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Ants use directionless odour cues to recall odour-associated locations

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Abstract Ants are key model organisms in the study of navigation and memory formation. Many ants learn food locations very quickly and with high accuracy. But can individual ants learn multiple separate food locations, associate them with a cue, and then correctly recall the food location and navigate towards it when later presented with that cue? In this experiment, we sequentially trained *Lasius niger* foragers to two scented feeders at either end of a T-maze. The next day, an odour cue corresponding to one of the food sources was presented to the ants in the air, on the substrate and via trophallaxis with nest mates. Trained foragers accurately navigated to the correct side of the T-maze (89 % correct decisions), but only after the first 10 min of testing. This demonstrates the ability of ants to perform associative recall, forming clear associates between odour cues and food locations and using these associations to navigate to food sources. We also found that trained ants in the first 10 min of testing showed no preference for the correct side (57 % correct decisions), which may be related to the motivational state of the ants tested. Ants with different motivational states (whether they are 'scouting' or 'recruited') made use of route memories in a completely different manner. This highlights the importance of taking account of motivational states when performing behavioural experiments.

Keywords Foraging · Route memory · Associative learning · Motivational states · Odour cues · Ants

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Introduction

Most social insects are central place foragers, and their foragers have ample opportunity to gather information about the environment around their nest. Social insects can memorise the location of a resource and make repeated trips to it. Honey bees and ants have also been shown to have further remarkable learning abilities. They can, for example, learn to associate unrelated stimuli (e.g. linking sight to smell) and make generalisations (e.g. that two shapes are 'the same' or 'different') (Srinivasan et al. 1998; Giurfa et al. 2001; Dupuy et al. 2006; Avarguès-Weber and Giurfa 2013). Foragers can learn the location of a feeder after only a single visit (Harrison et al. 1989; Grüter et al. 2011) and may retrieve such memories weeks or even months later (Rosengren and Fortelius 1986; Salo and Rosengren 2001). Linked to this is the phenomenon of site fidelity (or 'Ortstreue'), where workers repeatedly visit a particular site or route for extended periods of time (Rosengren and Fortelius 1986; Quinet and Pasteels 1996). Many insects, especially central place foragers such as ants and bees, learn visual information and use this information to navigate repeatedly to important locations via habitual routes (Judd and Collett 1998; Collett et al. 2003; Graham et al. 2007). This suggests that honey bees and ants can store multiple sub-routes and appropriately recall the information to guide a particular sub-route when required. The importance of recalling food locations is highlighted when route memories are put into conflict with social information. In honey bees and in most species of ants tested, when social information (pheromone trails or the waggle dance) conflicts with memories of a food location, foragers follow their memories (Fourcassie and Beugnon 1988; Harrison et al. 1989; Grüter et al. 2008, 2011). Indeed, in one experiment, when the ability of honey bee colonies to use directed recruitment is disrupted by laying hives on their side, such colonies fare almost as well as undisrupted colonies in temperate habitats, but not in Asian

tropical habitats (Dornhaus and Chittka 2004). This difference was due to the distribution of foraging sites in the two habitats, with foraging being more clustered in the tropics.

The ability of honey bee colonies to forage successfully in some situations without directed recruitment may be in part related to the strengths of honey bee memory. The ability to memorise food locations and form multiple simple associative memories via classical conditioning has also been demonstrated in solitary insects (e.g. Raubenheimer and Tucker 1997; Ofstad et al. 2011). However, honey bees can not only correctly memorise the location of multiple feeders but also associate them with a time of day or an odour (Wahl 1932; von Frisch 1967). Up to two such locations can be correctly recalled when exposed to the appropriate directionless odour stimulus (Reinhard et al. 2004). If honey bee foragers are trained to a feeder scented with an odour, then trained to another feeder scented with a different odour, the foragers will exit the hive and search in the appropriate location depending on which odour is puffed into the hive. The correct feeder location memory can also be retrieved when the appropriate odour is presented by another forager, via trophallaxis. This will allow the foragers in a colony to respond rapidly to changing forage situations, without the need for directional recruitment. The ability of storing multiple locations and recalling them appropriately is well matched to honey bee ecology, as honey bees must respond to a foraging environment that changes many times throughout the day, and their food sources are very often scented with specific floral odours. However, this ability may not be limited to honey bees. Many ants also rely heavily on semi-permanent food sources, including colonies of phloem-feeding Homoptera, flowers and extra-floral nectaries (Hölldobler and Wilson 1990). *Camponotus* workers have been shown to be able to associate an odour with the presence of food and, after training, can use this association to follow an odour-marked branch to a food source (Helmy and Jander 2003; Dupuy et al. 2006). In this study, we ask whether black garden ant (*Lasius niger*) foragers are capable of memorising multiple locations and accurately recalling the appropriate memory when presented with an associated cue.

Methods

Study species

We studied six *L. niger* colonies collected in the Ludwig-Maximilian University campus in Munich. Colonies were housed in plastic foraging boxes (40×30×20 cm high). The bottom of each box was covered with a layer of Plaster of Paris. Each foraging box contained a circular plaster nest box (diameter 14 cm diameter, height 2 cm). The colonies were queenless with 1,000–2,000 workers and small amounts of brood. Queenless colonies forage, make pheromone trails and care for brood, and are frequently used in foraging experiments (Devigne and Detrain 2002; Evison et al. 2008). Colonies were fed three times per week with Bhaktar diet, a mixture of egg, agar, honey and vitamins (Bhatkar and Whitcomb 1970). Colonies were deprived of food for 4 days prior to a trial in order to achieve uniform and high motivation for foraging. Water was provided ad libitum.

The aim of this study was to examine whether *L. niger* workers can simultaneously store multiple route memories and retrieve the appropriate memory when a cue is provided. Three experiments were carried out in this study. For clarity, the methods and results for each experiment will be presented sequentially. All experiments were conducted in two parts: a training phase and a testing phase. In the training phase, we provided individual ants with a route memory of two distinct feeding locations on a T-maze, with each location linked to a specific odour cue. In the testing phase, a directionless odour cue was provided, and the route memory retrieval of individual ants was tested by asking which direction they chose at the T-maze. The training phase was identical in all three experiments, but the testing phase differed between experiments.

Training phase

Twenty *L. niger* workers were allowed access to a plastic T-maze consisting of a 15-cm-long stem and a 22-cm-long head, both 2 cm wide. The T-maze was covered in paper. To the left of the T-maze, a bright light was placed, and the experiment was conducted in an open laboratory environment, so as to ensure the ants had sufficient salient landmarks to learn. At the end of one arm of the T-maze, a feeder containing 1 molar sucrose syrup was placed. The syrup was either flavoured with lemon essential oil if it was placed on the left or rosemary essential oil if it was placed on the right. The essential oils were added to the sucrose solution at a ratio of 5 μl per 100 ml of sucrose solution. In preliminary studies, ants showed no aversion to either flavoured solution. Of the 20 ants to first find the feeder, nine were marked with individual coloured paint dots on the abdomen as they fed, and the remaining 11 were marked with a tenth colour. The 20 ants were then allowed to make repeat return visits to the feeder, and the number of visits made by the individually marked ants was tracked. All other ants were prevented from entering the T-maze using a drawbridge linking the T-maze to the nest box. Once all the individually marked ants had made three return visits to the feeder, the feeder was removed. After at least 1 h, so as to prevent recently learned route memories interfering with further learning (Menzel 1979), a second feeder flavoured with the other scent was placed on the other side of the T-maze. The marked ants were then allowed to make three further return visits to this feeder. As the marked ants made their fourth return visit to the second feeder location,

they were collected and placed in a separate box containing water, a shelter, but no food. This was done to prevent the paint marks from being groomed away by nest mates in the nest. The marked ants were stored in this box overnight. The T-maze was cleaned with ethanol, and the paper overlays were replaced.

Testing phase

Experiment 1—triggering retrieval of appropriate route memories using odour cues

Methods In the testing phase, the ants were first tested without an odour cue (a control) and then tested with an odour cue. The day after the training, the marked ants were returned to their colony, and the colony, including untrained ants, was given free access to the T-maze for at least 45 min. Fifteen minutes before the start of a trial all, ants on the T-maze were returned to the colony, and the access bridge to the T-maze was removed. Using a separate bridge attached directly to a feeder, 20 unmarked ants were allowed to feed at unscented 1 M of sucrose solution feeder and return to the nest. These returning ants deposited pheromone and performed recruitment behaviours and trophallaxis with other ants, and thus acted to inform the other foragers that food is present. However, since a separate bridge and feeder were used, no pheromone was deposited on the bridge leading to the T-maze, or on the T-maze itself. Thus, although the ants had been recruited in the nest, no directional cues from pheromones were available to them. A line of distilled water was drawn over a fresh access bridge (as a control for an odour cue, see below), and this bridge was then used to give access to the T-maze. The number of marked and unmarked ants crossing a 'decision line' 3 cm from the centre of the T-maze bifurcation was then counted each 10 min for 60 min. Counted ants were immediately removed from the apparatus using an aspirator. After the 60 min of data collection, the collected ants were returned to the nest and the paper overlays were replaced by fresh overlays. After this control treatment, access to the T-maze was again given with no feeders being present on the maze. The colony, including the trained ants, was allowed to explore the unrewarding T-maze for at least 2 h, after which the test treatment began.

The second phase was similar to the first, with 20 unmarked ants being given access to a sucrose feeder via a separate access bridge. However, in this trial, the sucrose syrup was flavoured with either lemon or rosemary essential oil. Thus, the 20 ants returned scented syrup to the colony and shared it with nest mates via trophallaxis. A line of scented water (1 μl scent per 20 ml distilled water) was drawn using a pasture pipette over a fresh access bridge, which was then used to give the colony access to the T-maze. Lastly, a bottle of the same scent was left open underneath the access bridge. Thus, even if ants had not received a trophallactic interaction from a returning forager, they would sense the odour cue in the environment, without the odour cue having a directional component. As above, the number of ants that crossed the decision lines was counted every 10 min for 60 min. At the end of the experiment, the marked ants were permanently removed from the colony, and all other ants returned to the colony.

Statistical analysis for all experiments Statistical analyses were carried out in R2.15.1 (R Development Core Team 2012) using generalised linear mixed models (GLMMs) (Bates et al. 2013). Following Forstmeier and Schielzeth (2011), we included in the tested models only factors and interactions for which we had an a priori reasons for including, which were treatment (treatment or control) and training (trained or naive), or were clearly strongly affecting our results, i.e. time (first 10 min or last 50 min), and all the interactions between these factors. To test whether the training order affected recall, training order (odour presented=odour trained first or odour presented≠odour trained first) and its interaction was added as an additional factor in a separate model. Colony was added as a random effect. Data were modelled using a binomial distribution with the logit link function. Interactions were explored using sub-setting. For example, to explore an interaction between treatment and training, the data would be split into trained and untrained ants, and then the effect of treatment was tested in both these subsets. All *P* values are corrected using the Benjamini-Hochberg method (Benjamini and Hochberg 1995) to control for false discovery rates. In control experiments, where no odour is presented, the side of the T-maze indicated by the odour which would be presented in the test phase was arbitrarily defined as the 'correct' side.

Results When data collection began, there was an initial burst of activity with many ants entering the maze. This burst in activity dropped to a low rate of ants entering the maze after the first few minutes. This can be seen by comparing sample sizes for naive ants in the first 10 and last 50 min (see Figs. 1, 2 and 3).

Trained ants chose the direction implied by the odour stimulus with high accuracy (89 % correct choices) in the last 50 min of the experiment. However, the accuracy of trained ants was much lower in the first 10 min of the experiment (57 % correct choices) and not different from untrained ants or ants which were not provided with an odour cue (see Fig. 1). This can be seen from a significant three-way interaction between odour cue provision (scent or no scent), training (trained or untrained) and time within the experiment (first 10 min or last 50 min) ($Z=-5.454$, $P<0.0001$). When no odour cue was presented, there was no effect of either training
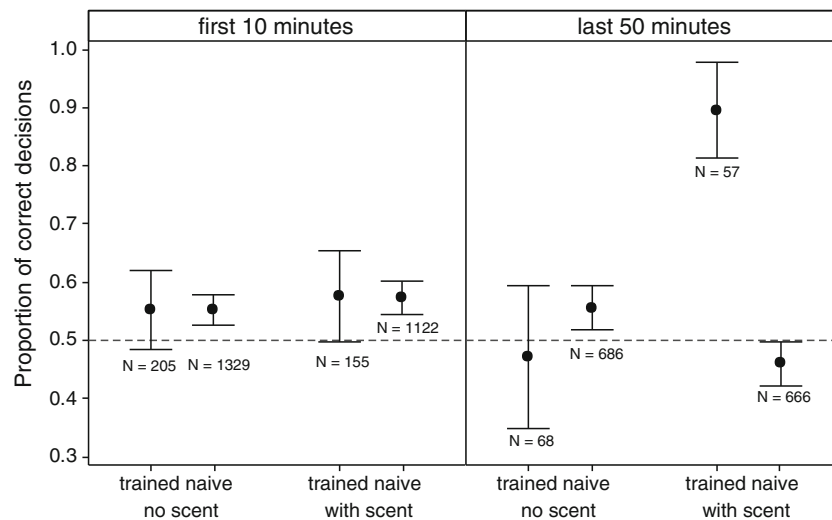
Fig. 1 Proportion of ants choosing the correct branch on a T-bifurcation depends on whether the ants were previously trained, whether a scent cue was presented and when during the experiment the ants made their decision. Trained ants had made three visits on the previous day to a lemon-scented feeder on the left branch of a T-maze and three visits to a rosemary-scented feeder on the right side of a T-maze. They were then presented with either lemon or rosemary odour the following day. Choices were considered correct if they were left with lemon and right with rosemary odour. *Dots* are means and *whiskers* are 95 % confidence intervals

($Z=-0.211$, $P=0.833$), experiment time ($Z=-1.561$, $P=0.256$) or their interaction ($Z=1.305$, $P=0.256$). However, when an odour cue was present, there was a significant interaction between training and experiment time ($Z=-5.482$, $P<0.0001$): in the first 10 min of the experiment, training did not have a significant effect on whether ants choose the correct branch of the T-bifurcation ($Z=0.038$, $P=0.97$), while in the last 50 min, training had a strong positive effect on the chance of ants choosing the correct branch ($Z=5.735$, $P<0.0001$). There was no significant effect of training order on whether or not ants made a correct decision: ants which were trained first to the side corresponding to the odour which would eventually be presented were neither more nor less accurate than ants first trained with the odour that was not presented the next day ($Z=0.115$, $P=0.923$).

Experiment 2—triggering retrieval of appropriate route memories using odour cues, with added home-range markings

Motivation To confirm the results of experiment 1, we repeated the experiment again. In experiment 1, ants were allowed to explore the maze for ca. 45 min before the control treatment and ca. 2 h before the test treatment. This may have resulted in a differing level of home-range markings: as *L. niger* foragers walk, they passively deposit cuticular hydrocarbons on the substrate, which can affect ant behaviour (Yamaoka and Akino 1994; Devigne et al. 2004; Czaczkes et al. 2011).

Methods To reduce the difference in home-range marking strengths between the control and test treatment, the paper

Fig. 2 Proportion of ants choosing the correct branch on a T-bifurcation depends on whether the ants were previously trained, whether a scent cue was presented and when during the experiment the ants made their decision. In this experiment, the ants were able to mark the apparatus with home-range markings for ca. 24 h before use
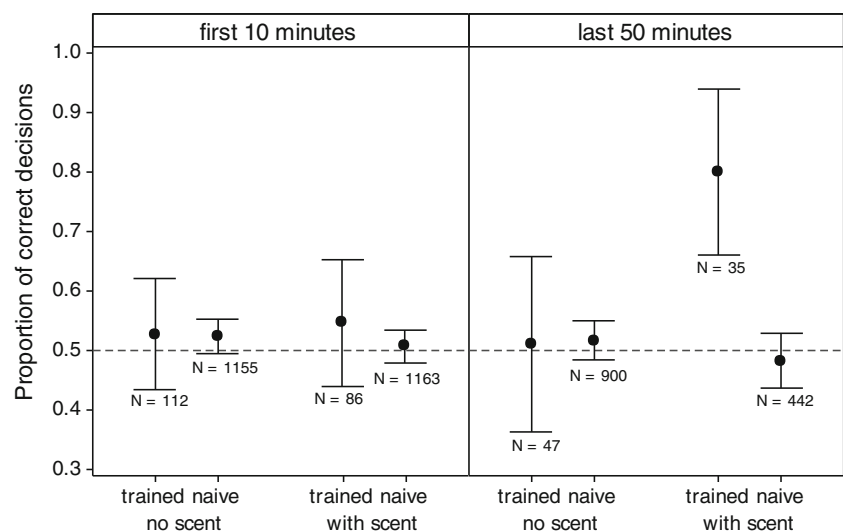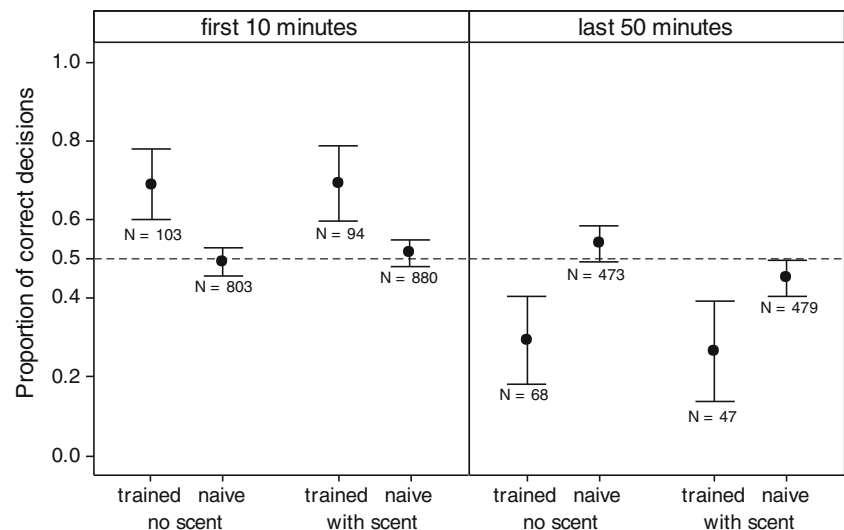
Fig. 3 Proportion of ants choosing the correct branch on a T-bifurcation depends on whether the ants were previously trained, whether a scent cue was presented and when during the experiment the ants made their decision. In this experiment, no recruitment was allowed before testing. The ants are thus in a very different 'motivational state' to those in experiments 1 and 2

substrate used for the bridge and T-maze overlays were left overnight in the nest boxes of the colonies to be tested, where they were explored by the ants and thus marked with home-range markings. Otherwise, experiments were carried out identically to the previous experiment.

Results The results of this experiment are qualitatively identical to the results of experiment 1 (see Fig. 2), although the *P* values are somewhat larger due to smaller sample sizes. We found a significant three-way interaction between odour cue provision, training and time within the experiment ($Z=2.416$, $P=0.0403$). When no odour cue was presented, there was no effect of either training ($Z=-0.105$, $P=0.916$), experiment time ($Z=-0.343$, $P=0.916$) or their interaction ($Z=0.369$, $P=0.916$). However, when an odour cue was present, there was a significant interaction between training and experiment time ($Z=-2.729$, $P=0.0171$): in the first 10 min of the experiment, training had no significant effect on whether ants chose the correct branch of the T-bifurcation ($Z=-1.004$, $P=0.345$), while in the last 50 min, training had a strong positive effect on the likelihood of ants choosing the correct branch ($Z=3.368$, $P=0.00104$).

Experiment 3—triggering retrieval of appropriate route memories using odour cues without recruitment

Motivation An enigmatic aspect of the results from experiment 1 was that learning only became apparent after the first 10 min of the experiment. A possible explanation for this would be that in the first 10 min, the trained ants which are tested had been actively recruited by the 20 ants allowed to return to the nest with food via a separate bridge. The ants in the first 10 min may therefore have been in a motivational state of a recruit. The ants in the last 50 min may not have been recruited and thus may have been in a 'scouting' motivational

state. As none of the ants had found food on the apparatus that day, none of them could be in an actively foraging motivational state, i.e. shuttling back and forth from a known food source. Ants in different motivational states may respond to cues or signals differently (Witte and Maschwitz 2002; Buehlmann et al. 2012, 2013).

Methods In this experiment, we tested the possibility that motivational states are affecting ant behaviour by repeating experiment 1, except that no recruitment was allowed: no ants were allowed to feed and return to the nest before the experiment began (or during the experiment, as in the previous experiments). As there was no evidence from experiment 1 that the order in which the odours were trained affected memory recall, in this experiment, the odour the ants were trained to first was also always the odour presented in the experiment.

Results The results are puzzling and different from those of experiments 1 and 2. In the first 10 min of the experiment, trained ants were likely to choose the correct side, while naive ants showed no preference. However, this was true both during the test phase of the experiment *and during the control treatment*, in which the ants did not have any information about where the odour would eventually be presented. Exactly the reverse pattern was seen in the last 50 min. Again, the naive ants did not show a side preference, but the trained ants were likely to choose the wrong side during both the test *and control* treatments (see Fig. 3). This pattern can be seen in a significant interaction between training (trained or untrained) and time (first 10 min or last 50 min) ($Z=5.179$, $P<0.0001$). In the first 10 min, trained ants were significantly more likely to choose the correct branch ($Z=4.786$, $P<0.0001$), and in the last 50 min, trained ants were significantly more likely to choose the incorrect branch ($Z=-4.239$,

$P<0.0001$). Treatment (whether an odour was presented or not) had no significant effect ($Z=0.033$, $P=0.973$) neither did a three-way interaction between treatment, training and time ($Z=-0.535$, $P=0.916$).

Discussion

The results of experiments 1 and 2 show that *L. niger* foragers are indeed capable of storing two different food locations in their memory, associating each location with a separate cue (in this case, an odour) and responding appropriately to this cue if presented in the future by recalling the appropriate memory. Scents distributed during trophallaxis or in the air can trigger recall of specific route memories. Ants presumably use this ability in the field, where memories may be triggered by odour cues in the form of food returned by nest mates or mass blooming of scented flowers, for example. Honeydew from different aphid colonies may also have idiosyncratic odours. As the memory of the ants was tested 22–26 h after training, our results also show that route memory in *L. niger* is stable for long periods of time, even after only a very limited number (3) of visits to a feeder.

The apparent lack of any recall in the first 10 min of experiments 1 and 2 is intriguing. Why did the ants seem to ignore their memory in the first 10 min after recruitment and access to the maze? One possibility may have been that memory is only relied upon when other cues, such as strong home-range markings, are present. Strong home-range markings may act as a reassurance, as trail pheromones do (Czaczkes et al. 2011), making ants more likely to attempt to rely on their own memories rather than to explore. The ants in the first 10 min may have deposited enough home-range markings by running on the maze so as to trigger memory use in subsequent ants. However, we controlled for home-range marking effects in experiment 2 by allowing ants to walk over the substrate for ca. 24 h, which should have resulted in uniform strong home-range markings. Nonetheless, the results from experiment 2 are almost identical to those of experiment 1. A second possibility is that ants have to be in a certain motivational state before using cues to recall route memories. We had hypothesised that successful foragers returning to the nest may put the other ants in a 'recruited' motivational state. In honey bees, bees which follow a waggle dance and are thus 'recruited' will use this as a signal to reactivate their own memories. They will fly out of the nest in search of food in the location they last foraged successfully, even if the dance is directed towards a different location (Grüter et al. 2008). We had thus hypothesised that in ants, recruitment would act to reactivate route memories. However, perhaps this is not the case, and recruited ants attempt to follow or search for a pheromone trail, completely shutting off any attempt to follow route memories. However,

this seems unlikely, since when pheromone trail and route memory information conflict, *L. niger* foragers preferentially follow route memories (Grüter et al. 2011). Nevertheless, perhaps in the first 10 min, ants in a 'recruited' motivational state were tested (those ants that had been successfully recruited, so climbed onto the apparatus quickly), and in the last 50 min, ants in a 'scouting' motivational state were tested. These were thus ants which had not been recruited and so, only by chance, entered the apparatus while searching randomly for food or have 'given up' searching for a pheromone trail and switched to using route memories. To test this, we performed experiment 3. However, the results of experiment 3 could neither confirm nor rule out motivational state as the cause of the 'first 10 min' effect. Lastly, it may be that in the first 10 min, a small proportion of the outgoing ants had deposited fresh pheromone on the apparatus. While rare, it is known that in starved colonies, a small proportion (27 %) of recruited *L. niger* ants which receive food via trophallaxis will deposit pheromone even on their first outgoing journey to the food source (Mailleux et al. 2011). There is also evidence that the simple presence of trail pheromone, even without a directional information component, can act as a 'reassurance' to route memory (Czaczkes et al. 2011, 2012). Thus, it is possible that the ants in the first 10 min deposited small amounts of pheromone on the apparatus, which had the effect of enhancing memory use by the ants in the last 50 min. Unfortunately, we could not experimentally test this possibility.

The results of experiment 3 are also surprising. In the first 10 min, trained foragers significantly chose the 'correct' direction more often and significantly chose the 'incorrect' direction in the last 50 min, even in control trials. The only possible explanation must be linked to the training order of the ants, as in experiments 2 and 3 trained ants were always first trained to the 'correct' side (the odour which would be presented the next day) and then trained to the 'incorrect' side. Thus, the results of experiment 3 could also be rephrased as 'in the first 10 min of the experiment, trained ants chose the side to which they were trained first, and in the last 50 min, trained ants chose the side to which they were trained second'. However, in experiment 1, the odour which was trained first was the odour presented during testing in only half of the trials, and we found no significant effect of training order on recall or trail choice. Why training order should only have an effect when no recruitment is involved is unclear. Nonetheless, these results do demonstrate that motivational state can have a very strong effect on behaviour and memory recall.

In terms of social insect foraging experiments, we think a clear distinction should be made between workers which are 'scouting' (not recruited, are not shuttling back and forth to a food source and are searching for food), 'recruited' (have experienced recruitment of some sort and are outgoing in search of food) and 'following memory/foraging' (have been

to the food source recently, are shuttling back and forth to a food source). For example, differences in motivational states are likely to explain the different results reported for pheromone trail following in *L. niger* in the studies by Grüter et al. (2011) and Czaczkes et al. (2012), suggesting that the response of foragers to signals, as well as cues, can be affected by motivational states. In the study by Grüter et al. (2011), many of the tested ants were likely in the 'following memory/ foraging' motivational state, hence showed poor trail following (62–70 % accuracy), while in the work of Czaczkes et al. (2012), naive ants were tested, which were likely in the 'recruited' motivational state, hence the higher trail following (75–85 % accuracy on a more challenging maze). The consistent difference in our experiment between the behaviour of ants during the first 10 min and the last 50 min is conspicuous and not easy to explain. This difference may be indicative of different sub-groups of ants or ants in different motivational states. For example, Witte and Maschwitz (2002) found that worker army ants only reacted to pygidial gland secretions during emigration and only reacted to poison gland secretions during a raid. Buehlmann et al. (2012, 2013) report that returning desert ants ignore nest-odour plumes unless they have path integration information suggesting they are near the nest, but outgoing ants do not ignore food-related odour plumes. Similarly, in our experiment, ants in one motivational state or sub-group may respond more rapidly to the sudden presence of a new area to explore and/or recruitment, while ants in another motivational state or sub-group may respond more slowly or not at all. Thus, the ants in the first sub-group may enter the maze and be measured within the first 10 min, and this same sub-group may also ignore route memories and have a preference for searching for the first-trained food location. Ants in the second sub-group may enter the maze more slowly (thus mostly in the last 50 min), follow route memories well and have a preference for searching for the last food location they were trained to.

Overall, our results highlight the complexity of ant navigation and the interplay of experience, private information (memory), social information (recruitment) and motivational state. While the ultimate reasons behind the '10-min effect' and the motivational state effects are still unclear, the importance of motivational states is very clear. It is now clear that that motivation should be explicitly considered when performing any cognitive or behavioural tests. Ignoring such effects may lead to unclear or incomplete results and perhaps even the drawing of false conclusions.

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