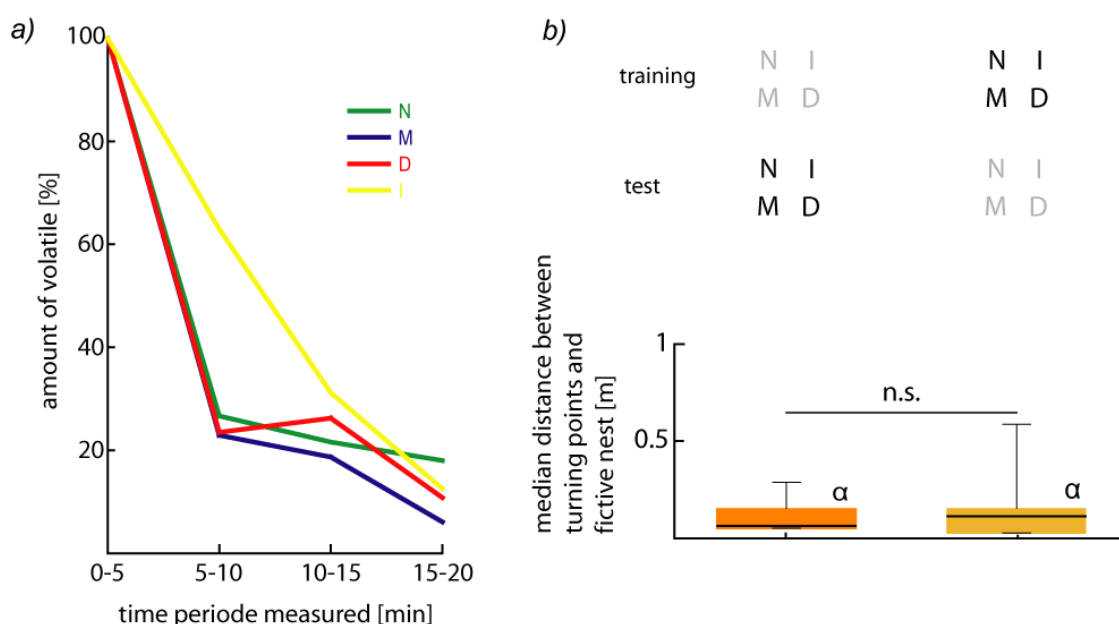
Supplementary Material Figure 1: (*a*) Normalized amounts of volatiles measured for time periods between reapplications of odour array. (*b*) and (*c*) Effect of changing volatile concentration between training and test runs on search accuracy. Grey caption depicts old arrays, i.e. with lower amounts of volatiles; black caption depicts freshly reapplied arrays, i.e. with higher amount of volatiles. 15 ants per plot. For figure details, see Fig. 4.

Could antennation of the ground provide the ants with gustatory information of the odour sources?

Supplementary Movie 1: Side view of running ants. Running ants keep their antennae in front of the head above ground. Antennation of the ground during running and during the nest search was not observed. Therefore, the knowledge of the distribution of surrounding odour sources is very likely based on olfaction rather than on taste.

Manuscript III

Memory goes crossmodal: visual and olfactory navigation in ants

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How is a crossmodal representation of the surroundings memorized? The desert ant *Cataglyphis fortis*, which has so far been a model organism for studying visual orientation [1], was recently shown also to use environmental olfactory landmarks in homing [2]. This makes it an excellent system in which to study the interplay of visual and olfactory information in memory formation. We performed homing experiments with *Cataglyphis* ants in the Tunisian desert using olfactory and visual cues singly or in combination. We found that a compound visual-olfactory cue was learned considerably faster than the singly presented elements, but was represented in separate sensory memory compartments. However, after multiple learning experiences with the compound cue only this compound elicited a robust response, while elements did no longer evoke any behavioural response. Thus, this study documents a striking change in associative strength between elements and compound depending on the degree of experience. Such a mechanism might prevent confusion arising from environmental ambiguity and thus help *Cataglyphis* to navigate within visually cryptic habitats.

Navigating subjects often deal with the problem of ambiguous landmarks. Landmark ambiguity can be reduced either by increasing sensory sensitivity, or by integrating information deriving from several modalities [3]. The desert ant *Cataglyphis fortis* has so far been a model organism for studying visual orientation alone [1]. However, it was recently shown that, in addition to the visual panorama, *C. fortis* also memorises environmentally-derived olfactory nest-defining cues and uses this information for navigation [2]. The present study investigates how compound bimodal cues are learned and stored in the brain of this navigator.

We performed field experiments to investigate how *Cataglyphis* perceives a compound visual-olfactory cue, and whether this cue is memorized as a compound crossmodal unit or as its individual elements. We trained ants to forage in an open channel in order to test their ability to associate the nest entrance with a specific cue. The inconspicuous entrance hole in the training channel floor was marked by a compound visual-olfactory cue or by one of its unimodal elements (Fig. 1). None of the cues was innately attractive to naïve animals (Fig. S1). Individually-marked ants were caught at the feeder and released for their homebound run in a test channel that was lacking an exit hole but was equipped either with the bimodal cue or with one of its unimodal elements. The median distance between the first six turning points of the ants' stereotyped nest search and the position of the nest-defining cue was used as a measure of search accuracy (Fig. S2), i.e. the degree of associative learning [2]. We tested ants with different degrees of experience; 1-run, 5-run and 15-run ants (Fig. 1).

To find out whether the bimodal cue was learned as a compound crossmodal unit or as its individual elements we tested compound-trained animals after their first outbound run with either the unimodal elements or with the bimodal compound. We found that already after one single outbound run compound-trained ants concentrated their search on the unimodal elements as accurately as on the bimodal compound, (Fig. 1B, C, D). Furthermore, in comparison with the unimodal training situations the elements of the compound were learned considerably faster in a bimodal training background. Hence, in the early training phase the two sensory stimuli were acting as independent elements that crossmodally enhanced each other's detection, but were individually sufficient to evoke a response (Fig. 1B, C, E, F). The benefit of multisensory training in subsequent unimodal tests has been shown for humans [4] and *Drosophila* [5]. The ants' accelerated acquisition of the compound cue thus supports the general rule of enhanced perception [6, 7] and learning [8, 9] of bimodal signals.
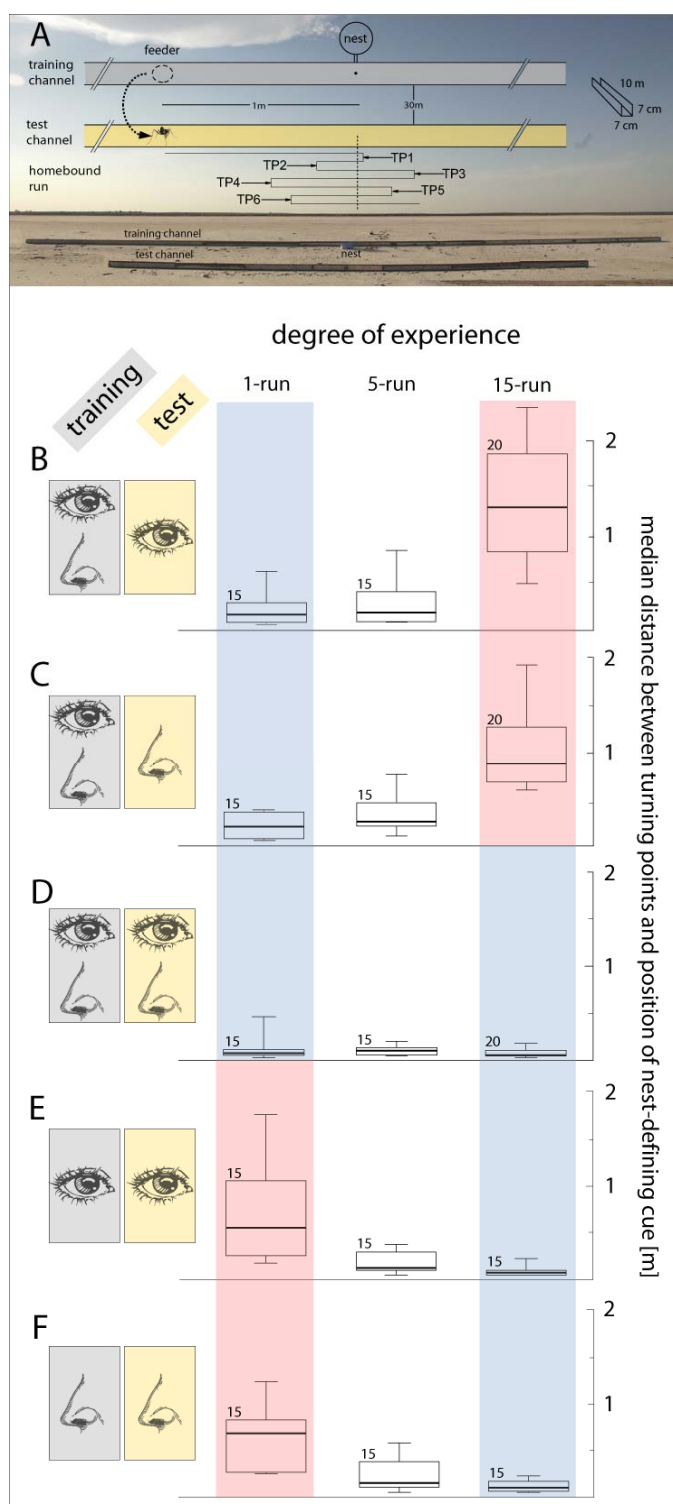
Figure 1: A. Training, test paradigm, and data acquisition. The training channel with the exit to the nest (black dot) contained a nest-defining cue. Visual cue, two black odourless pieces of cardboard on the channel walls; olfactory cue, an invisible drop of diluted indole applied directly in front of the entrance hole (reapplication every 20 min ensured presence at all times); bimodal compound cue, both elements present simultaneously. Dotted line, position of nest-defining cue in test channel; zigzag line, example specimen homebound run (shown outside the channel; for additional specimen runs see Fig. S2); TP1-TP6, first six turning points of the nest search; median distance between turning points and position of the nest-defining cue reflect the ants' confidence in the position of the nest, i.e. the degree of associative learning [2]. B-F. Search accuracies of bi- or unimodally trained ants, tested with bi- or unimodal cues after different degrees of experience, 1-run ants (i.e. tested after their first outbound run but before they could accomplish their first inbound run), 5-run ants and 15-run ants, trained and tested with visual (eye), olfactory (nose) or compound bimodal cue (eye+nose). Box plots, median distances between turning points and the nest-defining cues; black line, median; box, interquartile range; whiskers indicate the 90th and 10th percentiles. Numbers on the upper left corner of the box show sample size. Blue and red colours represent statistical differences within and between the 1- and 15-run experiments (same colour, p > 0.05, different colours, p < 0.01, Kruskal-Wallis test with Dunn's *post hoc* analysis). Data of 5-run ants are included to demonstrate the learning curve. Each ant was tested only once.

However, continued training with the bimodal compound degraded the localization abilities of ants tested with the elements, while the ants' response remained high to the compound (Fig. 1B, C, D). Two hypotheses might explain this striking change in associative strength of the elements:

a) The unitary representation of the elements entered into a single association with the reinforcing stimulus, i.e. increasing the degree of experience with the compound landmark produced a "fusion" of two elements into a single crossmodal unit.

b) Alternatively, the compound cue was learned as a unit after the first experience and animals generalized from the salient compound cue to its elements. With increasing experience the animals learned the compound context, which then inhibited the generalization to the elements when they were not presented in this context (for a review on associative learning of compounds see [10]).

Although we cannot falsify any of these hypotheses we could demonstrate that learning of compound landmarks under field conditions improves the navigational skills of desert ants considerably. Navigating subjects are often confronted with similar, i.e. ambiguous visual landmarks [3], which could become unequivocal if a second modality is added. Therefore, the use of bimodal cues can prevent confusion arising from environmental ambiguity.

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Supplemental Data (Manuscript III)

How were the channels prepared and the cues applied?
The channels were composed of open aluminum channel modules (length, 1m, width, 7cm, height, 7cm). The channel floor was covered with quartz sand. For each training situation we used different nests. That means that an ant nest that had been used for training with a visual cue, was never used for training with another nest-defining cue again. At the nest in the training channel we always used the same channel module containing the nest-defining cue. In the test channel we exchanged the modules containing the cue according to the test situation. Therefore, a channel module with an olfactory cue was never used as a visual test module or as a compound module and vice versa.

Were the visual and olfactory cues innately attractive to naïve animals?

To test whether the visual and olfactory cues affect the search runs of naïve animals we tested animals that had never experienced either cue before in the test channel, where a visual, an olfactory or the bimodal compound cue had been installed. These groups were compared to a control group of animals that were provided with no external sensory cue, but had to rely on their path integration only. As search accuracies of naïve ants tested with and without cue were statistically identical the cues were neither attractive nor repellent to naïve ants (Fig. S1).

Figure S1: Search accuracies of naïve ants tested with visual (eye), olfactory (nose), a compound bimodal cue (eye+nose), or without any cue. Box plots, median distances between turning points and the nest-defining cues; black line, median; box, interquartile range; whiskers indicate the 90th and 10th percentiles. Greek letters depict statistical differences between the groups (same letter, $p > 0.05$, Kruskal-Wallis test with Dunn's *post hoc* analysis). Each ant was tested only once.

Example specimens' runs

To clarify how we analyzed the medium distances between turning points and the position of the nest-defining cue, we present some example specimen runs. To improve the visualization, the runs are projected in two dimensions outside the channel (Fig. S2).

Figure S2: Example specimen runs. Search accuracies of bi- or unimodally trained ants, tested with bi- or unimodal cues after different degrees of experience, naïve ants (i.e. tested before the cues were installed in the training channel), 1-run ants (i.e. tested after their first outbound run

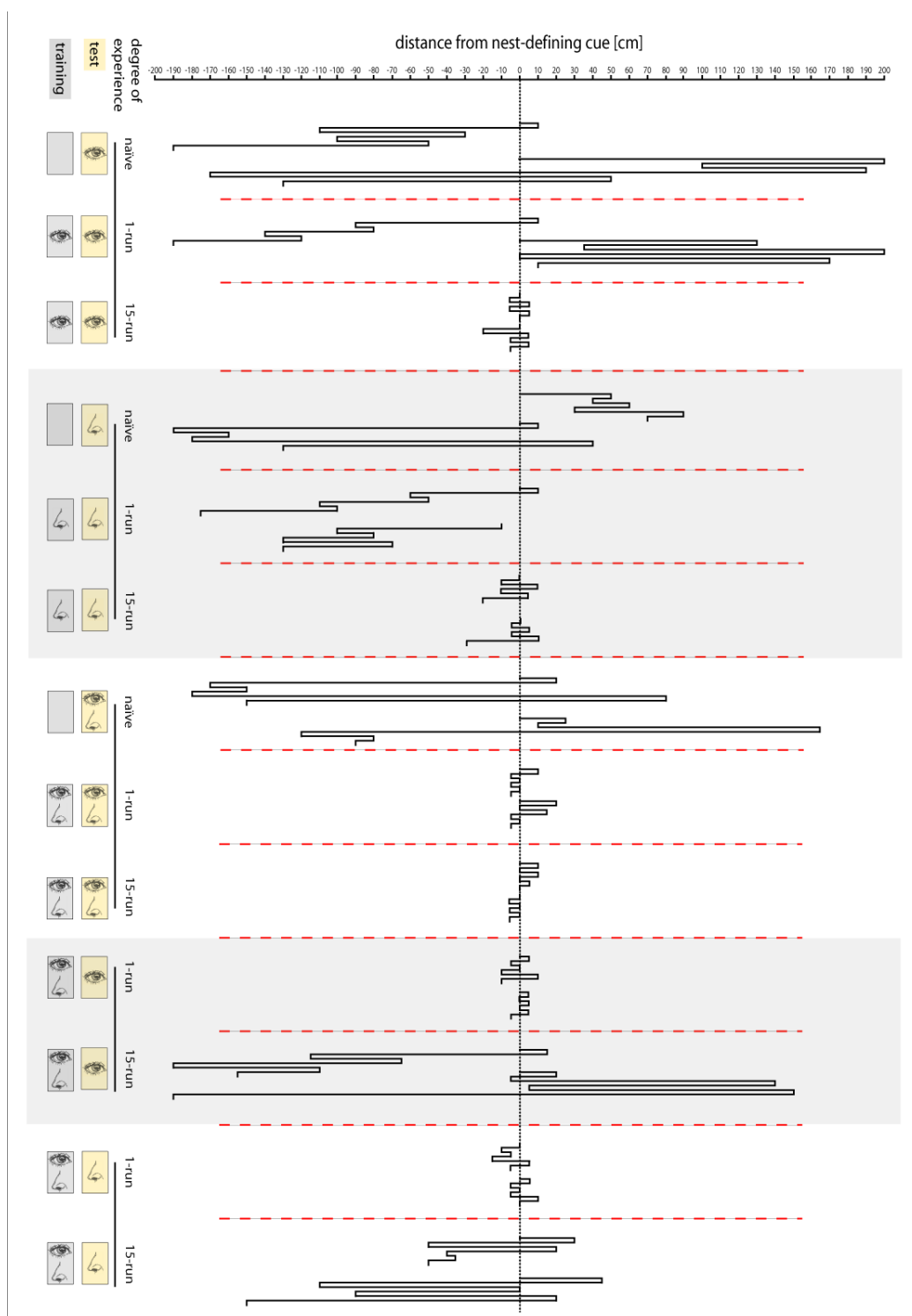with installed cue, but before they could accomplish their first inbound run) and 15-run ants, trained and tested with visual (eye), olfactory (nose) or compound bimodal cue (eye+nose). Black dotted line, position of nest-defining cue. The first six turning points after the ants passed the nest for the first time were taken for analysis. Each example displays two runs. In each example run, time is running from top to bottom.

A nest search narrowly pacing around the position of the nest-defining cue results in a short median distance between turns and cue, whereas a broad search pattern is reflected in a long median distance. This is comparable to studies concerning the accuracy of the path integrator with regard to the foraging distance. The accuracy of the path integrator decreases with increasing foraging distance. Simultaneously the search pattern broadens with increasing foraging distance (Merkle et al. 2006). This means that the more secure an ant is concerning the position of its nest, the narrower is the search pattern displayed. Similarly, in our case the narrowness of an ant's search reflects the search accuracy. Again, the more secure an ant is about the position of its nest, i.e. the better the nest-defining cue has been memorized, the narrower is the search pattern.

General Discussion

The world provides a multiplicity of volatiles that can be used by organisms to find food, a suitable oviposition site, or a mating partner. Animals orientate according to olfactory cues over a range of spatial scales. Most investigations into olfactory guided orientation have focused on short-range localisations of odour sources related to food, oviposition sites and partner finding (Murlis et al., 1992). In these cases, the volatiles of interest are usually emitted by the food source or the oviposition site itself, or are produced by conspecifics, i.e. pheromones. Therefore, in most cases of odour-guided orientation, the chemicals are innately attractive to naïve animals and do not have to be learned. Exceptions are known for *Hymenoptera*, e.g. honey bees or parasitic wasps, which are famous for their impressive learning abilities. Honey bees are known to show great fidelity to a particular species of productive flower. This demands an ability to learn associatively and to remember specific blends. Indeed, honeybees are highly specialised in learning and discriminating between floral volatiles in a variety of experimental paradigms (Bitterman et al., 1983; Hammer & Menzel, 1995). Parasitic wasps, *Microplitis croceipes* (Braconidae), e.g., on the other hand, can locate their hosts by orientation to host-associated volatiles released from frass and larval feeding damage (Drost *et al.*, 1986). The polyphagous nature of its host, which occurs on more than 200 plant species (Fitt, 1989) confronts the parasitoid with a wide variety of potential host habitats. The parasitoid's ability to learn olfactory cues experienced in association with host products (Lewis & Tumlinson, 1988) can thus serve as an effective strategy to cope with this variability.

The hostile habitat of the species studied in the present work, the desert ant *Cataglyphis fortis*, is extremely demanding. The hot and featureless saltpans in the Sahel are a perilous place for the solitary foraging ants. It is therefore absolutely essential for them to return rapidly and reliably to the safe nest after a foraging run. Additionally to the egocentric path integration system (e.g. Wehner, 1982; Wehner, 2003) *C. fortis* masters this task by learning and memorising the visual surroundings of the nest in order to pinpoint the entrance (Wehner et al., 1996). Due to its amazing visual orientation system, the desert ant became a model organism in which to study visual navigation. Here, we show that *C. fortis* is additionally equipped with a sophisticated olfactory orientation system.

Olfactory Landmarks

The first and second manuscripts of this thesis add a new aspect to the field of odour-guided orientation as they for the first time show that a habitat – in the present case Tunisian saltpans – provides place-specific blends that can be learned, distinguished, and used as olfactory landmarks by the ant, *C. fortis*. Not only were the ants able to distinguish between odours, they could also distinguish a learned odour from a blend background. In this way the main characteristics of a functional landmark are provided. The use of landmarks for homing has so far been thought to be restricted to visual navigation, for example in insects such as wasps (Collett & Rees, 1997), solitary bees (Fauria & Campan, 1998), honey bees (Cartwright & Collett, 1983), and ants (Wehner & Raeber, 1979) but also in pigeons (Wallraff *et al.*, 1989), and octopuses (Mather, 1991). Therefore, the results presented in Manuscript I add a new aspect not only to desert ant orientation but to the field of navigation in general.

Additionally, the finding that ants learn and distinguish odours does not only have relevance from a navigational point of view. Most organisms are able to discriminate among diverse odours and blends. These olfactory stimuli are often important for the organization of feeding, mating, and social behaviours, as well as for the processes of learning and memory that are associated with these behaviours. Therefore, the question of how individual odours and blends are perceived by an animal and how they are processed in the brain has become one of the main questions in olfactory research. The possibility of training ants to associate their nest with a specific odour or blend and of testing them with similar odours and blends facilitates an in-depth investigation of odour and blend processing and discrimination in future experiments.

Olfactory Scenery

Until now, environmentally-derived odours were mainly discussed in relation to large-scale navigation (Wallraff, 2004; DeBose & Nevitt, 2008). In particular, the ability of birds to navigate their way homewards by deducing positional information from atmospheric trace gases has been discussed (Wallraff, 2004). Indeed, analyses of volatiles demonstrate that the atmosphere contains spatial olfactory information (Wallraff & Andreae, 2000). Simulated navigation based on this olfactory landscape revealed that a homeward direction could be estimated for an array of several sites based on this knowledge (Wallraff, 2000). However, the difficulty of manipulating odours on such a

large scale complicates an in-depth investigation into how birds learn and use the olfactory landscape for navigational means. The results are comparable with the PID analysis of the olfactory array in Manuscript II (Steck *et al.*, 2010), where we found place-specific blends, though on a much smaller scale than described by Wallraff and colleagues (Wallraff & Andreae, 2000). Having shown that the desert ants can use environmental odours allows the manipulation of the olfactory scenery on a much smaller scale. Manuscript II describes the ants memorising the olfactory scenery surrounding the nest entrance and using this knowledge for homing. This is new to the field of insect odour-guided orientation, insofar as these results provide the first evidence that the odour source, i.e. the location of highest concentration, is not the goal, but that ratios of blends are used to pinpoint a goal relative to surrounding odour sources. At the beginning of the last century, Forel (1910) predicted that insects might be equipped with a topochemical sense and thus, are able to "read" the topographical information conveyed by olfactory landmarks (Forel, 1910). Forel proposed that places might be found by means of a 'topochemical sense', by which foraging ants are able to navigate making use of local 'chemical emanations' (Forel, 1928). This idea was later picked up (von Frisch, 1947) (Helmy & Jander, 2003) and evidence suggest that ants can associate their learned chemical place recognition with a navigational decision, namely the switching from horizontal locomotion to vertical climbing.

The results in Manuscript II indeed suggest that *C. fortis* is fitted with a topochemical sense as it learns the olfactory scenery and uses this information for homing. Remarkably, *C. fortis* not only memorises the olfactory landscape surrounding the nest entrance but also the specific location of an odour source, i.e. whether it had been on the left or on the right when homing (Steck *et al.*, 2010). We should of course also consider the possibility that the ants additionally integrate directional information deriving from the compass, i.e. the olfactory scenery is learned with respect to external compass bearings. Future studies should aim to investigate whether the information derived from the olfactory landscape is embedded into a directional reference scale provided by the ants' skylight compass, as was shown for visual arrays (Cartwright & Collett, 1983; Wehner et al., 1996; Åkesson & Wehner, 2002).

Stereo Sense of Smell

Generally, a stereo sense of vision, audition or olfaction, respectively, implies a simultaneous integration of bilateral sensory input. The two slightly different projections

of the world arise from different positions of the sensors. A sound source, for example, can be localised because the pressure waves emitted reach each ear at a slightly different phase and/or with a slight difference in intensity (Masterton *et al.*, 1975; Masterton & Imig, 1984). The difference between the two sides is integrated into a spatial impression. For humans an analogy for directional smelling was suggested some time ago (Vonbekesy, 1964; Kobal *et al.*, 1989). The extraction of spatial information from smell for scent-tracking was then shown in a variety of mammals, e.g. in rats (Wallace *et al.*, 2002),(Rajan *et al.*, 2006), dogs (Thesen *et al.*, 1993), and humans (Porter *et al.*, 2007). Humans, for example, accurately identify the direction of an odorant source. Temporal differences of about 0.1ms and variations in intensity of 10% of birhinally presented odorous stimuli were sufficient to create the impression that the source of the smell was located laterally to the medial plane (Vonbekesy, 1964). Increased brain activity in the superior temporal gyrus – a region known to be implicated in spatial auditory and visual localisation as well (Calvert, 2001) – was shown in humans during olfactory localisation (Porter *et al.*, 2005). This result suggests that a certain brain region is responsible for the spatial impression of the world in humans, irrespective of the sensory modality addressed.

Before trying to assess an analogous region in the insect brain, we might ask whether insects have a spatial perception of the surrounding world at all. An initial answer can be obtained by having a look at the insects' visual system. Bees and ants obviously lack stereopsis and focusing mechanisms in their eyes. Therefore, information about the third dimension is gained only during motion, in the form of motion parallax. Experiments with cylindrical landmarks of different sizes and with different distances to a goal revealed that size and distance are indeed confounded (Cartwright & Collett, 1983; Wehner et al., 1996). Hence, these insects possess only a two-dimensional perception and memory of images, or photographic snapshots, instead of three-dimensional representations of their surrounding.

However, with respect to the insects' olfactory sense, suitable experimental paradigms to study the spatial perception of the olfactory surrounding, using a stereo sense of smell, were still lacking. Studying the morphology and physiology of the insects' olfactory system was aimed at discovering what requirements needed to be fulfilled in order to process bidirectional sensory input. In *Drosophila*, stimulation of one antenna results in ipsi- and contralateral activation in the antennal lobes because the majority of sensory neurons project to analogue glomeruli in both antennal lobes (Stocker et al., 1990;

Kazama & Wilson, 2008). This property would seem to constrain the capacity for gradient tracking. However, fruit flies were shown to discriminate an intensity gradient between the two antennae (Borst & Heisenberg, 1982; Duistermars *et al.*, 2009). Lateralisation of the flies' sensory system, i.e. the fact that sensory signals from the left antenna, after stimulation from left, contribute disproportionately more to odour tracking than signals from the right, was thought to enable gradient tracking (Duistermars et al., 2009). Lateralisation of the olfactory system was also shown for Hymenoptera at sensory (Letzkus *et al.*, 2006) and processing level (Sandoz & Menzel, 2001; Sandoz *et al.*, 2002). In addition, contrary to the situation in *Drosophila*, in *Hymenoptera* odours activate the antennal lobes only ipsilaterally (Sandoz et al., 2002), which might be an additional prerequisite for processing bidirectional input. Indeed, directional smelling, i.e. comparing odour intensities across the two antennae, was shown to be used in honey bees (Martin, 1965) and ants (Hangartner, 1967).

What is the difference between "bilateral olfactory input" and a "stereo sense of smell"? When the olfactory system of *Drosophila* larvae, which usually receives bilateral input, is restricted to unilateral input, the larvae cannot pinpoint odour sources anymore (Louis et al., 2008). This could rather be a result of a decreased signal-to-noise ratio than of a loss of stereo smell. In contrast, the signal-to-noise-ratio hypothesis seems to be inappropriate to explain our results as the loss of one antenna did only compromise the ants' ability to navigate within the complex olfactory landscape but did not compromise the ants' ability to locate a point odour source. Similarly, unilaterally antennectomized *Lasius* ants loose their ability to follow a pheromone trail precisely (Hangart.W, 1967). Instead they tend to turn towards the intact side, suggesting that they use klinotaxis, i.e. taking volatile samples through space and over time (Fraenkel & Gunn, 1961). Again, bilateral olfactory input would facilitate this task. It is, however, not the main mechanism. Our results that unilaterally antennectomized ants loose their ability to orientate in an olfactory landscape, whereas they are still able to pinpoint a simple odour point source, can be explained best by the use of a stereo sense of smell.

Thus, we found that *C. fortis*, so far known for its remarkable vision-based orientation system, is additionally equipped with a sophisticated chemical sense of orientation. Further interesting questions arose from the possible interplay of these two modalities, vision and olfaction.

Crossmodal Learning and Memory

The surrounding world is always a diversified space, usually addressing more than one modality. Moreover, in animal communication multimodal stimuli strongly influence perception, processing and finally, of course, the behaviour of organisms (Rowe, 1999). On this basis, navigating subjects should also be able to take advantage of the multimodal environment to disambiguate landmarks. In robot science such possibilities have been discussed theoretically, as the confusion of landmarks – known as landmark aliasing – can circumvent any navigation by autonomous agents (Whitehead & Ballard, 1991; Frese, 2006). Studies on, for example, *Drosophila* (e.g. (Chow & Frye, 2008; Duistermars & Frye, 2008; Frye & Duistermars, 2009)), cats (e.g.(Meredith & Stein, 1983)), and humans (e.g.(Seitz *et al.*, 2006; Hecht *et al.*, 2008a)) have already shown that cues of different modalities can enhance each other's conspicuousness when they are presented together. However, a suitable organism for testing for increased navigational performance in the presence of multimodal cues needed to be found. As *Cataglyphis* can associate its nest both with visual (Wehner & Raeber, 1979) and olfactory cues (Steck et al., 2009a), i.e. use visual and olfactory landmarks, this species is suitable for investigating multimodal navigational enhancement. Therefore, in Manuscript III I describe how a navigation subject was able to learn and memorise a bimodal stimulus in order to disambiguate landmark information. I found accelerated acquisition for bimodal cues compared to unimodal ones. This is in accordance with findings in chicks and bumblebees, where sound or odour accelerates visual discrimination learning (Rowe & Guilford, 1999; Kulahci et al., 2008). The coincidence of different modalities not only affects learning but can even increase innate responses of, for example, pollinators to flowers (Hebets & Papaj, 2005; Goyret *et al.*, 2007). Generally, multimodal signals are more conspicuous, i.e. they can be perceived more easily and rapidly by the receiver (Waeckers & Lewis, 1994; Rowe, 1999; Hecht *et al.*, 2008b). These findings are supported by physiological studies with *Drosophila* (van Swinderen & Greenspan, 2003) and cats (Meredith & Stein, 1983), where the mere presence of an odour (flies) or an auditory signal (cats) increases the physiological response of the brain to visual stimuli. Similarly, in *Drosophila*, olfactory signals improve the salience of visual stimuli and thus enhance optomotor control (Chow & Frye, 2008; Duistermars & Frye, 2008). Increased salience of bimodal signals is further supported by our finding that the ants learned a unimodal cue much more rapidly in a bimodal context than in a unimodal context. This benefit of multisensory training has also been shown in humans, who learned a visual motion-detection task more rapidly when exposed to an audiovisual training procedure

(Seitz et al., 2006; Shams & Seitz, 2008). Likewise, in *Drosophila* bi-sensory training produced accelerated acquisition. Simultaneous olfactory and visual stimuli lowered the threshold for detecting each of the compound stimuli on its own (Guo & Guo, 2005).

Most striking, however, was the effect that after lengthy training, the ants apparently fused visual and olfactory information into a bimodal memory. After having internalised a landmark to be composed of both a visual and an olfactory cue, neither the isolated visual cue nor the isolated olfactory cue was recognised as the nest-defining landmark any longer. Certainly, natural selection does not allow the small brain of *Cataglyphis* to provide space and energy for computational and storage resources that are not of immediate use to the navigator, particularly because in energetic terms the brain is by far the most costly organ of an organism (Martin, 1981). Thus, the fusion of the olfactory and the visual cue after lengthy training is new and might point towards an economic strategy for storing multimodal cues. Further studies should investigate the persistence of the bimodal memory compared to unimodal memories.

Both results from Manuscript III indicate the increased navigational value of multimodal cues and this now allows the implementation of multimodal sensors in artificial agents. Furthermore, the effect of fused visual and olfactory memory is also interesting from a physiological point of view. In ants, optical imaging methods – i.e. methods to visualise neural activity in a living animal's brain – have been established for two ant species (Galizia et al., 1997; Zube & Roessler, 2008), which are closely related to *C. fortis*. *C. fortis* uses both vision and olfaction for the task of pinpointing the nest entrance. Furthermore, the calyces of the hymenopteran mushroom bodies exhibit a characteristic and conspicuous subdivision into the collar, which receives visual input, and the lip region, which receives olfactory input (Gronenberg, 2001). Therefore, *C. fortis* has the potential to become a model organism for investigating crossmodal information processing in the mushroom bodies, the first processing centres of the brain that receive visual and olfactory sensory input.

Conclusion

The hostile habitat of *Cataglyphis fortis* pushed the evolution of a sophisticated and reliable navigational machinery in this insect. The main tools for homing are path integration for long-distance navigation and landmark guidance in the vicinity of the nest. The present thesis reveals that *C. fortis* additionally is equipped with an advanced

olfactory orientation system. *C. fortis* is able to make use of olfactory landmark information and to navigate in a complex olfactory landscape. Additionally, our evidence suggests that *C. fortis* is fitted with a stereo sense of smell. Beyond that, we found that *C. fortis* is a suitable organism in which to study cross modal learning and memory.

The physiological basis of the conspicuous odour-guided orientation described in the present thesis is most probably conserved in many insect species. However, the striking behaviour of *C. fortis*, i.e. the reliable motivational state of the foragers, their impressive learning capability and the considerable flexibility in coupling and decoupling the different modules and referential systems to and from each other creates a unique scenario in which to study the function of higher brain centres. Results gained in *C. fortis* might be subsequently extrapolated to other model organisms gifted with genetic tools, allowing the exploration of the molecular basis of odour-guided behaviour.

Summary

The world provides a multitude of volatiles which can be used by organisms to successfully exploit their surroundings. Volatiles are emitted by the goal itself, i.e. the food source, the oviposition site, or the mating partner. Environmentally-derived odours and blends are mainly discussed in relation to large-scale navigation of birds and fishes. The species used in the present thesis to study odour-guided orientation is the desert ant *Cataglyphis fortis*. At first sight it is not obvious why *C. fortis* is a suitable organism in which to study the use of volatiles for navigation, as it has so far been a model organism for investigating visual orientation alone. *C. fortis* ants forage individually for dead arthropods in the inhospitable salt-pans of Tunisia. Locating the inconspicuous nest after a foraging run of more than 100 meters demands remarkable orientation capability. As a result of high temperatures and unpredictable food distribution, desert ants do not lay pheromone trails. Instead, path integration is the fundamental system of long-distance navigation. This system constantly informs a foraging ant about its position relative to the nest. In addition, the ants rely on visual landmarks as geocentric navigational cues to finally pinpoint the nest entrance. Apart from the visual cues within the ants' habitat, we found potential olfactory landmark information with different odour blends coupled to various ground structures. Here we show that desert ants can use olfactory information in order to locate their nest entrance. Ants were trained to associate their nest entrance with a single odour. In a test situation, they focused their nest search on the position of the training odour but not on the positions of non-training odours. When trained to a single odour, the ants were able to recognise it within a mixture of four odours. The use of environmentally-derived odours has so far only been discussed in relation to large-scale navigation, suggesting positional information from an atmospheric olfactory landscape. We artificially created a complex olfactory environment around the nest entrance, and investigated the ants' ability to navigate according to it. We show that the ants are able to orientate in a complex olfactory environment and to locate the nest entrance within a two-dimensional olfactory array. Ants were trained to memorise the nest entrance relative to four odours that were applied at the corners of an invisible quadratic array. In a test situation, the ants pinpointed the fictive nest only when the odours were present at their learned positions. Our results suggest that the ants had learned the olfactory scenery around their nest. Furthermore, unilaterally antennectomized ants could not pinpoint the nest within a two-dimensional array. Hence, this kind of orientation depends on the simultaneous input of both antennae, i.e. on a stereo sense of smell. Until now, insects

and mammals, including humans, have only been known to use bilateral sensory input to follow a concentration gradient of an odour. Our evidence suggests that desert ants require a stereo sense of smell to make use of the olfactory scenery around their nest for homing.

These findings make the desert ant a promising candidate in which to study the interplay between visual and olfactory information in navigating subjects. We therefore investigated how *Cataglyphis* perceives a compound landmark consisting of a visual and an olfactory cue, and whether the compound landmark is memorised as one fused bimodal unit or as two independent unimodal stimuli. Our results show the transition from individually-learned unimodal cues to a unified compound memory. While our data provide further evidence for the general rule of enhanced crossmodal perception (the bimodal cue was learned five to ten times faster than the unimodal one), the fusion of visual and olfactory memories into a bimodal unit has never before been demonstrated. This strategy might prevent confusion arising from environmental ambiguity and thus help *Cataglyphis* to navigate within visually ambiguous habitats.

Zusammenfassung

Unsere Umwelt ist voller Gerüche, anhand derer sich ein Organismus orientieren kann. Meist werden die Duftstoffe vom Ziel selbst abgegeben und dienen daher der Mehrheit der Insekten als Orientierungshilfe, Futter, einen geeigneten Eiablageplatz oder Partner zu finden. Umgebungsdüfte, das heißt Düfte, die weder im Zusammenhang mit Futter noch mit Fortpflanzung stehen, wurden bis jetzt hauptsächlich als Orientierungshilfe für Langstrecken diskutiert, wie sie zum Beispiel Vögel oder Fische zurücklegen.

In der vorliegenden Arbeit wird die Verwendung von Umgebungsdüften in Form von olfaktorischen Landmarken erforscht. Die Tunesischen Wüstenameise *Cataglyphis fortis* erscheint auf den ersten Blick vielleicht nicht geeignet, geruchsgeleitete Orientierung zu untersuchen, da sie bis jetzt dank ihrer visuellen Orientierungsmechanismen Berühmtheit erlangte. Arbeiterinnen der Wüstenameise entfernen sich während ihrer Futtersuche auf gewundenen Pfaden und über erhebliche Distanzen von ihrem Nest. Im Gegensatz zu den meisten anderen Ameisenarten werden bei der Gattung *Cataglyphis* keine Pheromonspuren verwendet. Grund dafür sind die kleinen, zufällig verteilten Futterstücke und die hohen Bodentemperaturen, bei denen Pheromone schnell verdampfen würden. Der Rücklauf zum unauffälligen Nesteingang erfolgt auf dem kürzesten Weg mittels Wegintegration. Hierbei werden en route die zurückgelegten Distanzen und die eingeschlagenen Winkel kontinuierlich zu einem Heimvektor aufintegriert. Während ein Sonnenkompass zur Bestimmung der Richtung dient, wird die zurückgelegte Strecke mit Hilfe eines Schrittintegrators, der die Laufbewegung der Beine verarbeitet, bestimmt. In unmittelbarer Nähe des Nestes verlassen sich die Ameisen auf visuelle Landmarken. Nebst visuellen Landmarken fanden wir im unwirtlichen Lebensraum der Wüstenameisen stabile, ortsspezifische Duftmischungen. Da deren Komponenten von den Ameisen wahrgenommen werden können, schließen wir daraus, dass die untersuchten Bodenstrukturen potentiell als olfaktorische Landmarken genutzt werden können. Mit Hilfe eines Feldversuches konnten wir zeigen, dass *C. fortis* in der Lage ist, die Assoziation zwischen einem spezifischen Umgebungsduft und dem Nesteingang zu lernen und diese Information zu nutzen, um nach Hause zu finden. Ebenso vermochten die Ameisen, den gelernten Duft aus einer Mischung unbekannter Düfte zuerkennen.

Die Verwendung von atmosphärischen Umgebungsdüften und deren Mischungen, sog. „Duftlandschaften" wurde bei Tauben untersucht. Wir erstellten künstlich eine

Duftlandschaft, die jedoch in weitaus geringeren Dimensionen den Nesteingang umgab, um in einem weiteren Feldversuch zu untersuchen, ob Wüstenameisen ebenfalls in der Lage sind anhand solcher komplexer Duftmuster zu navigieren. Die Ameisen mussten im Training die Position des Nesteingangs in mitten einer zweidimensionalen Anordnung von Duftquellen lernen. Im darauffolgenden Test fanden wir, dass *C. fortis* sich innerhalb dieser komplexen Duftlandschaft orientieren und anhand der ortsspezifischen Duftmischungen den Nesteingang zielgenau orten konnte. Diese Fähigkeit verlor sich, sobald die Positionen der Duftquellen zwischen Training und Test geändert wurden. Folglich hatten die Ameisen die komplexe Duftlandschaft bestehend aus mehreren, den Nesteingang umgebenden Duftquellen gelernt. Darüber hinaus fanden wir, dass Wüstenameisen mit einem Stereogeruchssinn ausgestattet sind, da zur erfolgreichen Orientierung innerhalb der Duftlandschaft zwei Antennen nötig waren. Bis anhin konnte ein Stereogeruchssinn nur bei Säugetieren gezeigt werden. Die Tatsache, dass *C. fortis* nebst visueller Information auch komplexe Geruchsinformation als Navigationshilfe verwenden kann, ermöglicht das Studium des Zusammenspiels dieser beiden Sinneseindrücke in einem navigierenden Organismus.

In einem dritten Versuchsaufbau untersuchten wir, wie *C. fortis* eine aus visuellem und olfaktorischem Reiz zusammengesetzte Landmarke lernt und abspeichert; als zwei einzelne Stimuli oder als kombinierter Stimulus. Wir fanden die generelle Regel, dass kombinierte Signale auffälliger sind und dadurch schneller gelernt werden, auch in den Ameisenexperimenten bestätigt. Neu hingegen ist die Erkenntnis, dass die isolierten Stimuli der zusammengesetzen Landmarke zu Beginn noch einzeln erkannt werden, dann jedoch zu einem kombinierten Sinneseindruck verschmelzen. Dieses Verschmelzen von visueller und olfaktorischer Information zu einer intermodalen Landmarke könnte den navigierenden Ameisen helfen, äußerlich ähnliche Landmarken voneinander zu unterscheiden, indem sie mit einem Geruch verknüpft werden.

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Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as coauthors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

Jena, January 8, 2010   ....
                                                        Kathrin Steck

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