Honeybee Cognition: Aspects of Learning, Memory and Navigation in a Social Insect

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Kognition bei Honigbienen: Aspekte zu Lernverhalten, Gedächtnis und Navigation bei einem sozialen Insekt

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Submitted by

Mario Pahl

From
Miltenberg

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Submitted on: …………………………………………………………..……

Members of the *Promotionskomitee*:

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Primary Supervisor: Prof. Dr. Jürgen Tautz

Supervisor (Second): Prof. Dr. Shaowu Zhang

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Journal of Experimental Biology 209, 4420-4428.

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Circadian timed episodic-like memory - a bee knows what to do when, and also where.
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Table of Contents

Summary

Honeybees (*Apis mellifera*) forage on a great variety of plant species, navigate over large distances to crucial resources, and return to communicate the locations of food sources and potential new nest sites to nest mates using a symbolic dance language. In order to achieve this, honeybees have evolved a rich repertoire of adaptive behaviours, some of which were earlier believed to be restricted to vertebrates. In this thesis, I explore the mechanisms involved in honeybee learning, memory, numerical competence and navigation.

Honeybees have the ability to flexibly change their preference for a visual pattern according to the context in which a discrimination task is carried out. In **Chapter 1**, I investigate the effect of the time of day, task, as well as both parameters simultaneously, as contextual cues in modulating bees' choices between competing visual stimuli. Three experimental series were carried out on bees trained to forage in a two-choice Y-maze and return to the hive through another two-choice apparatus. The results of the first series of experiments indicate that bees can reverse their stimulus preference following midday breaks, as well as overnight breaks, at the feeder and at the hive. The results of the second set of experiments show that trained bees can reverse their stimulus preference in just a few minutes, depending on whether they are going out to forage or return to the hive. The third experimental series revealed that, while reversing stimulus preference at the feeder and the hive entrance following midday breaks and overnight breaks, bees can simultaneously choose opposing stimuli at the feeder and the hive within each testing period. Thus, training can impose a learnt stimulus preference on the bees' daily circadian rhythm. This study demonstrates that bees possess a sophisticated memory, and are able to remember tasks within a temporal context. Honeybees can thus 'plan' their activities in time and space, and use context to determine which action to perform, and when.

In **Chapter 2**, I explore how the colour, shape and location of visual stimuli could be memorized within a time frame. The experimental bees were trained to visit two Y-mazes, one of which presented yellow grating stimuli in the morning (vertical rewarded), while the other maze presented blue grating stimuli (horizontal rewarded) in the afternoon. After the

bees had learned to visit the correct maze and choose the correct stimulus according to the time of day and maze location, several transfer tests were carried out. The trained bees could solve tests in which 1) all contextual cues from the training session were present, 2) the colour cue of the stimulus was removed, 3) the location cue was removed by testing the bees in a new maze at a neutral position, 4) the location cue and the shape cue of the stimuli were removed, and 5) the location and colour cues of the stimuli were removed. The results reveal that honeybees can recall the memory of the correct visual stimuli by using spatial and/or temporal information as contexts. The results also allow conclusions about the relative importance of colour, shape, location and circadian time in decision making. The bees' ability to integrate elements of circadian time, place and visual stimuli is akin to episodic-like memory as described in food-caching birds, and we have therefore named it 'circadian-timed episodic-like memory'.

Numerical abilities have been studied mainly in vertebrate species, and in **Chapter 3** I attempt to shift the balance more in favour of the invertebrates. Honeybees, by virtue of their other impressive cognitive features, are prime candidates for investigations of this nature. Using the delayed match-to-sample (DMTS) paradigm, the limits of the bees' ability to match two visual stimuli solely on the basis of the shared number of present elements were tested. After the experimental animals had learned the basic DMTS task in a modified Y-maze, they were able to discriminate patterns containing two or three elements. Without any additional training, they could distinguish stimuli containing novel objects, and even stimuli containing three or four elements. However, the bees could not discriminate higher numbers such as four versus five, four versus six, or five versus six. A series of control experiments confirmed that the bees were not using lower order cues such as the colour or configuration of elements, combined area or edge lengths of elements, or illusory contours formed by the elements. The number four as a limit is not only found in bees; most animals that have been shown to possess some form of numerical competence are able to discriminate up to four, but not more. The implications of this limited sense of number are discussed in this thesis. This is the first report of number-based visual generalisation in an invertebrate.

Honeybees frequently fly large distances to and from vital resources, and communicate the locations of those resources to nest mates by a symbolic language. In

Chapter 4, I explore the limits of forager bees' navigational capabilities, and discuss the mechanisms facilitating this amazing feat. Foraging bees memorise landmarks and the skyline panorama, use the sun and polarized skylight as compasses, and integrate distances and angles of their outbound flight paths in order to keep a constantly updated homeward vector. In the experiments described in this thesis, foraging bees were caught upon return to the hive, equipped with a radio frequency identification (RFID) chip, displaced to various locations in a black box, and released in order to record their homing times and homing rates from familiar and unfamiliar locations. This procedure ensured that the bees had to rely on previously memorized features of the landscape in order to fly back to the hive, as their homewards vector was zeroed, and they could not perceive distance or direction during displacement. The results reveal that homing rate, speed and maximum homing distance depend on release direction. Bees released east of the hive were more likely to return home, and arrived at the hive faster, than bees released in any other direction. The findings suggest that such large scale homing, from up to 11km in the eastern direction, is facilitated by global landmarks acting as beacons, and possibly the entire skyline panorama.

The findings acquired in this thesis show that honeybees are not the simple reflex automats they were once believed to be. The level of sophistication I found in the bees' memory, their learning ability, their time sense, their numerical competence and their navigational abilities are surprisingly similar to the results obtained in comparable experiments with vertebrates. Thus, we should reconsider the notion that a bigger brain automatically indicates higher intelligence.

Zusammenfassung

Honigbienen (*Apis mellifera*) furagieren an vielen verschiedenen Pflanzenarten, und navigieren über große Distanzen zu wichtigen Ressourcen. Die räumliche Lage von Futterquellen und potentiellen neuen Nistplätzen teilen sie ihren Nestgenossinnen mithilfe einer symbolischen Tanzsprache mit. Um all dies leisten zu können, haben sie ein reiches Repertoire von adaptiven Verhaltensweisen evolviert. Mehr und mehr Verhaltensweisen, die man nur bei Vertebraten vermutet hätte, werden auch bei der Honigbiene entdeckt. In meiner Dissertation habe ich einige der Mechanismen erforscht, die beim Lernverhalten, der Gedächtnisbildung, der numerischen Kompetenz und der Navigation eine wichtige Rolle spielen.

Honigbienen können ihre Präferenz für visuelle Stimuli in Abhängigkeit des Kontextes ändern, in dem eine Aufgabe ausgeführt wird. Im **ersten Kapitel** dieser Dissertation stelle ich vor, wie sich die Tageszeit, die Aktivität, sowie beide Parameter gleichzeitig, als Kontextsignale auf die Entscheidungsfindung der Bienen auswirken. Die Bienen wurden dressiert, an der Futterstelle in ein Y-Labyrinth zu fliegen und sich dort zwischen zwei gegensätzlichen visuellen Stimuli – einer belohnt, einer unbelohnt - zu entscheiden. Auch am Stock hatten sie die Wahl zwischen zwei entgegengesetzten visuellen Reizen, von denen jeweils nur einer Einlass in den Stock gewährte. Die Ergebnisse des ersten Experiments zeigen, dass Bienen ihre Stimulus-Präferenz an der Futterstelle sowie am Stock zeitabhängig umkehren können, also vormittags Stimulus A wählen und nachmittags den entgegengesetzten Stimulus B bevorzugen. Im zweiten Experiment wurde gezeigt, dass Bienen ihre Stimulus-Präferenz aktivitätsabhängig innerhalb weniger Minuten ändern können: sie wählen Stimulus A an der Futterstelle, und bei der Heimkehr zum Stock wenige Minuten später bevorzugen sie Stimulus B. Auch das schwierigste, dritte Experiment konnten die Bienen lösen: sie lernten, während sie ihre Stimulus-Präferenz an Stock und Futterstelle zwischen Vor- und Nachmittag umkehrten, auch abhängig vom Hin- und Heimflug entgegengesetzte Stimuli zu wählen. Die Bienen hatten also gelernt, ihre Stimulus-Präferenz mit ihrem circadianen Rhythmus abzustimmen: Sie wählten vormittags A an der Futterstelle und B am Nesteingang, und nachmittags B an der Futterstelle und A am

Nesteingang. Die Ergebnisse zeigen, dass die Honigbiene ein hoch entwickeltes Erinnerungsvermögen besitzt, und in der Lage ist, Aufgaben in einem temporalen sowie aktivitätsabhängigen Kontext zu speichern. Bienen können ihre Aktivitäten also räumlich und zeitlich ‚planen‘, und den Kontext nutzen um zu entscheiden, was wann zu tun ist.

Im **zweiten Kapitel** zeige ich, wie Farbe, Form und Position visueller Stimuli in einem zeitlichen Rahmen gespeichert werden können. Bienen wurden dressiert, zwei Y-Labyrinthe anzufliegen: vormittags Labyrinth A, in dem ein Stimulus aus vertikalen gelben Streifen belohnt wurde, und nachmittags Labyrinth B, in dem ein Stimulus aus blauen, horizontalen Streifen belohnt wurde. Nachdem die Bienen gelernt hatten, zur richtigen Tageszeit am richtigen Labyrinth den belohnten Stimulus zu wählen, wurde eine Reihe von Transfer-Tests durchgeführt. Die dressierten Bienen konnten ohne weiteres Training Aufgaben lösen, in denen 1) alles so war wie während des Trainings, 2) die Farbe des Stimulus entfernt wurde, 3) der Test an einem neutralen Ort in einem dritten Labyrinth stattfand, 4) der Test an einem neutralen Ort stattfand und die Form des Stimulus entfernt wurde, und 5) der Test an einem neutralen Ort stattfand und die Farbe des Stimulus entfernt wurde. Die Ergebnisse zeigen, dass Bienen sich an den korrekten visuellen Stimulus erinnern, in dem sie räumliche und/oder zeitliche Informationen als Kontext verwenden. Außerdem erlauben die Ergebnisse Schlussfolgerungen über den relativen Einfluss von Farbe, Form, Ort und circadianer Zeit auf die Entscheidungsfindung. Die Bienen integrieren also Informationen aus circadianer Zeit, Ort und visuellen Stimuli. Die Fähigkeit, sich zu erinnern was, wann und wo zu tun ist, weist Ähnlichkeiten zu dem episodischen Gedächtnis auf, das bei Futter versteckenden Vögeln nachgewiesen wurde. Deshalb haben wir es ‚circadian gesteuertes episodisches Gedächtnis‘ genannt.

Die Fähigkeit, die Anzahl von Objekten zu erkennen und für Entscheidungen zu nutzen, wurde bisher hauptsächlich an Vertebraten untersucht. In **Kapitel 3** dieser Dissertation bemühe ich mich, die Balance etwas in Richtung der Invertebraten zu verschieben. Honigbienen sind aufgrund ihrer vielseitigen kognitiven Leistungen gute Kandidaten für die Erforschung der ‚numerischen Kompetenz‘. In einem modifizierten Y-labyrinth wurden Bienen dressiert, visuelle Stimuli nur auf der Basis der Anzahl der enthaltenen Objekte abzugleichen. Nachdem die Bienen die grundlegende Regel des Abgleichens gelernt hatten, konnten sie Stimuli unterscheiden, die 2 oder 3 Elemente enthielten. Ohne zusätzliches Training waren sie sogleich in der Lage, neue Stimuli, die neue, noch nie gesehene Objekte enthielten, zu unterscheiden. Sie konnten sogar Stimuli unterscheiden die 3 oder 4 Objekte enthielten, ohne die Anzahl 4 jemals im Training gesehen zu haben. Hier lag allerdings die Grenze ihrer Fähigkeit, Stimuli anhand der Anzahl der enthaltenen Objekte zu unterscheiden: Stimuli mit 4 und 5, 5 und 6 oder 4 und 6 Objekten konnten sie nicht zuverlässig differenzieren. In einer Reihe von Kontrollexperimenten konnte gezeigt werden, dass die Bienen die Stimuli nicht anhand von anderen Reizen wie der Farbe, Anordnung, Fläche, Kantenlänge und illusorischen Konturen der Objekte unterschieden, sondern wirklich deren Anzahl benutzten. Bei den meisten Tieren, die auf ihre numerische Kompetenz getestet wurden, liegt das Limit der erkennbaren Anzahl bei 4. Die Implikationen dieses gemeinsamen Limits werden in dieser Arbeit diskutiert. In dieser Arbeit wurde zum ersten Mal numerische Generalisierung bei Invertebraten demonstriert.

Honigbienen fliegen große Strecken zu wichtigen Ressourcen und zurück zum Nest, und können ihren Stockgenossinnen die Lage dieser Futterquellen sogar in einer symbolischen Sprache mitteilen. In **Kapitel 4** untersuche ich die Grenzen dieser Navigationsfähigkeit, und diskutiere die Mechanismen, die bei der Navigation über lange Distanzen eine Rolle spielen. Sammelbienen prägen sich Landmarken und die Silhouette des Horizonts ein, benutzen den Sonnenstand sowie polarisiertes Himmelslicht als Kompass, und integrieren Flugdistanz und Flugwinkel, um den Vektor zurück zum Stock ständig zu aktualisieren. In den hier beschriebenen Experimenten wurden Sammelbienen bei ihrer Rückkehr zum Stock abgefangen, und mit einem Radiofrequenz-Identifikationschip (RFID) markiert. Danach wurden sie in einer verschlossenen Box zu verschiedenen Auslassorten gebracht und freigelassen, um ihre Heimkehr-Raten und -Zeiten aus bekanntem und unbekanntem Gebiet zu messen. Durch diese Vorgehensweise wurde sichergestellt, dass sich die Bienen nur anhand ihrer Erinnerung an Eigenschaften der Umgebung orientieren konnten. Ihr heimwärts-Vektor war null, da sie am Stock abgefangen wurden. Zudem konnten sie während des Transports keine Richtung oder Distanz wahrnehmen. Die Ergebnisse zeigen, dass die Rate, die Geschwindigkeit und die maximale Distanz, aus der Bienen wieder zum Stock zurückfinden, von der Himmelsrichtung zum Stock abhängen. Bienen, die im Osten freigelassen wurden, fanden öfters und aus größerer Entfernung zurück, und waren schneller wieder am Stock, als Bienen, die in andere Richtungen freigelassen wurden. Die Ergebnisse zeigen, dass die Navigation auf große Distanzen, bis zu 11km in der östlichen Richtung, durch globale Landmarken und die Silhouette des Horizonts ermöglicht wird. Wie diese Landmarken und das Panorama genutzt werden können, um aus großer Distanz zurück zum Stock zu finden, wird in dieser Arbeit diskutiert.

Die Ergebnisse, die in meiner Dissertation erzielt wurden, zeigen dass Honigbienen keineswegs die einfachen, reflexgesteuerten Organismen sind, als die sie lange Zeit angesehen wurden. Die Komplexität die ich im Gedächtnis, der Lernfähigkeit, dem Zeitsinn, der numerischen Kompetenz und der Navigationsfähigkeit der Bienen gefunden habe, ist erstaunlich ähnlich zu den Ergebnissen, die in vergleichbaren Experimenten mit Vertebraten erzielt wurden. Deshalb sollten wir die allgemeine Annahme, dass ein größeres Gehirn automatisch höhere Intelligenz bedeutet, überdenken.

General Introduction

When talking about cognitive processes in animals, the honeybee does not immediately come to mind. In the public opinion a fox is considered clever, dogs can be good learners, elephants have good memory, and some parrots can even imitate language. Our closest relatives, the great apes, are usually considered to be second only to humans when it comes to intelligence. The octopus is the only invertebrate to which we usually attribute some form of curious cleverness, while insects, the largest group of invertebrates, are largely regarded as hardwired reflex automats, without flexible behaviours. Not surprisingly, most of the traditional studies on animal cognition have focused on vertebrates like birds, rats and monkeys, e.g. animals that possess relatively large brains. In the last 30-odd years, however, this imbalance has begun to change. Many researchers have started to realize that understanding how small brains operate is an important step on the way to an understanding of bigger brains like ours (Pahl et al., 2010). In this thesis, I intend to further elucidate some aspects of cognition in the small brain of the honeybee.

Since '*cognition*' is an elusive term with different meanings depending on the context in which it is used, a definition is first required. Animals are constantly exposed to a stream of sensory information that must be successfully harnessed to improve their chances of survival and reproduction. The way in which animals go about doing this, e.g. the mechanisms by which animals acquire, process, store, and act on information from the environment in a flexible and adaptive sense, can be termed *cognition* (Shettleworth, 1998; Giurfa, 2003). The research I conducted for this thesis focuses on the latter part of this sequence of events, namely the manner in which honeybees respond to information through observable behaviours.

Why study honeybees?

A bee brain (Figure 1) contains less than a million neurons (Witthöft, 1967); about five orders of magnitude less than a human brain, which possesses an estimated 85 billion neurons (Williams and Herrup, 1988). However, it would be a mistake to assume that more neurons and larger brains are a prerequisite for complex behaviours. Honeybees have evolved a sophisticated *eusocial* division of labour, in which each bee flexibly carries out different tasks depending on its age and the colony's needs. Arranged in the order of occurrence, these are: cell cleaning, capping the brood, caring for brood, brood heating, tending to the queen, receiving & storing nectar, honey production, removal of detritus, pollen packing, comb building, ventilation, entrance guarding and foraging. Outside the hive, bees fly fast and with precision, navigate large distances to food sources, effectively manipulate different flowers to extract nectar and pollen, and return to communicate those food locations and potential new nest sites to nest mates using a *symbolic dance language* (von Frisch, 1967; Seeley, 1995).

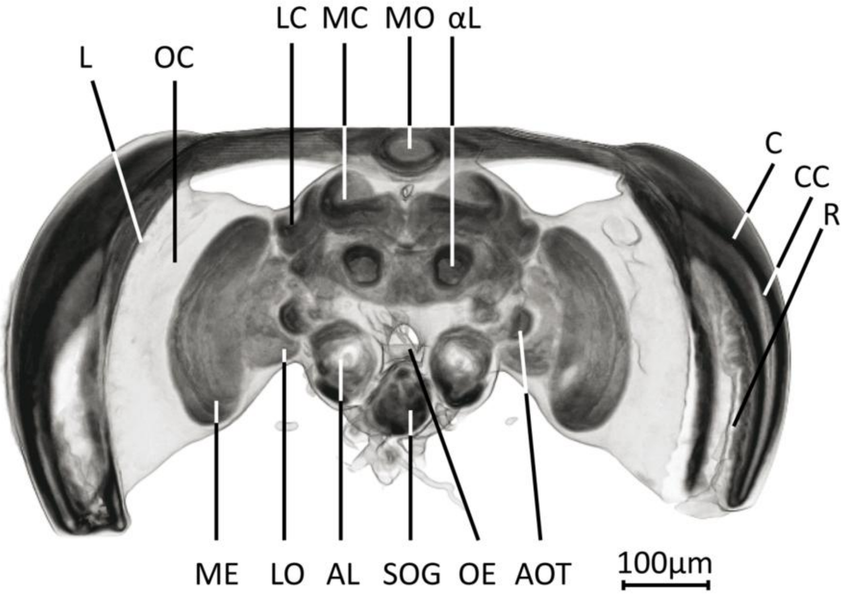

Figure 1: X-ray µCT of the honey bee head, frontal view. AL: antennal lobe, AOT: anterior optic tubercle (Kenyon's optic body) C: cornea, CC: crystalline cone area, LC: left lateral calyx, αL: α lobe, L: lamina, LO: lobula, MC: left median calyx, ME: medulla, MO: median ocellus, OC: outer chiasma, OE: oesophagus, R: retina, SOG: suboesophageal ganglion. Adapted from Ribi et al. (2008).

As central place foragers, honeybees can easily be observed in the hive or at an artificial feeding station. This life style makes honeybees highly suitable for studying the mechanisms of learning, memory and navigation (Pahl et al., 2010). In a free-flight experimental setup, a bee can learn a new odour in just a few rewarded visits to a food source, a new colour in about 5 visits, a new visual pattern after 20-30 visits and a new route to a food source in only 3-4 trips (Zhang, 2006). The underlying navigational skills that make this behaviour possible require efficient information processing and storage mechanisms which, in turn, allow bees to display perceptual and 'cognitive' abilities that are surprisingly rich, complex and flexible (Menzel and Mueller, 1996; Srinivasan et al., 1998a; Srinivasan et al., 1998b; Collett and Collett, 2002; Giurfa, 2003; Zhang and Srinivasan, 2004b; a; Pahl et al., 2010).

The sensory world of the honeybee

As the acquisition of useful information from the outside world is the first step in the cognitive sequence of events defined above, it is pertinent to consider the two main sensory systems of the honeybee – the *eyes* and the *antennae*. Most of the honeybee brain is devoted to processing the sensory input from these structures (Brandt et al., 2005), and it follows that vision and olfaction play critically important roles in honeybee cognition. These two systems are briefly described below.

Vision

The honeybee's *compound eyes* (Figure 2) cover a large part of the head, and consist of approximately 5500 *ommatidia* each (Stuerzl et al., 2010). Since each ommatidium contributes one pixel to the bees' 280° field of view, its visual *acuity* is relatively low. However, since more ommatidia are aimed straight ahead than into the visual periphery, the *resolution* in the frontal field of view is much higher than in the lateral field.

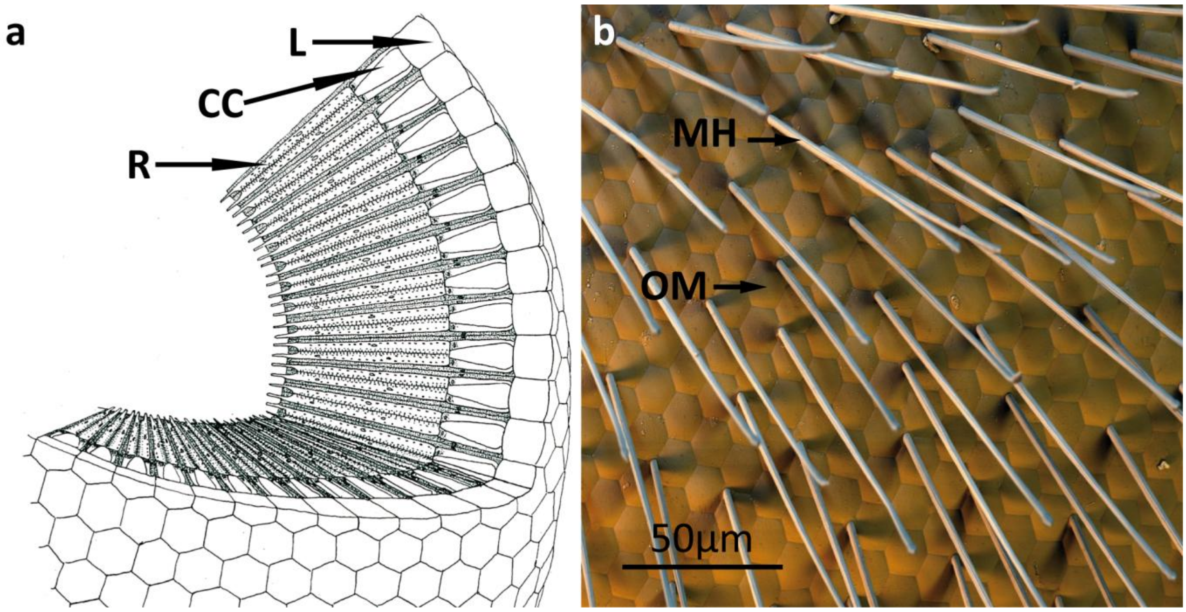

Figure 2 a: Section of the worker compound eye. The eye is built up from individual ommatidia (OM), each with its own lens (L), crystal cone (CC) and photoreceptor cells (R). Adapted from Goodman (2003). b: Lateral view on the compound eye. Mechanoreceptive hairs (MH) lie between ommatidia (OM). Picture courtesy of Stefan Diller.

The bees' ommatidial *frontal acceptance angle* has been measured at 2.6° (Laughlin and Horridge, 1971; Labhart, 1980), compared to about 0.016° in the human fovea (Land, 1999). The low resolution and the eyes' small stereo base limits binocular *depth perception* to very close objects, if it exists at all in honeybees (Labhart, 1980). Thus, a forager has to inspect a flower closely to make out its exact shape. Stürzl et al. have developed a 280° field of view catadioptric imaging system, and developed a model to remap the camera image according to the bee's spatial resolution. This is, at his stage, the best estimate of how honeybees perceive their visual environment. Some of the results are shown in Figure 3.

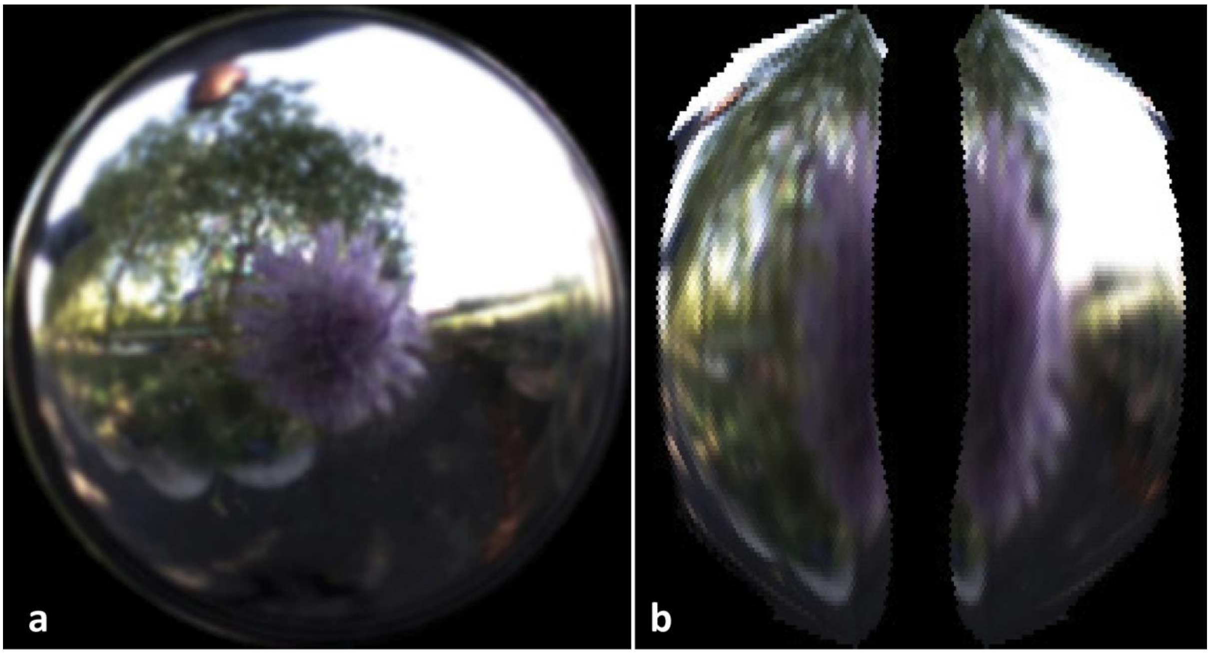

Figure 3: Mimicking honeybee eyes. a: 280° camera image. b: Image mapping according to the bee's ommatidial angles. The clover flower is significantly enlarged due to the higher resolution in the centre of the eyes. Adapted from Stürzl et al. (2010).

The wider acceptance angle, while limiting spatial resolution, means in turn, that bees are much more tolerant to *image motion*. In the human eye, the image begins to blur at an angular speed of $1°s^{-1}$, and all high spatial frequency information is lost at only $3°s^{-1}$. Bees, however, tolerate angular velocities of $100°s^{-1}$ without significant resolution loss. (Land, 1999; Srinivasan et al., 1999). Almost one century ago, later Nobel laureate Karl von Frisch showed, with a simple and elegant behavioural experiment, that honeybees have *trichromatic colour vision* (von Frisch, 1914). With receptors sensitive to ultraviolet (UV), blue and green light (Menzel and Blakers, 1976), the bees' *visual spectrum* is shifted into the shorter wave spectrum as compared to the human colour space. Thus, the colour red appears black to bees, but they can see UV light. The short wavelengths UV receptor is also involved in the detection of linearly polarized light in the dorsal rim area of the compound eyes (Rossel and Wehner, 1986). The perception and use of polarized light is further described below. Many blossoms carry UV-reflecting patterns called *stigmata*, and bees can use this information to discriminate flowers (Chittka et al., 1994). The green receptor is, apart from colour vision, used for *edge-* and *movement detection* (Lehrer et al., 1990), including distance estimation by *optic flow* (Chittka and Tautz, 2003).

In addition to its compound eyes, the bee also features three simple eyes arranged in a triangle on the top of its head, the dorsal *ocelli*. Unlike the compound eye or the vertebrate lens eye, these are not designed for the reception and analysis of spatial information. The high aperture dioptrics, combined with a wide field lens, makes the ocelli highly light sensitive, at the expense of acuity. During flight, the forward facing ocellus is aimed at the frontal horizon, while the lateral ocelli look at the sideward horizon. They detect rapid changes in light levels, such as in pitch and roll manoeuvres, and thus facilitate *flight stabilisation* (Goodman, 2003).

Olfaction

As the research conducted for this thesis focuses on visually guided behaviours, I will only briefly describe the bees' olfactory domain. Olfaction is important for navigation, foraging and social behaviours in the hive. Floral scents guide honeybees to profitable flowers to forage on, and they can amplify spatial memories of these food locations. Once a scent is associated with a food source, re-encountering this scent inside the hive (for example on a returning forager who has been collecting pollen on the same flower species) is enough to trigger the navigational memories, and cause the bee to revisit the food site (Johnson and Wenner, 1966; Reinhard et al., 2004).

Inside the hive, the *queen pheromone* prevents worker bees from laying haploid eggs and rearing other queens from inseminated eggs, as long as the active queen is young and healthy. Nurse bees can distinguish between queen and worker eggs based on their scent, and destroy eggs from other worker bees (Koehler, 1950). Since each hive has its own special bouquet of hydrocarbons on the bees' cuticulae, they can distinguish between nest mates and foreign bees. The 60000 olfactory receptor cells on the antennae are able to discriminate a nearly unlimited number of odours (Vareschi, 1971). Interestingly, the sensillae are concentrated on the right antenna, and bees respond to odours better when they are trained on their right antenna. This kind of *functional lateralization* is a phenomenon well known in humans and other vertebrates, and has recently been studied in honeybees as well (Letzkus et al., 2006).

Learning & Memory in honeybees

Honeybee memory

The cellular and molecular bases of memory are, despite of the substantial research conducted in this area, still poorly understood. The relatively simple, modular brain of the honeybee may prove to be a valuable window into the basic processes of memory formation (Chittka and Niven, 2009). In insects, the separate visual, olfactory and tactile pathways meet in the mushroom bodies (MB). These centres of integration are the primary sites of learning and memory (Menzel and Giurfa, 2001). Foraging experience leads to a volume increase in the honeybees’ mushroom bodies caused by dendritic outgrowth and branching of the MB’s Kenyon cells (Fahrbach et al., 2003). Recent research has shown that even a single olfactory long term memory, as acquired in PER conditioning, leads to synaptic reorganisation in the MB neuropil (Hourcade et al., 2010). Several phases of memory occur sequentially in the honeybee. The short term memory (STM) is created in a single learning trial, and can lasts for a few minutes. Zhang et al. (2005) have measured the time a bee can keep a visual pattern in working memory in a DMTS experiment. They found that the memory of the stimulus decays exponentially, and lasts for up to 8 seconds. Mid-term memory (MTM) is formed after multiple conditioning trials, and lasts for several hours, a time span in which the bee could be back in the hive or on a successive foraging trip (Menzel, 2009). Long term memory (LTM) can be subdivided into an early (eLTM) and a late (lLTM) stage. eLTM occurs 1-2 days after learning, and involves the translation of new proteins. lLTM follows, and depends on transcription and translation processes. There is evidence that the multiple stages of memory rely on independent pathways: The inhibition of the NR1 subunit of the NMDA receptor in honeybee brains via RNA interference impairs the formation of MTM and eLTM, but not lLTM (Müssig et al., 2010). Congruently, treatment with the transcription-inhibitor actinomycin D leaves STM and MTM intact, but impairs the formation of LTM (Hourcade et al., 2009). In the experiments described in this thesis, the honeybees memorized colours, shapes, times of day, numbers, rules and landmarks over several days in order to solve the tasks we presented them with.

Associative learning

Since the Pavlovian paradigm of *classical conditioning* has been applied to honeybees (Kuwabara, 1957; Bitterman et al., 1983), it is known that bees are capable of elemental forms of learning. Honeybees can be trained to form simple links between two stimuli (in the case of classical conditioning) or between a stimulus and a response (operant conditioning) in a well-controlled way by using the proboscis extension response (PER) paradigm (Giurfa, 2003). In this experimental paradigm, the bee is harnessed in a tube so that the antennae and mouth parts can move freely (Figure 4b). Touching the antennae with a sucrose solution (unconditioned stimulus, US) causes the bee to extend the proboscis in anticipation of food. Scents alone do not elicit the PER in untrained animals. However, when an odour (conditioned stimulus, CS) is presented just a few seconds before the US, the bee forms an association between the US and the CS (forward pairing). Now, the scent alone can elicit the PER (Bitterman et al., 1983). Associative learning can also be demonstrated in free flying bees, where bees learn to fly to a certain rewarded stimulus (i.e. yellow arm of a maze) and to avoid the unrewarded stimulus (i.e. the blue arm of a maze). A maze setup is shown in Figure 4a.

Higher learning

The PER paradigm can be used to study higher, *non-elemental forms of learning* as well. In non-elemental learning, the individual stimuli appear rewarded as often as unrewarded, and the configuration of those stimuli has to be learned in order for a bee to be successful. In the following, I will give some examples of non-elemental learning paradigms that bees can solve. In negative patterning (A+, B+, AB-), the bee learns to respond to the single stimuli A and B, but not the combined stimulus AB. In biconditional discrimination (AB+, CD+, AC-, BD-), the experimental bees learn to respond to only two of the four (or more) possible stimulus combinations. Feature neutral discrimination (AC+, C-, AB-, B+) requires the bee to learn that B and the compound AC predict a reward, but not C and the compound AB (Giurfa, 2003). One example of a learning paradigm that bees cannot solve is the transitive inference task. Benard and Giurfa (2004) concluded that memory constraints prevent the bee from forming transitive associations.

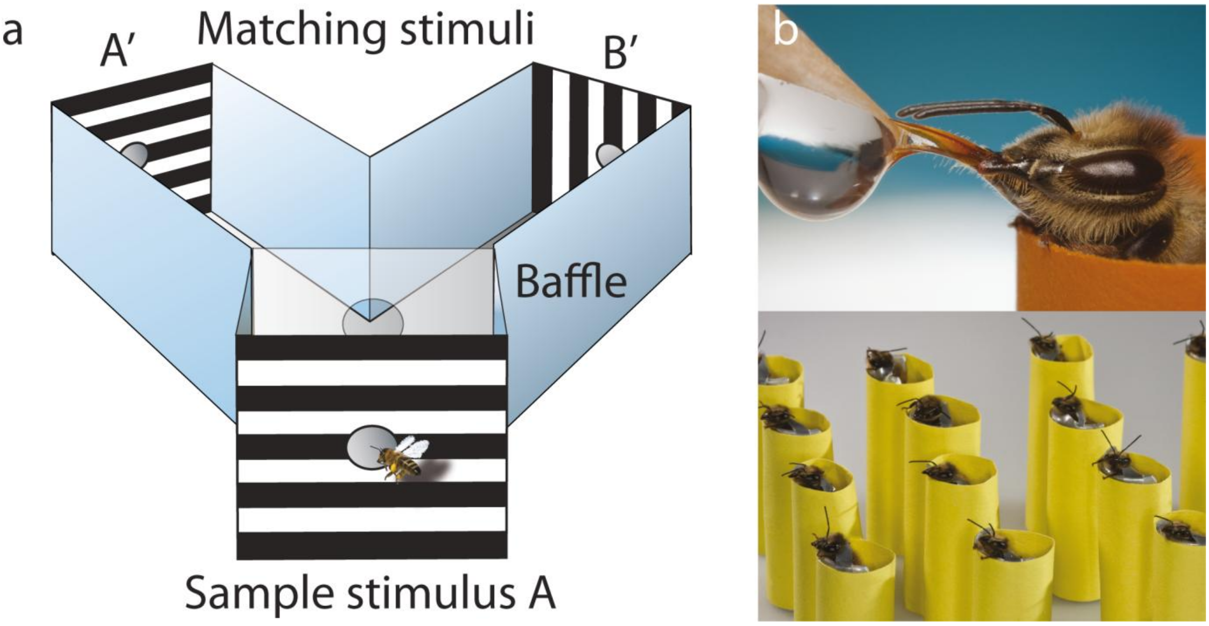

Figure 4 a: Delayed match-to-sample setup in a Y-maze. In order to get a reward, the bee has to memorize sample stimulus A, store it in working memory, and recall it when deciding between one of the two matching stimuli A’ and B’. The baffle prevents the bee from seeing the sample and reward stimuli at the same time. A’ leads to a reward in this example. Adapted from Pahl et al. (2010). b: Proboscis extension response (PER) conditioning in harnessed bees. Touching the antennae with sucrose solution (US) elicits PER in naïve bees, while an odour does not. After forward pairing of the US with an odour (CS), the scent alone leads to PER in the trained bee. Pictures in b courtesy of Helga R Heilmann.

Studying *free flying bees* has the advantage of avoiding the effects of stress experienced in the harnessed bee situation. In this more natural setup, higher learning mechanisms like *categorisation*, *context learning* and *rule learning* can be studied. Bees are easily trained to forage at artificial sucrose feeders, where each visiting bee can be individually marked, and its behaviour observed at the feeder and inside the hive (von Frisch, 1967). Bees can also be trained to visit feeders that are, step by step, moved further into a maze, in which the bee has to make one or more choices between visual stimuli or odours to gain access to the feeder (Figure 4a). The maze can be simply Y-shaped, when a single discrimination task has to be performed (Srinivasan and Lehrer, 1988), or more complex, when the bee is trained to navigate an extensive labyrinth according to symbolic cues or rules (Zhang et al., 1996). A widely used setup to investigate mechanisms of learning and working memory is the *delayed match-to-sample* (DMTS) paradigm (Figure 4a). Established for vertebrate models in cognitive studies such as monkeys (Damato et al., 1985), dolphins (Herman and Gordon,

1974) and pigeons (Roberts, 1972), it has been adapted and used for honeybee studies as well (Srinivasan et al., 1998b). In DMTS experiments, a bee is presented with a sample stimulus A at the maze entrance, followed by a delay (the length of the tunnel to the decision chamber), and is then presented with two (or more) matching stimuli, A' and B'. In order to receive a reward, the animal has to memorize A, and after the delay (e.g. flying through the 1m long tunnel for five seconds), choose the matching stimulus identical to the sample, A' in this case. If B is presented at the entrance, B' is rewarded in the decision chamber (Figure 4a). Having learned this rule, the bee is required to transfer her learned matching rule to novel stimuli, which it has never encountered during training. Both long term and working memory are required for the task: long term memory for the matching rule, and short term memory for the sample pattern (Pahl et al., 2010).

Cognitive strategies

All animals must make decisions throughout their lives when interacting with their surroundings. For honeybees, decision making is necessary inside the hive, for instance when following a waggle dance, and outside the hive when locating a food source, foraging, flying back to the hive or searching for a new nest site. The first evidence that prior experience is involved in visual recognition came from a study by Zhang and Srinivasan in 1994. The results demonstrated that both top-down and bottom-up processes are crucial in pattern recognition: prior experience can facilitate pattern recognition, when the sensory information alone is insufficient in naïve animals to solve a task (Zhang and Srinivasan, 1994). Three important top-down mechanisms that inform decision making in honeybees, as evidenced by behaviour observed in free flying animals, are categorisation, rule learning and contextualisation (Pahl et al., 2010).

Categorisation

Foraging bees need to remember routes to and from different food sources (see also Chapter 4 of this thesis). There can be little doubt that they use some kind of neural 'snapshot' to remember and recognise landscapes and landmarks on these routes (Collett and Cartwright, 1983; Judd and Collett, 1998). However, it is hard to imagine that the bee's brain can store a whole series of images from a travelled route in its restricted memory. One way of coping with this problem is categorisation. This is an information processing strategy in which objects or events are grouped together into categories, so that a similar response can be made to all members of the category (Keller and Schoenfeld, 1950; Troje et al., 1999). Thus, the bee is not restricted to respond only to stimuli it has already encountered, but it can develop a set of appropriate reactions, and generalize across all stimuli that match certain criteria (Wasserman, 1993). The lifestyle of honeybees requires them to remember a number of different patterns, such as the shape of the hive, shapes of nectar bearing flowers and shapes of prominent landmarks (Pahl et al., 2010). In order to compress such image information, and to extract the general identifying features of a pattern, honeybees have evolved the ability to extract orientation (Van Hateren et al., 1990), radial symmetry (Horridge and Zhang, 1995) and bilateral symmetry (Horridge, 1996) including the orientation axis (Giurfa et al., 1996) of visual stimuli. Other characteristics of images such as colour and size can be extracted and memorised as well, without having to store the entire image (Horridge et al., 1992a; Ronacher, 1992). In 2004, Zhang and colleagues showed that bees are capable of categorising similar images of natural scenes as well. In a variant of the DMTS paradigm, where the bees were presented with four matching stimuli, the experimental animals learned to form categories of star-shaped flowers, circular flowers, images of whole plants and images of landscapes (Zhang et al., 2004). Chapter 3 of this thesis will deal with categorisation and generalisation of number (Gross et al., 2009).

Rule learning

The ability to learn general rules for dealing with often-encountered situations is adaptive for an animal, as it removes the need to re-assess situations and try out new strategies each time. Especially in situations that can be categorised by attending to real or abstract cues, the animal can apply a learned set of behaviours (Pahl et al., 2010). Honeybees are great candidates for the study of the acquisition of abstract rules. After all, the waggle dance is the most complex known abstract communication system outside human language. Several studies show that honeybees can not only learn to use visual and olfactory stimuli as signals that indicate a particular action to be performed, but also other abstract cues that are specific to particular experimental situations (Srinivasan et al., 1998b). In 2001, Giurfa et al. showed that honeybees are able to learn the concept of 'sameness' in a DMTS task as described above and in Figure 4. They can even learn the concept of ‘difference’ in a *delayed non-matching to sample* task. In a maze setup similar to that in Figure 4a, the bees were trained to match colours, and transfer-tested on black and white patterns. The bees could solve this transfer, and even performed well in a transfer to the olfactory domain. A new group of bees learned to not-match colours, e.g. they chose blue when yellow was the sample and vice versa. These bees could also transfer the non-matching rule to black and white gratings (Giurfa et al., 2001). The fact that bees can even master abstract inter-relationships was one of the ideas that inspired the study about numerosity discrimination described in Chapter 3 of this thesis. A different study by Zhang and colleagues conducted in 2005 used the DMTS paradigm to measure working memory in honeybees. By varying the length of the tunnel between the sample and the matching patterns, they could vary the delay between the time the sample was memorised and the decision for a matching pattern. The choice level dropped to random at 8.68 seconds, after a flight through 4.75 metres of tunnel (Zhang et al., 2005). Zhang and colleagues also conducted several studies on navigation in complex mazes, in which bees had to learn rules in order to solve the maze. The bees learned to simply follow colour marks, but also to use colours as symbols indicating turns, e.g. ‘turn left at the green stimulus and turn right at the blue stimulus’ (Zhang et al., 1996).

Context learning

Contextual cues are dependent on the environment and the motivation. They can facilitate memory retrieval, when the context in which the memory was encoded is replicated. Thus, context cues help to carve up the world into distinct regions, and help animals cope with possible confusions (Colborn et al., 1999; Fauria et al., 2002; Cheng, 2005; Dale et al., 2005). Collet and Kelber found in their 1988 study that honeybees can retrieve the right landmark memory by the context in which the landmark is placed (Collett and Kelber, 1988). Bees can also change their response to a visual pattern according to whether the stimulus provides access to the hive or the feeder (Gadagkar et al., 1995). Dale et al. demonstrated that honeybees and bumblebees can learn to treat the same visual and olfactory target in different ways in various spatial, temporal or motivational contexts. Such contextual influences are important because they allow honeybees to flexibly adapt to many different situations (Dale et al., 2005). Contextualisation can be seen as the complementary strategy to categorisation: While categories contain different objects or situations that elicit the same behavioural response, the context in which an object or situation is encountered can alter the behavioural response to it. A bee can learn, for example, that dandelion contains nectar in the morning, but not in the afternoon. Thus, using the time of day as a context, a honeybee forager will land on a dandelion flower in the morning, but ignore it in the afternoon and keep searching for clover, which provides nectar in the afternoon but not in the morning (Linné, 1751). How time and motivation act as contextual cues is investigated in Chapter 1 of this thesis (Zhang et al., 2006), and in Chapter 2 I explore how circadian time and location can be used as contextual cues, facilitating the bees' decision making in foraging (Pahl et al., 2007).

The use of number

Numerical competence, or a sense of number, used to be attributed exclusively to humans. However, since Otto Koehler trained his pigeons and Jackdaws to distinguish between clusters containing two or three seeds (Koehler, 1941; 1950), we know that the ability to assess number is not unique to humans. Subitizing is the instant recognition of the number of objects presented, without sequentially counting them. This works precisely with up to four objects, but the precision declines for higher numbers (Jevons, 1871). This distinction of four or more seems to be more or less constant in all animals that have been shown to possess some form of numerical competence, e.g. (Davis, 1984; Kilian et al., 2003; Hunt et al., 2008) and human infants (Feigenson and Carey, 2005). For numbers beyond four, sequential counting, and therefore at least a rudimentary understanding of language, seems to be required, and has indeed been suggested for grey parrots that 'count' up to 6 (Pepperberg, 2006) and chimpanzees, which 'count' up to 9 (Tomonaga and Matsuzawa, 2002). The honeybee, by virtue of its many other cognitive merits, is a prime candidate for investigations of this nature. In Chapter 3 of this thesis, I investigate the bees' use of the number of objects to discriminate between visual stimuli.

Navigation

Central place foragers like honeybees have to keep up a constant flow of resources from the environment to the colony. Therefore, they have to find the way to a food source, and bring the resources back to the nest as efficiently as possible. The different strategies animals have evolved to tackle this problem have been especially well studied in social insects. One aspect makes the honeybee particularly suitable for navigation studies: its famous *dance language*, in which a bee informs her nest mates about the *direction*, *distance* and *attractiveness* of a food source. This symbolic form of communication is performed in total darkness inside the hive on a vertical comb. In the *round dance*, the bee signals a food source up to 50m around the hive, without directional information. In the *waggle dance*, the dancing bee moves in a series of alternating left- and right hand loops, roughly shaped like a figure of eight. In the waggle phase at the end of each loop, the bee shakes her abdomen vigorously from side to side. The *direction* to the resource is encoded in the angle between the vertical gravity line and the axis of the waggle, which corresponds to the angle between the azimuth of the sun and the food source. The duration of the waggle phase communicates the *distance* to the food source (von Frisch, 1967). The 'liveliness' of the dance communicates the *attractiveness* of the food source, which depends on the quality of the nectar, the distance to the source, the handling time of the flower and the colony's current needs. It is signalled by the number of loops performed, and the inter-waggle duration (Seeley et al., 1991; Seeley et al., 2000). This dance can be videotaped and analysed, and thus forms a unique window into the spatial representation of locations in the honeybee brain.

The celestial compass

The direction to a resource is measured by the bees' *celestial compass*. When the sun is visible in the sky, it can be used as a direct directional reference. The dancing forager has to measure the angle between resource, hive and sun azimuth, and translate it to the vertical comb surface. A bee following and reading the dance has to decode it to obtain useful navigational coordinates. Interestingly, bees even compensate for the movement of the sun during the day: in a prolonged dance inside the dark hive, the dancing bee adjusts its dance angle according to the actual sun movement by means of an internal clock (Lindauer, 1954; 1960). During cloudy days, when the sun is not directly visible, bees use a backup system: the *polarized light compass*. Light from the sun is unpolarized, but when entering the earth's atmosphere it is scattered by particles smaller than the wavelengths of the light (which may be molecules or individual atoms). Light that is scattered perpendicular to its initial direction becomes maximally polarized, and creates a pattern across the whole sky. Bees are able to perceive this polarization pattern and can, from just a small patch of visible sky, infer the position of the sun (Rossel and Wehner, 1986). Thus, waggle dances on overcast days are just as precise as on sunny days. Scattered skylight is most polarized in the UV, so the bee's UV photoreceptors are the best instrument to detect the polarization pattern. In the dorsal rim area of the compound eye, the microvilli in each UV-photoreceptor are aligned in the same direction. Thus, the photoreceptor absorbs light maximally if the light's e-vector is parallel to its microvilli, and minimally if the e-vector is orthogonal to them (Srinivasan, 2011). The arrangement of photoreceptors in the ommatidia and the ommatidial arrangement in the dorsal rim of the compound eye facilitate the perception of polarization precise enough to calculate the sun's position from anywhere in the sky.

The bees' odometer

How does a bee measure the *distance* flown to a resource? Early studies suggested that the bee's odometer measured the energy expenditure during flight (von Frisch, 1951; Heran and Wanke, 1952). There was conclusive evidence to support this idea: bees that were made to carry additional weight signalled a greater distance in the waggle dance. The same was true for bees flying uphill; they signalled a greater distance than bees flying the same route downhill (von Frisch, 1967). Later studies however questioned that hypothesis, and suggested that the most important cue is the image movement over the eye the bee experiences during flight (Esch and Burns, 1995; Esch et al., 2001). This *optic flow* hypothesis is supported by several studies, in which bees were trained to fly through a tunnel in order to reach a sugar feeder. When the tunnel walls were covered by a low special frequency pattern (for example, horizontal stripes), the foragers returned and signalled in a round dance that food was available in a radius of 50m around the hive. If the tunnel walls were covered by high spatial frequency patterns (like vertical stripes), however, the bees experienced massive image motion very close to the eye and signalled a distance of about 200m in the waggle dance, although they had flown for only 6m through the tunnel (Srinivasan et al., 2000). These findings also explain the earlier observations where bees signalled a longer distance when an extra weight was attached, or when flying uphill: in both cases the bee was flying closer to the ground, which resulted in greater optic flow and thus a higher, subjective distance travelled. This also means that the distance measure is not absolute, but depends on the environment a bee travels through. The ambiguity in the distance information is not a problem though: the recruit following the dance will fly in the same direction, on the same elevation through the same environment, and thus experience the same optic flow as the dancer has communicated.

Path integration

After determining the way in which bees measure the angle and distance to a food source, we need to find out how bees integrate this information. Bees usually have to circumnavigate obstacles on the way to a resource, and this elongates the route and makes it harder to determine the direct angle. Nevertheless, dancing bees always signal the direct, 'bee-line' angle to the target, even after taking a twisted route to get there (von Frisch, 1967). Wehner and colleagues have shown the same phenomenon in the desert ant *Cataglyphis bicolor.* After finding food at the end of a long and twisted route, it heads home to the nest in a straight line, even in the absence of any landmarks in the environment which could mark the nest entrance (Wehner, 1992). Both animals use a *dead reckoning* system: they continuously integrate the *distance* travelled between turns and the *angle* of each turn, and thus constantly update the *homeward vector*. Path integrating animals constantly keep track of the own position relative to the nest in this way. In bees, the directional angle is measured by the celestial compass, and the odometric information is provided by optic flow (as described above). Obviously, errors accumulate in such a navigation system. In order to prevent this, the directional information can be reset or updated by familiar landmarks near the hive, or by global landmarks in the environment, so that errors do not accumulate excessively (Collett and Collett, 2000a; Collett and Collett, 2000b).

Chapter 1: Honeybee memory: A honeybee knows what to do and when

Abstract

Honeybees have the ability to flexibly change their preference for a visual pattern according to the context in which a discrimination task is carried out. This study investigated the effect of time of day, task, as well as both parameters simultaneously, as contextual cue(s) in modulating bees' preference for a visual pattern. We carried out three series of experiments to investigate these interactions. The first series of experiments indicated that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, at the feeder and at the hive. The second series of experiments showed that trained bees are able to reverse their pattern preference in just a few minutes, depending on whether they are going out to forage or returning to the hive. The third series of experiments demonstrated that trained bees can significantly reverse their pattern preference at the feeder and at the hive entrance following midday breaks, as well as after an overnight break; the bees could also learn to choose different patterns at the feeder and at the hive entrance within each testing period. The training thus imposed a learnt pattern preference on the bees' daily circadian rhythm. This study demonstrates that the bee with a tiny brain possesses a sophisticated memory, and is able to remember tasks within a temporal context. Honeybees can thus 'plan' their activities in time and space, and use context to determine which action to perform and when.

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Introduction

Foraging is essential to a honey bee colony’s survival. To forage successfully, a bee has to learn and remember not only the colour and shape of flowers that contain nectar and pollen, but also how to get to them (Menzel et al., 1996; Collett et al., 2003; Wehner, 2003). Since the species of flowers that are in bloom, say, in the morning are likely to be replaced by a different species at a different location in the afternoon, the bee needs, and has indeed evolved, an impressive ability to learn and memorize local features and routes, as well as the time of blooming, quickly and accurately. Thus, having found a nectar bearing flower at a particular time on a particular day, a forager can remember the task and the time at which it was completed, and visit the flower at the same place and time on the following day (Lindauer, 1960; Gould and Gould, 1988; von Frisch, 1993).

During evolution, honeybees have evolved sophisticated sensory systems and well-developed learning and memorizing capacities, the essential mechanisms of which do not differ drastically from those of vertebrates (Giurfa and Menzel, 1997; Giurfa, 2003; Zhang and Srinivasan, 2004b; a; Dyer et al., 2005). Honeybees also have a time sense, with which they can modulate their response to a local stimulus according to the time of day. Koltermann examined circadian memory rhythm in honeybees, and discovered that honeybees can learn scents or colours in a time linked process, and remember them in a 24h cycle (Koltermann, 1971). Circadian systems permit organisms to measure time for adaptively significant purposes (Moore-Ede et al., 1982). Bees synchronize their behaviour with daily floral rhythms, foraging only when nectar and pollen are at their highest levels. At other times, they remain in the hive, conserving energy that otherwise would be exhausted on non-productive foraging flights (Moore, 2001). Menzel et al. investigated whether and how contextual parameters, such as time of day and features characterizing the location, can be utilized to determine choice behaviour, and claimed that time of day or landmarks cannot by themselves elicit the conditioned response, but can control different behaviours (such as image matching, navigation, timing of motivation to forage) (Menzel et al., 1996). Work carried out in the laboratory of Robinson (Robinson et al., 1989) has shown that there are molecular processes occurring in the brain that influence the division of labour and biological clocks of social insects. With respect to contextual learning by the honeybee, it has recently been shown that honeybees and bumblebees can learn to modulate their responses to local stimuli according to contextual cues. Collett and Kelber (1988), for instance, found that honeybees can retrieve the right landmark-memory by the context in which the landmarks are placed. Honeybees can also learn to change their response to a visual pattern according to whether a stimulus provides access to the hive or to the feeder (Gadagkar et al., 1995). Colborn et al. examined the contextual modulation of visuomotor associations in bumblebees, and reported that different contextual signals are associated with approaching the nest or approaching the feeder, and that these contextual signals facilitate learnt associations between orientation detection and motor commands (Colborn et al., 1999). The modulation of such contextual cues can help honeybees to recognize landmarks from new vantage points (Zhang et al., 1999b; Collett and Collett, 2002) In recent studies, Chittka et al. found that some insects can modulate their response time to perform tasks on a relatively short time scale, depending upon their perceived difficulty and context of a task, in order to make a trade-off between the speed and accuracy of foraging (Chittka et al., 2003; Franks et al., 2003; Dyer and Chittka, 2004). Dale et al. demonstrated that honeybees and bumblebees can learn to treat the same visual and olfactory target in different ways in different spatial, temporal or motivational contexts. Such contextual influences are important in allowing honeybees to flexibly adapt to many different situations (Dale et al., 2005).

However, there has been little experimental work investigating bees’ abilities to modulate their behaviour in response to multiple contextual cues. In the present article, we report that honeybees can learn to simultaneously change their preference for a visual pattern with both the time of day, i.e. whether it is morning or afternoon, and the task at hand, i.e. whether the bee is flying to the feeder or returning to the hive.

Materials and methods

General

The experiments were conducted during the Australian summer at the Australian National University and during the following German summer at Würzburg University. The research project commenced in November 2004 of the Australian summer season, and was carried out in an all-weather bee flight facility (AWBFF) at the Australian National University's Research School of Biological Sciences. The facility consists of a modified glasshouse in which the internal temperature is regulated by a computer to maintain 24.0±5°C during the day and 17.0°C at night, with a relative humidity of ~45%. The experimental hive and a Y-maze setup were located in the AWBFF; the distance between the hive and the Y-maze was 8·m (Movie·1 in supplementary material). Further experiments were carried out in the following German summer season during July and August 2005 at the Bee Station of the Würzburg University, where an observation hive and a Y-maze setup were maintained in two small huts; the distance between the two huts was 24 m (Figs·S1–S2 in supplementary material). About 20 foraging bees (*Apis mellifera L.*) were marked individually at the beginning of each experiment, and trained to visit a feeder with a 0.5 $\mathrm{mol{\cdot}l^{-1}}$ sugar solution in the Y-maze. Bees entering the Y-maze were trained to choose one of two patterns (termed positive) which indicated the position of the feeder. Bees returning to the hive also had to choose between two patterns to access the hive, as only the entrance behind the positive pattern was open during training (Fig. S3 and Movie 2 in supplementary material). During the test periods, both entrances were open. Thus, bees could choose to access the hive through either entrance. An experimental bee had to learn two tasks in one foraging trip, i.e. making a choice in the maze to receive sugar water and then another choice to enter the hive. During training, the positions of the positive and negative patterns at the feeder and the hive were regularly swapped every 30 min, so that the bees could not use position as a cue to either find the feeder or access the hive. Similarly, the positions of the positive and negative patterns were interchanged every 10 min in the middle of testing periods. During the midday break, the visual patterns at the feeder and the hive were removed; the feeder was moved to outside the maze, and both entrances of the hive were opened. The bees could therefore continue to visit the feeder located in the front of the maze, and access the hive through either of the two entrances.

Visual stimuli

The visual patterns (18 cm x 18 cm) were printed on a colour laser printer (Fuji Xerox Document Centre C360 PS colour printer). Gratings oriented at 45° versus 135° in blue/white (see the inset in Fig. 1), sectors versus rings, vertical versus horizontal gratings in black/white or blue/white (see the insets in Fig. 2, Fig. 4 and Fig. S4 in supplementary material) and blue versus yellow colour patterns (see Fig. 3) were used for different experiments.

Training and testing procedures

The present study comprised three series of experiments, and training and testing procedures are described separately as follows:

Series 1

We investigated whether honeybees can modulate their preference for a visual pattern with the time of day.

Experiment 1 of this series was carried out during the Australian summer in the AWBFF at the Australian National University. Here, a pair of blue/white gratings oriented at 45° or 135° to the horizontal was used at the feeder and the hive. In our experimental setup, the choice of the 45°-oriented grating allowed a forager bee access to a feeder and entry to the hive in the morning. In the afternoon, however, a 135°- oriented grating at the feeder as well as the hive became the positive pattern. Experiment 2 of this series was carried out during the German summer at Würzburg University. In Experiment 2, the visual patterns were black/white sectors versus rings at the feeder, and the black/white vertical versus horizontal gratings at the hive. The sectors pattern at the feeder and the vertical grating at the hive were positive patterns in the morning. However, the rings at the feeder and the horizontal gratings at the hive were positive patterns in the afternoon. The tests commenced after 3·days of training for each experiment, and lasted for 3·days.

Series 2

We examined whether honeybees can reverse their pattern preference with the task at hand, i.e. foraging or returning home. Two experiments were conducted during the

Australian summer in the AWBFF at the Australian National University. The yellow pattern was the positive pattern at the feeder, but the blue pattern was the positive pattern at the hive in Experiment 1. The blue pattern at the feeder and the yellow pattern at the hive became the positive patterns in Experiment 2. The tests, that commenced after 3·days of training for each experiment and lasted for 3 days, were conducted at both the feeder and the hive, one in the morning at 11:00·h and another in the afternoon at 16:00 h for 20 min, over 3 days.

Series 3

We examined whether honeybees can simultaneously change their preference for a visual pattern with both the time of day, and the task at hand. The experiment was carried out at the Bee Station of the Würzburg University. In our experimental setup, the choice of a 'horizontal grating' stimulus allowed a forager bee access to a feeder, whereas a 'vertical grating' stimulus allowed entry to the hive in the morning. In the afternoon, however, a vertical grating at the feeder and a horizontal grating at the hive became the positive patterns. In order to facilitate learning this complex task, horizontal and vertical gratings in blue/white were used at the feeder, and gratings in black/white were used at the hive entrance during the training period and the learning test. Three days of trainings were followed by 3 days of learning tests (see Training and learning tests for experimental Series 3 in supplementary material). After the learning tests, the bees were trained to more complex tasks, in which horizontal and vertical gratings in black/white were used at the feeder as well. This was followed by 'critical' tests, which lasted for another 3 days. In the critical tests, the visual patterns at the feeder and the hive were the same, i.e. black/white gratings. The bees were trained continuously between each critical test.

In the experiments of Series 1 and 3, training in the morning started at 09:30 h, and lasted for 3·h, and the afternoon session started at 14:30 h, and lasted for 3·h as well. The tests were carried out four times a day: 09:30 am, i.e. early morning; 11:30 h, i.e. late morning; 14:30 h, i.e. early afternoon; and 16:30 h, i.e. late afternoon. The mid-day break lasted from 12:30 h to 14:30 h. The terms MF1 and MH1 (or MF2 and MH2) denote the early morning (or late morning) tests at the feeder and the hive, and AF1 and AH1 (or AF2 and

AH2) denote the early afternoon (or late afternoon) tests at the feeder and the hive, respectively.

Data analysis

During tests, the first choices of bees at the feeder and the hive were recorded. We performed analyses of variance (ANOVA) across all repeated tests for individual bees and for each type of experimental condition using the statistical software SYSTAT. Thus, the performance of each bee was evaluated separately by pooling its correct choices and visits over all repeated tests, and calculating the ratio of the number of correct choices to the number of visits. The average performance for a particular experimental condition was obtained by averaging choice frequencies across bees. The sample size (N) was the number of bees, rather than the number of individual choices, ensuring that the samples were truly statistically independent. Mean values of choice frequency, standard deviation and standard error of the mean (s.e.m.) were calculated. In the text and in the figures, performance is indicated by the mean choice frequency (± s.e.m.). We used nonlinear regression to approximate the average choice frequency with reference to the specified patterns in Series 1 and 3.

To check whether the task at hand affects pattern preference in Series 2, we carried out statistical tests for each experiment to check for significant differences in performance at the feeder and the hive. To check whether 'time of day', i.e. morning or afternoon affects pattern preference in Series 1 and 3, we carried out the following four statistical tests to check for significant differences in performance: (MF1 vs AF2), (MH1 vs AH2), (MF2 vs AF2) and (MH2 vs AH1). To check the same for 'task', in Series 3, the following four tests were done: (MF1 vs MH1), (MF2 vs MH2), (AF1 vs AH1) and (AF2 vs AH2). x^2 tests were used for all of these comparisons.

Checking bias at the hive and the feeder

As a control, side bias counting was carried out usually in the morning before the experiments started, to check whether the trained bees had developed any side bias in their choices. In the bias counting, the bees' choice performance was measured while two identical visual patterns were presented at the hive or the feeder.

Results

Series 1

Honeybees can learn to reverse their preference for a visual pattern with the time of day, i.e. morning or afternoon.

The first experiment in this series was carried out in the AWBFF at the Australian National University. The trained bees were tested in all of eight different types of test (MF1, MH1, MF2, MH2, AF1, AH1, AF2 and AH2). The results of this experiment, shown in Fig.·1, revealed that the bees modulated their preference to the 45°-oriented grating according to the time of day, both at the feeder (Fig. 1A) and at the hive (Fig. 1B). In the morning, the bees significantly preferred the 45°-oriented grating at the feeder (MF1 and MF2 in Fig. 1A; $P<0.001$) and at the hive (MH1 and MH2 in Fig. 1B; $P<0.001$). Their pattern preference gradually changed to 135° around midday, and reduced to the random choice level of 0.50 in the early afternoon test at the feeder (AF1 in Fig. 1A; $P>0.3$) and at the hive (AH1 in Fig. 1B; $P>0.90$). However, pattern preference was significantly changed to the 135° grating in the late afternoon test, at the feeder (AF2, in Fig. 1A; $P<0.001$) and at the hive (AH2, in Fig. 1B; $P<0.001$). When we compare the average choice frequency in favour of the 45° grating between MF2 and AF1 at the feeder, or MH2 and AH1 at the hive, the results show that the bees' pattern preference has significantly changed in the time between the late morning and the early afternoon tests ($P<0.001$). Similar conclusions can be drawn when we compare the performance in the late afternoon test (AF2 at the feeder, in Fig. 1A and AH2 at the hive, in Fig. 1B) to the following early morning test (MF1 at the feeder, in Fig. 1A and MH1 at the hive, in Fig. 1B). The trained bees significantly reversed their pattern preference from the 135° grating to 45° grating after an overnight break. The modulation of the average choice frequency in favour of the 45° grating can be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. The correlation coefficient for the sinusoidal curve is 0.98 at the feeder and 0.998 at the hive.

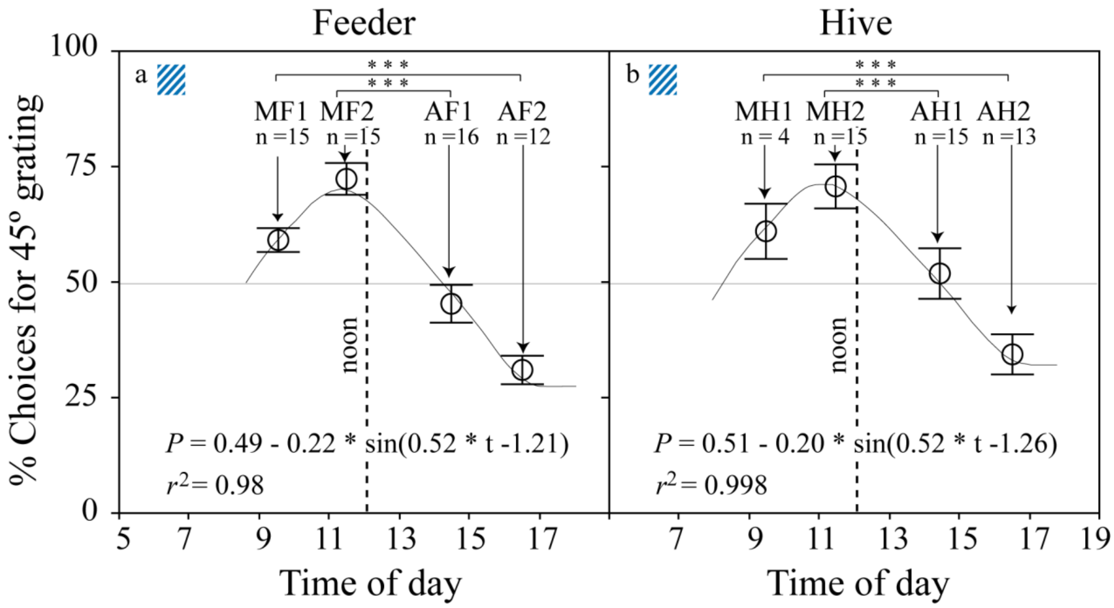


Figure 1: Results of experimental series I carried out in the AWBFF (indoor) at the Australian National University. A pair of blue/white gratings oriented at 45° or 135° to the horizontal was used at the feeder and the hive. The results indicated that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, both at (A) the feeder and (B) the hive. The modulation of the average choice frequency, with reference to 45° grating can be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12·h. The correlation coefficients are 0.98 for the feeder and 0.998 for the hive, respectively. Terminology for the test types: M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. ***p<0.001. See text for further details.

The second experiment in this series was carried out at the Bee Station in Germany. The bees were trained to simultaneously (i) choose the sectors pattern at the feeder to obtain a reward, and the vertical grating to access the hive in the morning; and (ii) the rings pattern at the feeder and the horizontal grating at the hive in the afternoon. The trained bees were tested in all of eight different types of test (MF1, MH1, MF2, MH2, AF1, AH1, AF2 and AH2). The results of this experiment, shown in Fig. 2, revealed:

(i) The average choice frequency for the positive pattern in the all of eight different types of test, except the MH1 test (Fig. 2C), is significantly different to the random choice level of 0.50 (P<0.001). (Fig. 2A,B,D–H). The average choice frequency in the early morning test at the hive (MH1) was 0.56±0.06 (P>0.03, N=14).

(ii) When we compare the average choice frequency between the early morning and the late afternoon tests, i.e. the MF1 (Fig. 2A) and AF2 tests (Fig. 2B) at the feeder, or MH1 (Fig. 2C) and AH2 tests (Fig. 2D) at the hive, we find that the bees’ pattern preference has significantly reversed in the time between the late afternoon test and the early morning test (P<0.001) after an overnight break.

(iii) When we compare the performance between the late morning test and the early afternoon test, i.e. MF2 (Fig. 2E) and AF1 (Fig. 2F) at the feeder, or AH2 (Fig. 2G) and AH1 (Fig. 2H) at the hive, we find that the bees' pattern preference has significantly reversed from the late morning test to the early afternoon test after the midday break (P<0.001).

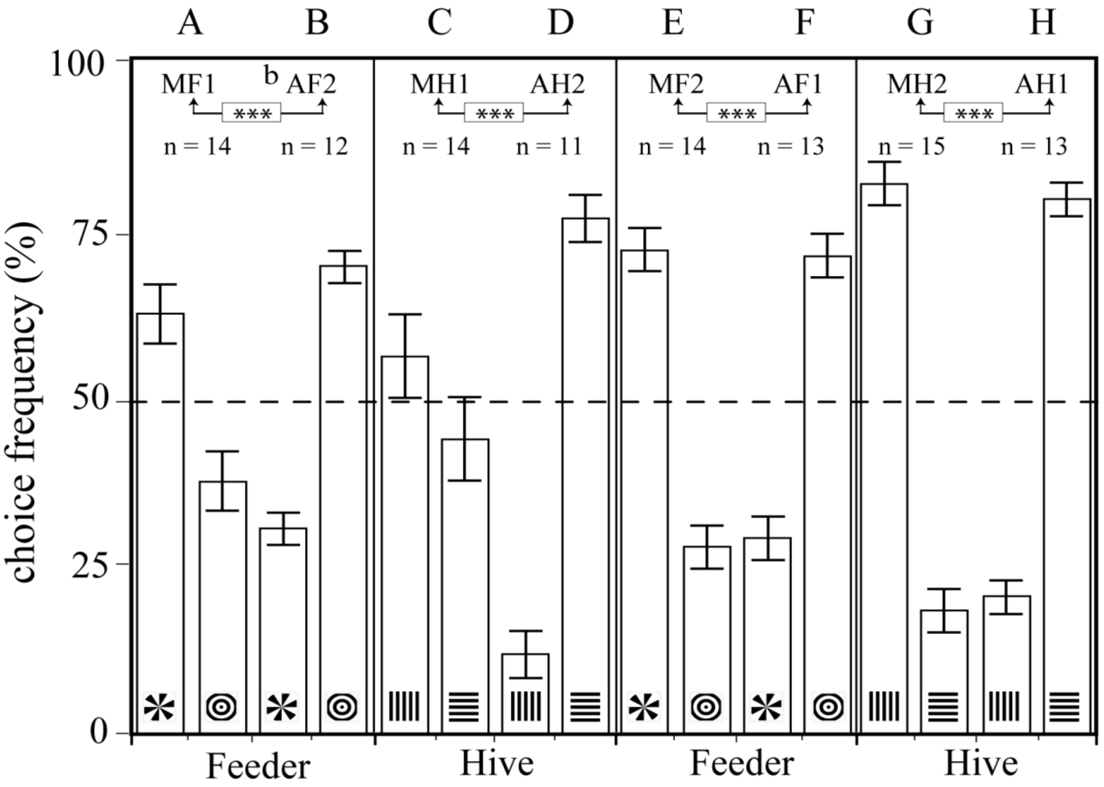


Figure 2: Results of Series I experiments carried out at the Bee Station (outdoors) at Würzburg University. The visual patterns were black/white sectors *versus* rings at the feeder, and black/white vertical *versus* horizontal gratings at the hive (patterns shown at the base of each bar). The results showed that trained bees can reverse their pattern preference following midday breaks, as well as an overnight break, both at the feeder and at the hive. Terminology for the test types: M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. ***p<0.001. See text for further details.

Series 2

Honeybees can learn to reverse their preference for a visual pattern depending on the task – foraging or returning home.

The experiments for this series were carried out in the AWBFF at the Australian National University's Research School of Biological Sciences. In the tests of the first experiment in this series, the bees showed a strong preference for the yellow pattern at the feeder with a statistically significant difference from the random choice level (0.93±0.03, N=11, P<0.001), but for the blue pattern at the hive (0.86±0.03, N=11, P<0.001; Fig. 3A). In the second experiment of this series, the bees showed a preference for the blue pattern at the feeder (0.75±0.03, N=8, P<0.001), but for the yellow pattern at the hive (0.58±0.05, N=8, P<0.05) (Fig. 3B). Thus, the trained bees could significantly reverse their preference for a visual pattern within a time interval of about 2·min on average, which included the flight time between the feeder and the hive and the time of drinking sugar solution on the feeder.

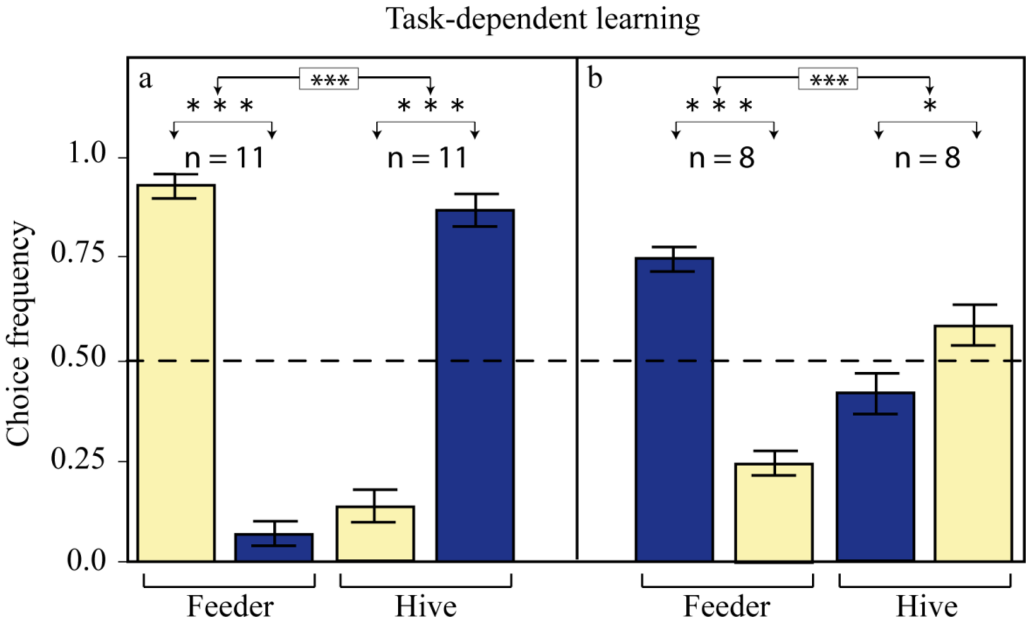


Figure 3: Results of Series 2 experiments carried out in the AWBFF at the Australian National University, which demonstrated that trained bees are able to reverse their pattern preference in just a few minutes, depending on whether they are flying out to forage or returning to the hive. ***p<0.001, *p<0.05. See text for further details.

Series 3

Honeybees can change their preference for a visual pattern simultaneously with the time of day as well as task at hand. In these learning tests, blue/white gratings were used at the feeder, while black/white gratings were used at the hive. The learning tests were carried out on the third day after training commenced. All eight tests were carried out. The results showed that preference for the positive pattern was significantly better than the random choice level of 0.50 in the learning tests (Fig. S4 in supplementary material). The critical test commenced after the learning tests had finished – now, the visual patterns at the feeder side and the hive side were the same, i.e. black/white gratings. The results showed that preference for the positive pattern was significantly better than the random choice level of 0.50 in all critical tests, namely MF1, MF2, AF1, AF2 (P<0.001, Fig. 4A) and MH1, MH2, AH1 and AH2 (P<0.001, Fig. 4B). Whereas the average choice frequency at the feeder was in favour of the horizontal grating in the late morning test (MF2, Fig. 4A), it reverted to the vertical grating in the early afternoon test (AF1, Fig. 4A), following the midday break (P<0.001). At the hive entrance, the average choice frequency favoured the vertical grating in the late morning test (MH2, Fig. 4B), but reverted to the horizontal grating in the early afternoon test (AH1, Fig. 4B) after the midday break (P<0.001). The trained bees therefore significantly reversed their pattern preference at the feeder and the hive entrance following midday breaks (P<0.001). Similar conclusions can be drawn when we compare the performance at the feeder in the early morning test (MF1, Fig. 4A) and the late afternoon test (AF2, Fig. 4A), and also at the hive when we compare the performance in the early morning test (MH1, Fig. 4B) and the late afternoon test (AH2, Fig. 4B). The trained bees significantly reversed their pattern preference at the feeder and the hive entrance after an overnight break (P<0.001). The modulation of the average choice frequency in favour of the horizontal grating can also be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12·h. However, the phase of the sinusoidal curve at the hive was shifted 180° in reference to the feeder. The correlation coefficient for the sinusoidal curve is 0.97 at the feeder and the hive. When we compared average choice frequencies at the feeder to those at the hive entrance within each testing period, i.e. early morning (MF1 in Fig. 4A to MH1 in Fig. 4B), late morning (MF2 in Fig. 4A to MH2 in Fig. 4B), early afternoon (AF1 in Fig. 4A to AH1 in Fig. 4B) and late afternoon (AF2 in Fig. 4A to AH2 in Fig. 4B), the bees were found to have reversed their pattern preference significantly (P<0.001, see inset table of statistical tests in Fig. 4A). Thus, the bees had learnt to choose a different pattern, depending on whether they were foraging or returning home.

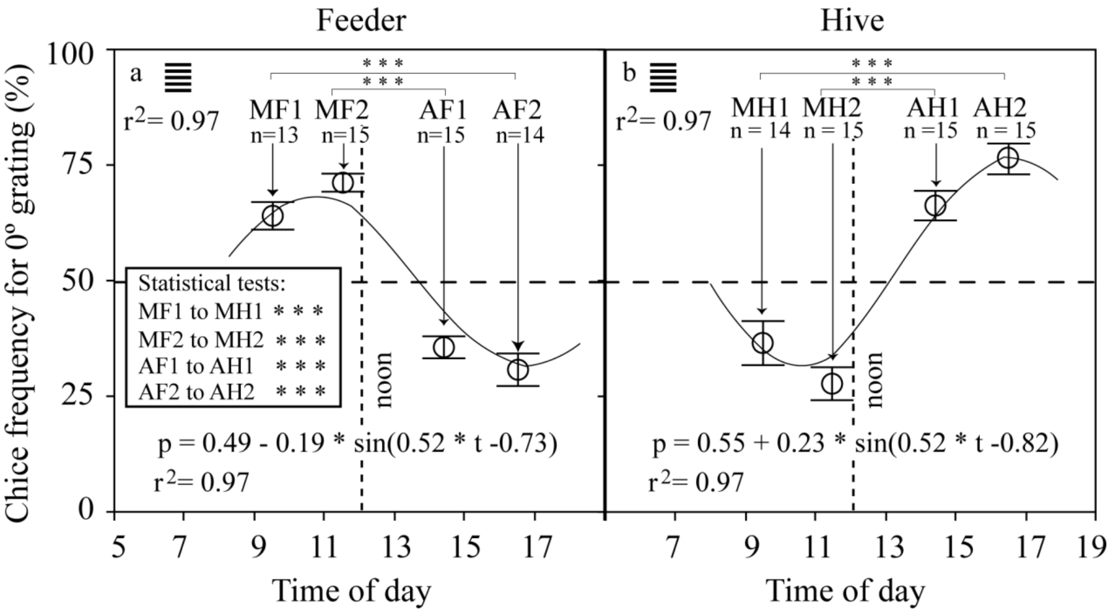


Figure 4: Results of the critical tests in Series 3 experiments carried out at the Bee Station at Würzburg University. The visual patterns were black/white horizontal *versus* vertical gratings at the feeder and at the hive. However, the horizontal grating at the feeder and the vertical grating at the hive were the positive patterns in the morning, whereas the vertical grating at the feeder and the horizontal grating at the hive became the positive patterns in the afternoon. Results for one pattern are given. The results showed that trained bees significantly reverse their pattern preference (A) at the feeder and (B) at the hive entrance following midday breaks, as well as after an overnight break. The trained bees also had a significantly different pattern preference at the feeder and at the hive entrance within each testing period. The modulation of the average choice frequency, with reference to the horizontal grating could be approximated very well by a sinusoidal curve with a frequency of 0.52, i.e. a period of 12 h. The correlation coefficient was 0.97 for both the feeder and the hive. However, the phase of the sinusoidal curve at the hive was shifted 180° with reference to the feeder. See text for further details. Terminology for the test types: M, morning; A, afternoon; H, hive; F, feeder; 1, early; 2, late. ***p<0.001.

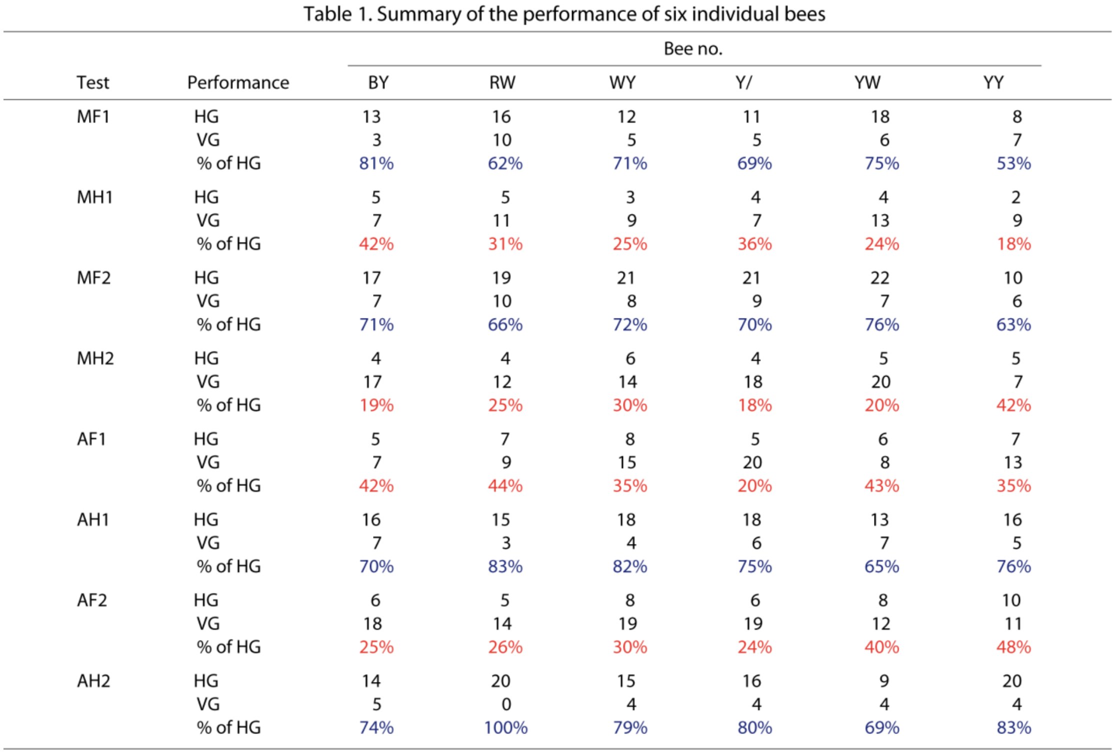


Table 1. Summary of the performance of six individual bees

Test	Performance	BY	RW	WY	Y/	YW	YY
		Bee no.					
MF1	HG	13	16	12	11	18	8
	VG	3	10	5	5	6	7
	% of HG	81%	62%	71%	69%	75%	53%
MH1	HG	5	5	3	4	4	2
	VG	7	11	9	7	13	9
	% of HG	42%	31%	25%	36%	24%	18%
MF2	HG	17	19	21	21	22	10
	VG	7	10	8	9	7	6
	% of HG	71%	66%	72%	70%	76%	63%
MH2	HG	4	4	6	4	5	5
	VG	17	12	14	18	20	7
	% of HG	19%	25%	30%	18%	20%	42%
AF1	HG	5	7	8	5	6	7
	VG	7	9	15	20	8	13
	% of HG	42%	44%	35%	20%	43%	35%
AH1	HG	16	15	18	18	13	16
	VG	7	3	4	6	7	5
	% of HG	70%	83%	82%	75%	65%	76%
AF2	HG	6	5	8	6	8	10
	VG	18	14	19	19	12	11
	% of HG	25%	26%	30%	24%	40%	48%
AH2	HG	14	20	15	16	9	20
	VG	5	0	4	4	4	4
	% of HG	74%	100%	79%	80%	69%	83%

Table 1: HG (or VG) denotes individual choices for the horizontal gratings (or vertical gratings); % of HG denotes choice frequency in % for the horizontal grating.

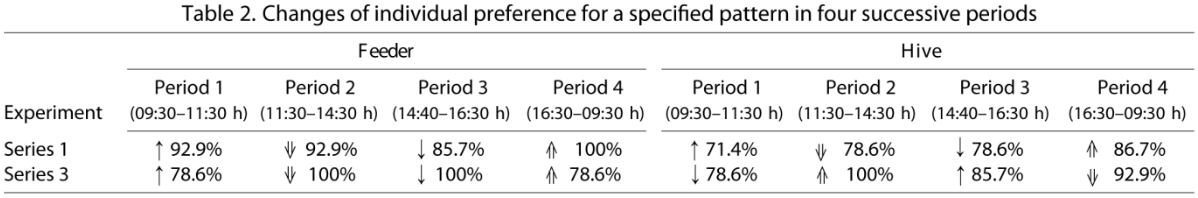


Table 2. Changes of individual preference for a specified pattern in four successive periods

Experiment	Feeder Period 1 (09:30–11:30 h)	Feeder Period 2 (11:30–14:30 h)	Feeder Period 3 (14:40–16:30 h)	Feeder Period 4 (16:30–09:30 h)	Hive Period 1 (09:30–11:30 h)	Hive Period 2 (11:30–14:30 h)	Hive Period 3 (14:40–16:30 h)	Hive Period 4 (16:30–09:30 h)
Series 1	↑ 92.9%	⇓ 92.9%	↓ 85.7%	⇑ 100%	↑ 71.4%	⇓ 78.6%	↓ 78.6%	⇑ 86.7%
Series 3	↑ 78.6%	⇓ 100%	↓ 100%	⇑ 78.6%	↓ 78.6%	⇑ 100%	↑ 85.7%	⇓ 92.9%

Table 2: The symbol ↑ denotes an increase in choice frequency in favor of the specific pattern; the symbol ↓ denotes a decrease in choice frequency; ⇓ denotes a tendency towards the specified pattern, and ⇑ denotes a tendency towards the opposite preference.

Consistency of performance between individual bees

We conducted ANOVA to check the homogeneity of the data before pooling and averaging individual bees' performances. However, a question that remains is whether the test results indicate a collective performance, driven by only a few bees that outperformed the others. Therefore, we compared the performance of individual bees in all types of tests in the critical test of Series 3. Table 1 summarizes the performance history of six individual bees; these bees were involved in all eight types of tests throughout the duration of the experiment. Table 1 convincingly demonstrates consistent performance between individual bees, in which all of the individuals reversed their pattern preference with time, when comparing MF1 to AF2, MH1 to AH2, MF2 to AF1 and MH2 to AH1; and also with the task, when comparing MF1 to MH1, MF2 to MH2, AF1 to AH1 and AF2 to AH2. Therefore, we can safely say that individual bees can reverse their pattern preference according to the time of day and the task, i.e. foraging or returning home.

Discussion

Training imposes pattern preference on the circadian rhythm

The results of Series 1 and 3 clearly demonstrate that a daily rhythm with sinusoidal variance of a period of 24 h exists in the honeybee, if we take into account the cessation of foraging activity for 12 h overnight (Koltermann, 1971; Kaiser, 1988; Bloch and Robinson, 2001; Moore, 2001; Sauer et al., 2004). Is it possible that this result is an artefact of averaging the performances of trained bees? How does the behaviour of individual bees compare with this pattern? To clarify this point, we carefully analysed the performance of individual bees; the results are summarized in Table 2, which shows that a large percentage of individuals varied their behaviour in four successive periods. In Series 1 at the feeder and at the hive, most individuals increased their preference for the specified pattern in period 1; this reduced to 0.50 or less in period 2 during the midday breaks, decreased further in period 3, and rose again to higher than 0.50 in period 4. A similar modulation of the choice frequency among individuals can also be observed in Series 3, with the difference that the direction of change at the feeder and at the hive within each period is reversed. Using nonlinear regression, we were able to fit a sinusoidal curve to the average choice frequencies of trained bees with reference to the specified pattern evaluated in the four daily tests. The tests were ordered around a daily cycle of 12 h, S. Zhang and others therefore giving a frequency of 0.52 to the sinusoidal curve. The results of the approximation show that the modulation of the average choice frequency in favour of a specified pattern in the four daily tests can be approximated very well by a regular sinusoidal pattern (Figs 1 and 4). In period 1 and period 3, the average choice frequency increased with reference to the specified positive pattern (note: the positive pattern was reversed in the afternoon). It could be caused by a difference in the choice-reversal rate of individual bees after overnight and midday breaks, as well as due to continuous training after the early morning and early afternoon tests. However, the 09:30 h test was commenced after an overnight break without re-training, and the 14:30 h test was commenced after midday breaks without re-training. Therefore, the performance level in these two tests could be only set by the biological rhythms. The zero-crossing point of the sinusoidal regression curve, predicting bees' pattern preference reversed at the feeder and at the hive, coincides with the midday and overnight breaks (Figs 1 and 4). A large percentage of individuals followed this trend (Table 2), as the experimental bees switched their preference from one pattern to the other, as dictated by their internal clock. We suggest that our training was able to impose the modulation of pattern preference on the biological rhythms of individual bees. Our findings were very consistent, in spite of the fact that experiments were carried out in different environmental conditions. The experiments began at the Australian National University, and were then repeated at the Würzburg University. We obtained the same results, in spite of the experiments being carried out both outdoors and within the indoor bee flight facility, which has a controlled ambience. There is relatively little UV light within the indoor bee flight facility, because the Perspex roof blocks most of it. The consistent results obtained indoors and outdoors indicate that the honeybees did not need to use polarized sunlight as a cue to change their preference according to time. However, there are slight differences in the results of the indoor and outdoor experiments. The bees gradually changed their pattern preference between the morning and the afternoon, immediately following a midday break in the indoor experiment of Series 1. In the case of the outdoor experiments, however, the bees significantly reversed their pattern preference immediately following a midday break in Series 3. A possible reason is that bees could not use polarized light and the exact sun position as cues in the indoor experiment, but were able to do so in the outdoor experiments of Series 3. However, as two different hives were used in the indoor and outdoor experiments, we cannot yet conclusively state that the presence of polarized light was responsible for the difference in the performance of the indoor and outdoor bees. Further experiments are required to clarify this matter.

The complexity of honeybee memory

In the experiments of Series 3, honeybees had to simultaneously change their preference for a visual pattern with both the time of day, as well as the task at hand. To date, this is the most complex task that honeybees in our laboratories have been trained to perform, since we started to explore this insect's cognitive capabilities at the Australian National University's Research School of Biological Sciences in 1996. In this task, the honeybees had to retrieve the exact memory for the decision not only by means of a single visuo-spatial input, but also using their biological clock and motivation as cues. In other words, the bees had to remember their responses to visual patterns within the context of the task to be performed, as well as the current time coordinates. In order to facilitate the learning of this very complex task, we adopted the method used by Collect and his colleagues: they trained bumblebees to discriminate between horizontally and vertically oriented gratings of black/white, in order to reach the feeder, and between different diagonally oriented gratings to gain access to their nest. Once the bees had been trained on these two tasks, they could rapidly learn new discrimination tasks, where they had to distinguish between horizontal and vertical gratings at both sites. Whereas they still approached the horizontal grating to reach food, they now had to choose a vertical grating in order to return to their nest (Colborn et al., 1999; Cheng, 2005). In our experiments, we introduced the additional contextual cue of colour difference at the feeder and the hive during the training phase and learning tests: the bees had to approach a blue horizontal grating to reach the feeder, and a black vertical grating to access their hive in the morning, and then reverse their preference at both sites in the afternoon. The trained bees learned these tasks well, and performed equally well in the learning tests. Afterwards, the bees were only briefly trained to the new, more complex tasks, following which the critical tests were carried out, where horizontal and vertical gratings of black/white were used at both sites. The bees performed very well in the critical tests, and could simultaneously change their preference for a visual pattern with both the time of day, and the task at hand.

An individual bee knows whether it is foraging or returning home

Foraging behaviour in honeybees can be modulated by juvenile hormone (Huang and Robinson, 1995), by demand for food in the colony (Seeley, 1997; Seeley and Visscher, 2004) or by a worker's nutritional state (Toth et al., 2005). Our experiments have demonstrated that individual bees can quickly reverse their pattern preference between an outward foraging flight and the return trip back to the hive. Thus, the forager knows what to do during both events. In our experiments, the distance between the hive and the feeder was very short, being only 8·m in the indoor experiments at the Australian National University and 24·m in the field experiments at the Würzburg University. The flight time between the hive and the feeder was a few seconds for the indoor experiments and 20 s for the field experiments on average. Each forager remained on the feeder drinking sugar solution for about 90 s. The experimental foragers could therefore change their pattern preference in about a few minutes, as their status changed from foraging to returning home. What could be happening in honeybee’s brain during this short period of time? Colborn et al. (1999) proposed that different contextual signals are associated with approaching the nest or approaching the feeder, and that these contextual signals facilitate learnt associations between orientation detectors and motor commands. Dyer examined the relation between motivation and vector navigation in honeybees, and found that the resetting of the path integration vector can be influenced by motivational cues associated with food deprivation (Dyer et al., 2002). If this is indeed the case, the brain dopamine level could be operating as a sub second modulator of food seeking (Roitman et al., 2004). It might even be that the motivations of foraging or returning home act as contextual cues, which can modulate the decisions available to a bee. Further changes in a bee’s status, such as an empty or a full stomach before and after obtaining a reward, could act as triggers that change a bee’s behaviour. The processes of learning and memory are undoubtedly more sophisticated in primates and mammals than in insects, but there seems to be a continuum in these capacities across the animal kingdom, rather than a sharp distinction between vertebrates and invertebrates. The abilities of an animal seem to be governed largely by what it needs in order to pursue its lifestyle, rather than whether or not it possesses a backbone (Zhang and Srinivasan, 2004b; a). The properties of learning and memory in insects have been shown to be well suited to the requirements of the tasks that they have to perform (Lynn et al., 2005; Cheng and Wignall, 2006). The present research demonstrates that the honeybee possesses a complex memory capable of memorizing tasks within a time schedule. Honeybees can ‘plan’ their activities in time and space, and use context to determine which action to perform and when.

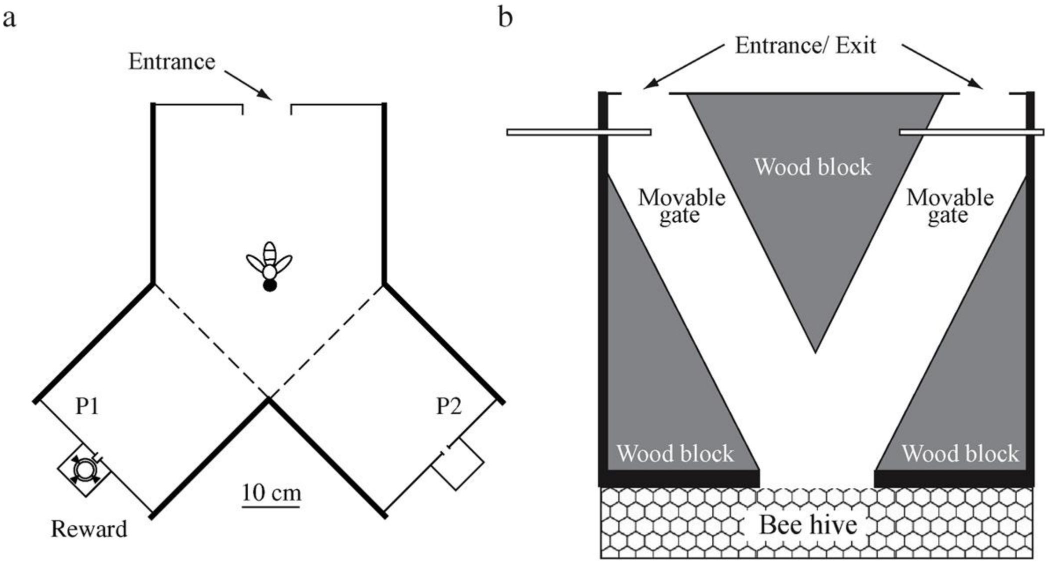
Acknowledgements

We thank Aung Si for critical reading of the manuscript, Dirk Ahrens and Paul Helliwell for help with beekeeping, Bill Speed for constructing the apparatus used in Canberra and Gerhard Eisenmann for constructing the apparatus used in Würzburg.

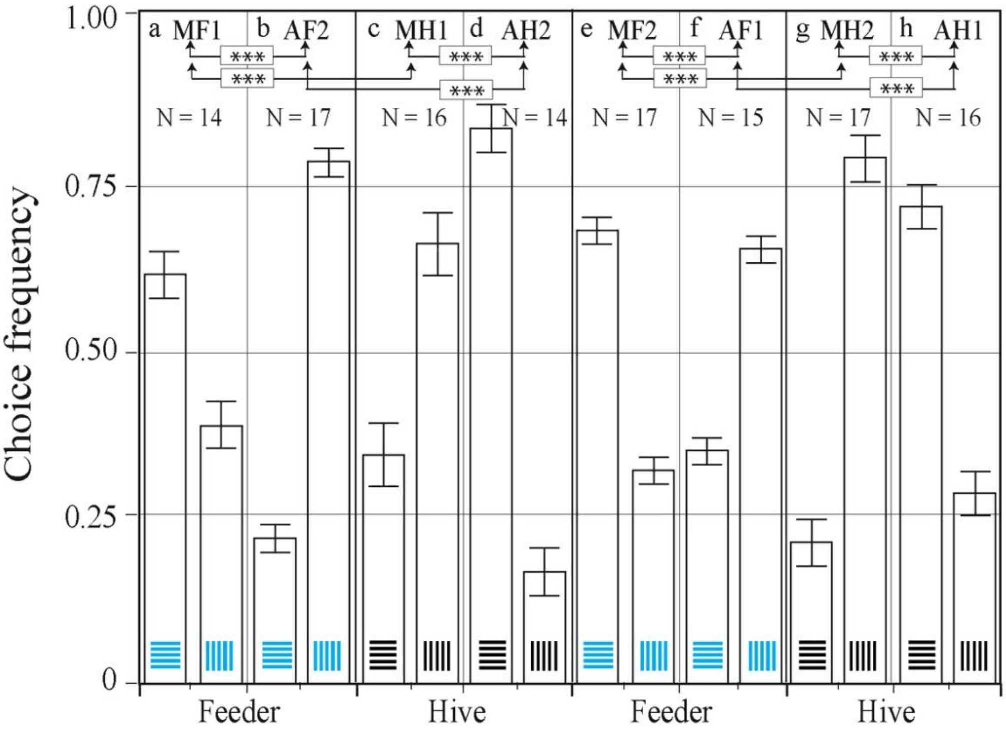
Supplementary material

Supplementary Figure 1: Bees returning to the hive that was maintained in this hut had to choose between two patterns to gain access to the hive.

Supplementary Figure 2: A Y-maze was set up in this hut.



Supplementary Figure 3: Experimental apparatus: (A) A Y-shaped, dual-choice apparatus at the feeder; (B) An entrance chamber, which split the access to the hive into two paths, was added to the entrance of a normal hive. Bees had to make a choice between two patterns for accessing the hive. Only the entrance behind the positive pattern was open during training.



Supplementary Figure 4: Results of the learning tests in Series 3 experiments carried out at the Bee Station at Würzburg University. The visual patterns were blue/white horizontal *versus* vertical gratings at the feeder, and black/white horizontal *versus* vertical gratings at the hive. The horizontal grating at the feeder and the vertical grating at the hive were the positive patterns in the morning, and the vertical grating at the feeder and the horizontal grating at the hive became the positive patterns in the afternoon. Honeybees could learn the tasks very well. The results show that trained bees could reverse their pattern preference following midday breaks, as well as overnight breaks, at the feeder and at the hive. The trained bees also had significantly different pattern preferences at the feeder and at the hive entrance within each testing period. The terms MF1 and MH1 (or MF2 and MH2) denote the early morning (or late morning) tests at the feeder and the hive, and AF1 and AH1 (or AF2 and AH2) denote the early afternoon (or late afternoon) tests at the feeder and the hive, respectively. See text for more details.

Supplementary videos

http://jeb.biologists.org/content/suppl/2006/10/25/209.22.4420.DC1/JEB02522Movie1.mp4

Movie 1. Indoor experiments carried out in an All Weather Bee Flight Facility (AWBFF) at the Australian National University's Research School of Biological Sciences.

http://jeb.biologists.org/content/suppl/2006/10/25/209.22.4420.DC1/JEB02522Movie2.mp4

Movie 2. Experiments were carried out during July and August 2005 at the Bee Station of the Würzburg University. Bees returning to the hive had to choose between two patterns to access the hive, as only the entrance behind the positive pattern was open during training but both entrances were open during the test periods.

Methods supplement: Training and learning tests for Series 3 experiments

In the learning tests, blue/white gratings were used at the feeder, and black/white gratings at the hive. All of the tests (MF1, MH1, MF2, MH2, AF1, AH1, AF2 and AH2) were carried out individually. Figure S4 shows that the preference for the positive pattern in all tests was significantly greater than the random choice level of 0.50 (p<0.001; Figure S4a,h). The average choice frequency at the feeder in favour of the blue horizontal gratings in the early morning test (MF1, Figure S4a) was 0.61±0.04, n=4; however, the average choice frequency in the late afternoon test (AF2; Figure S4b) was 0.22±0.02, n=17. The preference for the blue horizontal gratings was therefore significantly reversed between the MF1 and AF2 tests (P<0.001). At the hive, the average choice frequency in favour of the vertical gratings in the early morning test (MH1, Figure S4c) was 0.34±0.05, n=16; however, the average choice frequency in the late afternoon test (AH2, Figure S4d) was 0.83±0.04, n=14. The preference for the vertical gratings between the MH1 and AH2 test was thus also significantly reversed (p<0.001). The trained bees clearly reversed their pattern preference between late afternoon and early morning. Similar conclusions can be drawn from data from the feeder when we compare the performance between the late morning (0.68±0.02, N=17; MF2, Figure S4e) and the early afternoon tests (0.359±0.02, n=15; AF1, Figure S4f); as well as at the hive, when we compare the performance between the late morning (0.21±0.03, n=17; MH2, Figure S4g) and the early afternoon tests (0.71±0.03, n=16; AH1, Figure S4h).

Chapter 2: Circadian timed episodic-like memory- A bee knows what to do when, and also where

Abstract

This study investigates how the colour, shape and location of patterns could be memorized within a time frame. Bees were trained to visit two Y-mazes, one of which presented yellow vertical (rewarded) versus horizontal (non-rewarded) gratings at one site in the morning, while another presented blue horizontal (rewarded) versus vertical (non-rewarded) gratings at another site in the afternoon. The bees could perform well in the learning tests and various transfer tests, in which i). All contextual cues from the learning test were present; ii). The colour cues of the visual patterns were removed, but the location cue, the orientation of the visual patterns and the temporal cue still existed; iii). The location cue was removed, but other contextual cues, i.e. the colour and orientation of the visual patterns and the temporal cue still existed; iv). The location cue and the orientation cue of the visual patterns were removed, but the colour cue and temporal cue still existed; v). The location cue, and the colour cue of the visual patterns were removed, but the orientation cue and the temporal cue still existed. The results reveal that the honeybee can recall the memory of the correct visual patterns by using spatial and/or temporal information. The relative importance of different contextual cues is compared and discussed. The bees' ability to integrate elements of circadian time, place, and visual stimuli is akin to episodic-like memory; we have therefore named this kind of memory circadian timed episodic-like memory.

The original open access article is available at:

http://jeb.biologists.org/content/210/20/3559

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Introduction

Studies of the natural foraging behaviour of bees suggest that individuals have the capacity to learn and remember not only the colour and shape of flowers that are bountiful in pollen and nectar, but also how to get to them (Wehner, 1981; Chittka et al., 1993; Lehrer et al., 1995; Vorobyev and Menzel, 1999; Collett et al., 2003). The species of flowers that are in bloom, say this week, are likely to be replaced by a different species at a different location the next week, and different flower species have different peak times of nectar secretion during the day (Kakutani et al., 1989). So the bee needs not only spatial information, such as the features and location of the flowers, but also temporal information, and has indeed evolved an impressive ability to learn colours, odours, shapes and routes, within a time frame, quickly and accurately.

Bees can learn the time of day when flowers start secreting nectar. In an early study, when bees were trained to visit a feeder at a particular hour of the day, almost all of the trained bees visited the feeder during the hour-long reward period (Behling, 1929). This “Zeitgedächtnis” or time-sense persists for six to eight days, and thus can outlast short bad weather periods (Wahl, 1932). Koltermann (1971) also showed that bees can recall 9 different times per day, with an accuracy of 20 minutes. In his experiments, the bees could associate scents with an artificial feeder at a particular time (Koltermann, 1971).

Honeybee foragers possess a circadian rhythm, with an activity period during the day and a sleep-like state at night (Lindauer, 1975; von Frisch, 1993; Bloch and Robinson, 2001; Bloch et al., 2001; Moore, 2001). A special feature of the honeybees’ circadian rhythm is its flexibility. In typical circadian rhythms, a particular behaviour is fixed to a special phase of the cycle. The honeybee “Zeitgedächtnis” enables the bee to continuously adjust its behaviour according to its memory and the time of day (Chalifman, 1950; Lindauer, 1954; Wittekindt, 1955).

Honeybees have the ability to flexibly change their preference for a visual pattern according to the context in which a discrimination task is carried out. Context cues help to carve up the world into distinct regions, and so can aid animals to cope with possible confusions (Colborn et al., 1999; Fauria et al., 2002; Dale et al., 2005). Honeybees can learn to treat the same stimulus in different ways, depending on the context in which the stimulus is presented (Gould, 1987; Srinivasan et al., 1998a; Colborn et al., 1999; Zhang et al., 2006). Menzel et al. (1996) investigated whether and how contextual parameters, such as time of day and features characterizing the location, can be utilized to determine choice behaviour. They claimed that time of day cannot by itself elicit a conditioned response, but can control different behaviours, such as image-matching, navigation, timing of motivation to forage, and thus act as an occasion setter for a sensory-motor routine.

However, there has been little experimental work investigating bees' abilities to modulate their behaviour in response to multiple contextual cues in the spatial and/or temporal domain. In a previous study, we showed that honeybees are able to reverse their pattern preference according to the task at hand and the time of day (Zhang et al., 2006). In these experiments, the bees learned to make opposite decisions when foraging and when homing (task), and also between morning and afternoon (time). These contextual cues helped the bees to memorize the rules for navigating an experimental maze, and to recall the correct memory in the associated context. In the present study, we further investigated how the colour, shape and location of patterns could be memorized within a time frame, and examined the importance of different contextual cues.

Materials and methods

General

The experiments were conducted at the Australian National University, and the setup was located in a small greenhouse covered by an opaque PVC sheet. We added additional Styrofoam sheets beneath the PVC sheet above mazes A, B and C so that the mazes were always in the shadow, while ensuring a homogenous illumination of the mazes and some heat protection for the observers. The greenhouse was separated by two blinds into three compartments, so that the bees could not see maze A or maze B from the hive entrance. Nor could they see maze A from maze B and vice versa. The flight distance was approximately 4 m from the hive to maze A and maze B, and 2 m to maze C (see Fig. 1). The hive had two entrances at opposite sides, and was mounted on the wall, so that the bees were able to forage both inside and outside. At the beginning of each experiment, about 20 foraging bees (*Apis mellifera*) were individually marked and trained to visit a feeder with a

0.5 mol l^{-1} sugar solution in the Y-mazes. Bees entering the Y-mazes were trained to choose one of two patterns, which indicated the position of the feeder reward.

Maze setup

Three compound Y-mazes were used in the experiments. Each was made of four cylinders of 25cm height and 25cm diameter, and covered by a Perspex lid. The four cylinders were connected by holes, 4cm in diameter, through which the bees could fly from one cylinder to the next. The holes were positioned in the middle of the cylinder wall, halfway up from the base (12.5cm from both ends). The first cylinder carried two holes on opposite sides. The bee would enter through the entrance hole, and fly through the next hole into the second cylinder. The second cylinder had three holes, one serving as entrance, and two others, 90° apart, as exits leading to the next two cylinders. Each of the two holes carried a visual stimulus, between which the bees had to choose (Fig. 1). One of the two patterns indicated the position of the feeder reward. If the bee made a positive decision by flying through the correct pattern (termed positive), it would enter the third cylinder, and find a feeder with sugar solution as a reward. If the bee chose the wrong (termed negative) pattern, it found an empty cylinder, and was released to try again. A bee choosing between visual patterns could not see whether the next cylinder contained the feeder or not, because the feeder was placed on the floor of the maze, and a cardboard baffle was placed behind the entrance holes of the reward cylinders. This prevented the bees from seeing into the reward cylinder from the decision cylinder. The entrance of the decision cylinder also had a baffle to slow the bees down, which made observation easier, and gave the bees more time to look at the visual stimuli. This maze setup is well established in honeybee behavioural research (Srinivasan and Lehrer, 1988; Zhang et al., 1992; Zhang and Srinivasan, 1994; Zhang et al., 1995; 1996; 1999a).

During training, the positions of the positive and negative patterns at the mazes were regularly swapped every 30 minutes, so that the bees could not use position as a cue to find the feeder. Similarly, the positions of the positive and negative patterns were interchanged every 10 minutes in the middle of the learning tests and every 5 min in the middle of the transfer tests.

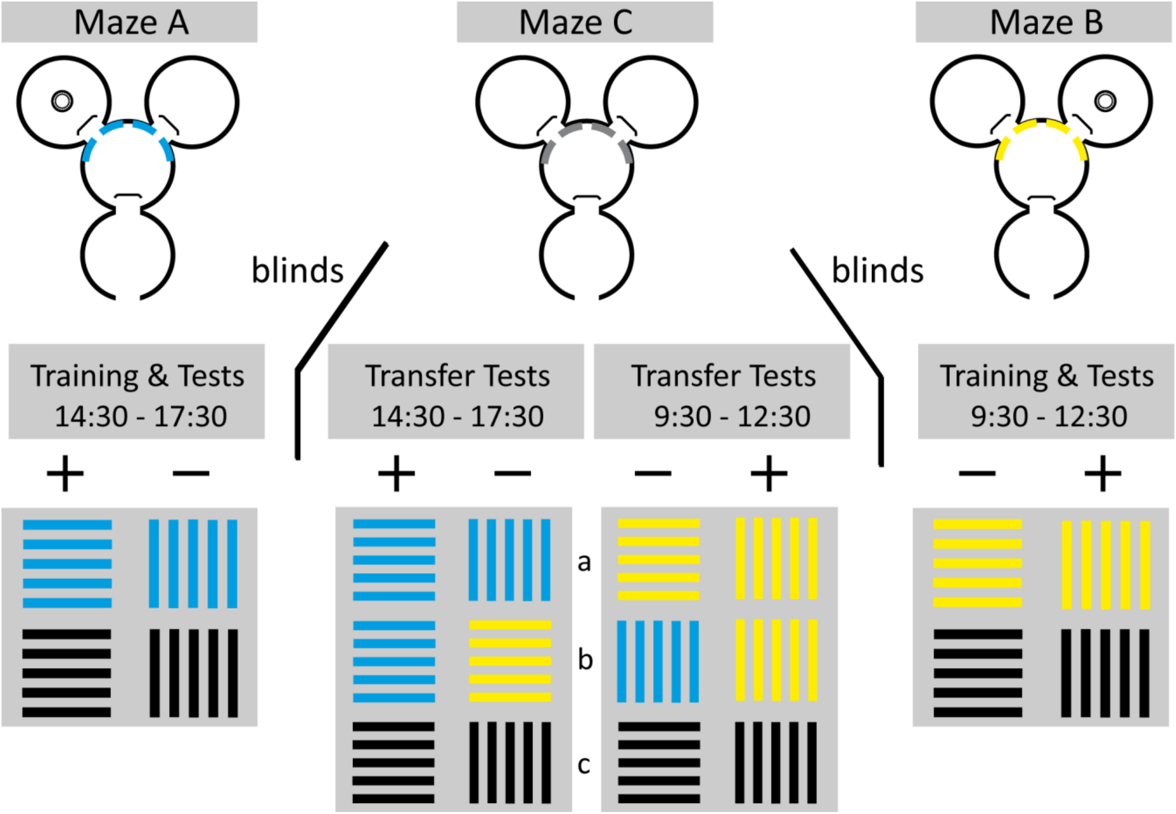


Figure 1: The experimental set-up and visual patterns. Yellow patterns were used in the training and learning test in the morning at Maze B; blue patterns were used in the training and learning test in the afternoon at Maze A; black patterns were used in experiment 1, the transfer tests at Maze A and B. Various transfer tests were conducted at Maze C: (a) experiment 2: yellow training patterns in the morning and blue training patterns in the afternoon; (b) experiment 3: blue and yellow vertical patterns in the morning and blue and yellow horizontal patterns in the afternoon; (c) experiment 4: black horizontal and vertical patterns in the morning and in the afternoon. The rewarded patterns were denoted by (+) and the unrewarded patterns were denoted by (–). See text for further details.

Visual stimuli

The stimuli were presented as 18cm x 18cm squares (grating patterns) or 18cm diameter circles (sector and ring patterns, details in supplementary material) on the exits of the second cylinder. They were printed on normal copy paper using a Fuji Xerox Document Centre C360 PS colour printer. The training stimuli in maze A were always blue/white, and the training stimuli in maze B were always yellow/white. The stimuli for the transfer tests at the mazes A and B were black/white. The stimuli for the transfer tests at maze C were blue/white, yellow/white or black/white. Horizontal versus vertical gratings (Fig. 1, 3, 4) and sector versus ring patterns (Fig. S1A, B in supplementary material) were used as visual stimuli with different groups of bees. The rewarded pattern, which provided access to a feeder, was termed ‘positive’, the unrewarded pattern was termed ‘negative’.

Training and testing procedure

The bees were trained for three days before testing began, and thus experienced the circadian reward pattern three times, *circa* an average of 20 rewards on each training pattern per bee. During training and testing, the number of bees in the apparatus was carefully controlled: if two or more bees were seen in the decision cylinder, all were released without a reward, and allowed to attempt the task again. Each bee that reached the reward cylinder, and collected the sugar solution in the feeder, was released by lifting the Perspex lid. Thus, it did not have to trace its way back through the maze. Training was carried out daily over two sessions. In the morning session (09:30 – 12:30), the bees were trained to forage at maze B. In the afternoon session (14:30 – 17:30), the bees were trained to forage at maze A. During the break from 12:30 to 14:30, and during the night, a feeder was placed in a neutral position to keep the bees motivated to fly inside the greenhouse. During the morning training at maze B, maze A did not contain a feeder, and the Perspex lids were open. Similarly, throughout the afternoon training at maze A, maze B did not contain a feeder, and the Perspex lids were open. During all learning and transfer tests, there was no difference between the mazes. Both mazes contained a feeder, and the Perspex lids were closed. The mazes were accessible to the bees at all times, except during the transfer tests at maze C.

During training, the yellow vertical grating provided access to the feeder at maze B in the morning, and the blue horizontal grating indicated the feeder position at maze A in the afternoon (Fig. 1). When the sector and ring patterns were used, the yellow sector pattern was positive at maze B in the morning, and the blue ring pattern was positive at maze A in the afternoon (Fig. S1A in supplementary material). Using the two colours blue and yellow at the two training mazes made learning easier for the bees, probably because cues stay longer in memory when offered in combination with other, simultaneously offered cues (Lindauer, 1970; Colborn et al., 1999; Cheng, 2005).

Data collection

During the learning tests and the transfer tests at Maze A or Maze B, both mazes were observed, and every positive and negative decision in the mazes was recorded. Only the first choice of each bee during one foraging flight was included in the data. The reward continued to be offered during all tests, to prevent bees from losing their motivation to visit the apparatus (Zhang et al., 1999a). The learning tests lasted for 20 minutes, and the transfer tests lasted for 10 minutes. In the middle of each testing period (after 10 minutes in the learning tests, and after 5 minutes in the transfer tests), the positions of the patterns were swapped in order to cancel out any effect of a possible side bias. All transfer tests were followed by at least 30 minutes of normal training at maze A or B. This shorter testing period, and subsequent training under normal conditions, ensured that the bees did not learn during the transfer test conditions. Each bee was allowed to make a maximum of three rewarded visits in each of the transfer tests, which is not enough to learn a pattern discrimination task. Additionally, there was a break of at least 24 hours before a transfer test condition was repeated. When testing the bees at maze C, mazes A and B were disassembled, in order to make the bees visit maze C.

Tests at mazes A and B

The performance of individual bees was recorded in the learning tests. During training, the bees learned for each of the mazes A and B where and when to go, and what to do there. The constant control of the learning level ensured that the bees were well trained throughout the transfer tests. These tests were repeated with a different group of bees, using the sector and ring patterns (Fig. S1A in supplementary material).

In experiment 1, we investigated whether honeybees can distinguish the patterns at two locations without the colour cue, using black patterns in mazes A and B (Fig. 1). If the bees were still able to choose the positive pattern, we could be certain that they had used the maze location cue (where), the shape cue (what), and the time cue (when), independently of the colour cue. These tests were repeated with a different group of bees, using sector and ring patterns (Fig. S1B in supplementary material).

Transfer tests at maze C

In experiment 2, we examined whether honeybees can choose the learned training patterns at a novel location, namely in maze C (Fig. 1a). The bees had never visited maze C before, and thus the maze location cue was excluded. They had to base their decision on the pattern colour and shape (what) and the time of day (when), transferring their knowledge of what to do in a certain timeframe to a new ‘where’.

In experiment 3, we examined whether honeybees can choose the trained colour independently of the location and shape cue. Yellow and blue vertical gratings were used in the morning, and yellow and blue horizontal gratings were presented in the afternoon (Fig. 1b). Thus, the bees could not use the pattern shape and the location cue for decision making. Since both patterns had the same (positive) shape, the bees had to decide between yellow and blue according to the time of day at the new location.

In experiment 4, we examined whether honeybees can choose the trained shape independently of the location and colour cue. We excluded the colour and location cues by presenting black gratings at maze C (Fig. 1c). The bees had to choose a pattern shape according to the time of day, without relying on pattern colour or maze location as cues.

Analysis of performance

We first performed analyses of variance (ANOVA) across all repeated tests for individual bees and for each type of experimental condition, using the statistical software SYSTAT 11 to check the homogeneity of the data. Once the data were found to be homogeneous, the performance of each bee was evaluated separately by pooling its correct choices and visits over all repeated tests, and calculating the ratio of the number of correct choices to the number of visits. Then, the average performance for a particular experimental condition was obtained by averaging choice frequencies across bees. The sample size (*N*) was the number of bees, rather than the number of individual choices, ensuring that the samples were truly statistically independent. Mean values of choice frequency, standard deviation and standard error of the mean (s.e.m.) were calculated. In the text and in the figures, performance is indicated by the mean choice frequency (± s.e.m.). In the analysis, we included only bees that visited both of the mazes regularly. A visit at the correct maze was counted when the bee entered the maze and made a decision. A visit at the wrong maze was counted when the bee entered the maze, and also when it hovered in front of the maze.

The performances in the morning and the afternoon tests were compared by GraphPad Prism statistical software, using Two-way repeated measurement ANOVA (Time: morning versus afternoon; Repeated measurements) to determine whether performance changed significantly during the time and between the repeated measurements. Post hoc comparisons were done by means of the Bonferroni t-tests which compared each repeated measurement in the morning and in the afternoon (for example, percentage of choices for the vertical pattern in the afternoon, compared with the percentage of choices for the vertical pattern in the morning). Control experiments were carried out at the end of experiments to test for a possible side bias. We conducted a simple dual choice test at Maze A and Maze B, for which 2x2 McNemar tests were used for statistical analysis

Results

The honeybees learned to forage at maze B in the morning and at maze A in the afternoon.

For a comparison of the bees' location preference in the morning and the afternoon, the total visits to Maze A and Maze B were recorded at the same time in each learning test and transfer test. The ratio of the number of visits at Maze B (or A) to the number of total visits was calculated for each test in the morning (or afternoon). Then, we averaged the ratios for all learning tests and also for all transfer tests in the morning and afternoon. The results are presented in Fig. 2. During the learning tests in the morning, the vast majority of visits (0.96 ± 0.01) from 16 tests were recorded at maze B (n = 16, total visits N = 559); whereas only a small number of bees (total 22 visits in 16 tests) approached maze A. These bees mostly confined their visit to a quick fly-over, only occasionally entering the maze. In the afternoon training, an equally large proportion of visits (0.97 ± 0.01) from 16 tests were recorded at maze A (n = 16, total visits N = 770; Fig. 2A); whereas a very small number of bees (total 17 visits in 16 tests) visited maze B. Here too, most visits were confined to a quick fly over, with only a few bees entering the maze. The bees clearly preferred maze B in the morning, and changed their location preference to maze A in the afternoon (t-test, d.f. = 30, $p<0.001$).

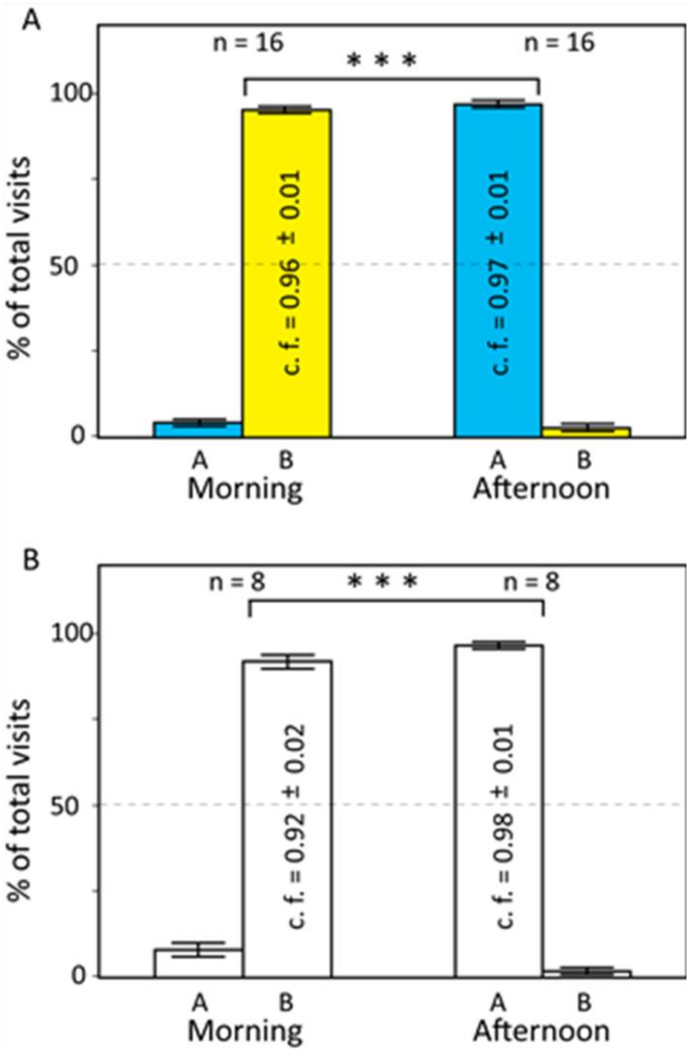


Figure 2: Trained bees change their preference to visit Maze A or Maze B from morning to afternoon. (A) Percentage of total visits at Maze A and B in the learning tests with yellow gratings in the morning at maze B and blue gratings in the afternoon at maze A; (B) percentage of total visits at Maze A and Maze B in the transfer tests with black and white gratings. *N*, number of repetitions of tests; values are means ± s.e.m. See text for further details.

The transfer test conditions (black patterns at mazes A and B) did not affect the bees' location preference. During the morning transfer tests, most visits (0.92 ± 0.06) from 8 tests were recorded at maze B (n = 8, total visits N = 124); whereas only a small number of bees (total 10 visits in 8 tests) approached maze A. In the afternoon transfer tests, most visits (0.97 ± 0.03) from 8 test times were recorded at maze A (n = 8, total visits N = 440; Fig. 2B), whereas a very small number of bees (total 13 visits in 8 tests) visited maze A. These bees visited the wrong maze, i.e. maze A in the morning and maze B in the afternoon and confined their visit to a quick fly-over, only occasionally entering the maze. The bees still preferred maze B in the morning, and reversed their preference to maze A in the afternoon (t = 39.1, d.f. = 14, p<0.001).

The bees learned to choose the yellow vertical grating at maze B in the morning, and the blue horizontal grating at maze A in the afternoon.

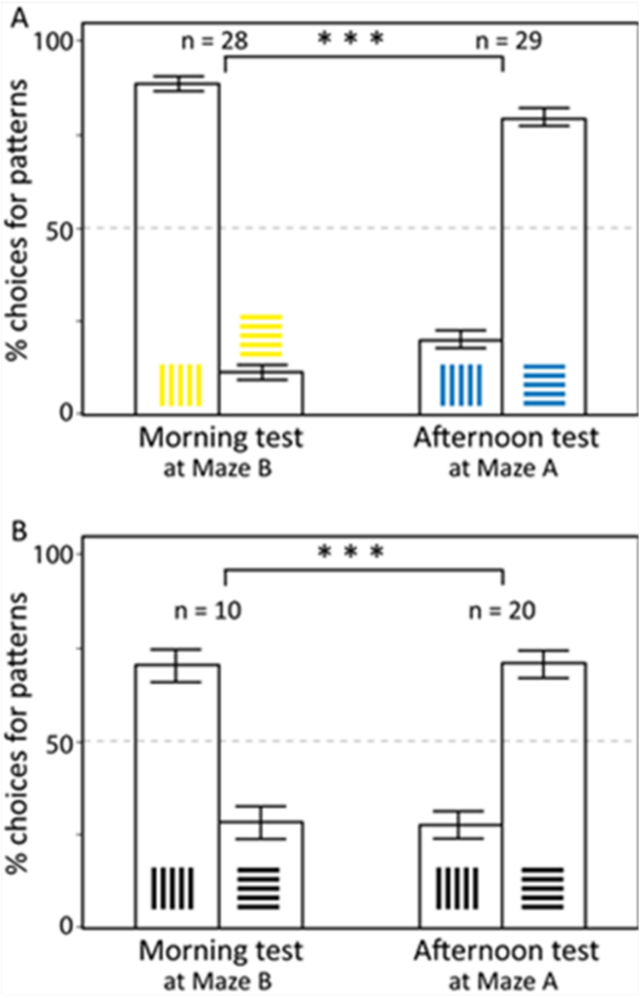


Figure 3: (A) Results of the learning tests at mazes A and B. The tested bees significantly reversed their pattern preference from the yellow vertical grating in the morning to the blue horizontal grating in the afternoon. (B) Results of experiment 1, the transfer tests with black patterns at mazes A and B. The tested bees significantly reversed their pattern preference from the vertical grating in the morning to the horizontal grating in the afternoon. *N*, number of individual bees attending the test; values are means± s.e.m. See text for further details.

The learning tests were conducted to ensure that the bees had a constant learning level throughout the experimental period. Once again, the bees had to fly to maze B in the morning, and choose the yellow vertical grating there. In the afternoon, they had to forage at maze A, and choose the blue horizontal grating. The results are shown in Fig. 3A.Analysis of variance showed no significant differences in the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1=5, d.f.2=84, F-ratio = 1.14, p = 0.362) or the afternoon sessions (ANOVA, d.f.1 = 13, d.f.2 = 75, F-ratio = 0.787, p = 0.672) at mazes A and B. When the bees visited maze B in the morning during training (Fig. 3A), they chose the yellow vertical grating in most of the visits (0.88 ± 0.02, N=28). In the afternoon at maze A (Fig. 3A), their choice frequency for the blue horizontal grating was 0.80 ± 0.02 (N=29). Results of ANOVA tests are as follows: d.f. (interaction) = 7, F = 0.573, p = 0.778; d.f. (time) = 1. F = 853.6, p < 0.001; d.f. (repeated tests) = 1, F = 1.737, p = 0.102. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. Thus, they learned to reverse their pattern preference between morning and afternoon. With this performance as a baseline, the transfer tests were started.

Experiment 1: The bees could distinguish black patterns at mazes A and B.

Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1 = 6, d.f.2 = 25, F-ratio = 1.584, p = 0.193) or in the afternoon (ANOVA, d.f.1 = 14, d.f.2 = 44, F-ratio = 0.52, p = 0.908). When the bees were tested for transfer to black gratings at mazes A and B (Fig. 3B), they chose the vertical grating at maze B in the morning in most of the visits (0.74 ± 0.04, N=10). In the afternoon at maze A, the choice frequency for the horizontal grating was 0.72 ± 0.04 (N=20). Results of ANOVA tests are as follows: d.f. (interaction) = 2, F = 0.05, p = 0.949; d.f. (time) = 1. F = 75.12, p < 0.001; d.f. (repeated tests) = 2, F = 2.257, p = 0.113. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees significantly reversed their pattern preference from vertical in the morning to horizontal in the afternoon, even without the colour cue, deciding for a grating orientation according to the time of day (when) and the maze location (where) **.**

Experiments 2 - 4 at maze C

Maze C had a neutral position between the training mazes A and B (Fig. 1), and the bees had never foraged in this maze before. To avoid any further learning at maze C during the transfer tests, the testing time was kept short, so that each bee did not visit more than three times in each testing session. Moreover, the transfer tests were followed by at least 30 minutes of normal training at mazes A and B.

Experiment 2: Honeybees can apply the learnt rules from mazes A and B to a new location within a temporal context.

In the first transfer experiment at maze C, the bees were tested with the usual training patterns, but with the location cue excluded. They encountered yellow gratings in the morning and blue gratings in the afternoon. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1 = 5, d.f.2 = 15, F-ratio = 1.334, p = 0.303) or in the afternoon

(ANOVA, d.f.1 = 7, d.f.2 = 6, F-ratio = 0.918, p = 0.55). The results of this experiment are shown in Fig. 4 A. The choice frequency for the yellow vertical grating in the morning was 0.94 ± 0.02 (N=18), while the choice frequency for the blue horizontal grating in the afternoon was 0.73 ± 0.06 (N=9). Results of ANOVA tests are as follows: d.f. (interaction) = 1, F = 2.736, p = 0.108; d.f. (time) = 1. F = 220, p < 0.0001; d.f. (repeated tests) = 1, F =0.283, p = 0.598. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. They reversed their pattern preference in the same (neutral) location, choosing a grating according to the time of day (when) and the pattern colour (what).

Experiment 3: Honeybees can use colour cues alone to make a correct decision at maze C within a temporal context.

In the next experiment at maze C, the bees were tested for colour preference by presenting blue and yellow vertical gratings in the morning, and blue and yellow horizontal gratings in the afternoon. Thus, we excluded the pattern orientation and the maze location cues. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1 = 9, d.f.2 = 12, F-ratio = 0.625, p = 0.756) or in the afternoon (ANOVA, d.f.1 = 7, d.f.2 = 8, F-ratio = 0.170, p = 0.985). The results of this experiment are shown in Fig. 4 B. The choice frequency for the yellow grating in the morning was 0.87 ± 0.04 (N=19). In the afternoon, the bees preferred the blue horizontal grating over the yellow horizontal grating, with a choice frequency of 0.95 ± 0.02 (N=11). Results of ANOVA tests are as follows: d.f. (interaction) = 1, F = 2.827, p = 0.102; d.f. (time) = 1. F = 344.5, p < 0.0001; d.f. (repeated tests) = 1, F =3.646, p = 0.065. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees were able to reverse their colour preference from yellow in the morning to blue in the afternoon in a neutral location, basing their decision in the maze on the temporal context.

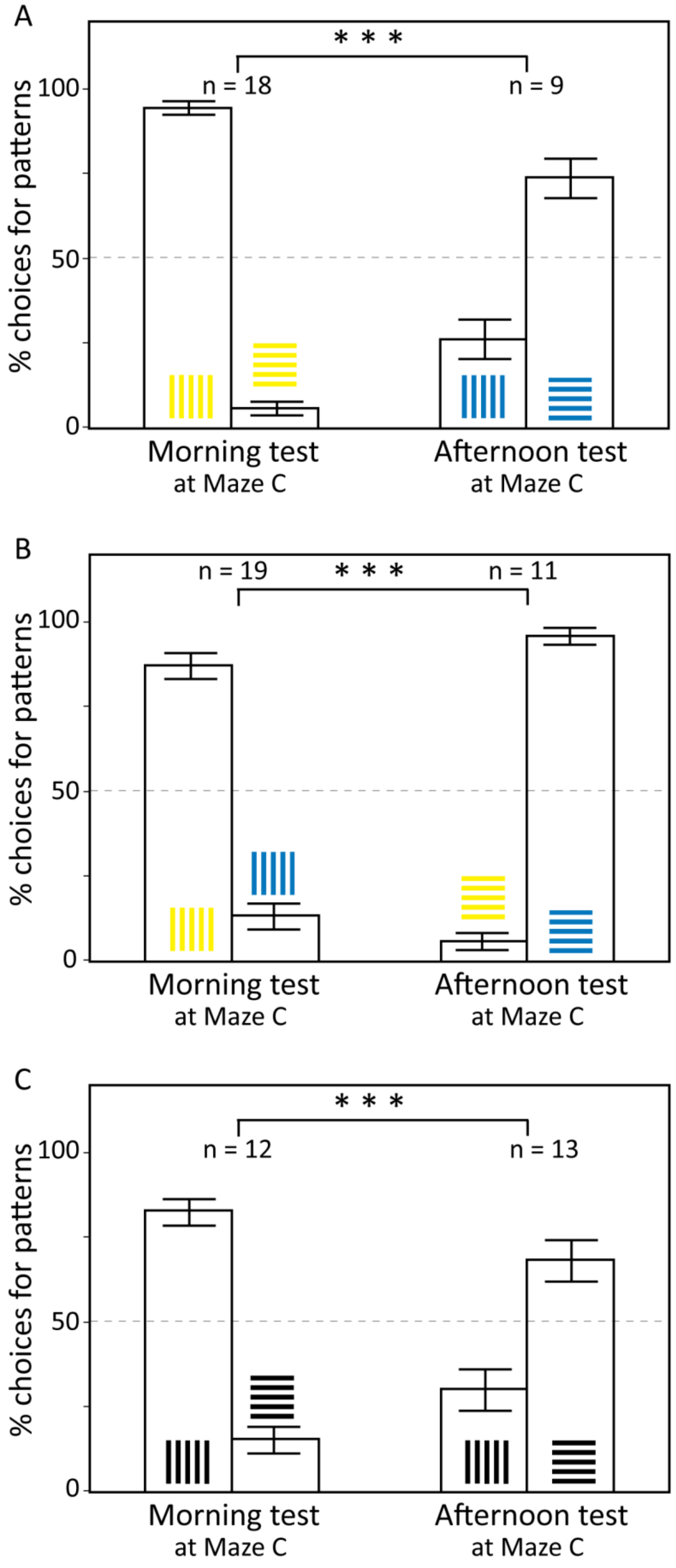


Figure 4: Results of experiments 2–4, the transfer tests at maze C. (A) Experiment 2: yellow and blue training patterns. The bees significantly reversed their pattern preference from the yellow vertical grating in the morning to the blue horizontal grating in the afternoon at the neutral location. (B) Experiment 3: yellow and blue patterns in the same orientation. The bees significantly reversed their colour preference from yellow in the morning to blue in the afternoon at the neutral location. (C) Experiment 4: black patterns. The bees significantly reversed their pattern preference from the vertical grating in the morning to the horizontal grating in the afternoon. *N*, number of individual bees attending the test; values are means ± s.e.m. See text for further details.

Experiment 4: Honeybees can use orientation cues without colour cues, to make a correct decision at maze C within a temporal context.

In the last experiment of this series, the location and colour cues were excluded by presenting black horizontal and vertical gratings at maze C (Fig. 4 C). The bees had to choose a grating orientation according to the time of day. Analysis of variance showed no significant differences between the performances of individual bees on all testing days either in the morning (ANOVA, d.f.1 = 10, d.f.2 = 8, F-ratio = 0.539, p = 0.822) or in the afternoon (ANOVA, d.f.1 = 12, d.f.2 = 10, F-ratio = 1.638, p = 0.221). In the morning, the bees preferred the vertical grating (0.84 ± 0.04, N=12). In the afternoon, they significantly reversed their pattern preference to the horizontal grating (0.69 ± 0.06, N=11). Results of ANOVA tests are as follows: d.f. (interaction) = 1, F = 1.631, p = 0.209; d.f. (time) = 1. F =

52.4, p < 0.0001; d.f. (repeated tests) = 1, F =0.243, p = 0.625. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees could reverse their pattern preference solely within a temporal context; no other cue was present to influence the bees' choices for the horizontal or vertical patterns. The same was true for the experiments with sector and ring patterns (see Fig. S2 in supplementary materials).

To negate the possibility that the bees had an unexpected preference for the vertical pattern, all experiments at the mazes A and B and the black pattern transfer test at maze C were repeated with a different group of bees and a set of central symmetric sector and ring patterns. The results are shown in Fig. S1 and S2 in the supplementary materials.

Control tests

Control tests were conducted to ensure that the honeybees did not develop a preference to a particular side of the maze. In these tests, the decision cylinders carried the same visual patterns on both sides, and no food reward. The bees' decisions for the left or right side were monitored.

At maze A, the choice frequency for the right side was 51.7%, while that for the left side was 48.3%. Thus, the bees did not have a preference for a particular side of maze A (2x2 McNemar test, d.f = 1, p = 0.961, see Fig. S3A in supplementary materials). At maze B, the bees chose the right side in 52.8% of the visits. The left side was chosen in 47.1% of the visits. There was no significant difference in the bees' choices for the left or the right side of maze B (2x2 McNemar test, d.f.=1, p = 0.936, see Fig. S3B in supplementary materials).

Discussion

Foraging at multiple feeding sites

Gallistel (1990) reported that the bee may simultaneously retain several different mnemonic constellations, each specifying different locations, with different sensory characteristics, and providing food at different times . In our experiments, during training, the bees learned to choose the yellow vertical grating at maze B in the morning, and the blue horizontal grating at maze A in the afternoon (Fig. 1, 2). The very low error rate in this training indicates that visiting two or more feeding places at fixed times is an easy task for foragers, and might be a common strategy in honeybees.

The aim of experiment 1 was to investigate whether honeybees can still find the correct pattern shape, without the colour cue. The bees extracted the orientation information of the coloured patterns, and applied it to the novel black patterns (Fig. 3A, B). The same was true for the sector and ring patterns (see Fig. S3B in supplementary materials). They showed a clear preference for the correct pattern at the respective maze and time of day.

Foraging at a novel feeding site

In all the transfer tests at the neutral maze C (experiments 2-4), the location cue was excluded. This setup further allowed us to artificially remove all other cues, except the temporal cue, as required. Under these conditions, the bees had to rely on their time-sense when deciding between visual patterns that possessed only partial features, such as colour or shape.

The aim of experiment 2 was to investigate whether honeybees can transfer learnt rules to a novel location. The data show that the bees recalled the rewarded patterns according to the pattern colour and the time of day, independent of the location cue (Fig. 3A). In this case, the colour and the time could prime the visual memory of shape, i.e. choosing the vertical grating if they saw yellow, and the horizontal grating if they saw blue, or choosing the vertical shape in the morning and the horizontal shape in the afternoon. The next experiment was carried out to check the importance of pattern colour.

Experiment 3 was conducted to check whether honeybees can transfer previously formed time-colour associations to a novel location. The high performance of the bees in this experiment (Fig. 4B) shows the importance of the colour cue in decision making. As the pattern orientation always suggested a positive pattern, the bees had to decide on a colour according to the timeframe. The bees recalled the correct colour according to the time of day from their memory.

In experiment 4, we tried to determine if honeybees can transfer previously formed time-shape associations to a novel location. The lower performance in this experiment (Fig.

4C), compared to the colour experiment (Fig. 4B), shows that this task was more difficult for the bees. However, they still significantly reversed their pattern preference between morning and afternoon. The location and the colour cue were not necessary for the bees to make a correct decision in this test. While the shape cues (orientation of gratings) certainly made the learning process easier, the bees successfully extracted and memorized the rule "choose the vertical grating in the morning and the horizontal grating in the afternoon", and recalled it to solve the task, independent of the pattern colour, and without a location context. The same is true for the additional experiments with sector and ring patterns (see Fig. S3C in supplementary materials).

The results confirm that bees would be able to forage from different kinds of flowers, at different times of the day, at the same feeding location. They could also select a particular kind of flower when visiting a new feeding location, recalling the memory of the most profitable flower species for a particular time of day.

These results cannot be an artefact caused by learning during a test, because it takes 20 to 30 visits for a honeybee to learn a geometric pattern (Zhang and Srinivasan, 1994; Zhang et al., 2004). The transfer test periods were kept short, so that each bee could make a maximum of three foraging trips per testing session. In addition, the ten minute testing period was followed by at least 30 minutes of normal training, which would also prevent a possible learning effect (Fischer, 1957).

Context learning

Contextual cues offer the possibility of treating the same stimulus in two or more different ways, thereby enabling the animal to interact more flexibly with its environment. In the case of the honeybee, contextual cues are essential for efficient foraging. When visiting a patch of flowers, the bees can decide on a profitable flower within the temporal context; i.e. choose the yellow flower in the morning and the blue flower in the afternoon, even when exploring new foraging territories.

Pattern memory can be primed by two locations, in which a bee that is trained to recognize one pattern at one site and another pattern at a second site will choose A+ over B- at site A, but B+ over A- at site B (Menzel et al., 1996). Bees' memories can also be primed by the surrounding panorama that includes spatial and colour contextual cues (Cheng et al., 1986; Collett and Kelber, 1988; Collett et al., 1997; Dale et al., 2005). A familiar nectar scent, encountered at the hive entrance before departing, can trigger specific route memories that expedite navigation to one of two different food sources (Reinhard et al., 2004). The limit of memorisable scent-feeder-associations seems to be two; to distinguish three different feeder–scent–combinations the bees need additional cues (e. g. colours) (Reinhard et al., 2006).

Finally, recent experiments have shown that honeybees are able to change their visual pattern preference in the presence of two cues: the time of day and the task at hand (Zhang et al., 2006).

In our present experiment, location cues were excluded by testing the bees at a neutral position (in maze C), the task at hand was always foraging, and the only reliable rule for the bees was the time-colour or the time-shape association. The bees chose the correct patterns at maze C in two different experiments, at a level significantly different from random choice. This indicates that bees can still find the correct visual pattern according to the time of day, when all other available cues are excluded.

The honeybees' internal clock

Animals ranging from bees to rats routinely record the time of day at which they have a noteworthy experience and make use of this record to time their subsequent behaviour (Gallistel, 1990). Bees need a precise time sense to compensate for the movement of the sun in their dance language, which is performed in the total darkness of the hive (Lindauer, 1960). Some 'marathon dancers' perform the recruitment waggle dance for hours, without leaving the darkness of the hive, accurately indicating the direction of a food source with respect to the sun's azimuth at any time of the day or night (Chalifman, 1950; Lindauer, 1954; Wittekindt, 1955). Dyer and Dickinson (1994) suggested that bees are innately informed of certain general spatial and temporal features of solar movement. Bees can synchronize their behaviour with daily floral rhythms, foraging only when nectar and pollen are at their highest levels. At other times, they remain in the hive, conserving energy that otherwise would be exhausted on non-productive foraging flights (Moore, 2001).

The greenhouse used in our experiments was covered by an opaque PVC sheet that was similar to the materials used to cover an extension of our All Weather Bee Flight Facility (AWBFF). We have discussed the effect of UV and polarized light on circadian rhythms in the honeybee in a previous publication (Zhang et al., 2006). However, we still measured the illumination spectrum in the greenhouse where the present experiments were carried out, and found that UV light was tremendously reduced to undetectable levels at the maze areas. In addition, the bees' performance was not affected by large changes in the weather. The weather record for the region shows that our experiments were sometimes carried out under complete cloud cover. Even then, the bees could still perform as usual. The consistent results obtained under different weather conditions indicate that the honeybees did not need to use the sun's position, UV or polarized sunlight as cues to change their preference according to time.

However, it is not clear if the honeybee has a time-sense governed by a circadian rhythm, connecting a specific memory to a certain phase in the 24h-cycle, or if it is also capable of measuring the elapsed time between two events. Interval-timing has been shown in vertebrates (Richelle and Lejeune, 1980; Gallistel, 1990; Babb and Crystal, 2005), and recently in an invertebrate from the same family as the honeybee, the bumblebee *Bombus impatiens* (Boisvert and Sherry, 2006). Further experiments are planned to investigate the question of interval timing in the honeybee. If honeybees show this ability, their memory of ‘what’, ‘where’ and ‘when’ could fulfil the behavioural criteria for episodic-like memory in nonhuman animals, as shown in the food caching scrub-jay *Aphelocoma coerulescens* (Clayton and Dickinson, 1998). Our experiments have shown that the honeybee links together in an integrated fashion the elements of circadian time (when during a day), place (where), and colour and pattern characteristics (what). This is akin to episodic-like memory, except that the temporal element is circadian time, instead of interval timing. We have named this kind of memory circadian timed episodic-like memory.

Cue ranking

In the learning tests (Fig. 3A and supplementary material Fig. S1A), the bees reached an average performance (morning and afternoon sessions) of 83% correct choices. Setting this as a baseline, and comparing it with the other tests where one or several contextual cues were taken out, we can compare the difficulty of the transfer tests, and thus determine the relative importance, to the bees, of the different cues .

In the transfer tests at maze C, the bees reached their best average performance of 91% in experiment 3, the colour discrimination task (Fig. 4B). The performance in the morning showed no significant difference between the learning test and the transfer test ($t = 0.258$, d.f. = 45, $p> 0.8$); however, in the afternoon, the performance in this transfer test was even better than that of the learning test ($t = 3.6$, d.f. = 38, $p > 0.001$), regardless of the missing location and pattern orientation cues. Thus, colour seems to be the most important visual cue for honeybee choice behaviour. These findings are consistent with previous reports that honeybees learn a new colour after about five visits, whereas they normally require 20 to 30 visits to learn a pattern (Zhang and Srinivasan, 1994).

Using the training patterns at maze C in experiment 2, the bees performed about the same as in the training tests at mazes A and B (83%, Fig. 4A). There was no significant difference in performance between the learning test and the transfer test in the morning ($t =2.01$, d.f. = 44, $p > 0.05$) and in the afternoon ($t = 1.25$, d.f. = 36, $p > 0.20$). Here, the only missing cue was the maze location. This cue seems to have had almost no effect on the bees' choice performance in small scale navigation, when other contextual cues were available. The results of this transfer test demonstrate that pattern colour and the time of day were enough to allow a baseline level of performance at a new location.

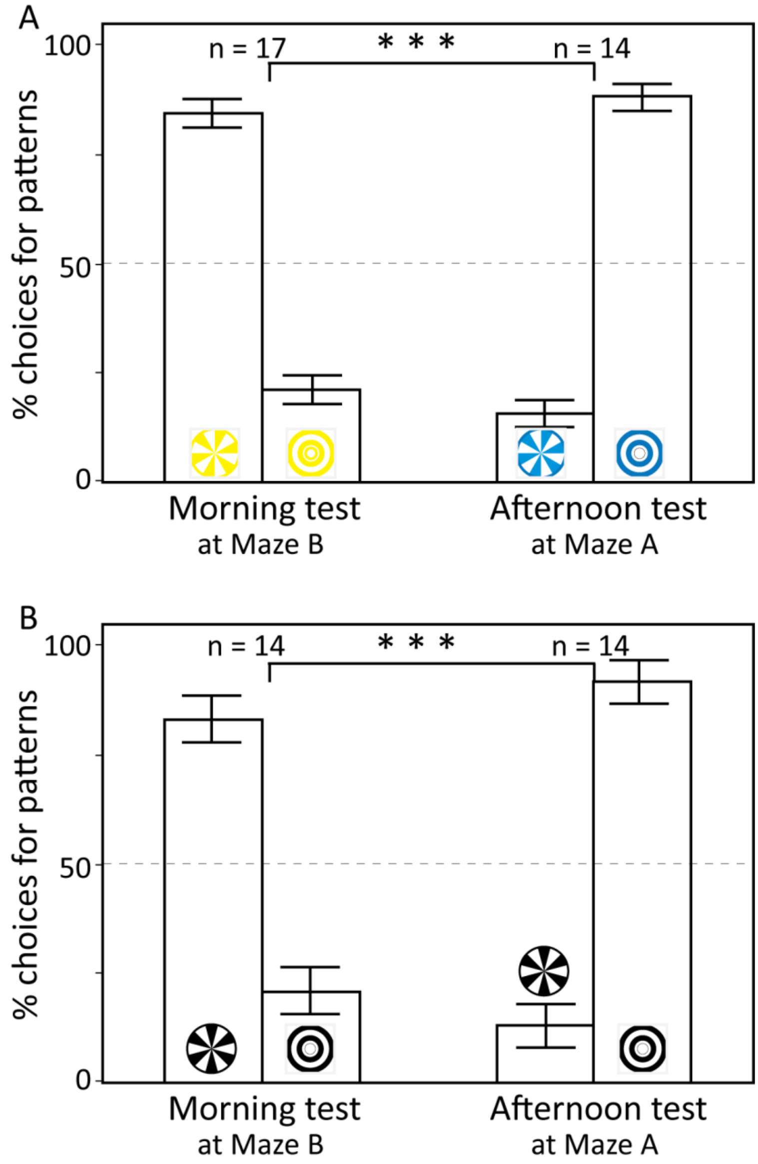
When the colour cue and the location cue were both taken out in experiment 4, the bees' average performance was reduced to 72% (Fig. 4C and supplementary material Fig. S2). The performance in the morning was slightly worse in the transfer test than in the learning test, but not significantly so ($t = 1.10$, d.f. = 38, $p > 0.2$). However, in the afternoon, the performance in the transfer test was slightly worse than that of the learning test ($t =2.1$, d.f. = 40, $p <0.05$). The results indicate that the shape cue is more difficult to use for the bees than the colour cue. Pattern orientation, or, in nature, the shape of different flowers, is thus more important than location for the bees' choice behaviour once they have reached their feeding site. The bees clearly used the former to distinguish between the patterns in all experiments except the colour discrimination task (where pattern orientation was unavailable).

Applying these findings to the natural situation, we can say that the colour and shape of flowers are the most important visual cues used by bees to choose between different flower species. When visiting different feeding sites, or different patches of flowers, they can recall their memory of the most rewarding species in conjunction with the time of day, and thus find the most profitable food source even at a new location.

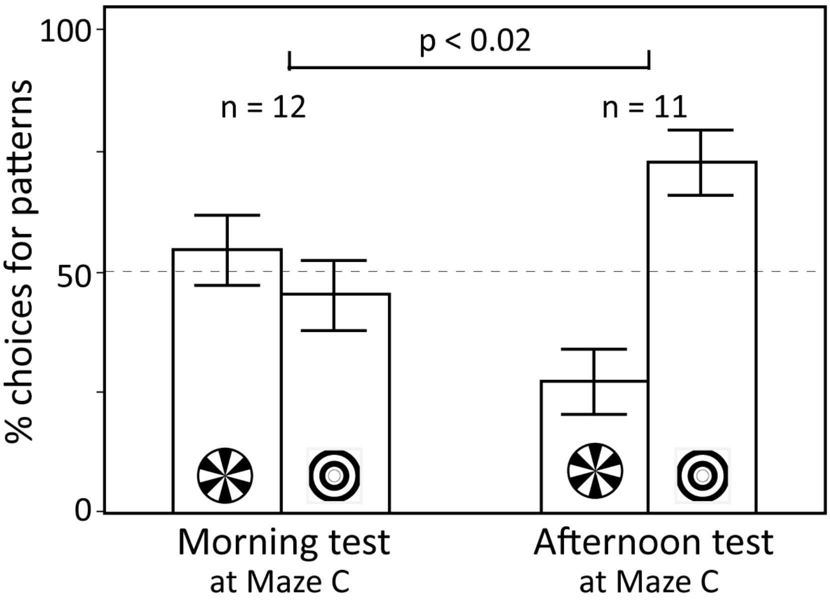
Acknowledgements

We would like to thank Aung Si for critical reading of the manuscript, Paul Helliwell for help with beekeeping, and Bill Speed for constructing the bee-tunnel and apparatus.

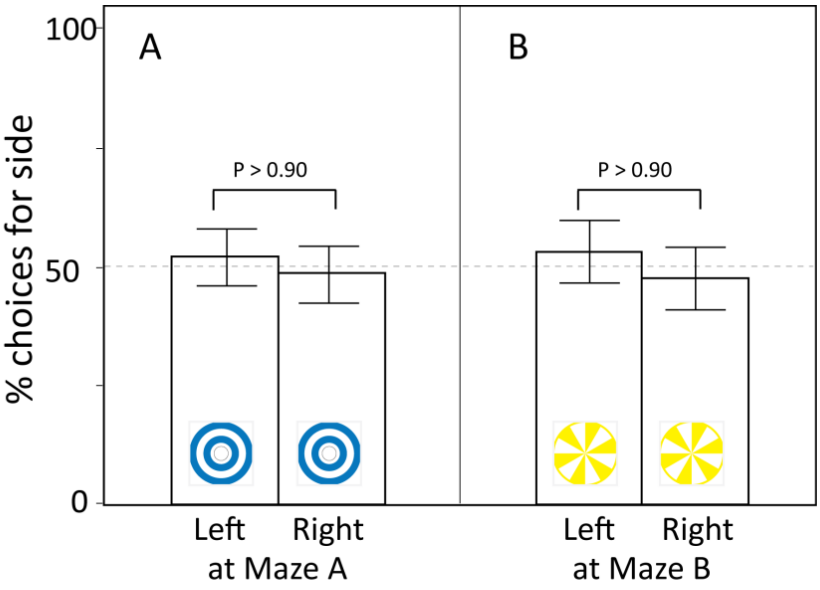
Supplementary Material



Supplementary Figure 1: Results of the training and transfer tests at mazes A and B with sector and ring patterns. (a) Results of the training tests at mazes A and B with yellow patterns in the morning and blue patterns in the afternoon. At maze B in the morning, the choice frequency for the yellow sector pattern was 0.80±0.03. At maze A in the afternoon, the choice frequency for the ring pattern was 0.85±0.02. Results of ANOVA tests are as follows: d.f. (interaction)=4, *F*=0.266, *p*=0.891; d.f. (time)=1, *F*=329.9, *p*<0.0001; d.f. (repeated tests)=4, *F*=0.243, *p*=0.913. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees clearly changed their preference from the sector pattern in the morning to the ring pattern in the afternoon (b) Results of the transfer tests with black patterns at mazes A and B. At maze B, the choice frequency for the sector pattern was 0.80±0.05 (*N*=14), while at maze A, the choice frequency for the ring pattern was 0.89±0.05 (*N*=14). Results of ANOVA tests are as follows: d.f. (interaction)=2, *F*=0.653, *p*=0.526; d.f. (time)=1, *F*=142.1, *p*<0.0001; d.f. (repeated tests)=2, *F*=0.067, *p*=0.935. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees had clearly changed their preference from the sector pattern in the morning to the ring pattern in the afternoon (*t*-test, *p*<0.001). Values are means ± s.e.m.; *N*, number of individual bees attending the tests. See text for further details.



Supplementary Figure 2: Results of the transfer test at maze C with black sector and ring patterns. The choice frequency for the sector pattern in the morning was 0.55±0.07. In the afternoon, the bees chose the ring pattern with a frequency of 0.74±0.07. Results of ANOVA tests are as follows: d.f. (interaction)=1, *F*=0.000, *p*=0.96; d.f. (time)=1, *F*=12.1, *p*<0.01; d.f. (repeated tests)=1, *F*=0.111, *p*=0.741. All the Bonferroni post tests showed performance in the morning and in the afternoon are significantly different. The bees had significantly reversed their pattern preference between the morning and afternoon testing sessions. Values are means ± s.e.m.; *N*, number of individual bees attending the tests. See text for further details.



Supplementary Figure 3: Results of the control tests at mazes A and B. (a) At maze A, the choice frequency for the right side was 51.7%, while that for the left side was 48.3%. Thus, the bees did not have a preference for a particular side of maze A (2×2 McNemar test, *p*=0.961, *N*=58). (b) At maze B, the bees chose the right side in 52.8% of the visits. The left side was chosen in 47.1% of the visits. The bees did not have a preference for a particular side of maze B (2×2 McNemar test, *p*=0.936, *N*=70). Values are means ± s.e.m.; *N*, number of individual bees attending the tests. See text for further details.

Chapter 3: Number-Based Visual Generalisation in the Honeybee

Abstract

Although the numerical abilities of many vertebrate species have been investigated in the scientific literature, there are few convincing accounts of invertebrate numerical competence. Honeybees, *Apis mellifera*, by virtue of their other impressive cognitive feats, are a prime candidate for investigations of this nature. We therefore used the well-established delayed match-to-sample paradigm, to test the limits of honeybees' ability to match two visual patterns solely on the basis of the shared number of elements in the two patterns. Using a y-maze, we found that bees can not only differentiate between patterns containing two and three elements, but can also use this prior knowledge to differentiate three from four, without any additional training. However, bees trained on the two versus three task could not distinguish between higher numbers, such as four versus five, four versus six, or five versus six. Control experiments confirmed that the bees were not using cues such as the colour of the exact configuration of the visual elements, the combined area or edge length of the elements, or illusory contours formed by the elements. To our knowledge, this is the first report of number-based visual generalisation by an invertebrate.

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Introduction

The numerical ability of non-human animals has long been a source of fascination and contention to members of the academic and lay communities alike. As early as the 1940s, Otto Koehler and his students were able to demonstrate that pigeons could be trained to peck at a cluster of seeds containing, for example, exactly three seeds, and ignore the cluster containing two. Jackdaws could also learn a matching-to-sample paradigm to recognise visual patterns with the “correct” number of dots, and obtain a hidden food reward (Koehler, 1941). Drawing inspiration from these humble beginnings, later researchers have been able to show that a wide range of vertebrate species such as raccoons (Davis, 1984), dolphins (Kilian et al., 2003), monkeys (Brannon and Terrace, 2000), songbirds (Hunt et al., 2008) and even salamanders (Uller et al., 2003) also possess some form of numerical competence. Indeed, studies on chimpanzees have uncovered the impressive ability of this species to order numerosities on a scale, even in the absence of a language faculty (Brannon and Terrace, 1998).

However, even a brief survey of the literature on animal numerical abilities will reveal a surprising asymmetry: mostly vertebrate species have been studied to date, leaving the numerical cognition of invertebrates largely unexplored. We intend to correct this imbalance in this paper, by reporting our novel findings on the numerical ability of the honeybee. Research in the last two decades has shown that honeybees possess impressive cognitive abilities, such as the capacity to match and categorise visual objects (Zhang et al., 2004), learn the concept of sameness and difference (Giurfa et al., 2001), associatively group and recall visual objects (Zhang et al., 1996), and carry out different tasks within a temporal context (Pahl et al., 2007). Indeed, an early claim that honeybees might be able to distinguish between flowers of different species by ‘counting’ the number of petals (Leppik, 1953), was probably confounded by the insect's ability to detect bilateral symmetry (Giurfa et al., 1996; Horridge, 1996) and categorize visual objects by their overall shape (Zhang et al., 2004). While it would be unwise to expect honeybees to perform tasks comparable to those attributed to chimpanzees, we thought it not unreasonable to expect at least a rudimentary form of numerical ability in this insect. After all, an estimate of the number of flowers visited on a foraging trip, weighed against the amount of nectar collected, could yield an estimate of the profitability of a food source (Skorupski and Chittka, 2006). At least one study has hinted at the possibility that foraging honeybees might be able to remember the number of landmarks encountered on the way to a food source (Chittka and Geiger, 1995). Bees could also be trained to match either the 'first' or 'second' sample pattern in a sequence of two, to the correct choice pattern (Zhang et al., 2005). Finally, a recent variant of the Chittka and Geiger study reported sequential counting of landmarks by bees flying to a food source (Dacke and Srinivasan, 2008).

We therefore set out to determine if any form of numerical cognition could be attributed to the honeybee. Using a y-maze setup, and a delayed-match-to-sample (DMTS) paradigm, we trained honeybees to make generalisations about the number of elements in a visual pattern, and distinguish between arrays composed of two and three elements. Having controlled for lower-order cues such as area and edge length, we find that the bees were using the number of elements in each pattern as a cue on which to base their decisions. While our results neither suggest that bees can 'count', nor that they can order numerosities, we believe that this is the first report of number-based visual generalisation in an invertebrate.

Material and methods

Basic training

A group of approximately 20 bees was trained in a modified y-maze apparatus to perform a basic DMTS task (Giurfa et al., 2001) with identical patterns of two versus three blue dots. Briefly, when a bee entered the apparatus, it encountered a sample pattern; say two blue dots; which it had to retain in its working memory. The bee had to then fly through a 1-meter long tunnel and then into a decision chamber, where it was presented with two choice patterns, only one of which was identical to the sample. The other pattern was composed of three blue dots. The bee had to choose the matching pattern (two dots), to obtain a reward of sugar solution from a hidden artificial feeder. A bee making an incorrect decision was released from the maze and allowed to try again. However, only the first-trial data for each bee were used. The positions of the choice patterns were swapped every ten minutes, to prevent the bees from developing a side preference. Thus each sample pattern was presented for twenty minutes during training. Every time the positions of the choice patterns were exchanged, they were also rotated by 180°, as was the sample pattern. Once training was completed on a particular sample (say, two dots), it was replaced with the competing sample (three dots); this sample was also presented for two ten-minute sessions. In all, each pair of competing patterns was presented to the bees in four orientations: two dots (upright), two dots (rotated), three dots (upright), and three dots (rotated). Training, including pre-training and training proper went on for a total of three days, by which time the bees were able to consistently solve the DMTS task. During the training proper, baffles behind the entrances of the two choice chambers (See Figure 1) completely prevented the bees from viewing the feeder in the reward chamber from the decision chamber. The learning curve was acquired during this period.

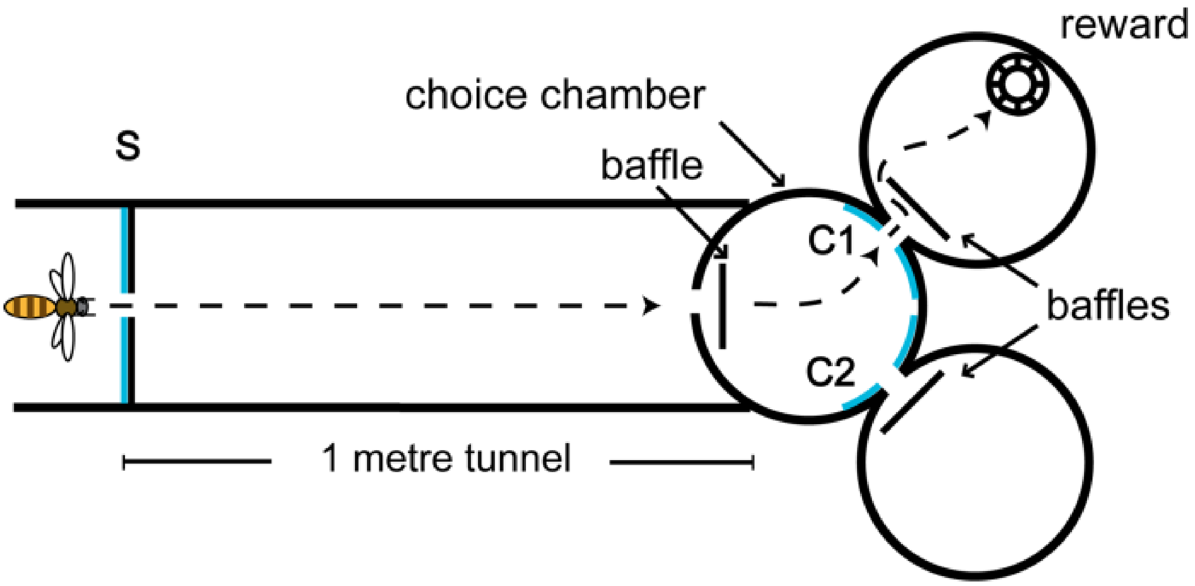


Figure 1: Layout of the Delayed Match-to-Sample (DMTS) experimental apparatus. The bee encounters and flies through the initial sample pattern (S) before traversing a 1m-long tunnel with a Perspex roof. There is a baffle behind the entrance of the decision chamber and baffles behind the entrances of the choice chambers. The baffles prevented the bees from experiencing the stimuli in the decision chamber until they had entered it, and from viewing the feeder from the decision chamber. Upon entering the choice chamber, she is presented with two choice patterns (C1 and C2), only one of which (C1 in this case) has the same number of dots as S. The bee must choose the matching pattern C1 in order to obtain a hidden reward of sugar solution.

Testing and data collection

A rewarded feeder was present in the 'correct' chamber at all times during testing. Testing was carried out in shorter, 5-minute blocks, as only the first choices of the bees (per test condition) were of interest to us. This procedure also had the effect of minimizing any additional learning that might have taken place during the test sessions. Every time the positions of the choice patterns were swapped, they were also rotated by 180° to ensure that the bees were not learning a particular configuration of elements (see Zhang et al. (2005) for further details). Non-choices, where a bee enters the choice chamber, but is unable to decide on a pattern for an extended period of time, occurred frequently in the early stages of training, but had ceased to be a problem by the time testing commenced. The experiment was halted for 30–40 minutes between each transfer test. During these breaks, another feeder, with a dilute sugar solution, was provided at the entrance of the tunnel (which was otherwise blocked). This procedure, combined with frequent transfers to novel sets of stimuli, improved the bees' performance, compared to a regime of prolonged, uninterrupted training on a single set of stimuli. Note, for example, that the bees frequently performed better in the transfer tests with novel stimuli than in the last two sessions of the learning curve.

As the y-maze had only two reward chambers, we took pains to randomize the position of the starting chamber in each transfer test. This prevented the bees from learning a rule like ‘go to the left chamber at the start of each test’.

Controlling the number of bees within the apparatus

We were careful, during the transfer tests, to limit the number of bees in the choice chamber to one at a time. This is important, because bees readily follow each other within the confined space of the apparatus, and can also distract one another during the decision-making process. To a large extent our experimental design ensured that the y-maze was never visited by a surplus of bees during any given transfer test. The extended breaks between each transfer test, combined with the weaker sugar solution offered at the maze entrance during these breaks, had the effect of temporarily reducing the traffic of our 20 trained bees between the maze and the hive. Moreover, during the occasions when more than one bee did enter the decision chamber, the experimenter would open the lid of the chamber, and let the excess bees out of the maze. These bees would then have to return to the maze entrance to try again. Thus we ensured that bees were making independent decisions.

Control tests for olfactory cues

In order to exclude the possibility that olfactory cues were influencing the bees' decisions, we carried out an additional control experiment. A group of bees was trained specially for this purpose. The bees were trained to solve a basic DMTS task with a set of patterns containing two and three blue dots that were the same as used in the previous training (Figure 2A), and were then, after they had reached a high plateau, tested on new patterns with randomised two and three dot configurations. During the transfer tests, the three cylinders of the maze apparatus (Figure 1) were replaced with fresh ones, and no reward was present in the end cylinders. The testing period was kept short (2–3 min), to make sure that each bee would encounter the unrewarded transfer test situation only once, since it is similar to the punishment for making a wrong choice for the bee, and thus leads to negative learning. Each of those tests was followed by a long training period to keep the bees motivated to visit the maze.

Statistical analysis

We performed ANOVA using the statistical software SYSTAT for checking the homogeneity of the data (Systat Software, Richmond, CA). Next, the performance of each bee was evaluated separately, by pooling its first choices. The mean choice frequency was calculated as follows: the first choice of each bee in a given test condition, if correct, was scored 1, and if wrong, was scored 0. Each bee provided one data point in the first test configuration (with the patterns upright), and then another in the second test configuration, when the feeder was moved into the other chamber, and the patterns were rotated by 180°. A bee could therefore achieve an average score of 0%, 50% or 100% in a transfer test. The average scores of all bees involved in the test were averaged for an overall indication of performance. The Student t-test was used to determine whether performance was significantly better than random choice. Two types of Student t-tests were performed: the first type of test was to check whether the bees made decisions according to the sample pattern, namely whether their performance was significantly different from random choice; the second type of test was to check whether the bees reversed their preference after the sample was changed.

Results

The experiments were carried out repeatedly several times in Germany and in Australia during 2006 to 2008.

Training on the basic DMTS task

Over a period of three days, a group of approximately 20 bees were able to learn the basic DMTS paradigm, where they had to match one of two choice patterns to a previously encountered sample pattern (See the experimental apparatus in Figure 1). In particular, bees could choose a pattern of two or three blue dots that exactly matched the sample pattern in every way, in order to obtain a sugar reward. The ANOVA tests revealed that the data collected across all blocks and across all bees were homogeneous ($p>0.05$). The exact p values for each block are summarised in Supporting Table S1. Figure 2A shows the learning curve of the experimental bees in the six training blocks; performance is seen to plateau at approximately 70% correct choices after the 4th block. Figure 2B shows the percentage of incorrect second choices following a positive first choice. The percentage of incorrect second choices of the 1st block was 50.0%; this declined to a low 24% by the last block.

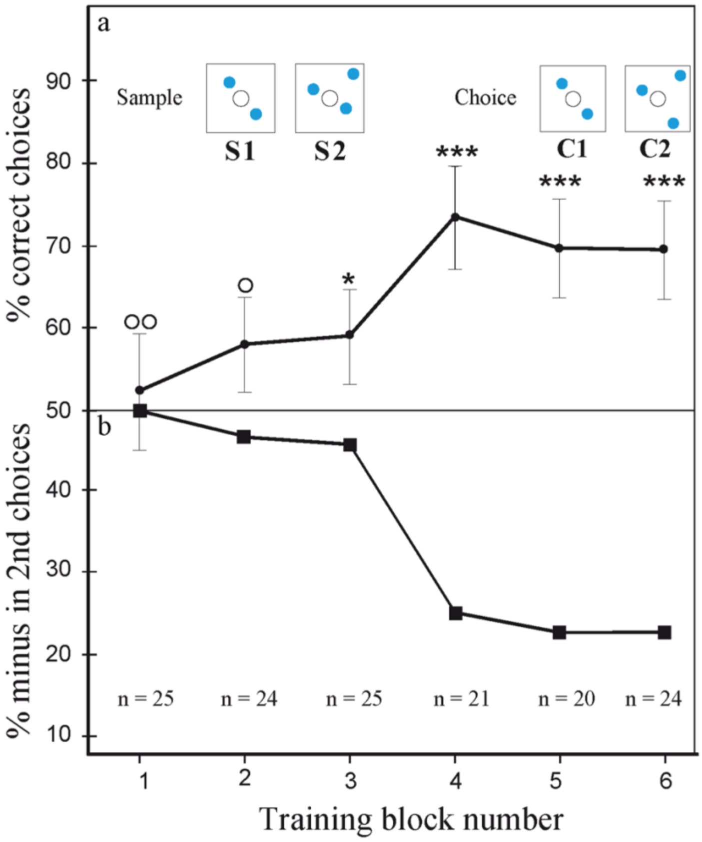


Figure 2: (a) Learning curve for bees trained on a basic delayed match-to-sample (DMTS) task. Each 'block' represents two twenty-minute sessions of training (one for each sample S1 and S2). Bees were considered to be trained in this task when their performance reached a stable plateau (approximately 70% correct choices). n denotes number of bees per condition. Error bars show standard error. * denotes statistically significant difference at p<0.001, * denotes p<0.05. (b) The incorrect second choices of bees in each of the training blocks, following a positive first choice.**

Transfer test on patterns containing dots in randomised orientations

Once the bees' performance in the basic DMTS task had stabilised, they were presented with new patterns, in which the configuration of dots was randomised. There were 19 bees in the 1st half of the transfer test when the sample was three blue dots, and 17 bees in the 2nd half of the transfer test, when the sample was two blue dots (as denoted in Figure 3a). An individual bee visited the apparatus during a transfer test for four times on average (one visit per configuration). The bees were able to carry out this more difficult task, and attained a score of 70% (significantly different to 50%, $p<0.01$) for the three-dot-sample, and 79% (significantly different to 50%, $p<0.001$) for the two-dot-sample. The performance was significantly reversed after the sample pattern was swapped from the two-dot sample to the three-dot-sample ($p<0.001$, Figure 3a). The same notations are used in all other figures. This experiment gave the first indication that the trained bees might be using the number of elements in the visual arrays as a cue to perform the matching task.

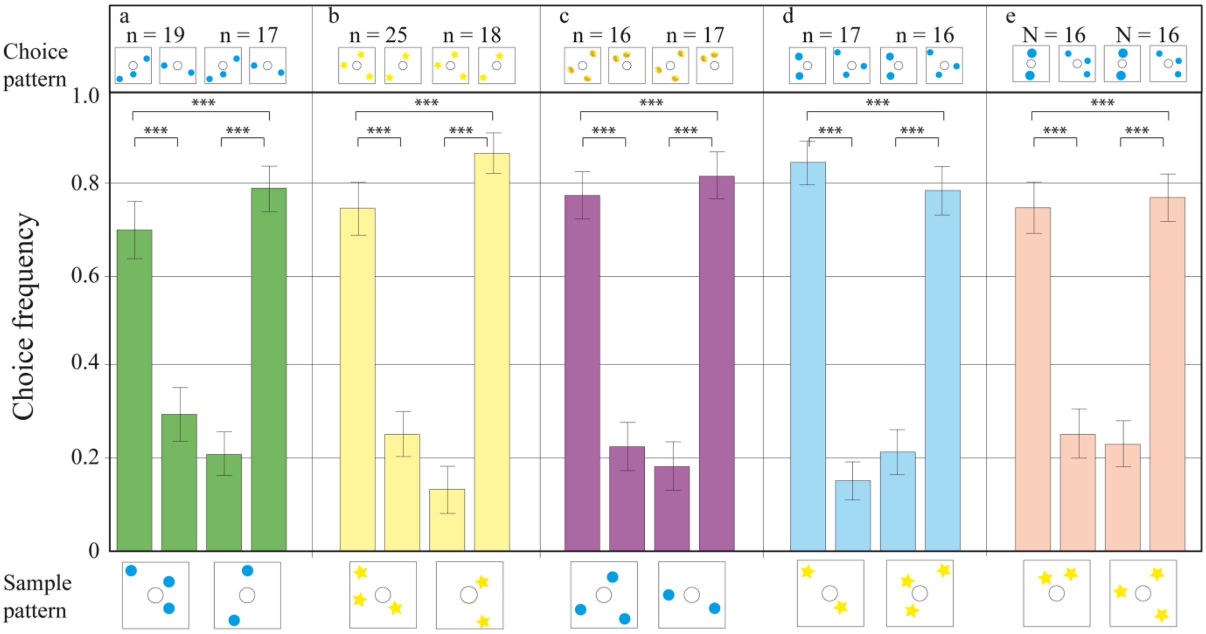


Figure 3: Results of transfer tests with various pattern configurations. The pattern below each pair of bars is the sample and that above each bar is the choice pattern; the y-axis gives the choice frequency. The data represent the pooled first choices (from each foraging trip) of individual bees. (a) The configuration of dots on the sample and choice patterns is randomised. (b) The blue dot patterns in (a) are replaced with yellow stars, to see if bees can transfer their matching ability to different, unknown stimuli. (c) The sample and choice patterns are composed of different elements. (d) The choice patterns are modified so that the total area of the elements is equal. (e) The choice elements are modified so that the total edge length of the elements is equal. n = number of bees per condition. Error bars show standard error. * denotes statistically significant difference at p<0.001, ** denotes p<0.01, * denotes p<0.05 and ○ denotes p>0.05.**

Transfer tests with novel stimuli

We then tested whether the same trained bees could transfer the rule "match the number of items" to a totally novel set of stimuli. Once again, when the bees were shown a sample pattern containing two stars, for example, they were able to convincingly match it with a choice pattern also containing two stars (Figure 3b). This experiment was repeated with further sets of novel stimuli, such as two versus three yellow lemons, with much the same result (see Supporting Figure S1a and Supporting Table S2 for statistical analysis). Then, an additional level of abstraction was introduced, by making the elements of the sample and choice patterns different. Now, the bees encountered a sample pattern of three blue dots, for instance, which they had to match to a choice pattern composed of three yellow lemons, again in random configurations. Here too, the bees performed remarkably well, using the number of items to identify the matching, rewarded pattern (Figure 3c).

Reversing the order of the patterns, i.e. yellow lemons as the sample and blue dots as the choice patterns, did not affect performance (See Supporting Figure S1b).

Control tests for lower-order cues

To control for the possibility that the bees could be using cues such as the edge lengths or combined areas of the visual items, we presented them with stimuli where these cues, in the sample and choice patterns, had been equalised. The bees were still able to choose the pattern with the right number of dots, even when the areas (Figure 3d) and edge lengths (Figure 3e) of the choice patterns were the same.

Transfer tests with novel numerosities

Next, we investigated if the bees could transfer their ability to discriminate between two and three, to arrays of three and four items, the latter being a value they had not previously encountered during the experiment. The bees could successfully carry out a three-to-three match, when the competing stimulus contained four elements (Figure 4a to c). However, they were not able to consistently do a four-to-four match, at a level significantly above chance, when the competing stimulus contained three elements. Thus, there seemed to be a definite limit to their ability to extrapolate to higher numerosities: their performance in discriminating four versus five, five versus six and four versus six was also not above chance in all tests. The bees were not able to decide for a choice pattern according to the numerosity of the sample (Figure 5a to e).

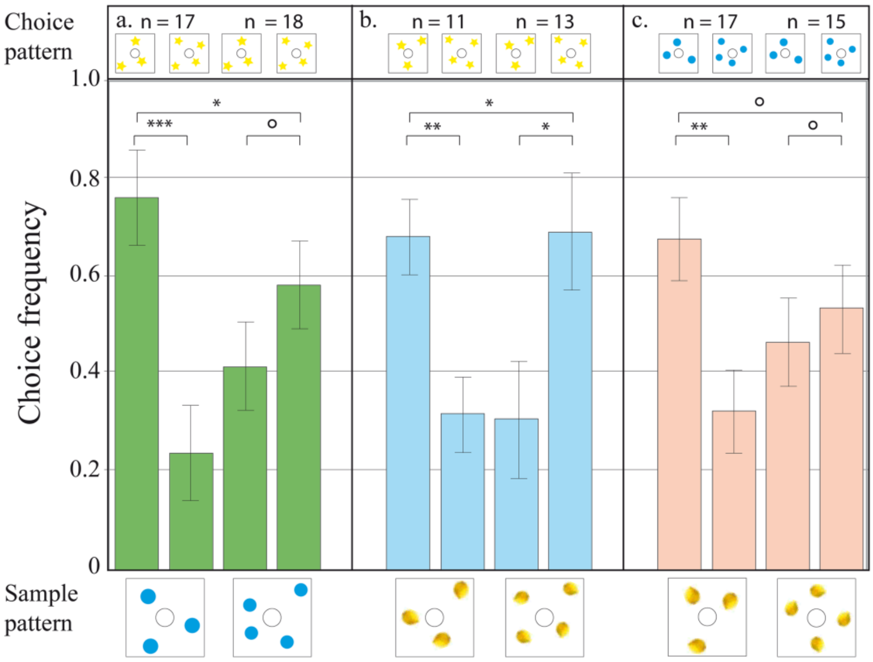


Figure 4: Results of transfer tests to determine the trained bees' ability to discriminate between three and four without any prior training on patterns with four elements. The notations used here are the same as those in Figure 3.

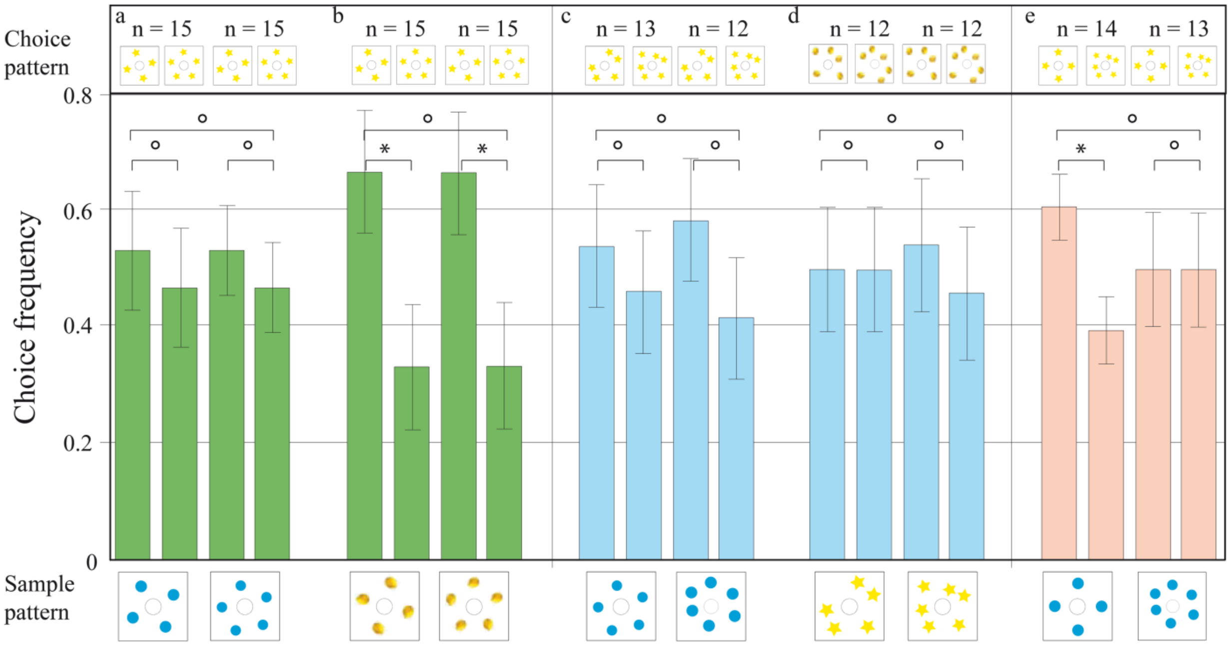


Figure 5: Results of transfer tests to determine our trained bees' ability to discriminate between patterns containing higher (unknown) numbers of elements. (a–b) Bees trained to discriminate between two and three are tested on patterns with four and five elements. (c–d) Discrimination by the same set of bees between five and six. (e) Discrimination by the same set of bees between four and six. The notations used here are the same as those in Figure 3.

Control test for illusory contours

Since bees are able to detect illusory contours (Horridge et al., 1992b), we carried out another series of control experiments where the elements in a visual array were always arranged in straight lines of equal length (Figure. 6a to d). This prevented the bees from using the overall shape described by the elements (i.e. a triangle versus a straight line) to carry out the matching task. Once again the bees were able to match the right number of elements, even in mixed arrays (i.e. when the arrays were composed of mixtures of elements, and there were no elements in common between the sample and choice patterns, as in Figure 6c and 6d).

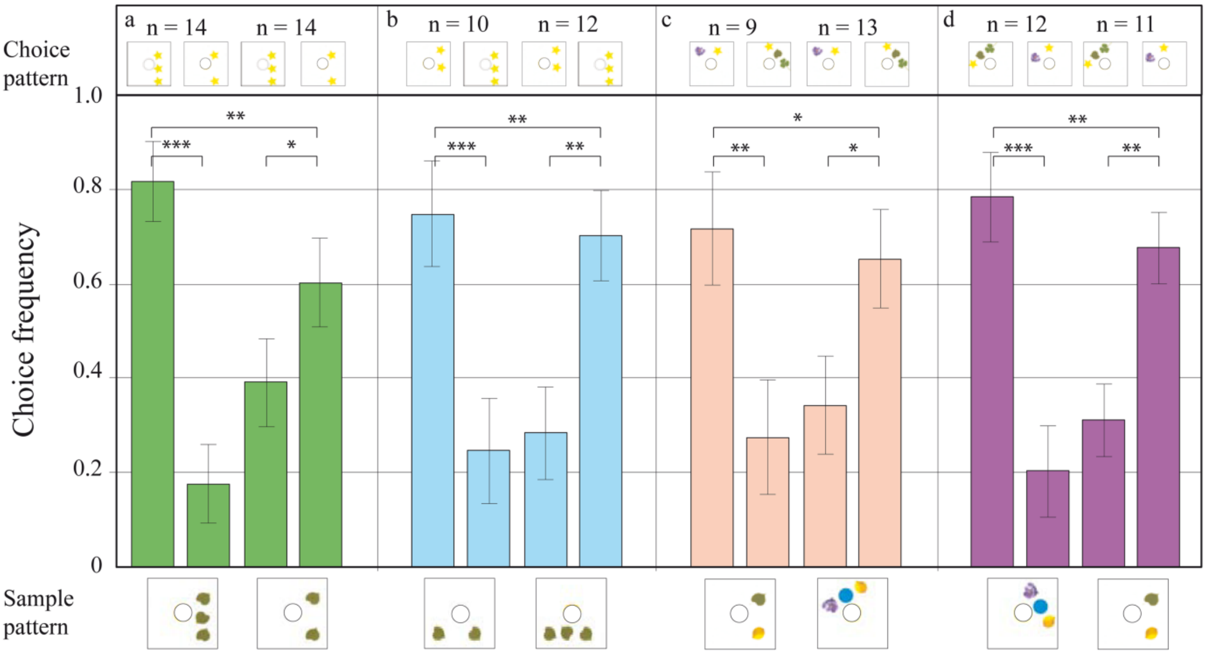


Figure 6: Results of tests to determine our trained bees' ability to discriminate between patterns containing elements arranged in straight lines of equal length. (a) The sample and choice patterns are both oriented vertically. (b) The sample and choice patterns have different orientations. (c) and (d) the sample and choice patterns have different orientations, and are composed of different elements. Note the 'misdirecting' cues: the purple flower in (c) and the dark-green leaf in (d). The notations used here are the same as those in Figure 3.

Control tests for olfactory cues

In order to exclude the possibility that olfactory cues of the feeder were influencing the bees' decisions, we carried out an additional control experiment (See material and methods). When the three dot sample was presented, the bees preferred the three dot choice pattern (0.78±0.15 of the decisions), at a level significantly different to random choice (n = 9, total 17 visits, p<0.001). When the two dot sample was presented, they significantly preferred the two dot pattern. The choice frequency of 0.74±0.10 for the two dot pattern is also significantly different to random choice (n = 11, total 23 visits, p<0.001). The bees significantly reversed their preference when the sample pattern changed (n = 12, total 40 visits, p<0.001). The use of new maze cylinders and the absence of a feeder behind the correct pattern did not impair the bees' ability to solve the task, showing that olfactory cues do not play a role in the bees' decision making in our experimental paradigm.

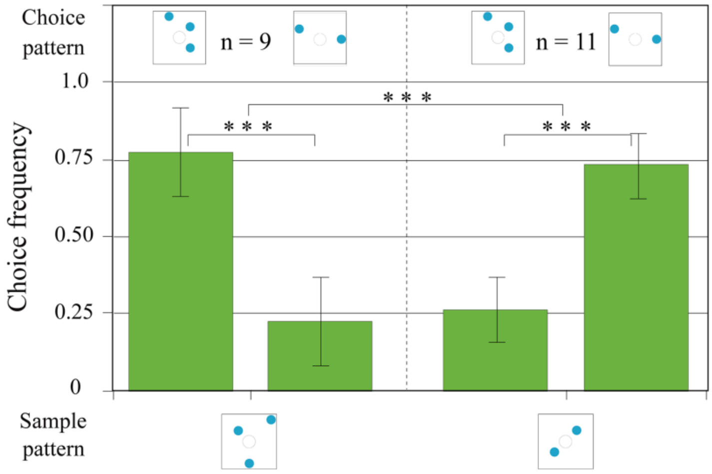


Figure 7: Results of the control tests for olfactory cues The notations used here are the same as those in Figure 3.

Discussion

Our results clearly demonstrate that honeybees can use the number of elements in a visual pattern, to match a choice stimulus with a sample stimulus in a DMTS paradigm. First, we were able to confirm earlier findings that bees are able to learn the abstract concept of 'sameness' (Giurfa et al., 2001). Using this as a starting point, we then tested bees on progressively more challenging sets of stimuli, where only the number of elements in each stimulus was kept constant. While the first training experiment only required the bees to match patterns that were identical in every respect, we subsequently showed that bees could transfer the matching rule even to stimuli where the elements (the blue dots) were in different, random orientations. They were able to match stimuli which contained novel elements, also in random orientations, and to match sample and choice stimuli that contained different elements. Our control experiment for illusory contours confirms that the bees were not using the overall shape described by the elements as a cue.

Given any one set of sample and choice patterns from our experiment, it would be quite reasonable to suggest alternative hypotheses for the bees' performance: bees could indeed be using lower-order visual cues, or relying on accidental features shared by the sample and rewarded choice pattern. After all, ants can use ambient light levels within a nest cavity to estimate the number of nest entrances, while evaluating a potential residence (Franks et al., 2006). However, our protocol involved training a single group of approximately 20 bees on a standard DMTS task, and later testing them sequentially with the entire set of novel patterns, where only the number of elements was kept constant (See Supporting Figure S2 for a list of all the patterns used). The entire experiment was repeated twice with different sets of bees. Over the course of the testing procedure, a bee that had successfully matched three blue dots to three yellow lemons (arranged in random configurations) might, 40 minutes later, be required to match two green leaves to two yellow stars (arranged in straight lines of equal length). If the bees were employing lower-order or accidental features, it would have had to re-teach them for each new set of patterns, which would have taken a few trips to the apparatus in each condition. Instead, our bees were mostly able to solve such tasks immediately, as evidenced by the first-choice data of individual bees presented in Supporting Tables S3 a & b and S4.

We also tried to make the sample and rewarded choice patterns as dissimilar as possible in terms of element configuration, and also deliberately tried to induce the bees to choose the wrong pattern in some experiments. Thus, in Figure 6c, the three-element sample pattern and the incorrect two-element choice pattern both contain a purple flower, while the dark-green leaf in the two-element sample pattern (Figure 6d) serves the same purpose. Still, the majority of bees ignored such obvious (misdirecting) cues, and chose the pattern with the right number of elements. Finally, our observation that bees can adapt well to novel visual stimuli (in terms of element type and orientation) containing the same number of elements, but not to those containing a novel number of elements, indicates that element number was a salient cue.

The presence of a feeder during all stages of testing could be considered a departure from a standard memory testing protocol. The advantages of unrewarded testing include the certain exclusion of olfactory cues from the feeder, and the prevention of learning during the tests. Such testing conditions are essential only when bees are trained to a simple task, where individual bees have to go through the transfer test only once. However, as mentioned above, we wanted to ensure that the same group of trained bees kept visiting the maze throughout the duration of the experiment, *i.e.* over the complete series of transfer tests. Had we put them through unrewarded tests, many of the trained bees would have lost their motivation after a few attempts, and stopped visiting the apparatus. After all, in our experimental paradigm, the experience of an unrewarded test, where the bee makes a correct decision but doesn't find a feeder behind the correct choice pattern, is similar to the punishment for making a wrong decision, and thus equivalent to negative training. One could argue that bees might not be able to solve the task without the help of olfactory cues, although these non-visual stimuli alone are not sufficient to support correct choices. However, as mentioned later in this section (‘The absence of olfactory cues’), it has been conclusively shown that the presence of a feeder during a test does not lead to false positives in the bees' choice data. If olfactory cues did exist, the bees should have found the feeder in the case of our four vs five or four vs six dot experiments as well. In the control experiment for olfactory cues, a new set of bees was trained to the basic DMTS task, and then tested in fresh maze chambers without a feeder. The data show that the bees are able to choose the correct number of elements according to the sample pattern without the presence of a feeder in the final chamber (see methods section and Figure 7 for details). In Figure 2, we show that the percentage of incorrect second choices following a positive first choice in the 1st block was 50.0%, which declined to a low 24% by the last block. This unequivocally supports the absence of olfactory cues at the feeder. In addition, we made the following observation at the end of the complete series of transfer tests, in which i) the two choice patterns and the sample pattern were identical; ii) there was a feeder with sugar water behind one choice pattern and a feeder with only water behind the other choice pattern; and iii) the positions of the two feeders were swapped after 5 min., which is half the normal testing period. The visiting frequency at the two feeders during the 10 min. observation period was 20: 17 (the feeder with sugar water to the feeder with water). There is no significant difference from random choice level (Chi = 0.003, P>0.90).

Moreover, it is highly unlikely that the bees' performance is somehow predicated on the additional training they might receive by the attainment of a reward during the testing procedure. The reliable learning of a pattern takes about 15–20 rewarded exposures (e.g. Giurfa et al. (1999)), but in our experiment, the average bee would only be able to visit the feeder a maximum of four times during a test. In any case, any learning that did take place during our tests would only be reinforcing the basic DMTS rule of ‘match the right choice pattern to the sample’; this is in no way contrary to the aims of our study. Even if the bees learned the details of a particular pattern, that would also not invalidate our data, as 1) only the first choice of a bee per test condition was analysed, and 2) a radically different novel test pattern would be presented to the bees in the following test condition (see methods and above). The analysis of incorrect second choices (Figure 2b) showed that in the early stages of training, up to 50% of bees were choosing the negative pattern on their second visit, even though their first choice was correct. This is more evidence that the bees did not use scent as a cue. The frequency of this type of error declined with training.

As mentioned in the Introduction, an estimation of relative numerical quantity could be extremely useful to foraging honeybees: combining information on the degree of stomach distension along with the number of flowers visited on a foraging trip could provide bees with an index of the profitability of a food source. Honeybees can recognise images of complex natural scenes (Dyer et al., 2008), and may be able to use them as potential landmarks. The number of landmarks encountered on a foraging trip, or found near the hive, could be useful in navigation (Chittka and Geiger, 1995; Skorupski and Chittka, 2006). Number generalisation could also help in estimations of the number of blossoms on a branch and/or, the number of bees on a blossom, thereby allowing a new forager to decide whether to forage at that location, or look for a new one. It has been suggested that both duration and numerosity may be represented mentally in animals through the same mental magnitudes, i.e. through real numbers (Gallistel and Gelman, 2005). As the ability to measure time intervals was recently demonstrated in the bumblebee (Boisvert and Sherry, 2006), there is a pleasing sense of symmetry in our demonstration of the honeybee's ability to distinguish between visual arrays of two and three elements, using only element number as a cue, and to even transfer this ability to discriminate between completely novel stimuli containing three and four elements.

The only unifying feature of all the patterns used in our tests was that they contained the same number of elements that the bees had initially been trained on. We are not proposing here that the bees were ‘counting’, *sensu stricto (Meck and Church, 1983)*, that they possess mathematical competence (Boysen and Berntson, 1989), or that they were able to order the abstract concepts of ‘two’ and ‘three’ on a scale of magnitude (Brannon and Terrace, 2000). We conclude that the bees are able to make generalisations about patterns based on the number of elements, and transfer this ability to discriminate between two and three to new situations. Our most intriguing result was their ability to match three-element stimuli, when the competing stimulus was a four-element pattern. However, their performance in the four-five, five-six and four-six comparisons was not above chance. This last result further supports our conclusion that the bees were indeed using element number to decide which chamber of the y-maze to enter: if the bees had been using lower-order cues such as edge length, dot density, or the area of the dots or background (or even odour cues), one would have expected them to perform just as well in this condition, as they had in previous conditions. Instead, the bees performed well only when at least one of the patterns in the decision chamber contained a number on which they had been previously trained (i.e., two or three). As early as 1871, Jevons proposed that the maximum number of items that a human could accurately estimate with just a moment's exposure lay ‘half-way between’ four and five (Jevons, 1871). More recently, Cowan (2001) presented an impressive amount of evidence to support his claim that, due to attentional limitations, the number of items that humans can hold in their short-term memory and subsequently recall is four, or very close to it . Since the DMTS paradigm is partly a test of the honeybees' short-term memory (which displays temporal decay (Zhang et al., 2005)), it is possible that the mechanisms elucidated by Cowan have a bearing on our results. There is, in addition, evidence that human infants rely on mechanisms of object-based attention and short-term memory to represent small numbers of objects: they can discriminate arrays containing 1, 2, or 3 objects, but fail with arrays greater than 3 (Feigenson and Carey, 2005). This upper limit also seems to apply to rhesus monkeys (Hauser et al., 2000). However, these last results are not directly comparable to ours, as the former represent the spontaneous choices of experimental subjects, whereas the latter are the consequence of extensive training. Moreover, the observation that our bees could distinguish between two and three, but not four and six, indicates that performance was not dictated by the ratio difference in set sizes, which, in contrast, seems to be the case in human infants, at least for large numerosities (Xu et al., 2005).

Another intriguing finding from our study is the improved performance of our trained bees in the transfer tests, in comparison to the last sessions of training on the basic DMTS task. We hypothesize that the reason for this effect is the novelty of the test patterns – after three days of training on the same set of visual patterns, the bees were presented with patterns of increasing novelty in the transfer tests. We noticed that by the last stages of training, bees would often proceed past the sample and choice patterns, and into the (correct or incorrect) reward chamber with only a cursory scan of the patterns. When presented with novel test patterns, however, bees would regularly spend more time scanning them, and were frequently seen to approach each element in a pattern, before passing into the next chamber. Chittka et al. (2003) have shown that the more time an individual bumblebee invests in making a decision, the more accurate are its responses (Chittka et al., 2003). In addition, Heisenberg et al. (2001) have reported the phenomenon of ‘selective attention’ in flies, with tethered *Drosophila* able to preferentially attend to one of two competing stimuli. Van Swinderen (2007) showed that *Drosophila* reacted to novel visual patterns, and that mutants deficient in genes implicated in short-term memory also suffered from attention deficits (van Swinderen and Flores, 2007). It is possible that our bees, too, not only attend to novel stimuli for longer, but also achieve improved memory scores as a result.

Recent research has revealed that perceived numerosity is susceptible to adaptation, in the same way as the primary visual properties of a scene, such as colour, contrast, size, and speed (Burr and Ross, 2008). Numerosity can therefore be considered an independent primary visual property which, as our results demonstrate, can also be apprehended by honeybees. Our study therefore suggests a fruitful line of investigation for the future, as the limits of this and other invertebrates' cognitive abilities remain to be determined.

The absence of olfactory cues

A possible role of olfactory cues as a confounding factor in experiments such as ours has been excluded here as in former experiments. Van Hateren et al. (1990) and Zhang et al. (1996; 1999a; 2004; 2005) have carried out tests to address this very question, and found that the presence of a hidden feeder behind one of a set of identical choice patterns (in a similar, but more elaborate y-maze setup in the 1996 study, and in a maze much like the present one in the 2005 study) does not in the least influence the probability of a bee choosing that pattern. To address this question, we have conducted an additional experiment to control for olfactory cues. A group of bees was trained to the basic DMTS task with visual patterns containing two and three blue dots, and then tested on patterns with randomised dot orientations in fresh maze chambers and without a feeder behind the correct pattern. The bees were still able to solve the task (see results section and Figure 7 for details). These data also show that the presence of a hidden feeder does not influence the bees' choice of a particular pattern in our experimental setup.

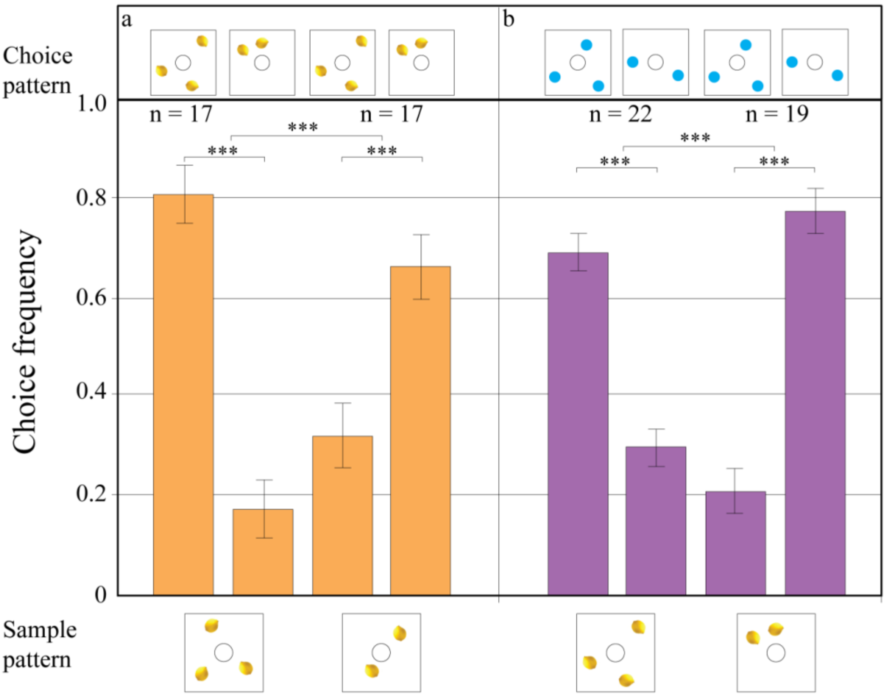
The feeder in our experiments was found and visited by bees, which presumably would have left scent marks in that choice chamber. However, this did not make that chamber any more attractive to subsequent bees.

When honeybees opened their Nasonov gland in our experiments, this was clearly visible to the experimenters. This rarely happened at all, and if it did, we removed the bees from the maze. If the feeder had carried any scent, from the Nasonov pheromone or otherwise, the bees would have been able to solve any task we presented to them, no matter what item numbers were visible on the visual patterns. However, they were not able to do a 4 to 4, 5 to 5 or 6 to 6 match, thus demonstrating the complete absence of olfactory cues.

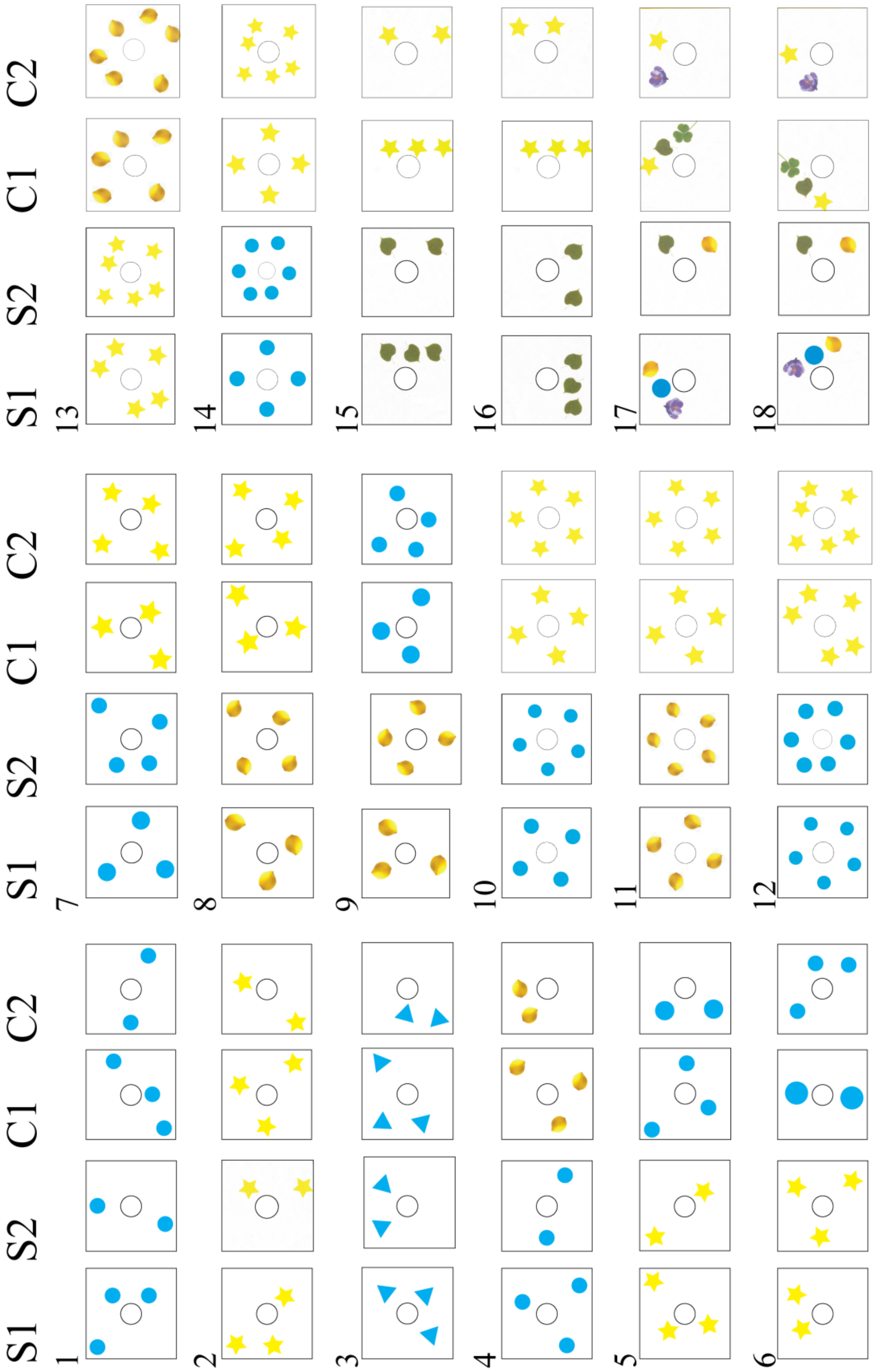
Acknowledgements

We would like to thank Dirk Ahrens-Lagast (Würzburg) and Paul Helliwell (Canberra) for help with beekeeping.

Supplementary material



Supplementary Figure 1: Results of transfer tests with further sets of novel stimuli. (a) The yellow stars in Figure 3b are replaced with yellow lemons; (b) Reversing the order of the patterns in Figure 3c, i.e. yellow lemons as the sample and blue dots as the choice patterns. The notations used here are the same as those in Figure 3.



Supplementary Figure 2: All sample and choice patterns used in the learning tests and various transfer tests. Each group of bees was tested on a large number of patterns, both in the orientation shown above, as well as rotated 180.

Block No.	Source	Sum-of squares	d.f.	Mean-square	F ratio	p
Block 1	Bees	5.358	22	0.244	0.965	0.518
	Error	15.148	60	0.252		
Block 2	Bees	5.699	20	0.285	1.226	0.271
	Error	12.315	53	0.232		
Block 3	Bees	5.365	21	0.255	1.023	0.452
	Error	14.733	59	0.250		
Block 4	Bees	2.442	15	0.163	0.674	0.795
	Error	11.106	46	0.241		
Block 5	Bees	2.289	15	0.153	0.651	0.816
	Error	10.311	44	0.234		
Block 6	Bees	4.599	17	0.271	1.257	0.255
	Error	11.839	55	0.215		

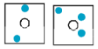
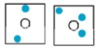
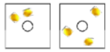
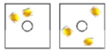
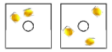
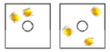
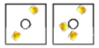
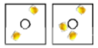
Supplementary Table 1: In each block Bees denotes the variance inherent in the performance score plus variance attributed to an individual bee's variation; Error denotes only the variance inherent in the performance score; d.f. lists degrees of freedom for the specified conditions; F-ratio is the Mean-Square for Bees divided by the Mean-Square for Error. The P value is probability of exceeding the F-ratio when the group means are equal.

	Test Type 1		**Test Type 2**
Figure 2	**Sample 3**	**Sample 2**	**Reversing preference tests**
Figure 2a	t=3.4;df=18, p=0.003	t=5.7,df=16,p=0.0000	t=6.6, df=34, p=0.0000
Figure 2b	t=4.6,df=24,p=0.0002	t=7.29,df=17,p=0.0000	t=8.1, df=41, p=0.0000
Figure 2c	t=5.5,df=15,p=0.0000	t=6.3,df=16,p=0.0000	t=7.9,df= 31, p= 0.0000
Figure 2d	t=8.01,df=16,p=0.0000	t=5.45,df=15,p=0.0000	t=9.3, df=31, p= 0.0000
Figure 2e	t=4.3, df=15, p=0.0006	t=4.9, df=15, p=0.0002	t=6.55,df=30, p=0.0000
Figure 3	**Sample 3**	**Sample 4**	**Reversing preference tests**
Figure 3a	t=3.86,df=32,p=0.0005	t=1.26, df=34, p=0.21	t=2.60,df =33, p= 0.014
Figure 3b	t=3.39,df=20,p=0.0029	t=2.25, df=24, p=0.034	t=2.51, df=22, p=0.020
Figure 3c	t=2.93,df=32,p=0.0062	t=0.51, df=28, p=0.615	t=1.68,df=30, p=0.1027
Figure 4	**Sample 4**	**Sample 5**	**Reversing preference tests**
Figure 4a	t=0.29, df=14, p=0.78	t=0.38, df=14, p=0.72	t=0.00023,df=28,p=0.999
Figure 4b	t=1.6, df=14, p=0.06	t=1.61,df=14, p=0.06	t=0.00, df=28, p=1.0000
	Sample 5	**Sample 6**	**Reversing preference tests**
Figure 4c	t=0.37,df=12, p=0.78	t=0.76, df=11, p=0.46	t=0.81, df=23, p=0.43
Figure 4d	t=0.00, df=11, p=1.0	t=0.35, df=11, p=0.73	t=0.25, df=22, p=0.80
	Sample 4	**Sample 6**	**Reversing preference tests**
Figure 4e	t=1.96,df=13, p=0.07	t=0.00, df=12, p=1.00	t=0.97, df=25, p=0.34

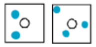
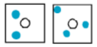
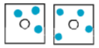
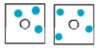
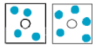
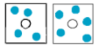
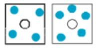
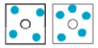
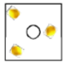
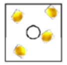
Figure 5	**Sample 3**	**Sample 2**	**Reversing preference tests**
Figure 5a	t=3.78, df=13, p=0.002	t=1.2, df=13, p=0.25	t=3.4, df=26, p=0.005
Figure 5b	t=2.3, df=9, p=0.004	t=2.2, df=11, p=0.05	t=3.2, df=20, p=0.004
Figure 5c	t=1.8, df=8, p=0.10	t=1.54,df=12, p=0.149	t=2.3, df=20, p=0.003
Figure 5d	t=3.1, df=11, p=0.01	t=2.6, df=10, p=0,026	t=3.8, df=21, p=0.001
Figure S1	**Sample 3**	**Sample 2**	**Reversing preference tests**
Figure S1a	t=5.49,df=16, p=0.000	t=2.6,df=16, p=0.001	t=5.4,df=32, p=0.000
Figure S1b	t=5.21,df=21, p=0.000	t=6.3,df=18, p=0.000	t=8.3,df=39, p=0.000

Supplementary Table 2 summarises the details of the student t tests for Figures 2–5 and Figure S1. Test Type 1 was to check whether the bees made the decisions according to the sample pattern, namely whether their performance was significantly different from random choice; Test Type 2 checked whether the bees reversed their preference after the sample was changed listed under Reversing preference tests. For each test, student t, df (degree of freedom) and p values are given in the table.

Supplementary table S3a

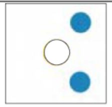
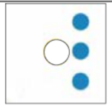
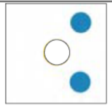
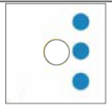
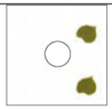
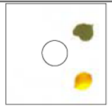
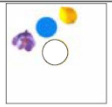
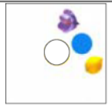
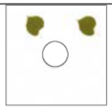
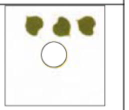
	Transfer 2v3-1				Transfer 2v3-2				Transfer 2v3-3				Transfer 2v3-4				Transfer 2v3-5				Summary		
Choice patterns																							
Rewarded pattern	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	2+	3+	%
Bee 02	+	+	+	+	+	+	+	+	+	-	+		-	+	+	-	+	+	+	+	8+,2-	8+,1-	84
Bee 12	+	-	+	+		+	+	+	-	+	-	+		+					+	+	4+,2-	7+,1-	79
Bee 1 4	+	+	-		+		+	-	+	+	-	+	+	+	+	+	-	+	+	+	8+,1-	6+,4-	74
Sample pattern																							

Supplementary table S3b

	Control-same areas					Control-same edges					Transfer 3v4					Transfer 4v5					Control 4v5-1				
Choice Patterns																									
Reward pattern	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	%	2+ 0° (R)	180° (L)	3+ 0° (R)	180° (L)	%	3+ 0° (R)	180° (L)	4+ 0° (R)	180° (L)	%	4+ 0° (R)	180° (L)	5+ 0° (R)	180° (L)	%	4+ 0° (R)	180° (L)	5+ 0° (R)	180° (L)	%
Bee 02	+	+	+	+	1.0	+	+	+	+	1.0	+	+	+	+	1.0		-		+	0.5	+	-	+	+	75
Bee 12	+	+	-	+	0.75	+	+	+	+	1.0	+	+	-	+	0.75	-		-	+	0.3	-	+	+	+	75
Bee 14	-	+	+	+	0.75	+	+	+	-	0.75	+	+	+	-	0.75	-	+	-	+	0.5	+	+	+	-	75
Sample pattern																									

Supplementary Table 3 a & b: Individual performance records of three bees (from a group of about twenty) trained in an experiment performed in December 2006. The first choice of each bee within a ten-minute testing block is shown (+ correct choice, − incorrect choice). A blank cell indicates that the bee did not visit the apparatus during that block. These bees were chosen as they were involved in all steps of the transfer tests. 3+ and 2+ indicate the number values of the rewarded patterns, while 0 and 180 indicate pattern orientation within a ten-minute block (as each set of sample and choice patterns was tested in two orientations). R and L mean that the reward was in the right or left arm of the y-maze. Each pair of 3+ and 2+ columns represents a different set of novel test stimuli. The choice patterns and the sample pattern for each set of the tests are shown respectively above and below the choice performance.

Supplementary Table 4

	Transfer tests 1				Transfer tests 2				Transfer tests 3				Transfer tests 4				Transfer tests 5				Transfer tests 6			
Choice Patterns																								
Rewarded pattern	2+		3+		2+		3+		3+		2+		2+		3		3		2		2+		3+	
	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)	0° (R)	180° (L)
Bee 10	+	+	-	+	+	-	+	+	+	+	+	-			+	+	+	+	+					
Bee 16		+	+	+	+	+	-	+	-	+	-	+				+	+	+					+	+
Bee 21	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	-	+	-	+	+
Bee 31	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+
Bee 60	-	+	+	+	+	+	+	-	+	+	+	-			+		+	-	+	-	+	+	-	+
Sample pattern																								

Supplementary Table 4: Individual performance records of five bees (from a group of twenty) trained in an experiment performed in April 2007. The notations used here are same as in Table S1.

Chapter 4: Large Scale Homing in Honeybees

Abstract

Honeybee foragers frequently fly several kilometres to and from vital resources, and communicate those locations to their nest mates by a symbolic dance language. Research has shown that they achieve this feat by memorizing landmarks and the skyline panorama, using the sun and polarized skylight as compasses and by integrating their outbound flight paths. In order to investigate the capacity of the honeybees' homing abilities, we artificially displaced foragers to novel release spots at various distances up to 13km in the four cardinal directions. Returning bees were individually registered by a radio frequency identification (RFID) system at the hive entrance. We found that homing rate, homing speed and the maximum homing distance depend on the release direction. Bees released in the east were more likely to find their way back home, and returned faster than bees released in any other direction, due to the familiarity of global landmarks seen from the hive. Our findings suggest that such large scale homing is facilitated by global landmarks acting as beacons, and possibly the entire skyline panorama.

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http://www.plosone.org/article/info:doi/10.1371/journal.pone.0019669

Pahl, M., Zhu, H., Tautz, J. and Zhang, S. (2011). Large Scale Homing in Honeybees. *PLoS ONE* **6**, e19669.

Introduction

Honeybee foragers have to provide a constant flow of nectar, pollen, water and propolis to the colony. The navigational information necessary for their frequent long distance flights is acquired from celestial and terrestrial cues. In order to keep track of the current position relative to the goal, forager bees employ several strategies. When first leaving the hive, young foragers perform systematic flight manoeuvres, backing away from the hive in a series of increasing arcs (Zeil et al., 1996). During those **orientation flights**, the animals memorize the hive itself, local landmarks surrounding the hive and global landmarks around the area (von Frisch, 1967; Capaldi and Dyer, 1999; Capaldi et al., 2000). When flying between nest and food source, the bee can then match the memorized cues with the actual visual environment (Cartwright and Collett, 1983). The flight distance is estimated by **optic flow** experienced by the bee on the outbound route (Esch et al., 2001; Tautz et al., 2004). When forced to fly in a non-beeline, i.e. around large obstacles like mountains, honeybees employ a **dead reckoning** system which constantly updates the distance and direction to the hive. Thus, in the waggle dance, the dancer communicates the straight line and distance to the resource, rather than the absolute distance flown around the obstacle (von Frisch, 1967). Using direct light from the sun and polarized skylight detected by specialized ommatidia in the eye’s dorsal rim area (Labhart, 1980), the honeybee’s **celestial compass** is able to measure angular movement relative to a reference direction, the solar meridian (Rossel and Wehner, 1986). As a compass-backup for cloudy days, the skyline panorama is memorized together with the solar ephemeris function (Dyer and Gould, 1981a; Towne and Moscrip, 2008). En route to a goal, **familiar landmarks** can break down a trip into several segments to improve accuracy (Srinivasan et al., 1997), and panoramic cues allow the recognition of landmark cues that, in turn, trigger local vectors (Collett et al., 2002). These systems are flexibly applied to the task at hand. Chittka and colleagues have shown that when foraging by familiar landmarks, honeybees are able to suppress their path integration system, even when those landmarks are displaced. Alternatively, when forced to forage in a novel location without learnt landmarks, they use path integration without landmarks to navigate back to the hive (Chittka et al., 1995).

Homing after displacement to unfamiliar regions has been investigated in various hymenopterans such as solitary sphecid wasps, *Cerceris tuberculata* (Fabre, 1879; 1882) and

Cerceris hortivaga (Tsuneki, 1965), social wasps, *Polistes gallicus* (Ugolini, 1985) and *Vespa orientalis* (Towne and Moscrip, 2008), solitary bees, *Dasypoda altercator* and *Osmia* sp.(Capaldi and Dyer, 1995; Guedot et al., 2009), the social bees *Bombus terrestris* (Goulson and Stout, 2001) and *Apis mellifera* (Uchida and Kuwabara, 1951; Becker, 1958; Southwick and Buchmann, 1995), and several ant species (reviewed in Collett et al. (2007)) for more than a century. Homing success in flying hymenopterans usually declines with increasing displacement distance, but the rate of decline is quite different between species. The maximum distance from which bees return after displacement varies widely from 200m in *Pithitis smaragdula* (Abrol and Kapil, 1994) to 23km in *Euplusia surinamensis* (Janzen, 1971; Wikelski et al., 2010) , and is believed to be a good indicator for a species' maximum foraging range (van Nieuwstadt and Ruano-Iraheta, 1996). In studies on honeybees, the maximum homing distance ranges from 6km (Becker, 1958) to 9.2km (Southwick and Buchmann, 1995). To further investigate the honeybees' navigational abilities, we captured pollen foragers that had just returned to the hive, artificially displaced them in a black box to various destinations, and measured the time each bee took to come back home. Thus, we deprived the bees of any distance or directional celestial information about the release location in relation to the hive. The bees had to rely on knowledge they already had about the landscape.

Human observation can only be carried out reliably for a few hours at a time, which makes it difficult to gauge the behaviour of large numbers of foragers over a long study period, such as days or weeks. It is precisely to overcome such difficulties that some researchers have turned to miniature signalling devices that can be attached to the thorax of individual bees, thereby allowing their behaviour to be monitored automatically. One such technique involves the use of harmonic radar, with which the exact trajectories of individuals can be monitored over short periods of time, up to 1000m from the radar device (Menzel et al., 2005; Wikelski et al., 2010). We decided to use radio frequency identification (RFID) tags to be able to record the incoming and outgoing flights of many individual foragers at once, and over a time period of several days. While flight trajectories were not recorded, the small size of the RFID tags ensured undisturbed behaviour of the bees, and no range limit in picking release sites. This was an improvement on previous techniques (Uchida and Kuwabara, 1951; Becker, 1958; Southwick and Buchmann, 1995), because the exact return times and subsequent flight behaviour of many individual bees could be measured, without the need of constant human observation. Even bees returning outside of normal observation hours and after several days in the field were recorded. Each tag was coded with an individual ID, which was logged by a receiver every time a tagged honeybee passed near it. Identification number, time and direction of movement were recorded by the receiver every time a forager returned home after an artificial displacement.

Materials and Methods

Experimental bees

The experimental *Apis mellifera ligustica* bees were housed in a two-frame observation hive containing approximately 3000 animals, connected to the outside via a Perspex tunnel. The hive box was situated indoors in the Australian National University's native animal enclosure (35° 16' 49.09''S, 149° 06' 41.68''E, elevation 563m). Each bee was tested only once.

Experimental procedure

Pollen-carrying bees were captured upon return from a foraging trip at the hive entrance and briefly immobilized on ice, so that a RFID tag with known id number could be glued to each bee's thorax with shellac glue from a queen marking kit. Groups of 20 tagged bees were then kept in cages with *ad libitum* access to 50% sucrose solution. The cages were transported to the respective release sites in dark Styrofoam containers so that the bees did not derive any directional information before the experiments began. The preparations were conducted in the morning, so that the experimental bees could be released in the early afternoon. At the respective release sites, the cages were opened at one side, and the bees were given 5 minutes to take off. The bees then spiralled upwards in wide circles until they were lost from view; homing trajectories could therefore not be determined. Animals which had not left the cage after 5 minutes were excluded from the experiment. Approximately two hours passed between the bees' capture and release. Upon return to the hive, the bees' identity and homing time were recorded by the RFID receivers at the hive entrance.

RFID system

Each bee was equipped with a RFID tag on the thorax (2.0 x 1.6mm, 2.4mg, Microsensys mic3-TAG 64-D). All tags carried a unique 64 bit number, which allowed us to individually track the experimental bees' flight behaviour. Two RFID receivers (Microsensys 2k6 HEAD) attached to the hive tunnel recorded each in- and outbound flight of the tagged bees.

Landscape of the experimental area

The experimental area is shown in the satellite map in Figure 1, and the surrounding panorama as seen from the hive is shown in Figure 2. We released groups of bees in the four cardinal directions in various distances from the hive. In the **eastern direction**, the bees were released in rural areas (up to 3300m distance), on top of and behind the 830m high Mount Ainslie (MA, 4400m to 7800m distant), and further away (up to 13000m) behind MA. Black Mountain (BM, elevation 810m) was visible from the rural areas and from the top of MA (4400m away), but not from the release spots further away, where MA blocked the direct line of sight. We chose a line of release spots slightly north easterly from the hive, in order to use the peak of MA as a visual barrier for the bees at the distant release spots behind the mountain. The release spots in the **western direction** were chosen in a way similar to the eastern ones, i.e. to have the large visual barrier of BM between the hive and the distant release spots. Behind the 1400m spot on top of BM, the mountain was still visible from all release spots, but from a different angle than the one the bees were used to. MA was not visible from behind BM. In the **northern direction**, the bees were released in rural areas at a maximum distance of 7000m from the hive. BM and MA were visible from all spots, although from an unfamiliar angle. In the **south**, the line of release spots crossed Lake Burley-Griffin (LBG). Bees homing from 800m to 1500m distance were released from a boat. BM and MA were visible from all releases up to the 5000m spot on top of Red Hill (RH), but not from the spots behind RH at 6 and 7 km.

Weather

Experiments were conducted solely in fine weather conditions. On all experimental days, the average temperature was between 25 and 35°C, the sky clear or partly overcast with a visibility of at least 10km. The wind usually blew from the north-east with an average speed of 15 km/h.

Data analysis

The homing rate for each release spot was determined as the number of returning bees divided by the number of released bees. The time between take-off at the release site and the first reading of each bee at the hive was determined to be the individual homing time. Median homing time was calculated for each release across all bees returning on the same day. Bees returning on the next day were excluded from the homing time analysis, but not from the homing rate analysis. Homing speed was calculated for each bee returning on the same day, as the release distance divided by the individual homing time. This measure does not represent flight speed, as it includes searching, resting and refuelling on the way.

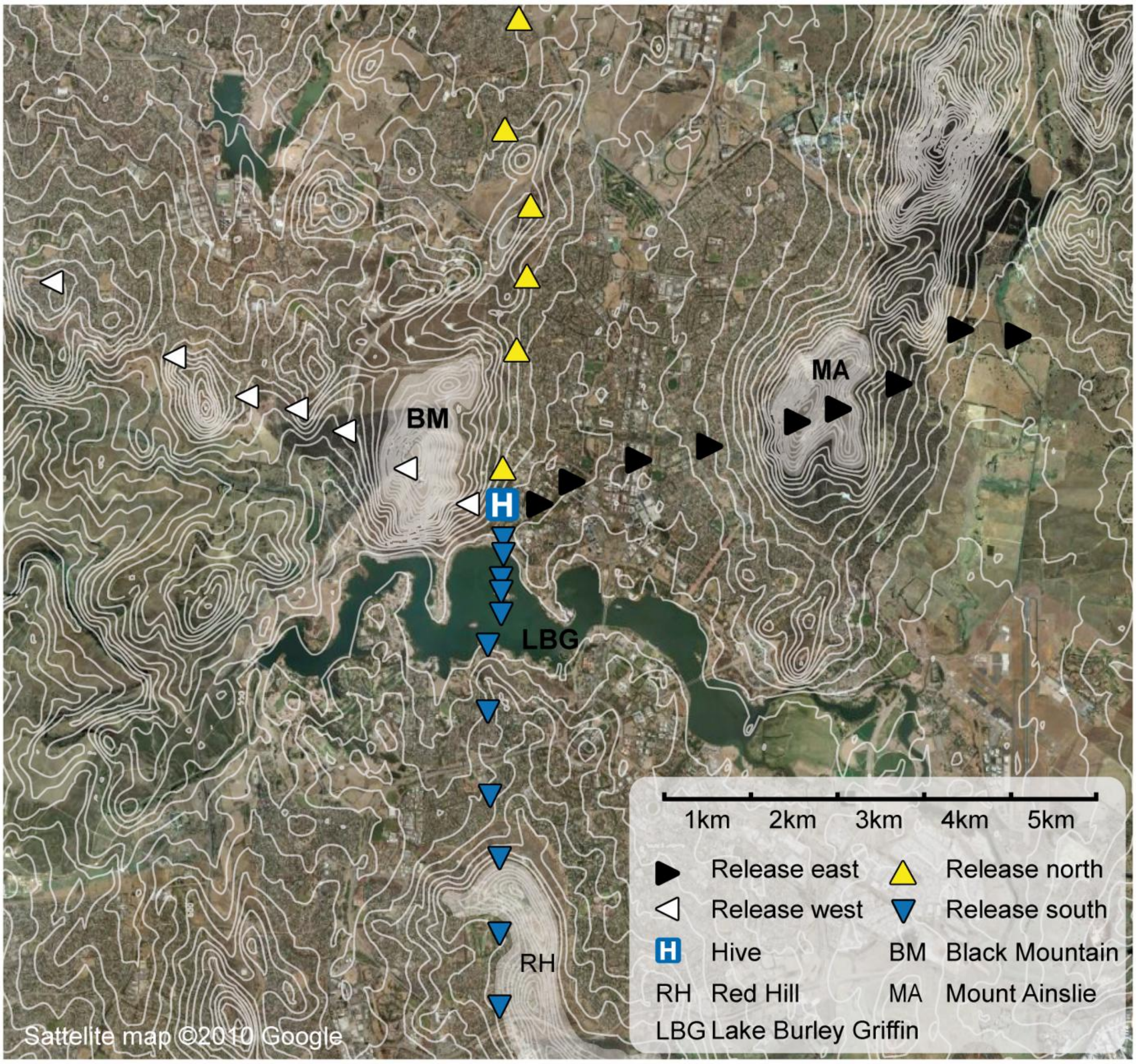

Figure 1: Map of the experimental area. 20 bees were released at each marked spot. White lines show terrain contour, and white areas denote hills blocking the direct view to the vicinity of the hive. Up, down, left and right-pointing triangles indicate releases in the north, south, west and east, respectively.

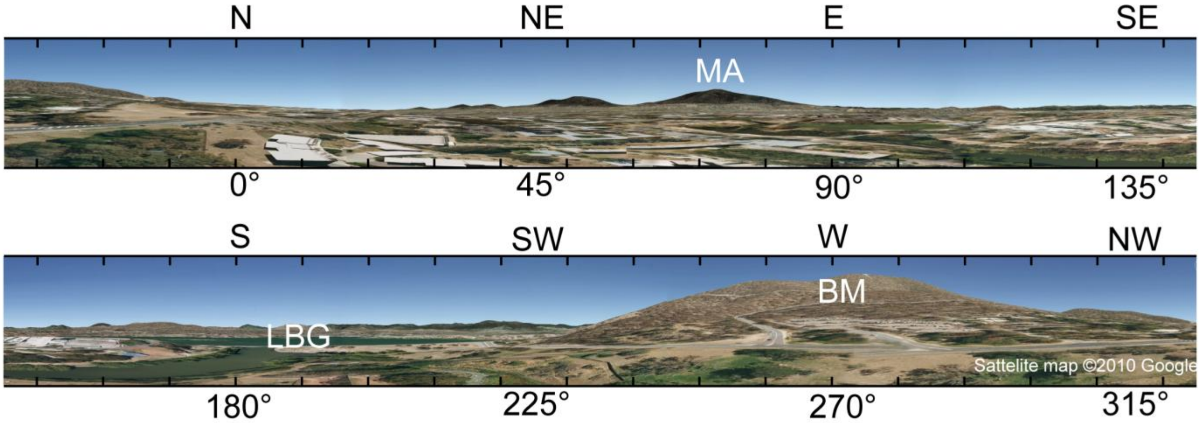

Figure 2: Panoramic view of the experimental area, as seen from the hive. Buildings and trees are flattened; the viewpoint elevation is 15 m. Note the distinctive shapes of Black Mountain (BM) in the west and Mount Ainslie (MA) in the east. Lake Burley Griffin (LBG) lies south of the hive.

Results

Homing rate & homing time

In all four directions, there was a negative linear relationship between homing rate and distance, and a positive relationship between homing time and distance (Fig 3 and 4). There was no significant deviation from linearity in homing rate (Runs test, $p_{(east)}=0.825$, $r^2_{(east)}=0.877$; $p_{(west)}=0.700$, $r^2_{(west)}=0.824$; $p_{(north)}=0.800$, $r^2_{(north)}=0.809$; $p_{(south)}=0.955$, $r^2_{(south)}=0.707$) or homing time (Runs test, $p_{(east)}=0.788$, $r^2_{(east)}=0.933$; $p_{(west)}=0.500$, $r^2_{(west)}=0.754$; $p_{(north)}=0.667$, $r^2_{(north)}=0.899$; $p_{(south)}=0.222$, $r^2_{(south)}=0.569$) in any of the four directions. Consequently, the data were analysed by linear regression.

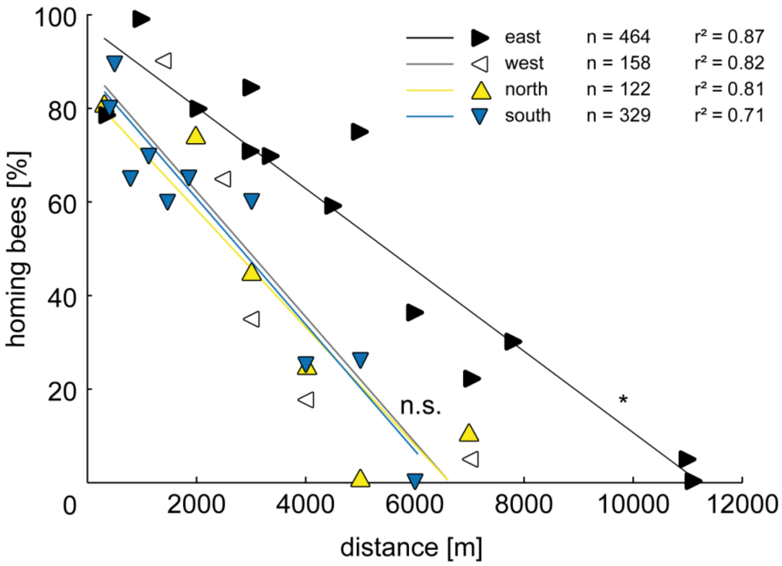

Figure 3: Homing rate in the four cardinal directions. Beginning at 80–100% close to the hive, the proportion of returning bees declines to 0% at around 6 km in the west, north and south, and at 11 km in the east. Homing rate from the eastern direction is consistently higher than from north, west and south (Comparison of slopes, p<0.033. Each point is based on 20 bees). Up, down, left and right-pointing triangles indicate releases in the north, south, west and east, respectively.

In the **east**, the maximum homing rate was recorded at 1000m, from where all bees returned. The maximum homing distance was 11000m (Fig 3), and the maximum homing speed of 50.51 ± 9.07 $m \cdot min^{-1}$ was recorded from the 5000m spot (Table 1). In the **western** direction, a maximum of 90% returned from the 1400m release on top of BM, probably due to the exceptional view of the surrounding area from the mountain peak. The maximum homing distance was 7000m (Fig 3), and the maximum homing speed of 31.01 ± 12.35 $m \cdot min^{-1}$ was reached at 4000m (Table 1). **North** of the hive, the highest homing rate was reached at 300m, where 78.9% of the bees returned at a speed of 33.75 ± 12.43 $m \cdot min^{-1}$ (Table 1). The maximum homing distance was 7000m (Fig 3). In the **south**, the highest percentage of bees returned from the 520m release at the lake shore (89.5%, Fig 3). The fastest homing flight from south was recorded at the 520m spot, where the bees returned at an average pace of 65.00 ± 11.66 m·min^{-1} (Table 1).

Figure 3 shows that the best-fit lines for the **homing rates** from west, north and south do not differ significantly from each other (linear regression; slopes: f=0.012, DFn=2, DFd=18, p=0.988; elevations & intercepts: f=0.059, DFn=2, DFd=20, p=0.943). Thus, the data were pooled and compared to the eastern direction. There was a significant difference between the best-fit lines for the homing rates from the east and the pooled data from west, north and south (linear regression; slopes: f=4.958, DFn=1, DFd=32, p=0.033).

Similarly, Figure 4 shows that the best-fit lines for the **homing times** from west, north and south are not significantly different from each other (linear regression; slopes: f=0.014, DFn=2, DFd=13, p=0.986; elevations and intercepts: f=0.172, DFn=2, DFd=15, p=0.843). Accordingly, the data were pooled and compared to the eastern direction. Linear regression showed a significant difference between the elevations and intercepts of the best-fit lines (f=7.489, DFn=1, DFd=27, p=0.011), but not between the slopes (f=1.996, DFn=1, DFd=26, p=0.170).

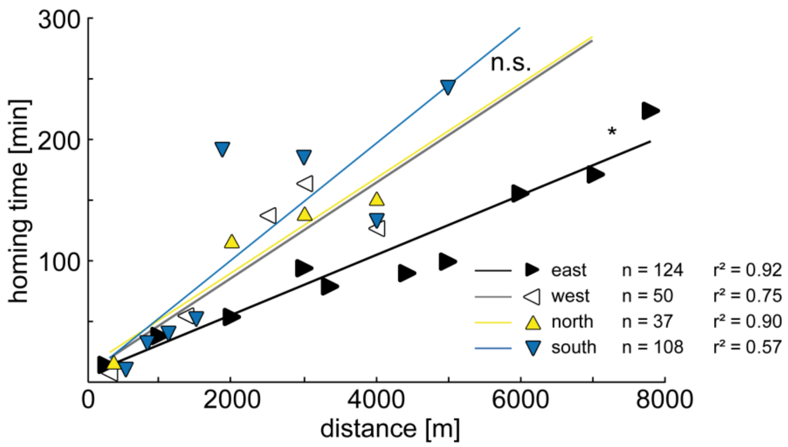

Figure 4: Homing time in the four cardinal directions. Bees returning from the east take less time than bees returning from the west, north and south (comparison of elevations and intercepts, p = 0.011). Up, down, left and right-pointing triangles indicate releases in the north, south, west and east, respectively.

Homing speed

The average **homing speed** of bees returning from the west, north and south was around 25 m·min^{-1}, about 10 m·min^{-1} slower than the homing speed from the east (Fig 5). The speeds from the west, north and south did not differ from each other (ANOVA, p=0.697). Consequently, they were pooled and compared to the homing speed from the east, which was significantly higher than the speeds of bees returning from the west, north and south (t=14.379, df=317, p<0.001).

	East	West	North	South
Median homing speed [m·min^{-1}]	36.90 ± 3.26	24.27 ± 3.69	23.53 ± 5.01	26.43 ± 3.89
Maximum homing speed	50.51 ± 9.07	31.01 ± 12.35	33.75 ± 12.43	65.00 ± 11.66
at distance	5000m	4000m	300m	520m
$n_{(\text{returned in 24h})}$	124	50	37	108
$n_{(\text{returned})}$	154	64	48	128
$n_{(\text{released})}$	464	158	122	329

Table 1: Homing speed and number of released bees. The median homing speed in the four release directions and the highest homing speed at the respective release distance are shown. The number of bees returning inside the 24 hours after release, the number of bees that returned at any time after release and the total number of released bees are noted.

In the southern direction, some bees were released over water. Figure 6 shows the terrain of the release spots up to 3000m from the hive, and compares the southern homing times to those measured for the other directions. The homing times for close distances up to 1500m were similar in all directions. When released on the opposite side of the lake, however, homing times increase drastically from an average of 52±12 min at 1480m to 193±25 min at 1870m; an almost fourfold increase in time, while the distance is only 400m further.

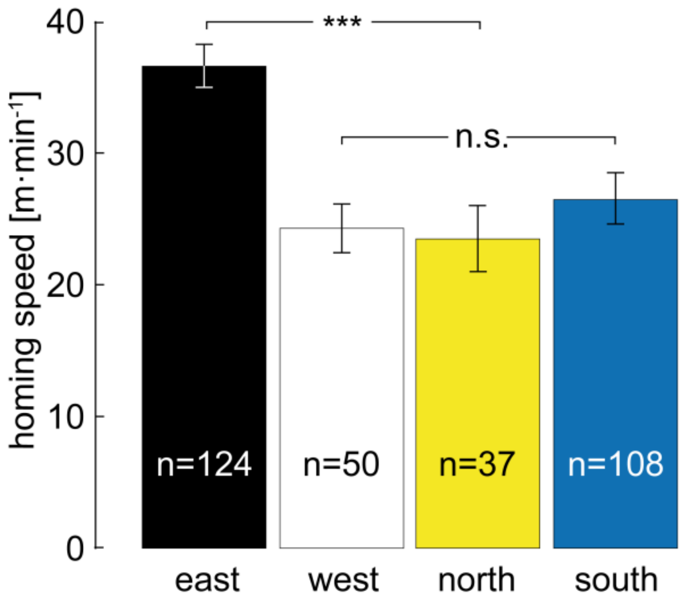

Figure 5: Comparison of homing speeds. Bees homing from the eastern direction return to the hive sooner than bees from the west, north and south. * Denotes p<0.001; n.s. = not significant. Error bars show SEM.**

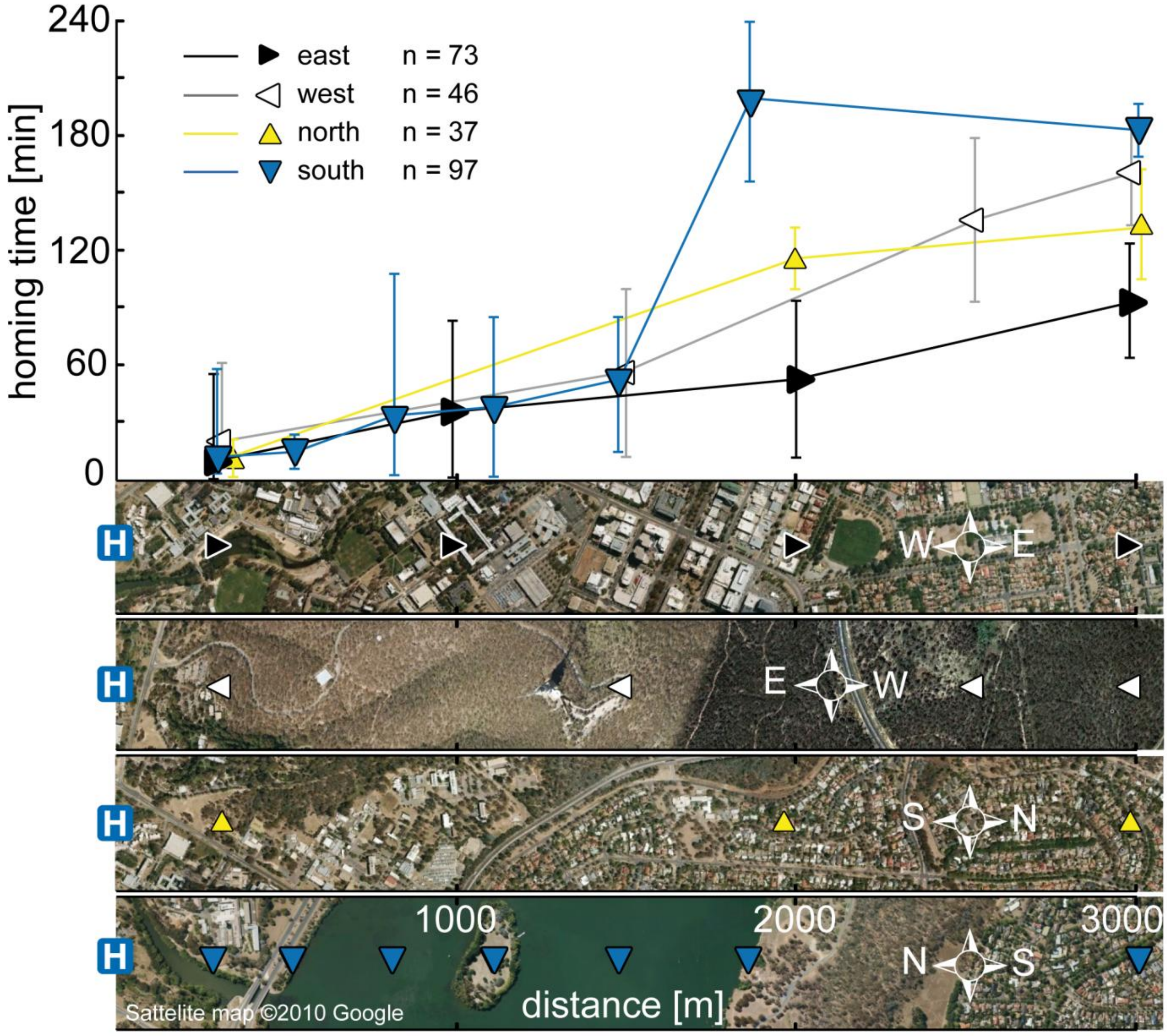

Figure 6: Homing time over land vs. water. The graph compares homing times from up to 3000 m in four directions. Map strips show release spots over different terrain: urban areas in the east, forests west of the hive, urban areas north and the lake south of the hive. 20 bees were released at each spot, and homing times were calculated from bees returning on the day of release. Error bars show SD.

Discussion

Many of the honeybees found their way back home even after blind displacement to unfamiliar areas, some of them from up to 11km. Our RFID setup monitored a large number of individual bees around the clock for many days. It produced precise measurements by recording the exact arrival time of each animal, and ensured that no late arrivals were missed.

Since the bees could not perceive the direction of movement during the displacement, compass information alone (be it from the sun, the polarization pattern in the sky, magnetic inclination or polarity) could not have guided them. Catching experimental forager bees upon return to the hive ensured that the bees' path integrator was set back to 0, and thus had no influence on the bees' homing direction. Local landmarks around the hive were not visible from release spots further than 500m away, and even global landmarks like BM were not always visible (on the release sites further than 4000m in the east).

The typical honeybee foraging range depends on the abundance of food, water and propolis around the hive. Most resources are collected within a 600 - 800m radius, although distances of 2km are still common, and bees may even travel 5km in some situations (Visscher and Seeley, 1982; Southwick and Buchmann, 1995). Only in extreme experimental conditions of food and water deprivation do bees venture to maximum distances of 13km (Eckert, 1931). However, the experimental hive was situated only 300m from the Canberra National Botanical Gardens, a year-round source for pollen, nectar and propolis. Thus, it is unlikely, but not impossible, that the bees knew the areas beyond the lake, behind BM and beyond MA. How could they find the way back? When bees leave the hive for the first time, they perform orientation flights, a series of steadily increasing arcs in which they familiarize themselves with the surrounding area (reviewed by Zeil et al. (1996)). Those trips are essential for successful homing; bees artificially displaced before the first orientation flight have trouble finding their way back home even from a 50m distance (Becker, 1958). The hive itself, the surrounding local landmarks and global landmarks from the horizon panorama are memorized to make sure they find their way back home after the first foraging trip. Bees learn the sun's pattern of movement in relation to the entire landscape panorama around their nests, enabling them to extract the solar ephemeris function even on cloudy days from the surrounding skyline (Dyer and Gould, 1981b; Towne and Moscrip, 2008). Ants have recently been shown to use the panoramic skyline to determine the homewards direction after artificial displacement (Graham and Cheng, 2009). Despite the difference in scale, it is likely that bees can use similar visual cues for homing after displacement.

Most foragers in our study had no trouble flying back from the close release spots in a 1500m radius around the hive. On this small scale, familiar local features can guide bees towards frequent foraging routes or directly to the hive (Menzel et al., 2000). Especially high homing rates were recorded from the release site 1400m west on top of BM, from where 90% of the bees returned due to an exceptionally good view of the area surrounding the hive, and the 1000m eastern release, from where all bees returned. Earlier studies have looked at homing from different directions only in close distances up to 2000m. They found no difference in homing success or homing time between different directions close to the hive (Uchida and Kuwabara, 1951; Becker, 1958), consistent with our results.

On a medium scale, up to about 4000m, the homing rates from the eastern releases are much higher than from the other directions (Fig. 3). Bees homing from the east spend less time finding their way home than bees homing from the other directions (Figure 4). The panorama between the two mountains BM and MA is familiar to the bees, since the orientation flights are performed in this area. Thus, BM could act as a beacon, guiding bees towards the hive. Bees familiar with the area could also have vector memories associated with global landmarks like BM and MA. Retrieved in the right panoramic context, memories encoding distance and direction to the nest could guide the bees home or to the next familiar path segment (Collett et al., 2002). The directional component of the vector could either be provided by the polarization compass, or the panorama itself. Another possible mechanism is the use of the entire skyline panorama (Graham and Cheng, 2009; Philippides et al., 2011). The bees could home in towards the hive by minimizing differences between the stored, familiar panorama around the hive, and the actual surrounding view, e.g. flying away from MA westwards to BM (Zeil et al., 2003; Stürzl and Zeil, 2007). The distinctive shape of BM as seen from the hive (Figure 2) could also be directly used as a landmark beacon. Southwick and Buchmann (1995) released bees at a 3900m distance from their hive in the four cardinal directions. In a flat, featureless experimental area, where the maximum homing distance was 5600m, they found no difference between the homing rates in the four directions, probably due to the missing panoramic cues. In a mountainous experimental area, where bees returned from up to 9200m, they studied only one release direction, south-east along a mountain ridge. In this area, with a prominent panoramic skyline around the hive, they might have found significant differences between the release directions as well.

On the larger scale, further than 7000m distant, only bees from the east successfully returned home. BM is not visible from the release spots further than 4400m in the east, since MA is blocking the view. Even so, 30-40% of the bees returned from the releases behind MA. Mechanisms similar to those operating in the medium scale could be at work here: by flying towards a mountain in the west, the released bees would fly to MA first and then continue towards BM, where familiar local features eventually take over and guide the bees to the hive. This would also explain the lower homing rates from the other directions: flying west towards the next mountain from those release sites would only take the bees further away from the hive.

The flight time for the homing trip increased with distance. Flying at a pace of 15 km/h, even the most distant release spots were easily reachable after a 60 minute flight. However, the homing times were always much higher than expected at the usual travel speed of a bee. Sometimes, e.g. from the 11000m spot in the east, it took several days for a bee to return to the hive. Homing times, e.g. from the 3000m spots, varied between 78min from the east and 280min from the south. This indicates that the time spent searching for the correct heading is much longer than the actual travel time, and significantly different for each direction. The actual distances travelled by the bees, were they constantly flying at 15 km/h, could be as much as 19.5km from the east and 70km from the south. To cover such distances, the bees would have to drink nectar to refuel on the way, since a crop load of 20µl 1.3M sugar solution will keep a bee flying for just about 25min, or 7km (Hanauer-Thieser and Nachtigall, 1995).

Bees homing from the southern release spots on the lake took as much time as those homing from equal distances from the other directions (Figure 6). When released from the opposite shore, however, homing speed decreased from 28.47 ± 6.39 $m*min^{-1}$ at 1480m (last release on the lake) to 9.69 ± 3.14 $m*min^{-1}$ at 1870m (first release on opposite shore). It is unlikely that the bees were just flying slower from the release on the southern lake shore, since homing speed is no measure of flight speed, but includes searching, resting and refuelling time. The two release sites were only 400m apart, have the same elevation and share a similar view of the surrounding area. Moreover, a comparable percentage of bees found the way back to the hive (60% from 1480m and 65% from 1870m), indicating that the bees did not have more trouble locating the hive from the opposite lake shore. Since bees are generally hesitant to fly over water (Heran and Lindauer, 1963; von Frisch, 1967), they most likely chose the detour over land along the shore from the 1870m spot, and took the direct route from the release on the lake.

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General Discussion

A constant influx of resources is essential for a bee hive. Honeybee foragers maintain a steady flow of nectar, pollen, propolis and water to meet the needs of the colony. At the same time, forager bees act as the colony's sensory units, carrying information about the location, quality and attractiveness of resources and potential nest sites, to the hive. The interaction between hive bees and foragers leads to an integration of the incoming information, so that the resource flow is constantly adjusted to the colony's needs. 145 million years of co-evolution with angiosperm plants (Hu et al., 2008) have resulted in sensory systems well adapted to this task, as discussed in the general introduction to this thesis. Bees face complex decisions in order to forage as efficiently as possible. In this work, I highlight some of the mechanisms involved in the bees' decision making.

The honeybees' visual and olfactory systems allow for precise navigation and flower discrimination, and thus for the bees to be flower constant (von Frisch, 1967; Seeley, 1995). Pollinators sticking to one type of flower are obviously advantageous for plants, because it facilitates pollination from the same species. The advantage for bees is not as clear though. Recorded for the first time some 2300 years ago by Aristotle, flower constancy puzzled Charles Darwin (Darwin, 1876) and the reason for bees to stay with one type of flower at a time is still a matter of debate (Raine and Chittka, 2007). There are multiple hypotheses for flower constancy, ranging from a reduction in handling time on consecutive visits to constraints in short-term memory and interference effects in learning (Chittka et al., 1999). The sophisticated cognitive processes described in this thesis make constraints in learning and memory unlikely causes for flower constancy (Zhang et al., 2006; Pahl et al., 2007; Gross et al., 2009; Pahl et al., 2010; 2011).

Visual and olfactory properties are not the only cues separating different flower species. Flowers open and close their blossoms at regular times during the day, as the Swedish taxonomist Carl von Linné observed more than 250 years ago (Linné, 1751). The regularity of this circadian rhythm in plants inspired him to design a flower clock, showing the time of day according to the flower species opening or closing their blossoms. He found suitable plants for every hour between 4 am and 10 pm, but his dial would fail because the species are in bloom during different times of the year (von Frisch, 1967). A recent study found that Linné's floral clock would be slow without pollinators, because some flowers close their blossoms much later if they are not pollinated (Fründ et al., 2011). Moreover, it is not only the opening and closing times of blossoms that follow a circadian pattern. Beutler and Kleber found that the amount and concentration of nectar varies over time in a species-typical way (Beutler, 1930; Kleber, 1935), and the same is true for pollen (Parker, 1925). Thus, time is a factor of great importance for nectar and pollen collectors (von Frisch, 1967). Bees would profit from a time sense not only to compensate for the sun's movement during the waggle dance, but also in order to visit the flowers during their peak nectar- and pollen production times. This sense of time was first described by August Forel, who found bees waiting at his coffee table just before breakfast and afternoon tea in anticipation of sweet marmalade (Forel, 1910). His observation inspired the further investigation of the bees' biological clock by von Frisch's student Ingeborg Behling (1929) and later by Rainer Koltermann, who found that he could train bees to remember up to 9 different times during a day when he presented a scented sucrose feeder (Koltermann, 1971).

In Chapter 1 of this thesis, I show that bees can use time as a contextual cue, setting two competing visual stimuli in different contexts, when simultaneously observing a task-dependent rule (i.e. choosing A at the feeder and B at the hive in the morning, and B at the feeder and A at the hive in the afternoon). The experimental bees learned to treat the two stimuli differently in the morning and in the afternoon, as well as when flying to the feeder and returning to the hive. The training imposed a learnt stimulus preference on the bees' circadian rhythm, demonstrating that honeybees possess a sophisticated memory which is able to remember tasks within a temporal context (Zhang et al., 2006). They could use this ability to treat stimuli differently during navigation to a food source and on the way back to the hive, as well as during foraging on at least two different times of the day, in order to be at the right place at the right time. 'Planning' activities within a temporal and spatial frame of reference could enable foragers to use resources more efficiently.

In Chapter 2, I investigate the role of temporal and spatial information as contextual cues. In the training for this experiment, the bees learned to choose stimulus A at maze one in the morning, and stimulus B at maze two in the afternoon. With this performance as a baseline, we could observe the bees' performance while selectively removing shape, colour and/or location information. By testing the animals in a new maze in a neutral location, the spatial context was removed. Manipulating the visual stimuli, we selectively eliminated colour or shape information. The results demonstrate that colour and shape are the most important visual cues when bees decide between flowers. The absence of the spatial cue did not impair the bees' performance; they still showed a significant preference for the rewarded stimulus according to the time of day. When visiting different feeding sites, or even when a new flower patch is discovered, previous experience enables bees to choose the most profitable flower according to the time of day (Pahl et al., 2007).

The findings described above make it highly unlikely that memory constraints or interference effects during learning could be the reason for flower constancy in honeybees. The bees' ability to integrate elements of visual stimuli, place and circadian time (what, where and when) is akin to episodic-like memory as described in food-hoarding birds (Clayton and Dickinson, 1998). We have therefore named this kind of memory 'circadian-timed episodic-like memory' (Pahl et al., 2007). Recently, Prabhu and Cheng showed that honeybees trained on colours for only one day can already use temporal information in decision making. This indicates that bees might have a natural tendency towards inferring a circadian pattern on unclear reward situations (Pahl et al., 2007; Prabhu and Cheng, 2008a). They also found that, when similar training is conducted on the olfactory domain, bees prefer only the odour that was most recently associated with a reward (Prabhu and Cheng, 2008b). Thus, bees have two ways to assess the reward probability when encountering a novel situation, which may cause conflicting interests. However, in a forager bee's natural environment, colour and scent are rarely experienced separately from each other. More experiments are required to examine how the bees' circadian preference pattern is modulated when colour and scent are coupled. Another open question remains regarding the nature of the bee's biological clock. I have shown that bees link information to circadian times, but do they also have a way to measure intervals between two occasions? This ability has been shown in bumblebees (Boisvert and Sherry, 2006), and it is highly likely that honeybees possess the same skill.

The use of number in the animal kingdom has been investigated in several vertebrate species (listed in the general introduction and in Chapter 3), but few convincing studies exist for invertebrates so far (Chittka and Geiger, 1995; McKee, 2004; Franks et al., 2006; Dacke and Srinivasan, 2008). In Chapter 3 of this thesis, I attempt to shift the balance more in favour of the invertebrates. There are two basic mechanisms to assess the exact number of objects in a stimulus: subitizing and true counting. True counting requires subjects to (1) produce a standard sequence of number tags, (2) apply a unique number tag to each item to be counted, (3) remember what already has been counted, and (4) know that the last number tag tells how many objects are there (Gallistel, 1988). This is clearly not what the bees were doing in our experiment. Subitizing is the instant recognition of the number of objects without sequential counting. Stanley Jevons found, in his paper from 1871, that the maximum number of beans in a box he could estimate correctly, after a brief presentation not allowing for sequential counting, was 4. With higher numbers, the amount of errors increased rapidly (Jevons, 1871). The honeybee is the first invertebrate species where a numerical ability has been convincingly demonstrated, and the process by which it achieves numerical discrimination is most likely subitizing (Gross et al., 2009). The fact that its sense of number has a similar extent as the subitizing abilities reported for many vertebrate species, including human infants (Linnel and Fluck, 2001), hints towards a highly conserved mechanism; one quite separate from real counting (Gross, 2011a). I would expect to find it in many more nervous systems (e.g. cephalopods), once adequate experimental paradigms have been developed. Our results indicate that numerosity is treated by the bees as one more primary visual feature of a scene, along with colour, contrast, size and speed (Burr and Ross, 2008; Gross et al., 2009). The distinction between four and more has had, in fact, a great influence on human written numbers. In pre-classical Roman times, the numbers one to five were written I, II, III, IIII and V. Later, the number four was replaced by IV (five minus one). Accordingly, in ancient China, the symbols for one to five were I,II,III,IIII and X. Other examples include south Arabia, the Mayas in middle America and the Vikings in northern Europe (Gross, 2011b). These languages show that up to four symbols can be instantly recognized by subitizing, while higher numbers require sequential counting and are, for practical reasons, replaced by novel instantly recognizable symbols. Data on the adaptive value of numerical competence is rare, because most studies were conducted in laboratory situations. However, there are some examples of field studies: Food-hoarding robins have been shown to use information about the number of food items in a cache in the wild (Hunt et al., 2008). Lyon reported a spontaneous use of numerical information (egg counting) in a natural context, reducing the fitness costs of conspecific brood parasitism in American coots (Lyon, 2003). Lions base the decision to attack or retreat from a group of intruders on the number of roaring individuals (McComb et al., 1994). Honeybees could use their sense of number to recognize flowers by the amount of petals (Leppik, 1953), to navigate by the number of landmarks encountered (Chittka and Geiger, 1995; Dacke and Srinivasan, 2008), or to make foraging decisions according to the number of bees already present on a blossom (Gross et al., 2009). In Chapter 3 of this thesis, I explore the use of number by free-flying honeybees in a DMTS setup. This is, to my knowledge, the first report of numerosity discrimination in an invertebrate (Gross et al., 2009).

In order to properly service the needs of the colony, forager bees need to travel large distances between the hive and important resources. The mechanisms by which bees navigate are the celestial compass, a visual odometer, a path integrator as well as memorized local and global landmarks. The properties of those navigational tools are discussed in the general introduction to this thesis and in Chapter 4. In the large scale homing experiments described in the last chapter, the experimental bees were caught after returning to the hive from a pollen foraging flight, and transported to the release sites in a black box. Catching the bees upon return ensures that the bees' path integration vector was set back to zero. The transport in a black box guaranteed that no visual stimuli reached the experimental animals, and thus they could not perceive the direction or distance of the artificial displacement (Pahl et al., 2011). Once released at the destination, the bees had to rely on previously acquired knowledge about the spatial layout of the landscape in order to find the way back to the hive. With increasing displacement distance, the percentage of bees returning from the release spots declined, while the homing time increased, as expected. Surprisingly, the bees released in the eastern direction had higher homing rates, their maximum homing distance was much higher (11km vs. 7km) and the homing speed from the east was much faster than the speed of the animals released in the other directions. The homing performance of the animals was similar in all directions up to 1.5km, where almost all bees returned without trouble. The advantage in homing from the east became apparent at distances around 2 to 4km, and most pronounced at a distance of 7km up to 11km, from where only bees from the east returned. This is most likely due to the visibility of global landmarks like Mount Ainslie, Black Mountain and Lake Burley Griffin, which make up the panoramic skyline of the experimental area. How those can be used to find back to the hive is discussed in Chapter 4.

Do honeybees navigate according to a cognitive map? A map-like spatial memory would enable a bee to choose the shortest route between two random points in the charted area, without ever having travelled that route before. There has been a long going debate about a map-like representation of space in the honeybee, mostly spawned by two studies by James Gould. In the first study, the ‘lake experiment’, he observed that bees, which followed dances pointing to unlikely food locations (such as the middle of a lake) rejected those dances, and concluded that the dance followers judged the plausibility of those dances according to a cognitive map (Gould and Gould, 1982). In the second study, when he displaced bees departing from the hive and a feeder, their departure directions were recorded, and he concluded that the bees chose a map course rather than a compass course, i.e. did not rely on their path integrator but on a map-like representation of the experimental area (Gould, 1986; Gould and Towne, 1987). When Wehner and Menzel repeated the latter study, they found that the released bees followed compass courses rather than map courses (Wehner and Menzel, 1990), and concluded that Gould’s results were probably confounded by visual cues. Gould’s initial study was repeated by Margaret Wray and colleagues in 2008. They found no evidence that dance followers were rejecting dances for implausible locations, and concluded that Gould’s study should “no longer be cited as evidence that honeybees possess cognitive maps, ‘insight’ or ‘imagination’” (Wray et al., 2008). In Gould’s initial studies, visual cues like the horizon skyline were most likely used by the bees to find back to the hive. Randolf Menzel and his team revisited the question of a map-like memory in 2005 with a harmonic radar setup. Displacing bees that were just about to leave the hive or the feeder, they used the harmonic radar to record the flight trajectories of those bees. They found that the bees first departed on the initial course they had been flying before capture, then searched in spirals, and finally departed on straight courses towards the feeder or the hive. The authors interpret the course-setting at arbitrary points in the hive surroundings, and the decision between two goals, as evidence for a map-like organization of spatial memory (Menzel et al., 2005). The range of the harmonic radar setup was quite limited however (the experimental area was 800 x 800m), and although the authors controlled for obvious landmark cues, the bees were never further than 500m away from the target. The animals could have used the horizon skyline, which bees have been shown to memorize together with the solar ephemeris (Towne and Moscrip, 2008), to determine the direction to the feeder or the hive. In the study I present in Chapter 4, the bees actually behaved very similar to the bees in Menzel et al.'s experiment. We also observed the spiral search manoeuvres, in release distances up to 11km. If the bees were using a cognitive map of the area, we would expect them to fly straight to the hive after determining their position during the spiral search. The bees took much too long to return to the hive though. A straight flight should have taken them home in well under one hour, even on the furthest releases. Especially from the closer releases less than 3km distant from the hive in familiar foraging territory, a straight flight would have taken the bees home after minutes, and not hours as we recorded (see Figures 5 and 6 in Chapter 4). These results are more consistent with an extensive search for landmarks, with which vector memories may be associated (Collett et al., 2002). Cruse and Wehner have recently proposed a neural network model based on path integration and various landmark guidance mechanisms, which can explain homing after displacement in ants and bees as an emergent property arising from a decentralised system (Cruse and Wehner, 2011). This model introduces no new assumptions to explain the results obtained in insect navigation experiments. Occam's razor teaches us that Cruse and Wehner's more parsimonious model of decentralized memory is the best explanation we have so far, since a cognitive map is not required to explain the experimental evidence we have to date. However, honeybees continue to fascinate many scientists, and there may well be new evidence arising that cannot be explained by the simple model, and the cognitive map may have to be re-evaluated.

The findings acquired in this thesis show that honeybees are not the simple reflex automats they were once believed to be. The level of sophistication I found in the bees' memory, their learning ability, their time sense, their numerical competence and their navigational abilities are surprisingly similar to the results obtained in comparable experiments with vertebrates. Those cognitive abilities may have evolved out of the need for efficient information processing caused by the limited capacities in the bees' small brain. Thus, we should reconsider the notion that a bigger brain automatically indicates higher intelligence.

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Publications & Conference Contributions

Peer-reviewed articles:

Pahl M, Zhu H, Tautz J and Zhang S (2011):
Large Scale Homing in Honeybees.
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Gross HJ, **Pahl M**, Si A, Zhu H, Tautz J and Zhang SW (2009):
Number-Based Visual Generalisation in the Honeybee.
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Pahl M, Zhu H, Pix W, Tautz J. and Zhang SW (2007):
Circadian timed episodic-like memory - a bee knows what to do when, and also where.
Journal of Experimental Biology 210, 3559-3567.
Highlighted on the cover page of the 2008 JEB calendar.

Zhang S, Schwarz S, **Pahl M**, Zhu H and Tautz J (2006):
Honeybee memory: a honeybee knows what to do and when.
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Book chapter:

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Honeybee Cognition.
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International Conference Contributions:

Pahl M, Gross HJ, Si A, Zhu H, Tautz J, Zhang S (2010):

Number-based visual generalisation in the honeybee.

9th International Congress of Neuroethology in Salamanca, Spain (Talk).

Pahl M, Gross HJ, Si A, Zhu H and Zhang SW (2009):

Number-Based Visual Generalisation in the Honeybee.

International Ethological Conference in Rennes, France (Poster).

Pahl M, Zhang SW, Pix W, Zhu H (2008):

Celestial and terrestrial cues in honeybee navigation.

International Conference on Invertebrate Vision 2008 in Bäckaskog, Sweden (Poster).

Pahl M, Zhu H, Pix W, Tautz J, Zhang SW (2007):

Honeybee memory: Bees can schedule their activities in time and space.

Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung 2007 in Veitshöchheim, Germany (Poster).

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Honeybee memory: Bees can schedule their activities in time and space.

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