Maze Navigation and Route Memorization by Worker Bumblebees (*Bombus impatiens* (Cresson) (Hymenoptera: Apidae)

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Abstract Bumblebees move about their environments by flying and by walking. Most experimental studies have addressed navigation during foraging flights, but we presented our experimental bees with the challenge of learning to navigate while walking as they must do in nature within topographically complex spaces containing their nests. We trained bumblebee workers to navigate complex, nine-channel, mazes in the absence of specific visual, chemical or textural cues. They successfully navigated through complex multi-turn mazes (stereotypical "rat mazes") with several dead-ends by memorizing the entire sequence of appropriate turns, and their choice of correct first turn on entering the maze. Thus, their observed proficiencies indicated that the individual bumblebees had each memorized the maze by learning motor sequences which were not linked to visual, chemical or textural stimuli, and that their memories were triggered by contextual cues associated with the bees' positions in a sequence. Our findings have implications on natural ambulatory activities inside and outside the colony, and even in practical use as vectors of biological control agents.

Keywords Memory · cognition · navigation · memory deterioration · bumblebees · bees

Introduction

In orienting themselves in their environments, animals, especially central-place foragers (i.e. foragers that go from and to a specific location such as a hive or nest), memorize landmarks (visual, olfactory, tactile) and other information and retrieve stored information as necessary as they navigate. Animal navigation, and the cues involved, have been

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studied under manipulated and mapped environments in nature, in enclosed spaces as large as planetaria and as small as laboratory bench-top choice chambers and mazes. Such environments are useful tools to study route navigation and spatial learning in various subject animals (Munn 1950; Olton 1977; Gallistel 1990; Wehner and Menzel 1990; Collett and Zeil 1998; Healy 1998; Chameron et al. 1998; Anderson 2000; Reynolds et al. 2013). It is now well known that ants, bees and wasps are guided by olfactory and visual landmarks when following paths and travelling from and returning to their nests (Baerends 1941; Thorpe 1950; Janzen 1971; Rosengren 1971; Heinrich 1976; Collett 1992; Collett et al. 1992; Wehner 1992; Chittka et al. 1995; Wehner et al. 1996; Thomson et al. 1997; Ohashi and Thomson 2012; Lihoreau et al. 2012).

Bumblebees forage by flying to and from their nests and perform many tasks by walking. Within the nest, the queen, workers and even drones (Cameron 1985) attend the brood, maintain and expand the nest (Sladen 1912; Free and Butler 1959; Goulson 2010). Bumblebees build their nests in piles of stones, underground in cavities, and in buildings. In such places, they must walk and navigate through topographically simple to complex spaces, but few studies have addressed ambulatory navigation in bees. Our study used walk-through mazes to examine the abilities of bumblebees to learn to navigate while walking rather than flying.

There is a long history of maze learning studies in vertebrates, primarily rats, mice, and pigeons (Dale 1988; Anderson 2000). In contrast, few studies have used mazes to study navigation in invertebrates. Most of those studies have used simple, single bifurcation choice chambers (T- or Y-mazes) to examine the sorts of cues used in navigation (e.g. flat worms of the now-infamous Worm Runner's Digest (see McConnell 1966)), crayfish (Tierney and Andrews 2013), cuttlefish (Cartron et al. 2012), fruit fly *Drosophila* spp (Quinn et al. 1974; Hay 1975; Bicker and Spatz 1976; Tully 1984) and especially ants (Hymenoptera; Formicidae) (Goetsch 1957; Schneirla 1941; Bernstein and Bernstein 1969; Muller and Wehner 1988; Chameron et al. 1998; Wilson and Hölldobler 1990). These mazes have also been used to examine cues (visual, olfactory, tactile) used by eusocial bees (Hymenoptera: Apidae), mostly worker caste of western honeybees (*Apis mellifera*) (Menzel 1981, 1990; Kevan and Lane 1985; Zhang et al. 1998; 2000) and worker caste of bumblebees (*Bombus* spp.); (Chittka 1998; Dyer et al. 2007; Dyer et al. 2008; Han et al. 2010). Slightly more complex are multilateral choice chambers (Colton and Samuelson 1976) in which the subject animals face an array of choices, such as of colours (as with Kevan's (1979) multilateral visiometer), shapes (Lehrer et al. 1995), scent (Martin 1965) or drug-laced water (Pick and Yanai 1983). Bumblebees placed in a 12-arm radial choice chamber showed an ability to distinguish radial vs. concentric visual patterns (Plowright et al. 2006; Séguin and Plowright 2008).

Multiple-turn mazes (e.g. stereotypical rat-mazes (Honzik 1936)) have been rarely used to examine navigational capacities by invertebrates. Learning of complex mazes has been demonstrated in ants and bees; they have proven to be skilled at reaching a food source by navigating mazes, mostly with sensory (visual, olfactory, textural) cues provided at choice points (Schneirla 1929; Weiss 1953; Collett et al. 1993; Collett and Baron 1995; Zhang et al. 1996; Zhang et al. 1998, 1999; Chameron et al. 1998). Fruit flies (*Drosophila melanogaster*)

have also been shown to navigate multiple bifurcation mazes with illuminated end points (Hay 1975), in completed darkness (Bicker and Spatz 1976), and with textural cues (Platt et al. 1980). Zhang et al. (1996) studied flying honeybees' abilities to navigate complex labyrinths containing many "dead ends" that involved making a correct choice at each turn to achieve the reward. Zhang et al. (2000) explored worker honeybees' capabilities to navigate in flight through four types of mazes: constant turn, zig-zag turns, irregular and variable turns. Their results showed that the bees could learn to navigate all four types of mazes but performed best in the constant-turn mazes and increasingly poorly in the zig-zag mazes, the irregular mazes, and the variable irregular mazes. Although multi-turn maze learning and ambulatory navigation (such as we have used) may seem unnatural, it may be important in bumblebees' lives within and immediately beyond the nest.

Our Study Examines the Question

Can worker bumblebees navigate complex, multiple-turn, irregular (but not variable) mazes, consisting of several "dead ends" and involving making correct decisions to accomplish the goal of passing through the mazes?

General Methods

Foragers of *Bombus impatiens* (Cresson, 1863) (Hymenoptera: Apoidea) from queen-right colonies of 30–40 workers/colony (supplied by BioBest Biological Systems Canada [Leamington, Ontario]) were used in the experiments. We started with 5 colonies, but used only the three that had the greatest similarities in learning abilities at the very start (see below for similarity criteria (SCs)). We continued to monitor for inter-colony differences in navigation as our experiments progressed (as described below). When not being tested, colonies were provided with a constant supply of pollen prepared from honeybee collected pollen from the Honeybee Research Centre, University of Guelph and freshly made 50 % sugar syrup (w:w). Four different colonies were used in the experiments.

Experiments were conducted in indoor screened flight cages (2.15 m long×1.20 m wide×1.80 m tall) with grey floors. The flight cages were set up inside a flight room illuminated by 10, 60 W daylight fluorescent tubes set for 12 h light (when the experiments were made) and 12 h darkness. A moveable screen on one side of each cage allowed access. One bumblebee colony was connected to a small, outer cage (30× 23×20 cm) that served as a holding area (Fig. 1). The holding area was attached to the main flight cage (testing arena) by gated, wire-mesh tunnels that allowed control of the bees' entry to the flight cage and the maze. During experiments, bees exiting the hive could take only one route through the holding area to the testing arena (the gate on the diagonal route was kept closed). The gates between the holding area and the testing arena were manipulated as needed to allow single bees to enter the testing arena. Once in the testing arena, bees had to navigate through the maze to access a feeding area that was located 165 cm from entrance and exit points. The feeding area consisted of a green Styrofoam plate 45 x 35 x 5 cm with 8 holes that held microcentrifuge tubes
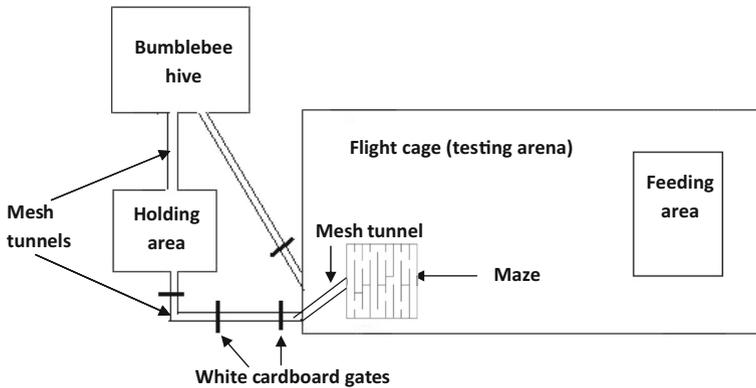
Fig. 1 Experiment setup showing hive, holding area, flight cage testing arena, feeding area and mesh tube (tunnels) routes with gates by which the bees were allowed to enter and exit the flight cage. The bees, in training or as trained, exited from the hive and could take only one route through the holding area to the testing arena in the main flight cage. The exiting bees were not allowed to use the diagonal route because the gate in it was kept closed. The gates after the holding area are opened and closed to allow single bees to enter the maze (Fig. 2) in the testing arena during trials. The bees returned to their hive from the testing area via the diagonal mesh tube route without having to navigate the maze, the gate of which was opened as necessary

(1.5 ml). The tubes, hidden from the bees, were filled with 50 % w:w sucrose solution (syrup) as the reward. The amount of syrup was not controlled, but was replenished as soon as it was exhausted. After foraging in the feeding area, bees were allowed to return to the hive via the diagonal route.

Description of the Maze (Fig. 2)

The maze was 13 x 13 x 2.1 cm with nine rows (channels). The path through the maze contains several dead ends and many turns to right and left. A prototype cardboard (non-washable) maze was made to make initial tests of experimental feasibility. The results were positive (below). Because it is known that bumblebees can leave chemical footprint as signals (Free 1987; Free and Butler 1959; Goulson et al. 2000;
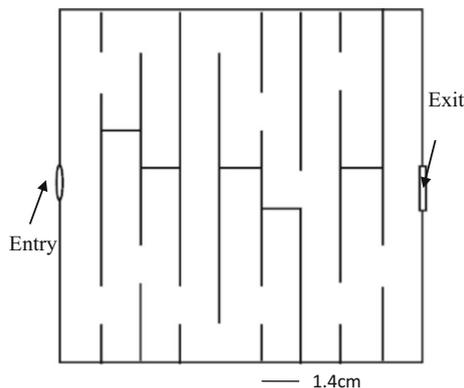

Fig. 2 Multiple-turn maze with several dead ends made from a solid block of white high density polyethylene so that it had the same white colour for the walls and the floor

Saleh et al. 2007; Wilms and Eltz 2008) we then had three identical, washable, mazes made to be used for the experiments reported. They were made of opaque white plastic (high density polyethylene) walls and floor and covered with a transparent Plexiglas lid. In this maze visual cues were absent as there was no colour difference between the walls and the floor. There may have been slight textural differences resulting from the machining that left circular marks on the floor and vertical marks on the walls and perhaps shadows (imperceptible to the investigators) from the diffuse lighting in the flight room. The mazes were sufficiently small and deep that bees within them would be unable to see over the opaque channels. Directly overhead, diffused light from the fluorescent illumination entered the testing arena through the screen roof remained the same in all experiments. The plastic mazes were washed with 70 % ethanol in water and wiped dry after each use to remove any water and alcohol soluble residues (e.g. footprint chemicals of straight-chain saturated and monounsaturated hydrocarbons 21–31 carbon atoms long (Goulson et al. 2000; Wilms and Eltz 2008)) left by the bees.

Prior to the start of the experiments, naïve bees were allowed to forage for syrup in the feeding area for 7–10 days to accustom them to foraging in the experimental arena, but without the maze. During this period, bees had direct access from the hive to the flight cage (i.e. through mesh tubes and holding area) and returning through the diagonal mesh tube. After this period of conditioning was complete, but prior to the start of the experiments, bees were marked individually on their thoracic dorsal surfaces with uniquely numbered and coloured tags (Opalith Plättchen, Christian Graze KG, Germany). Colonies, when not being tested, had constant pollen supplies and their diets supplemented with sugar syrup.

Experiment Procedure

First, the bees were restricted from flying directly to the feeder by having to crawl through a screen tube that opened into the maze. Thus, to forage, the subject had to pass through the maze to exit and then fly to the feeding area. Because some bees took a long time to accomplish the task, or failed to pass through the maze, a criterion of a maximum of 20 min to achieve success was established. Bees that failed to pass through the maze in 20 min were removed from the maze and eliminated from the experiment.

For the behavioural assay of the possible effects of trail-marking chemicals in the prototype cardboard maze, we timed how long it took for each of 15 bees (in the order of their entering the maze) tested to complete their passage through the maze and noted where in the order those bees that failed to navigate the maze came. We reasoned that if the first few bees to encounter the maze had the greatest difficulty and either failed or took longer than the last few bees, the latter bees might have had the benefit of a chemical trail left by previous bees. We tested that hypothesis with Spearman's Rank Correlation. We followed this protocol for all experiments thereafter.

For each successful individual, and each time it passed through the maze, we recorded choices of turning left or right, traveling time (by stop watch, $+/- 0.1$ s) to pass through the maze, and how many mistakes (mistakes measured as turning in the wrong direction and/or once having turned in the correct direction,

changing direction) were made. We also recorded the number of times each bee navigated the maze before it did so without error.

Maze Navigating Experiment

Each individual marked bee was allowed to enter the maze directly through the mesh tunnel leading to the maze in the flight cage. Again, each bee was left to learn its way through the maze without interference by the experimenter. For each individual bee, we recorded the first turn (left or right (correct)) and the amount time (as above) required to navigate through the maze was recorded for 20 passes through the maze (5 passes/ bee in the first day and 15 passes/ bee in the second day). We also noted the number of passes required before the bee could navigate the maze without error.

To assess that the three colonies tested comprised bees with similar in learning abilities (see Raine and Chittka 2008), we used 6 similarity criteria (SCs) derived from the results of our experiments. We compared SCs 1, 2 and 3 as the amounts of time it took the successful bees from each colony (12 of 15, 14 of 15 and 12 of 15 for colonies 1, 2 and 3 respectively) to pass through the maze on their first encounters with it, on their first 5 encounters and their last 5 encounters. SC 4 compared the learning curves by repeated measures ANOVA. SC 5 compared by the exponent of the power function model fitting the learning curve (Ritter and Schooler 2002) and SC 6 compared the ZPDs (Zone of Proximal Development) (after Vygotsky 1987) quantifies learning rates as the difference between a) the time taken to solve a problem at first encounter and b) the time taken after the solution to the problem has been learned (Mirwan and Kevan 2014) for each bee according to its colony of origin.

Data Analysis

Spearman's Rank Correlations were used to test the hypothesis that the order in which the bees entered the mazes was correlated with the time it took the bees to navigate through. If there were a correlation with the first bees taking longer than subsequent bees, then chemical trail marking could be invoked.

A simple chi-square test was used to assess if the bees chose to turn right (R) or left (L) on entering the mazes for the very first time (expectation 1:1::L:R) and in subsequent entries into the mazes (expectation for departure from 1:1::L:R as the bees learned how to navigate).

To test the hypothesis that the skill of the individual bees did not deteriorate overnight, the number of errors made by the bees on their final (5th) trial on Day 1 was compared with the number of errors they made on their first trial on Day 2, we used a pairwise t-test and chi-square tests.

After it was determined that the data sets conformed to the assumptions of normality of distribution, one and two way repeated measurement ANOVA (SAS 2014) was used to a compare the results of the experiments and to analyze our findings (i.e. to test the hypotheses that the colonies did not differ significantly from each other in terms of a) the time the individual bees from each colony

took to navigate through the mazes, b) the number of mistakes made by individual bees, from the three colonies, while traversing the mazes over 20 trials, and c) ZPD.

Results

Possible Influence of Trail-Marking

We found no correlation between the order in which the bees entered the prototype cardboard maze and the time it took the bees to navigate through (Spearman's Rank Correlation=−0.090; p=0.75). In all subsequent experiments in plastic mazes, we obtained similarly insignificant Spearman's Rank Correlations (for three colonies, analyses resulted in a Spearman's Rank Correlation ranging from −0.37 to 0.31 (p= 0.17 to 0.39).

Maze Navigating and Similarities (SCs) Between Colonies

Worker bumblebees (*B. impatiens*) (from colonies 1, 2, and 3) could navigate through the maze without reference to visual cues. With increasing experience, the bees became better and better as they came to navigate the maze at their maximum speeds (Fig. 3). There were no significant differences between the three colonies in the times it took the bees to navigate the maze for the first time (SC 1: $F_{2,14}$=0.45; P=0.65) nor for the first (SC 2: $F_{2,4}$=1.3; P=0.29) or last (SC 3: $F_{2,4}$=2.33; P=0.11) 5 trials. There were no differences in the times it took the bees from the different colonies to navigate the maze over the 20 trials (SC 4) (ANOVA repeated measures $F_{2,19}$=2.07; P=0.14) (Fig. 3). The exponents for the power function models of the learning curves were all similar (SC 5), ranging from −1.23 to–1.38 (Fig. 3). The ZPD values (SC 6) for the bees from each colony did not differ significantly either ($F_{2,11}$=0.15 ; P=0.86). As expected, there were significant differences between trials made during our experiments as the bees became increasingly adept ($F_{2,19}$=90.74; P<0.0001).

The same similarities between colonies can be seen in Fig. 4 for the numbers of mistakes (about 7 mistakes for inexperienced bees on their very first encounter within the maze) vs. less than 1 for experienced bees with perfection (no, or almost no, mistakes) after several trials. The number of mistakes that the bees made declined so that after 2 days and 17 trials they navigated the maze without errors, there were no significant differences between the 3 colonies of bees in the total numbers of mistakes they made over 20 trials ($F_{2,19}$=0.77; P=0.47) but, as noted above, there were significant differences between the trials ($F_{2,19}$=68.38; P<0.0001) (Fig. 4). Because overnight recall of learnt information or skills is imperfect in bumblebees (Keasar et al. 1996) we also examined, for the three colonies, the numbers of mistakes by the individual bees made on the first day for five trials and compared them with the first five trials on the second day. When the number of mistakes made by the bees on their final (5th) trial on Day 1 was compared with the number of mistakes they made on their first trial on Day 2, we found no significant differences over all colonies combined (paired t-test for each bee, t_{38}=1.84; p=0.074) nor in colonies 2 and 3 (χ^2=6.01; p=0.87,
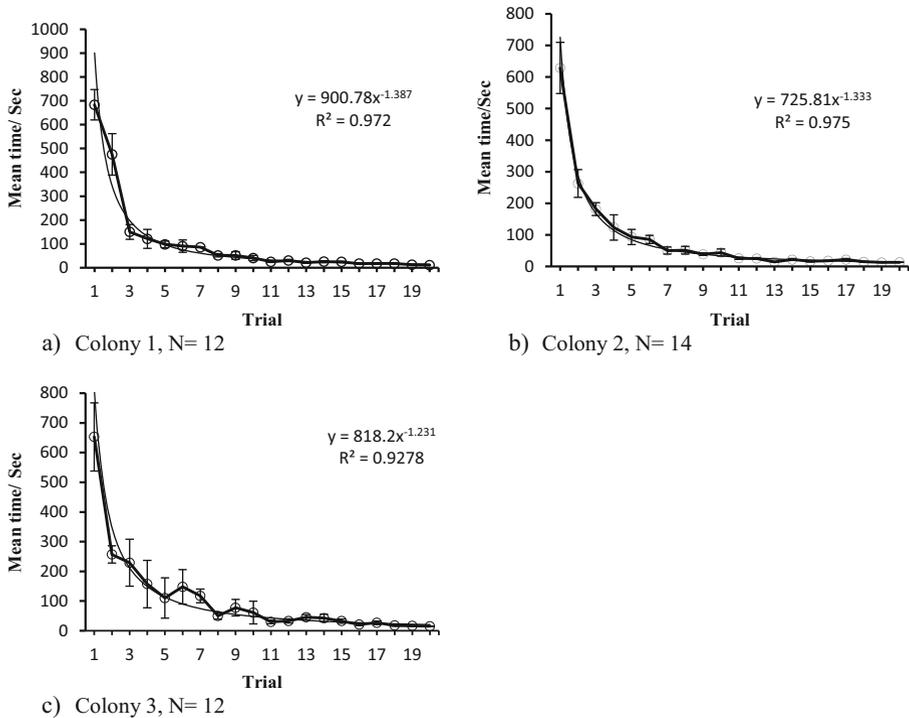
Fig. 3 The mean (±SE) of the durations (seconds) taken by the bees from three different colonies to navigate through the maze from their first encounter (trial 1) to their 20th trial together with the power function model equation and least-squares fit (R^2) for the learning curves

$\chi^2=10.27$; $P=0.50$ respectively), however, there was a significant difference in Colony 1 which showed more errors in the first trial of the 2nd day than on the 5th trial of the first ($\chi^2=27.84$; $P=0.009$).

The maze had the entry hole in the middle of its front wall. The bees had to choose first of all upon entering the maze between turning left or right (the correct direction for continued navigation through the maze). As expected, the inexperienced bees, regardless of colony, made random choices in their very first turns on entering the maze (expected errors by random choice 50 %) ($\chi^2=0.04$; $P=0.84$) (Fig. 5). Once the bees became experienced their error rate on entering the maze was non-random and to the right (correct) ($\chi^2=81.0$; $P<0.0001$) (Fig. 5).

Discussion

We have shown that worker bumblebees (*Bombus impatiens*) can learn to navigate by walking through complex mazes with multiple turns and several dead ends, as used in classical conditioning with vertebrates (Honzik 1936). Our results also indicate that the bees did not use chemical signals from possible trail-marking (Corbet et al. 1984; Free 1987). Throughout our
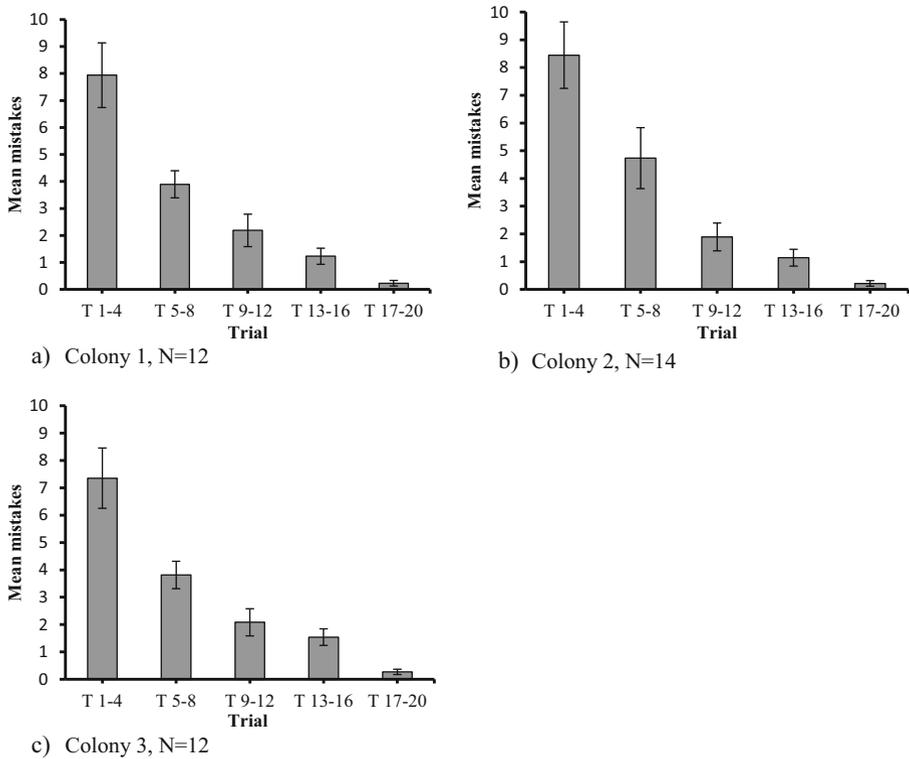
Fig. 4 The mean (±SE) numbers of mistakes made by bees from three different colonies when navigating through the maze. The findings are presented by groups of trials (trials 1–4 combined; 5–8 combined; to 17–20 combined)

experiments, our results indicate that the colonies did not differ in their capacities to learn to navigate.

Our results indicate that foragers of *B. impatiens* have the capacity to negotiate multi-turn maze in the absence of visual and chemical cues. Zhang et al. (2000) suggested that honeybees learn by using a fixed motor program, possibly linked to some form of path integration, as our results also suggest. There are few studies with bumblebees (*Bombus* spp.) and these studies used only simple bifurcation mazes (e.g. Chittka 1998; Chittka and Thomson 1996; 1997) to study cue discrimination (choice) and memory. We are not aware of complex maze-learning experiments made with bumblebees. However, the fascinating results obtained by Reynolds et al. (2013) may have similarities to our findings. They also found that worker bumblebees were able to improve their navigational performances in the complex environment of the field through sequential experiences whereby the subjects learnt, adapted and relearnt so as to reduce and eventually minimize overall travel distances and times while foraging at artificial nectar stations

The bees in our experiment with a complex maze of 9 channels, each with only one successful exit, took a long time (between 10 and 15 mins) at first to
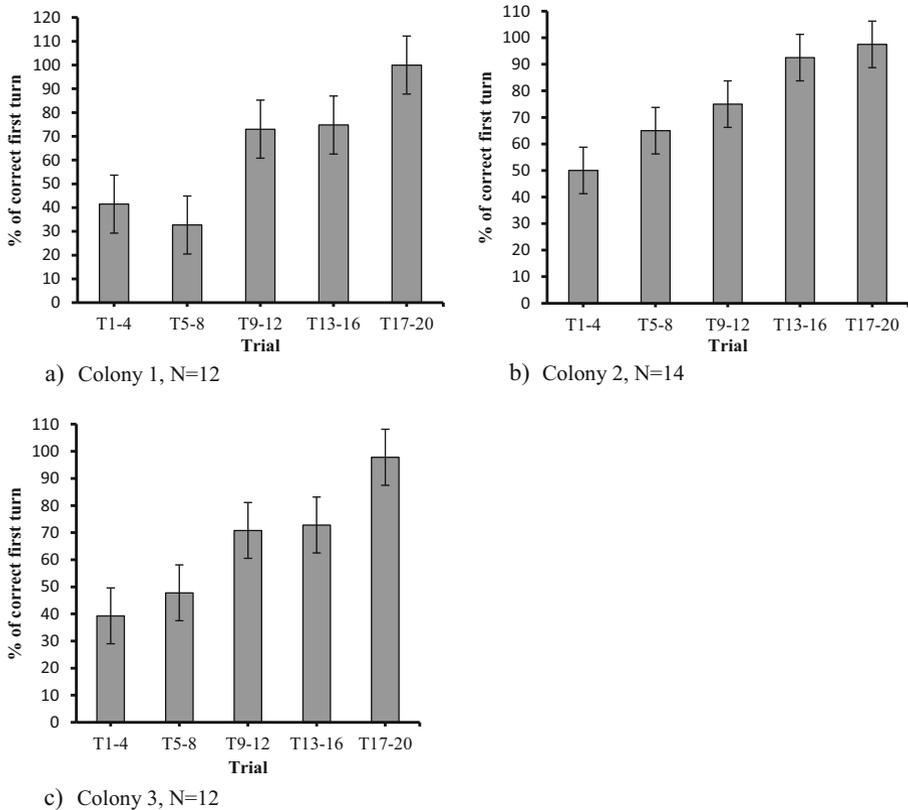
a) Colony 1, N=12

b) Colony 2, N=14

c) Colony 3, N=12

Fig. 5 The percent (±SE) of bee-choices for the correct (to the right) first turn by tested bees entering the maze. The findings are presented by groups of trials (trials 1–4 combined; 5–8 combined; to 17–20 combined)

pass through the maze, and required numerous of attempts to pass through maze without making mistakes, but improved their performances markedly after a few trials (Fig. 4). They navigated the mazes without mistake after 15–17 trials and did so in about 30 s. Even though one might expect some deterioration of performance overnight (Keasar et al. 1996), our results indicate that such was not a significant influence. Once bees had learned to navigate the mazes, there were no differences in their subsequent performances over 20 trials as measured by the number of errors the bees made within the maze or the time they took to successfully traverse it (Figs. 4 and 5).

Although bumblebees forage by flying to and from their nests, they perform many tasks by walking. Within the nest, the queen, workers and even drones (Cameron 1985) attend the brood, maintain and expand the nest (Sladen 1912; Free and Butler 1959). The Amazonian bumblebee, *B. transversalis* (Olivier) even forages for nest-building thatch materials along its established walking trails (Cameron and Whitfield 1996). It is well known that bumblebees build their nests in piles of stones, underground in cavities, and in buildings where they must walk and navigate through topographically simple to complex spaces. Thus, although multi-turn maze learning and ambulatory

navigation (such as we have used) may seem unnatural, it may be important in bumblebees' lives. The practical application of ambulatory navigation is exemplified in the technology that uses foraging bumblebees to deliver biological control agents against crop pests and pathogens from special, somewhat complex (one-way out and one-way in), walk-through dispensers on the hives (Kevan et al. 2008; Kevan et al. 2014).

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