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Effects of caffeine on olfactory and visual learning in the honey bee (*Apis mellifera*)

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Abstract

Although caffeine is known to improve alertness and arousal in humans and other mammals, its impacts on specific behaviours, including complex cognitive processes, remain controversial. We reasoned that the availability of an easily manipulable, but behaviourally complex invertebrate organism with a simpler nervous system would be beneficial to this field of research. We used a popular behavioural model, the honeybee, to evaluate the effects of caffeine on (1) the development of olfactory learning and (2) the performance in complex learning paradigms, including a 'delayed-match-to-sample' task and visual associative learning. To evaluate the efficacy of caffeine treatment, a variety of doses (0.4–400 ng/1 mg of body mass) were applied topically to tethered individuals. Behavioural testing was performed with either tethered or free-flying adult honeybees. We show that caffeine has *marked* cognitive effects in this species. In young honeybees, it reduces the age at which restrained individuals are able to learn an olfactory associative task, whereas in older, free-flying bees, caffeine improves both motivation and cognitive performance in complex learning tasks. Our results suggest that the honeybee model may be useful in explaining caffeine-related behavioural changes not only in this species, but also in mammalian systems.

Keywords: Honeybee; Caffeine; Cognition; Learning; Memory; Motivation

1. Introduction

Caffeine is arguably the most common psychostimulant drug used worldwide, and its impact on alertness, mood and general performance in humans is widely acknowledged (Fredholm et al., 1999; Smith, 2002). However, the scientific examination of the relationship between caffeine and specific behaviours in humans and other mammals has often produced inconsistent results (Smith, 2002; Nawrot et al., 2003). For example, a large number of studies prior to 1990 on the effects of caffeine on more complex cognitive processes failed to detect significant effects in human subjects (Smith, 2002). On the other hand, unequivocal beneficial effects on vigilance and cognitive performance in both rested and sleep-deprived individuals have been documented by numerous reports, including a study employing a specially developed visual vigilance task (Lieberman, 2003). In general, caffeine consumption increases alertness and vigilance in individuals, especially in situations where arousal is low (Smith et al., 1999; Beaumont et al., 2001; Brice and Smith, 2001; Mikalsen et al., 2001; Lieberman et al., 2002; Yeomans et al., 2002; Gruber and Block, 2003; Rogers et al., 2003). The arousal effect of caffeine extends also to invertebrates, with caffeine-treated *Drosophila* resting less than control flies in a dose-dependent fashion (Shaw et al., 2000).

The effects of caffeine on working memory, short-term memory (STM) and long-term memory (LTM) are less clear-cut than those on arousal, and seem to depend on the time of drug administration (pre-training, post-training or pre-test) and the testing paradigm employed. Higher levels of coffee consumption, for instance, correlate with improved performance in reaction time, verbal memory and visuospatial reasoning in humans (Jarvis, 1993; Hameleers et al., 2000), while a slow-release dose of caffeine has a positive action on a mathematical processing task involving both LTM and STM (Beaumont et al., 2001). Caffeine also counteracts the normal

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decline in memory performance that occurs during the course of a day in older adults (Ryan et al., 2002) and leads to better recall in older women with higher levels of lifetime caffeine consumption (Johnson-Kozlow et al., 2002). Caffeine, when administered immediately after training in mice, facilitates the retention of an inhibitory avoidance task (Kopf et al., 1999) and, in a dose-dependent manner, improves performance in repeated acquisition tasks, which assess motor learning and STM.

In contrast, Herz (1999) found no effect of psychoactive doses of caffeine on long-term verbal memory in humans, while neither Hudzik and Wenger (1993) nor Buffalo et al. (1993) were able to elicit any improvement in the delayed matching-to-sample performance of squirrel and rhesus monkeys respectively. Such inconsistencies can probably be attributed to a range of other factors, such as methodological differences, personality differences, the time of day (of testing) and the consumption of other psychoactive substances, such as alcohol, tobacco, etc. (Nawrot et al., 2003). There has also been some indication that natural genetic variation may be largely to blame for the varying responses of individuals to pharmacological agents: the survival time of *Drosophila melanogaster* individuals exposed to chronic ingestion of caffeine correlates not only with the sex, but also with the genetic makeup of the individual (Carillo and Gibson, 2002). Such contradictory results, arising from the investigation of the behavioral effects of a pharmacologically active substance in a complex, highly interconnected nervous system, are understandable, given the underlying circuitousness of the path from molecules to behavior. In this context, developing a simple and efficient animal model system with which to explore the effects of caffeine and other psychoactive drugs on behaviour may prove beneficial to this area of research.

The recent sequencing of the honeybee genome (Honeybee Genome Project, 2004) combined with the ease with which behavioral testing can be performed in this species (Zhang et al., 1999; Giurfa et al., 2001) make the honeybee a potentially exciting platform for evaluating the effects of drugs on nervous systems and to compare such results to similar treatments in humans. Honeybees can solve a surprising variety of cognitive tasks including those that have been traditionally associated with vertebrate animals. For example, bees can be trained to recognise categories of objects with shared features (Giurfa et al., 1996; Zhang et al., 2004), and learn abstract concepts such as 'sameness', 'difference' (Giurfa et al., 2001) and even 'sequence' (Zhang et al., 2005). In addition, the honeybee is the only known non-primate species that has evolved a symbolic system of communication, the so-called waggle dance.

Cognitive studies involving caffeine have largely been carried out on vertebrates, with attention focussing mainly on rats, mice, monkeys and humans. As a result, the cognitive effects of caffeine on invertebrate species, including insects, remain largely unknown. The first part of this study investigates the effect of caffeine on associative learning using the well-known proboscis extension reflex (PER) in young, tethered honeybees, as an easy and quick way of assessing the age-and dose-dependent effects of caffeine on long-term olfactory associative memory. In particular, we report that caffeine allows bees to learn olfactory associations at a much earlier age. The second part of this study examines the effects of caffeine on honeybees in a situation where they face a complex cognitive task, the so-called 'delayed-match-to-sample' (DMTS). This paradigm has been used to investigate principles of learning and memory not only in a number of vertebrate species including dolphins (Herman and Gordon, 1974) and monkeys (Salzmann et al., 1993), but also in honeybees (Giurfa et al., 2001; Zhang et al., 2005). The DMTS paradigm is useful in assessing working memory, as well as the ability of a subject to learn concepts such as 'sameness' or 'difference'. Finally, we examine the performance of honeybees in a Y-maze following caffeine treatment, to conveniently assess the bees' acquisition and long-term (4 and 8 days) memory of a visual association. The observed effects of caffeine administration are discussed in the context of what is already known about the behavioural effects of the drug in humans and other vertebrates.

2. Materials and methods

2.1. Experimental location

All treatments and testing on restrained bees were carried out at the Beehouse at the Research School for Biological Sciences, The Australian National University. All maze experiments were carried out within the All-Weather Bee Flight Facility at the RSBS, ANU. The only exception was the repeat DMTS experiment, which took place outdoors.

2.2. Organism and training paradigms

2.2.1. PER study

Individual frames of brood comb were removed from an experimental hive and placed in an incubator at 31 °C and 80% humidity overnight. Newly emerged bees from the previous night were collected everyday. A 2-μl drop of 100 mM caffeine dissolved in the organic solvent dimethyl formamide (dMF) was placed on the thorax of each bee to be treated immediately after emergence. Control bees were given only a 2-μl drop of dMF. The dose administered (~40 μg/bee or 400 ng/1 mg of body mass) is not directly comparable to quantities of caffeine used in experiments with vertebrate animals or to human consumption because the efficiency of cuticular penetration is not expected to be 100%. However, it was reasoned that, in insect behavioral studies, a non-invasive topical delivery is far superior to injections that often lead to increased mortality and/or microbial infections (Kucharski and Maleszka, 2003). Indeed, the survival of caffeine-treated bees was not different from that of the untreated ones. The bees were kept in wire mesh cages (at about 50–60 individuals per cage) and fed honey ad libitum until they reached the desired age.

The training protocol employed by Bitterman et al. (1983), with some modifications (Maleszka et al., 2000; Si et al., 2004) was adopted for the present study. Briefly, bees were tethered

in thin-walled aluminium tubes using strips of fabric-reinforced tape on the day prior to the day of training. Two different training protocols were compared in this study: in the two-scent protocol normally employed in our laboratory, bees were given three exposures to each of two odours (conditioned stimuli CS) at 6-min intervals. Limonene was paired with a rewarding sucrose solution (US), while natural vanilla was paired with a punishing salt solution. A 6-s interval was allowed between CS and US. Bees were tested with the two odours 24 h later. Bees that performed a PER to limonene, but withheld it on being presented with vanilla were scored as having responded correctly (see Si et al., 2004 for more details). In the one-scent training protocol, which is commonly mentioned in the literature, bees were given three exposures of limonene paired with a rewarding sucrose solution. Tests were carried out 24 h later and bees performing a PER on being presented with limonene were scored as learners.

Bees aged 2 days to 7 days post-emergence were trained with the two-scent protocol; the one-scent protocol was used for comparison on 3-day-old and 6-day-old bees only. A dose-dependence curve for caffeine was generated using 4-day-old bees and caffeine concentrations of 0.1 mM to 100 mM.

2.2.2. Maze experiments

Adult forager bees of unknown age from a two-box (eight frames each) hive were trained to an artificial feeder, containing 1.5 M sugar solution. The feeder was then gradually moved into the experimental apparatus (Fig. 2a) in steps of about 20 cm, and in the absence of any visual patterns, in order to teach bees the path to the final, reward chambers. Once the bees had learnt the path to the feeder, the visual pattern to be associated with the reward and the competing pattern were put in place. Caffeine-treated and control bees were trained to perform a DMTS task by first being made to fly through a 1-m long tunnel, at the entrance of which was placed a sample stimulus (Fig. 2a). Following the 1–2-s time delay caused by the flight through the tunnel, bees would enter a decision chamber, whose distal end bore two choice stimuli, one of which was identical to the sample stimulus. If the bee picked the matching choice stimulus, it would enter a reward chamber with a feeder containing sugar solution. The sample stimulus was changed after every 20-min training block; within each block, the position of the rewarding choice stimulus and reward feeder was alternated every 10 min between the two reward chambers of the apparatus. A similar training protocol was used to train bees in the visual association task, using a Y-maze (Fig. 3a). Here, bees would have to learn a single visual stimulus, which was always associated with a reward of sucrose solution. Again, the position of the rewarding stimulus and feeder was alternated every 10 min between the two reward chambers of the apparatus. Bees entering the wrong chamber were released through the top of the chamber and allowed to re-enter the apparatus, and make a second choice. In both the DMTS and Y-maze experiments, a minimum of 15 bees was marked for each of the caffeine-treated and control groups. This ensured that data would be obtained from a reasonably large number of bees (6–7 for each group) for the duration of the experiment.

A 2-μl drop of 100 mM caffeine dissolved in dMF was placed on the thorax of each bee to be treated (while drinking from the feeder), prior to training. Control bees were given only a 2-μl drop of dMF. Bees were treated after they had learnt to fly through the maze and find the feeder, but 1 h before being trained with any stimuli. The dose administered (~40 μg/bee or 400 ng/1 mg of body mass) is not directly comparable to quantities of caffeine used in experiments with vertebrate animals or to human consumption because the efficiency of cuticular penetration is not expected to be 100%. However, it was reasoned that in insect behavioral studies, a non-invasive topical delivery is far superior to injections that often lead to increased mortality and/or microbial infections (Kucharski and Maleszka, 2003). Indeed, the survival of caffeine-treated bees was not different from that of the untreated ones.

2.3. Data collection and analysis

2.3.1. PER experiment

The tethered bees' performance in the two-scent PER experiments were scored as described in Si et al. (2004). Bees in the one-scent experiments were scored as 'correct' if they extended their proboscis to the scent on testing. At least two experiments were carried out for each data point and the results pooled before statistical testing using the χ^2 test.

2.3.2. DMTS experiment

Two separate experiments were carried out with two different hives; each time, a new set of bees was treated and trained. Equal numbers of caffeine-treated and control bees (~15) were marked and treated at the beginning of each experiment. All bees used in the DMTS paradigm were given individual paint markings to aid in their identification during the process of data collection. The first choices of bees within the decision chamber were recorded. The proportions of correct choices were pooled for all bees in each category (i.e., caffeine-treated and control) to obtain a final percentage. The visit frequencies of caffeine-treated and control bees were also monitored and recorded. Twelve 20-min training sessions were completed over a period of 2 days; each training session comprised two 10-min blocks, where the feeder position was alternated.

χ^2 tests were carried out to test for statistical significance. In calculating the bees' performance over the course of the experiment, the first choices of bees for each group were pooled over the total number of visits, for all bees of that group, from all training sessions.

2.3.3. Y-maze experiment

Two separate experiments were carried out with adult forager bees of unknown age from a single hive. However, different sets of bees were used for each experiment. All bees were given individual paint markings to aid in identification and data collection. The first choices of bees entering the decision chamber were recorded. The proportions of correct choices were pooled across experiments for all bees in each

category (i.e., caffeine-treated and control) to obtain a final percentage. Seven 20-min training sessions were completed, each comprising two 10-min blocks, where the feeder position was alternated. Learning curves for the two conditions were generated, based on the scores from the training sessions. Long-term memory for both groups was tested in a 10-min retention test without a reward at 4 days and 8 days after training. Different groups of bees were used in the 4-day and 8-day tests.

3. Results

3.1. PER study

The administration of caffeine to newly emerged worker honeybees markedly reduced the minimum age at which an olfactory association could be reliably stored in long-term memory. While the control bees performed poorly (<30%) in a 24-h two-scent olfactory association task before the age of 6 days post-emergence, the caffeine-treated bees were attaining high scores (~60%) from the age of 3 days post-emergence (Fig. 1a, lines). This pattern was observed regardless of the training protocol: bees trained with a single-odour association also scored significantly higher at 3 days post-emergence, when treated with caffeine (Fig. 1a, bars). By the age of 6 days, both treated and control bees were performing equally well. Caffeine was also found to act in a dose-dependent manner, with concentrations of 10–100 mM bringing about the most improvement in the performance of the associative task in 4-day-old bees (Fig. 1b). On the basis of this result, we decided to use a 100 mM dose of caffeine in the following experiments.

3.2. DMTS study

The PER study revealed that a single dose of caffeine following emergence could improve performance in an olfactory association task in young foragers and allow them to recall olfactory associations at an earlier age. Might caffeine treatment also have a similarly positive outcome in adult foragers? In accord with previous studies on learning in honeybees (Zhang et al., 1999; Giurfa et al., 2001), the bees in our study were also able to successfully learn the DMTS

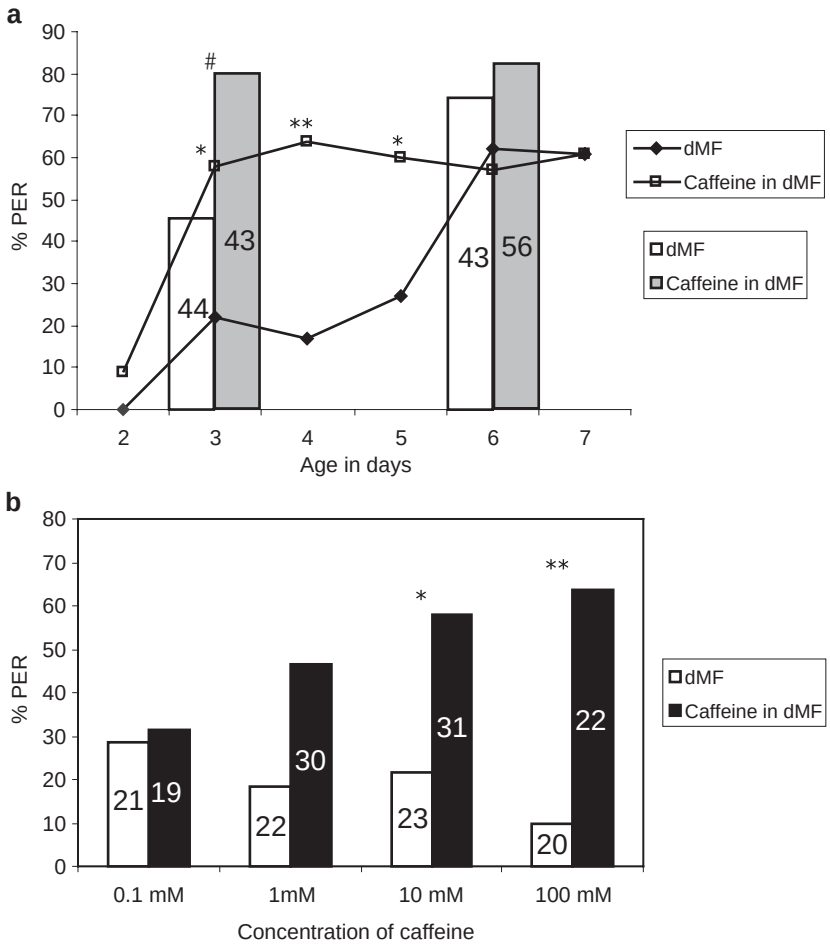

Fig. 1. (a) The effect of caffeine on the ability to learn and recall (after 24 h) an olfactory association in juvenile bees. The *y*-axis gives the proportion of animals that were able to recall the association when tested 24 h after training. Lines: performance following a two-scent training protocol. $^{*}P<0.05$, $^{**}P<0.01$, χ^2 test. Bars: performance following a one-scent training protocol. $^{\#}P<0.05$, χ^2 test. (b) Dependence of the level of PER conditioning on the concentration of caffeine administered in 4-day-old bees. The control (white bars) in all cases was dMF. The numbers on the bars give the number of bees tested in each condition. $^{*}P<0.05$, $^{**}P<0.01$, χ^2 test.

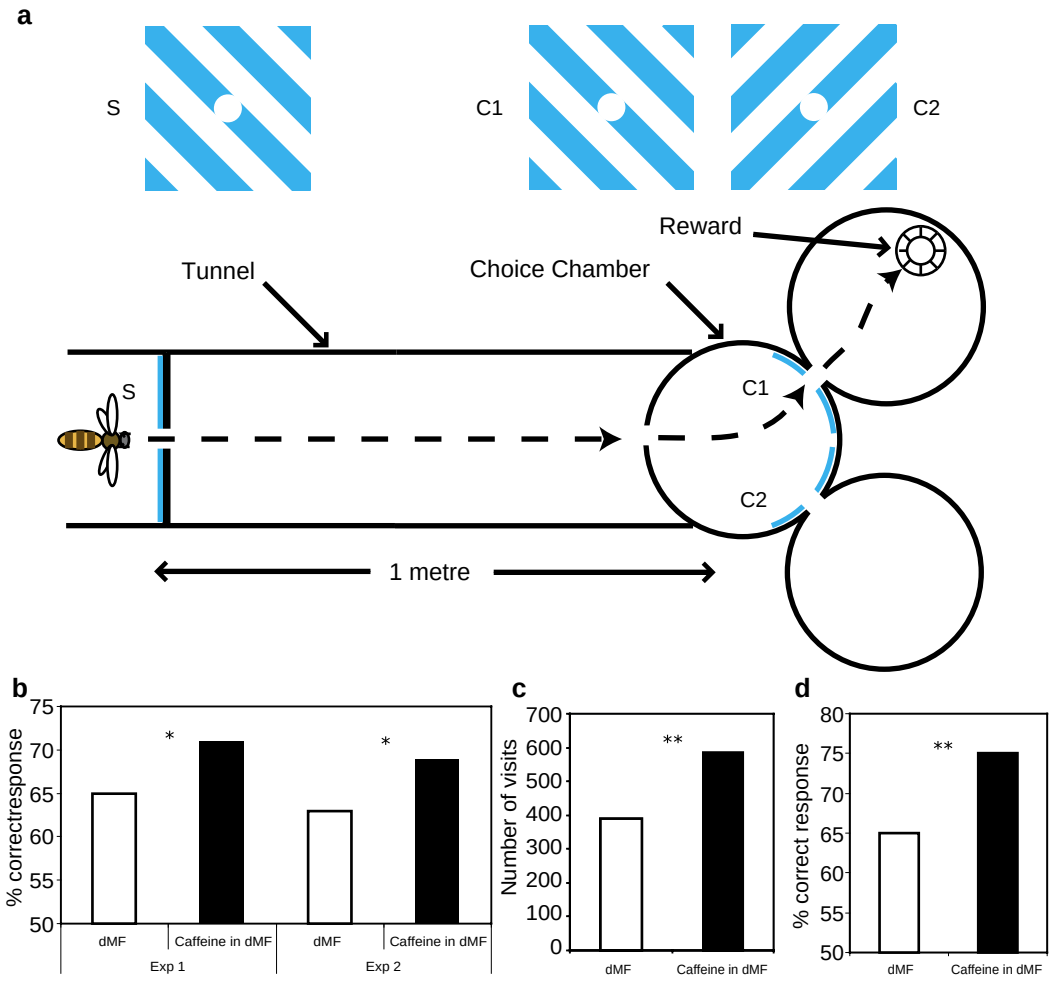

Fig. 2. (a) Layout of the delayed-match-to-sample (DMTS) experimental apparatus. The bee encounters and flies through the initial sample pattern (S) before traversing a 1-m long tunnel. Upon entering the choice chamber, she is presented with two choice patterns (C1 and C2), only one of which (C1 in this case) is identical to S. The bee must choose the matching pattern C1 in order to obtain a reward of sugar solution. (b) Caffeine-treated bees perform significantly better than controls in the DMTS task. (c) Caffeine-treated bees visit the experimental apparatus much more frequently than controls. Data are from Experiment 1. (d) Adjusted data from Experiment 1. Caffeine-treated bees perform significantly better even when the number of visits is equalised for both control and treated groups. Note: The DMTS experiment was carried out twice, once indoors in the climate-controlled All-Weather Bee Flight Facility at the RSBS and then repeated outdoors, using a different hive and completely different bees. The results obtained from both experiments showed a similar trend. *$P<0.05$, **$P<0.001$, χ^2 test.

task, i.e., the percentage of correct responses was significantly greater than a random-choice score of 50% (χ^2 test, $P<0.001$) (Fig. 2b). However, the caffeine-treated bees performed significantly better than the control bees (71% and 65% correct responses, respectively, χ^2 test, $P<0.05$). In addition, we observed that the total number of visits to our experimental apparatus over a 2-day period was much higher for the caffeine-treated bees than in the case of the controls (585 and 391 visits, respectively, χ^2 test, $P<0.001$) (Fig. 2c). This happened in spite of the fact that equal numbers of bees (~15) were marked and treated for each group before the start of the training procedure.

The enhanced performance of the caffeine-treated bees could have been due to their greater number of visits to the experimental apparatus (and hence more practice in performing the task). To determine if it really was the drug treatment that was improving the bees' performance, we compared the performance of the two groups after an equal number of visits (i.e., 391 visits each) (Fig. 2d). Under these conditions, the caffeine-treated bees were found to be performing even better (75% correct, χ^2 test, $P<0.01$).

3.3. Y-maze study

Given the result of the DMTS experiment, we decided to run a simpler visual association experiment using a Y-maze, to determine whether it was acquisition or retention of long-term memory that was being affected by caffeine. The learning curves for the Y-maze visual association task were not significantly different for caffeine-treated and control bees. Both sets of bees attained a maximum performance level of ~90% after just five 20-min training sessions (Fig. 3b and c). As in the DMTS experiment, caffeine bees made more frequent trips to the feeder than the controls (caffeine-treated, 605 trips vs. controls, 462 trips, χ^2 test, $P<0.05$, data not shown). The number of treated bees returning to the feeder on the eighth day

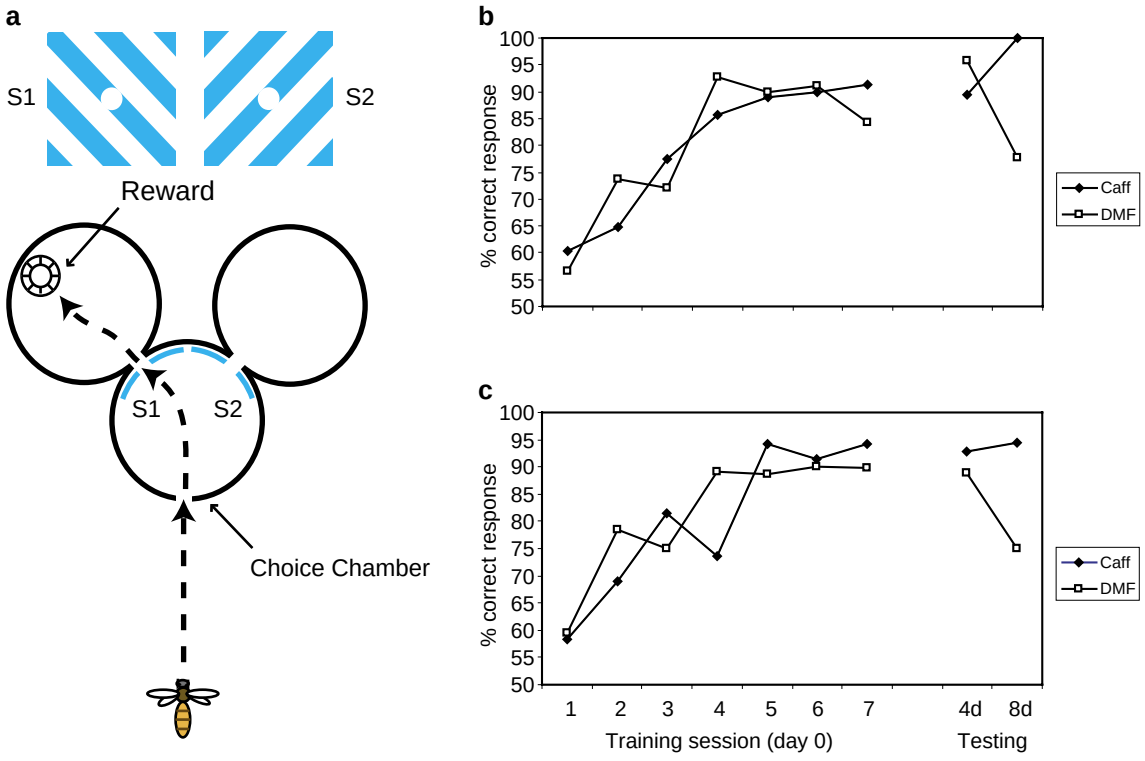

Fig. 3. (a) Layout of the Y-maze experimental apparatus. The bee enters the choice chamber and must choose between two competing patterns (S1 and S2). Only one (S1 in this case) leads to the chamber containing the reward. (b, c) Learning curves obtained in a visual association in a Y-maze from two separate experiments with different sets of bees. Bees were subjected to seven 20-min sessions on day 0, followed by 3 days of 'forgetting' time. The same bees were then tested for the long-term retention of visual memory on days 4 and 8. Caffeine-treated bees perform significantly better than controls on day 8. NS, no significant difference ($P>0.05$), *$P<0.05$, χ^2 test.

of the experiment was too low (five bees in Experiment 1 and six bees in Experiment 2) for even a ~20% difference in performance to be significant; however, pooling the data from two separate experiments performed on completely different sets of bees showed that the performances on day 8 were indeed different. In summary, the performance of bees on day 4 was unchanged, with both groups scoring >90%. However, on day 8, the control bees displayed a drop in performance to about 75%, while the caffeine-treated bees' score was as high as ever (χ^2 test, $P<0.05$).

4. Discussion

Very young (3-day-old) bees treated with caffeine on emergence attained significantly higher scores than the controls in a long-term olfactory association task. In addition, the control bees' performance did not reach such levels until they were 6 days old. The current data do not allow us to conclude whether the cause of this phenomenon is an actual improvement in memory formation and/or recall brought about by caffeine, or instead an accelerated development of the brain (and the olfactory lobes in particular). The honeybee olfactory system develops gradually in the first few days after emergence, with antennal cells showing maximal electrophysiological activity on day 4 (Masson and Arnold, 1984), and the proportion of individuals responding correctly in a single-trial short-term memory test crossing 70% only on day 6 (Morgan et al., 1998). Our control data closely follow this trend. Moreover, the authors of the latter study also reported that the percentage of bees failing to display the proboscis extension reflex in response to sugar–water stimulation of the antennae remained quite high (~30%) until day 4. Could this normal pattern of development have been disrupted by the administration of caffeine?

A recent molecular study on the gene expression changes caused by the administration of a similar dose of caffeine to ours and, at a similar age, revealed that in addition to genes involved in synaptic signalling, genes that are essential for cytoskeletal modifications (kinesin and microtubule motors), protein translation (ribosomal proteins, elongation factors), energy transfer and calcium-dependent processes were significantly upregulated in treated individuals (Kucharski and Maleszka, 2005). The products of these genes are necessary for the movements of organelles, microtubules or chromosomes along microtubules during cell division and for new protein synthesis. The altered expression of synaptic, cell division and energy metabolism genes are likely to be interrelated. Since most developmental and cell growth processes are initiated by calcium release from internal stores the upregulation of genes controlling calcium-dependent processes in caffeine-treated bees is of particular significance. Because the changes in adult brains are primarily concerned with growth, these results suggest that caffeine is able to somehow accelerate the developmental processes in the juvenile honeybee brain.

Similar changes have been noted in vertebrate species: chronic caffeine feeding to juvenile rats has also been shown to increase the DNA and protein content in certain brain regions, such as the hypothalamus and cerebellum, respectively (Nakamoto et al., 1991), while chronic postnatal caffeine treatment is able to cause a 20–30% increase in adult adenosine receptor levels (Marangos et al., 1984). At the cellular level, adding caffeine to hippocampal slices leads to calcium release from internal stores and the fast growth of new dendritic branches (Korkotian and Segal, 1999). Recent epidemiological and laboratory studies have also hinted at a possible, valuable neuroprotective role for caffeine: as a result, A_{2A} receptor blockade is now being pursued as a possible candidate for combating neurodegenerative diseases, such as Parkinson's disease and Huntington's disease (Schwarzschild et al., 2002).

In the past, the DMTS paradigm has proved useful in elucidating the effects of certain drugs like muscarinic agonists (Terry et al., 2002) and AMPA modulators (Buccafusco et al., 2004) both of which produce modest improvements in working memory in primates,. In our study, we not only successfully trained both the control and treated groups of bees to perform a DMTS task, as has been reported previously (Giurfa et al., 2001; Zhang et al., 2005), but also found that the performance of the caffeine-treated bees was slightly, but significantly better than that of the controls.

Caffeine might play a direct role in the improvement of the DMTS task in adult foragers by increasing the level of alertness or cognitive arousal, as has been shown to occur in humans (Herz, 1999; Brice and Smith, 2001; Ryan et al., 2002). At another level, the improved learning seen in the caffeine-treated bees might be a result of increased motivation brought about by the drug. The administration of caffeine through food has been shown to produce heightened activity both inside and outside the hive in honeybees, and enhanced mobility, sensitivity to external (acoustic) stimuli and phototropism in hornets (Ishay and Paniry, 1979). A significantly higher frequency of visits to the experimental apparatus in our study is likely to result in more reinforcements leading to enhancements in the encoding of new information. Equalizing the number of visits by both caffeine-treated and control bees only had the effect of improving the performance of the former. As training (and hence data collection) was carried out over a period of 2 days, the above result suggests that the caffeine administered prior to training was only effective during the first half of the experiment. Performance would have declined on the second day, as the effects of the drug wore off. We conclude from these results that caffeine, in addition to increasing motivation in foraging honeybees, is also able to significantly improve performance in a DMTS task. This is reminiscent of human studies showing that caffeine improves encoding of new information and counteracts the fatigue that develops over the test session (Smith et al., 1999).

The DMTS paradigm (requiring the learning of the 'matching rule', as well as temporary storage of the initial stimulus in short-term memory at each trial) is a much more challenging task, and therefore not directly comparable to the Y-maze paradigm. The nature of the DMTS task, however, would more likely allow any increase in alertness and cognitive arousal, brought about by caffeine, to lead to an improvement in performance. The Y-maze experiment in the present study showed that the acquisition of a visual association task is not affected by caffeine administration. Control bees in the Y-maze experiment showed a large, significant decline in the long-term retention of visual associative memory, while the caffeine-treated bees kept performing extremely well (~90% correct), even 8 days after training had ended.

This closely matches the results of a rat-based study that also found 48-h memory retention, but not memory acquisition to be positively affected by caffeine (Angelucci et al., 2002). Studies on the effects of caffeine in humans have also reported enhancements in long-term memory, although this effect is sometimes restricted to certain age groups (Hameleers et al., 2000; Schmitt et al., 2003).

The mechanism by which caffeine causes a stimulant effect in vertebrates is gradually being revealed: caffeine blocks adenosine A_{2A} receptors in the brain (Fredholm et al., 1999) and inactivates certain enzymes, such as protein kinase A and protein phosphatase 2A (Lindskog et al., 2002; Vaugeois, 2002). At the neurotransmitter level, a number of systems may be affected, including dopaminergic and cholinergic transmission (Schwarzschild et al., 2002). Unfortunately, the neurological basis of the resulting cognitive effects is as yet poorly understood. The cAMP-dependent transcription factor CREB is essential for the conversion of short-term memory to long-term memory in flies (Tully et al., 1994) and mice (Bourtchuladze et al., 1994). Recently, three novel compounds, thought to be caffeine analogues, have been shown to enhance the activity of CREB in vitro (Scott et al., 2002).

While the effects of caffeine on the honeybee nervous system remain to be investigated, it is certainly possible that, at the molecular level, caffeine in the honeybee acts in a manner similar to that in mammals. In addition to the above-mentioned effects on the dopaminergic system, caffeine has been shown in vertebrates to stimulate synapsin I and protein III phosphorylation and GABA release (Walaas et al., 1989), neuronal branching and the growth of dendritic spines (Korkotian and Segal, 1999) and, at larger doses, to lead to calcium mobilization and the inhibition of phosphodiesterase (Lorist and Tops, 2003). These effects closely echo the changes induced in the honeybee brain by caffeine (Kucharski and Maleszka, 2005). Such effects, both on gene expression and on physiology might explain the behavioural phenomena observed in the present study, including the apparent accelerated development, arousal and enhancement in learning ability. Further molecular, biochemical and behavioural investigations are needed to make clear the precise mechanisms by which caffeine causes marked changes in honeybee behaviour.

In conclusion, the cognition-and activity-modulating effects of caffeine in the honeybee suggest that this drug can be used as a powerful tool to investigate general principles for the organization of behaviour in this species. Additionally, the remarkable similarity in behavioral effects of caffeine between a simple invertebrate and complex mammals suggests that non-

invasive drug treatments that modify behaviors in an easily manipulable insect system can be explored to advance our understanding of the complexity of human behavior.

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