Enhanced cholinergic transmission promotes recall in honeybees[☆,☆☆]

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Abstract

The involvement of the cholinergic system in learning and memory in honeybees has been well established using olfactory conditioning. We examined the effect of Methyl Parathion (MeP), an acetylcholinesterase inhibitor of the organophosphate family, on the learning and recall of visual and olfactory discrimination tasks in honeybees. One of our expectations was to observe the effects induced by both the nicotinic and muscarinic systems, as the blocking of acetylcholinesterase should induce an increase in the activity of both systems. We were also interested in knowing whether the type of tasks could influence the results. The visual tasks involved learning to discriminate the orientation of gratings in a Y-maze; the olfactory task involved learning to discriminate odours in a proboscis extension reflex (PER) paradigm. The results indicate that MeP treatment enhances recall of learned tasks in the visual and olfactory domains, but it does not affect the acquisition phase in either domain. Surprisingly, MeP treatment led to muscarinic-like effects but failed to mimic the nicotinic-like effects already described in relation to learning phases in honeybees. Implications for the role of cholinergic pathways in learning and memory and the nature of their involvement are discussed, and an hypothesis relating to the organisation of the cholinergic system and the relationship between the nicotinic and muscarinic systems in honeybees is proposed. The results are also discussed in terms of their ecotoxicological consequences.

Keywords: Cholinergic system, organophosphates, *Apis mellifera*, discrimination learning, associative learning, nicotinic, muscarinic, pesticide, non-target species, anticholinesterase, ecotoxicology

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1. Introduction

The mechanisms underlying learning and memory have long interested researchers, and there has been considerable interest in unraveling the participation of a number of different neuromessenger systems in these processes (e.g. for review in the honeybee see Bicker (1999); Weinberger (2006); Farooqui (2007)).

In the mammalian cholinergic system, the nicotinic (Levin and Simon, 1998) and muscarinic (Fibiger et al., 1991) pathways have been linked to learning and memory. In mammals, for example, treatment with nicotinic agonists are known to improve performance on a variety of memory tasks, whereas nicotinic antagonists can impair memory function (Levin and Simon, 1998). In the honeybee, a popular invertebrate model in the study of learning and memory, work over the past ten years has demonstrated the involvement of the cholinergic and aminergic systems in learning and memory (Lambin et al., 2001; Cano Lozano et al., 2001; Gauthier et al., 1994; Cano Lozano et al., 1996; Cano Lozano and Gauthier, 1998; Hammer and Menzel, 1998; Guez et al., 2001, 2003; Gauthier et al., 2006). Most of these studies have used the well-known olfactory conditioning paradigm of the Proboscis Extension Reflex (PER) (Kuwabara, 1957) or its habituation (Braun and Bicker, 1992). Braun and Bicker (1992) used this paradigm to show that eserine, an acetylcholinesterase blocker, significantly altered the performance of bees in the habituation of the proboscis extension reflex (PER) by increasing the number of trials before the onset of habituation.

It has also been shown that nicotinic agonists such as Imidacloprid (a neonicotinoid) have a significant impact on the habituation of the PER in young bees (Guez et al., 2001, 2003). In 7 day-old bees, treatments with Imidacloprid led to an increase in the number of trials before habituation. In comparison with 8 day-old bees, the same treatments led to a decrease in the number of trials before habituation 15 minutes and 1 hour after treatment, and an increase in the number of trials 4 hours after treatment. In the case of older bees (of foraging age), Imidacloprid treatments led to a decrease in the number of trials before habituation 1 hour after treatment (Lambin et al., 2001). In addition, evidence obtained by using antagonists of the nicotinic system (Cano Lozano et al., 1996, 2001) and the muscarinic system (Cano Lozano and Gauthier, 1998; Cano Lozano et al., 2001) suggests the participation of the nicotinic system in acquisition and retrieval. Treatments with nicotinic antagonists compromise acquisition as well as recall (Cano Lozano et al., 1996, 2001). On the other hand, treatments with muscarinic antagonists compromise only the recall process, suggesting that the muscarinic system is involved solely in the recall process (Gauthier et al., 1994; Cano Lozano et al., 1996, 2001).

In the present study we investigated the role of the cholinergic system on learning and memory in the honeybee. We used Methyl Parathion (MeP), an insecticide of the organophosphate family, which is known to affect the insect cholinergic pathway by blocking acetylcholinesterase (*e.g.* Haynes, 1988; Fukuto, 1990). We examined the effect of MeP on acquisition and retention of learning by using two well-known associative learning paradigms. One paradigm involved training free flying bees to choose between two visual stimuli in a Y-maze by associating one of them with a food reward (revs. Wehner (1981); Srinivasan (1994)). The second paradigm involved the well-known olfactory conditioning of the PER (Kuwabara, 1957).

2. Materials and methods

2.1. Y-Maze experiments

All experiments were performed in the all weather bee flight facility at the Australian National University. Forager Bees (*Apis mellifera*) were first trained to enter the Y-Maze by visiting a feeder placed in the maze. The feeder was progressively placed further into the maze and its placement was alternated in both branches of the maze. The feeder was then placed in the reward box at the back of the apparatus (see Figure 1), and the bees were trained to enter the reward box in order to gain access to the feeder. The feeder position alternated in each reward box (grey targets were present at the front of each reward box and therefore could not be used to discriminate between the non-reward and the reward box). Getting the bees to enter the reward box was the longest part of the pretraining. Bees who were accessed the feeder at this stage were individually marked using non-toxic paint, and were trained to discriminate between two visual stimuli, each presented in one arm of a Y-Maze (Figure 1). The stimuli differed either in orientation (as in Figure 3) or in colour (as in Figure 6). In each experiment, one stimulus (termed the positive stimulus, +) was associated with a reward of sugar solution provided by a feeder placed in a box behind the stimulus. The other stimulus (termed the negative stimulus, −) carried no reward. Bees could access the reward by entering the box via a tube passing through the centre of the stimulus. During training, the positions of the two stimuli were regularly interchanged. This ensured that the bees learned to associate the reward with the positive stimulus, and not with a particular arm of the Y-maze.

Although we were using indoor facilities, the bees were also given access to the outside environment and so hive activity was still greatly influenced by outdoor weather conditions. However, it was not possible to run the control and treatment sets that were needed in order to account for hive and weather variations (etc) if bee were trained individually, so bees were trained simultaneously. For this reason, a training block was deemed to be complete when each stimulus had occupied each arm of the Y maze once and not after a specified number of trials for each individual bee. The total duration of the first training block was 30 minutes, with each stimulus remaining in a given arm for 15 minutes. The duration of each subsequent training block was 20 minutes, with each stimulus remaining in a given arm for 10 minutes. The choice performance of each marked bee was continuously monitored, starting from the commencement of the training. The performance was scored by recording the first choice of each bee after it entered the Y-maze. If a bee entered the arm containing the positive stimulus, it was regarded as a correct choice (such a visit invariably resulted in the entry of the reward box and reinforcement). An entry into the arm containing the negative stimulus was regarded as an incorrect choice. The choice frequency in favour of the positive stimulus was calculated separately for each block as $\frac{n_1}{n_1+n_2} \times 100$, where n_1 and n_2 denote the number of correct and incorrect choices, respectively. Control tests, carried out by temporarily removing the feeder from its usual location behind the positive stimulus, assured us that the bees were not choosing the correct stimulus on the basis of pheromone scents deposited on the feeder. Further details of training and testing procedures for Y-mazes are given in van Hateren et al. (1990).

Each training/testing experiment was carried out at least three times using a fresh set of bees each time. The figures show the choice frequencies in favour of the positive stimulus for each training block, obtained by pooling choices across bees and across repeated experiments.

2.1.1. Treatment

Bees were treated by topical application of 1 μl of MeP (Sigma) dissolved in DMSO (50 mg/l). The DMSO (solvent) functioned as a transport medium for the drug by facilitating the transfer of the drug through the insect cuticle. The drug was applied on the dorsal side of the thorax while the bees visited and drank at the feeder during a ten minute period prior to acquisition (Figure 3), prior to reacquisition (Figure 4 and 5) or prior to recall (Figure 6). Since preliminary experiments and previously published works showed that DMSO did not elicit any observable behavioural effects in free flying conditions (Guez et al., 2005), and given the experimental constraints that precluded the simultaneous use of a large number of bees in order to follow them accurately, controls with DMSO were not included. Pure DMSO has also been shown to have no effect on other aspects of honeybee behaviour, such as habituation (Guez et al., 2001, 2003). The only claim of an effect of DMSO on honeybee behaviour can be found in Lambin et al. (2001) on habituation, although this effect was obtained only when DMSO was mixed with a saline solution and not on its own (see also Guez et al. (2001, 2003).

Our choice of using topical application over injection was based upon two factors. Firstly, we were using honeybees in free flying condition which made the use of injections unpractical. As noted by Barron et al. (2007), topical application is technically easier and much less stressful than injection for the animal as it does not involve anaesthetic treatments. Secondly, injection and anaesthesia treatment in insects are known to cause an immune response and/or behavioural changes (Barron, 2000; Mallon et al., 2003; Pankiw and Page, 2003). Mallon et al. (2003) have also showed that an induced immune response in honeybee inhibited associative learning. Because we were studying the effect of MeP on associative learning in honeybees, treatment by injection was therefore ruled out and topical application was preferred (this argument was also valid for our olfactory conditioning experiments).

The dose of MeP used was chosen in function of the LD50 of MeP (topical applications) in summer bees (290 ng/bee, Dr. Jerry Bromenshenk, personal communication), and the results obtained in a previous experiment (Guez et al., 2005). In this previous work we observed complex time dependent effects for our lowest dose of MeP namely 10 ng/bee (29 time less than the LD50), compared to our highest dose of 50 ng/bee (6 time less than the LD50). The time variation of effects observed at the two doses was attributed to the fact that the critical concentration at the site of action was reached at different times after the application of the pesticide. To minimise this factor in the present experiments the 50 ng/bee dose was chosen. No mortality was observed at these doses for the duration of any of these experiments (present and past).

2.1.2. Statistical analysis

χ^2 tests were performed using the Systat 10 statistical package from SPSS Inc., to check for significant differences in choice frequency between the treated and control

groups. This was done separately for each training block. p values lower than 0.05 were considered as significant.

2.2. Olfactory conditioning using the proboscis extension reflex

Forager bees (*Apis mellifera*) were collected at a feeder and stored overnight in a wooden frame box (200 mm x 200 mm x 50 mm), covered with mesh. The queen was not included. Fresh honey was provided through 14 ml Falcon tubes with small holes pierced in the base. The tubes were introduced through two holes on the top of the mesh box. Each box, containing around 80 individuals, was kept overnight in an incubator at 31°C and 80% humidity.

On the following day the bees were immobilised on ice and mounted in thin-walled aluminium tubes (7 mm inner diameter) using a thin strip of fabric-reinforced tape (GAFFA) with the thorax exposed. After mounting, the bees were fed to satiation with 1 M sugar solution, and then placed along rows in a Perspex holder and kept overnight at room temperature (21°C).

Bees were trained to distinguish between a positive (reward-bearing, sucrose solution) odour and a negative (non reward-bearing, saturated NaCl solution) odour. The training procedure was as in Maleszka et al. (2000), but performed in one trial (see below). The training procedure was therefore a modified version of the well-known olfactory conditioning protocol of the PER, which usually involves training to a single odour (Kuwabara, 1957; Bitterman et al., 1983) and the 3 trials procedure proposed by Maleszka with a 'positive' and a 'negative' odour (Maleszka et al., 2000).

The reasons for using this modified procedure were two-fold. Firstly, using a one trial procedure allowed us two distinguish between acquisition effects, consolidation effects and recall effects of the treatment as the time course of the memory trace is well known (Erber et al., 1980; Muller, 1996; Menzel, 1999) in the bee for this type of conditioning (see Figure 2). Secondly, it is common using the classical 1-trial 1 'positive' odour procedure to obtain a 90% result for correct choices after 1 trial learning. Because the observed effect in visual discrimination was what appeared to be an improvement of recall, we needed the olfactory task to be harder in order to be likely to detect an effect. If we were observing a 90% or more correct choice in the control after 1-trial learning we would be unlikely to be able to detect any improvement generated by the treatment. Using a 2-odour discrimination task in 1-trial learning significantly increased the difficulty of our task enabling us to detect both improvement or deterioration of the performances.

The positive odour was natural lemon essence ($4\,\mu l/ml$), dissolved in 1 M sucrose solution, which constituted the reward. The negative odour was natural vanilla essence ($4\,\mu l/ml$), dissolved in saturated NaCl solution, which constituted the non reward solution (Maleszka et al., 2000). The training protocol was as follows. A drop of the positive odour/reward solution or negative odour/salt solution, emerging from the tip of a syringe needle, was waved in front of the antennae for 5 seconds (without contact). At this time bees that extended their proboscis spontaneously were recorded for both odours. This constituted the pre-training response (see Table 1). The antenna was then touched with the solution and the drop was provided for the bee to taste. This training was carried out only once, i.e. the bees were subjected to 1-trial learning.

Testing was always carried out 1 hour after the training to ensure it was well after the consolidation of the memory trace (see Figure 2). A test consisted of waving a drop of the positive odour solution and a drop of the negative odour solution in front of the antennae. Each drop was then presented, in turn, for 5 seconds. The order of the presentation (positive odour followed by negative odour, or vice versa) was randomised for each test and each bee was tested only once. The test stimuli carried no sugar reward or salt detriment, but possessed the same odour concentration as the training stimuli. At the end of each test (after presentation of both odours), the persistence of the reflex was checked by touching the bee antennae with a 1M sucrose solution. Data from bees that failed to respond to this stimulus with a proboscis extension were disregarded. We had expected that a failure to exhibit of the PER at this stage may have been the expression of a physiological fatigue that precluded the expression of learning, but this case did not present itself during the course of the experiment.

The test results were scored as follows. (i) If the positive odour elicited a proboscis extension, but did not with the negative odour, the test trial was regarded as having yielded a correct response. (ii) If both odours elicited a proboscis extension, the response was regarded as incorrect. (iii) If the negative odour elicited a proboscis extension but there was none with the positive odour, the response was regarded as incorrect (this response rarely occurred). (iv) If neither odour elicited a proboscis extension, the response was discounted. (Although this last case could be indicative of a memory/learning problem, the absence of a proboscis extension to either odour could also have been due to lack of olfactory sensitivity. Because the two possible cases could not be disambiguated it would have been inappropriate to label either of these as correct or incorrect responses and so they were omitted.) The frequency of correct responses in the tests was calculated as $\frac{n_i}{n_i+n_{ii}+n_{iii}} \times 100$, where n_i, n_{ii} and n_{iii} denote the number of responses in categories (i), (ii) and (iii), respectively.

As for before, each training/testing experiment was carried out at least three times, using a fresh set of bees each time. Training and testing were carried out with the experimental bee placed in front of a suction fan to extract the odours and thus prevent the air in the experimental area from becoming saturated with them.

2.2.1. Treatment

We used MeP (Sigma) in DMSO solution. Bees were treated by topical application of $1\,\mu l$ of DMSO alone or MeP in DMSO on the dorsal side of the thorax for a total dose of MeP of $50\,ng/bee$. The control bees were treatment free. Depending upon the particular experiment, the treatment was applied 10 minutes before the learning trial (acquisition), 5 minutes after the learning trial (consolidation), or 1 hour after the learning trial and 15 minutes prior to the test (post consolidation, recall), as shown in Figure 2. In all cases the bees were tested for odour discrimination 1 hour after training (see Figure 2).

2.2.2. Statistical analysis

The Systat 10 package from SPSS Inc. was used for data analysis. Data sets were analysed using a Kruskal-Wallis non-parametric test followed by a Mann-Whitney test for pairwise comparisons, where appropriate. Results were expressed by pooling the

data across at least three independent experiments. In all cases p values less than 0.05 were considered significant.

3. Results

In our investigation the dosage of MeP was chosen to be $50\,ng/bee$. This dosage was based on preliminary experiments which indicated that this concentration had subtle effects on memory mechanisms, without disrupting motor activity or the survival of treated individuals as during our past and present experiment no mortality was ever recorded using this dosage. (The LD 50 of MeP is ca. $0.29\,\mu g/bee$; Dr. Jerry Bromenshenk, personal communication.)

We began by asking whether MeP influenced the rate at which bees learn a visual discrimination task. Two groups of bees were trained in a Y-maze to distinguish between gratings that were oriented at 135°and 45°to the horizontal, by associating a food reward with the 135°orientation (Figure 3). One group was treated with MeP, 10 minutes prior to commencement of the training. The other group received no treatment and constituted a control. (For details of the treatment, training and test procedures, see "Materials and Methods".)

Both groups learned the task well. After 4 training blocks, the treated group as well as the control group chose the correct stimulus with a frequency of ca. 90% (Figure 3). The learning curves displayed by the two groups were statistically indistinguishable ($p > 0.05$ at each point). In each case, the rate of learning was similar to those reported in earlier studies of orientation discrimination in bees (Wehner, 1971; Lehrer et al., 1985). Thus we concluded that MeP, applied 10 minutes prior to training, does not affect the acquisition of a task.

Next, we asked whether MeP affected the recall of a learned task. To address this question, a group of bees were trained on the grating discrimination task for one day (Figure 4). Training was then discontinued for two subsequent days, but the bees were encouraged to continue to visit a feeder placed in front of the (now closed) entrance to the Y-maze. During this time, the bees did not receive any further training in discriminating between the two stimuli, because they did not view either stimulus when they visited the feeder. On Day 4, the Y-maze was reopened and the bees were trained on the same task again. One subgroup of these bees, however, was treated with MeP 10 minutes prior to the commencement of re-training. The other subgroup received no treatment and served as a control. (The bees were randomly assigned to either of these groups.) Both subgroups showed a reduction in performance at the beginning of Day 4, presumably because of some forgetting that had occurred over the past two days (Figure 4) or because of the 'Kamin effect' (Kamin, 1957), whereby the performance of an animal is reduced when tested at an intermediate duration. (That is, this effect is not observed if the test is either performed immediately or a long time after the learned task (e.g. see Klein and Spear (1973)). However, the MeP treated bees displayed significantly better performance at the beginning of Day 4, compared to the control bees ($p < 0.05$ for the corresponding data points in Block 5). This suggests that MeP enhances the recall of a previously learned discrimination task. Later on Day 4 both the treated and control groups showed a similar performance ($p > 0.05$ for all

blocks beyond Block 5). Evidently, by Block 6, the control group had caught up with the treatment group.

This finding, taken together with that of Figure 2, suggests that MeP facilitates recall of a previously acquired task but does not affect the process of acquisition or re-acquisition *per se*.

The third question we asked involved specificity. Does MeP only enhance the recall of cues that are very specific to a learned task, or does it also improve the bees reacquisition performance in a more general way? For example, can MeP facilitate recall of the general layout of the maze, and the general nature of the task that has to be performed in it, namely, a dual stimulus, forced-choice discrimination? We investigated this question by carrying out the experiment illustrated in Figure 5. On Day 1, bees were trained to discriminate grating orientation by associating a reward with the 135°grating. On Day 2, the same bees were trained on a different task, namely, discriminating between the blue and the yellow target. Before commencing the new training, the bees were randomly divided into two groups. One group was treated with MeP 10 minutes prior to the commencement of the training, while the other group served as a control. There was no significant difference in performance between the control and the treated groups during Day 2. In this experiment, the training stimuli used on Day 2 were entirely different from those used on Day 1, the only common information being the contextual cues provided by the maze. The absence of MeP-induced improvement in performance on Day 2 ruled out the possibility of performance improvement through an enhanced recall of contextual cues. Rather, the enhancement induced by MeP treatment in the previous experiment (Figure 4) was likely to have been task-specific.

Under what conditions is MeP most effective at eliciting recall? To examine this, we conducted a third experiment in which a group of bees were trained for two days (as before) on the orientation discrimination task. The trained bees were then divided into two subgroups. Each subgroup was tested once on Day 5 (i.e., 3 days after the termination of the training phase) and once on Day 7 (i.e., 5 days post-training). One subgroup was treated with MeP 10 minutes before each test, whilst the other subgroup received no treatment and served as a control. Between tests, the trained bees visited a neutral feeder, as described above.

The results (Figure 6) reveal that the MeP treated bees showed a significantly better performance than control bees when tested 3 days post training, but not at 5 days post-training. Thus, it appeared that MeP facilities recall when it is administered relatively soon after the learning phase (when the retention of the task is relatively high), but not when the retention has decayed to a lower level.

Finally, we asked whether MeP plays a role in the learning and retention of non-visual tasks. We chose an olfactory discrimination task and used the conditioning of the PER as the experimental paradigm. The advantage of using this paradigm is that it enables precise control of the instant at which reinforcements are provided and tests are made. Thus, one can easily examine the effects of administering MeP at various stages of the learning process – acquisition, consolidation, and recall (see Figure 1). We used a one-trial training procedure in which captive bees were trained to extend their proboscis in anticipation of a reward of sugar solution when they received a scent of lemon, and not to extend the proboscis when they received a scent of vanilla (during training vanilla was paired with a saturated solution of sodium chloride). Details of the training

and test procedures are given in "Materials and Methods". Three groups of bees were used. One group served as an untreated control, the second group was treated with just the solvent DMSO that was used in conjunction with MeP (and therefore constituted a carrier control or DMSO control), and a third group received the MeP treatment. In separate experiments we examined the effect of treatment just prior to acquisition, during consolidation, and after consolidation. In each case, the trained bees were tested one hour after the conditioning procedure. The timing of the treatments and tests are shown in Figure 1. In the tests, we measured the frequency of correct proboscis responses (extension in response to the previously rewarded scent and non-extension to the previously non-rewarded scent). The results of these experiments are shown in Table 1 and Figure 7. Table 1 compares correct responses recorded spontaneously before conditioning with the correct responses recorded after condition at test. The results presented confirm that learning took place, i.e. the performance of bees improved with training as the frequencies of correct responses after training were significantly higher than the level of spontaneous responses recorded for each group prior to the training.

MeP had no effect on the performance in the test when it was applied just prior to acquisition or during consolidation. However, it had a strong and significant effect when it was applied after consolidation (Figure 7). This indicated that MeP does not affect the processes of acquisition or consolidation (Figure 7). Its only effect is on recall, and, as in the visual tasks, MeP enhanced the recall of the learned discrimination. Furthermore, the results show that MeP effects are short lived, probably due to detoxification processes (see for example Abel et al., 2004; Clark et al., 1986). In comparison the DMSO group exhibited an average of 33% correct responses and there were no significant differences between the test results, regardless of when the DMSO was applied. The tests on the control group showed no significant variation, as expected, and the performance of the control group was not significantly different from that of the DMSO group ($p > 0.05$ in all cases).

4. Discussion

Taken as a whole, our findings suggest that Methyl Parathion facilitates the recall of previously acquired rules (for a theory of learning based on the acquisition of rules see Guez (2009)). However, it does not affect the learning of these rules or their consolidation. The experiments examining the discrimination of grating orientation (Figures 3-6) reveal that MeP had no effect on the rate or level of acquisition, but had a clear effect on recall. Bees performed significantly better in a previously learned task when they were given MeP just prior to testing them on the task. Thus, MeP enhances memory recall and does this in a specific manner (compare Figure 4 and 5). Figure 6 suggests that this enhancement occurs only during the early intermediate phase of retention (up to 3 days after training), and disappears at 5 days post-training. Furthermore, Figures 4 and 6 suggest that treatment with MeP either specifically prevents the occurrence of the Kamin effect (Kamin, 1957) or greatly attenuates it. Since the Kamin effect has been associated with the failure to recall rather than the failure to consolidate, this provides further evidence of an effect on recall (Klein and Spear, 1973). Nevertheless, in these visual discrimination experiments the time course of the memory formation in honey-

bees is not known. The problem is further complicated since the time of acquisition cannot be pinpointed in the first place.

In order to address this issue we performed an olfactory discrimination task in 1-trial. The advantage of using such a task is that the time course of memory formation is well known using this preparation (Muller, 1996; Menzel, 1999). It was therefore possible to distinguish acquisition effects from consolidation or recall effects by variating the time of treatment if it had an effect which was limited in time (see Figure 2 for more details). The olfactory discrimination experiment described in Figure 7 again revealed that MeP plays a role primarily in the recall of learned odour discrimination. It does not affect either the acquisition or consolidation of memory. Thus, our experiments suggest that MeP plays a similar role in the recall of tasks in at least two different sensory modalities, and that the cholinergic mechanisms that underlie recall may be similar in the visual and olfactory domains.

Our results are broadly consistent with other studies that suggest an important role for cholinergic systems in the mediation of recall (Gauthier et al., 1994; Cano Lozano et al., 1996; Cano Lozano and Gauthier, 1998; Cano Lozano et al., 2001). These authors demonstrated a degradation of the recall process by using antagonists of the nicotinic (Cano Lozano et al., 2001) or muscarinic cholinergic pathways (e.g. Gauthier et al. (1994); Cano Lozano et al. (1996); Cano Lozano and Gauthier (1998); Cano Lozano et al. (2001). Our results complement these findings in that they demonstrate an improvement of the recall process when an inhibitor of acetylcholinesterase is used to enhance cholinergic transmission. Furthermore, Shapira et al. (2001) showed that Methionate, another organophosphate, improved performance in an olfactory associative learning paradigm. Although their experimental procedure could not strictly distinguish between the effects of Methionate on acquisition, consolidation and recall, their results strongly suggest an effect on recall.

Our findings shed some light on the nature of the participation of the cholinergic system in learning and memory. Over the past decade agonists and antagonists have been extensively used in an attempt to unravel the role of the cholinergic system in associative and non-associative learning (such as habituation) in the honeybee. A range of muscarinic and nicotinic antagonist have been successfully used to ascertain the role of the different cholinergic systems in the various phases of learning, namely, acquisition, consolidation and recall, using the PER paradigm (Cano Lozano et al., 1996; Cano Lozano and Gauthier, 1998; Cano Lozano et al., 2001). These studies suggest that the muscarinic system is primarily involved in the recall process, whereas the nicotinic system is involved in acquisition as well as recall. The evidence for this is that muscarinic antagonists disrupt only recall, whereas nicotinic antagonists disrupt acquisition as well as recall (Gauthier et al., 1994; Cano Lozano et al., 1996; Cano Lozano and Gauthier, 1998; Cano Lozano et al., 2001).

In the present study we used MeP, an organophosphate which blocks acetylcholinesterase, and consequently increases the level of cholinergic transmission. One might expect that an increase in the cholinergic transmission would produce an opposite effect to that generated by antagonists of the nicotinic and muscarinic system and hence it would enhance acquisition as well as recall. However, our results reveal that MeP improves only the recall process and it fails to improve the acquisition process expected from an increased nicotinic transmission. Thus, MeP mimics only the effect expected

of a muscarinic agonist, and not that of a nicotinic agonist.

We envisage two possible explanations for the discrepancies observed in our experiments, and in the published findings described above. One possibility is that, in the cholinergic pathways that underlie learning and memory, the muscarinic receptors outnumber the nicotinic ones. As a consequence, increased activation of the muscarinic receptor system could dominate the effects of an increase in cholinergic transmission, and swamp the relatively small and opposing influence of any increased activation of the nicotinic receptor system. However, this hypothesis is in contradiction with published data on receptor populations. For example, Trimmer (1995) indicated that, contrary to the situation in human brains, the insect brain features more nicotinic receptors than muscarinic ones. This observation has also been corroborated for honeybees by Huang and Knowles (1990).

Another possible explanation could be based on the possible existence of two functionally different types of muscarinic receptors in the insect nervous system reported by Trimmer (1995). One is an excitatory, postsynaptic receptor while the other is an inhibitory presynaptic muscarinic autoreceptor, which has been shown to down-regulate the nicotinic transmission in Locusts (Breer and Knipper, 1984) or in the giant interneurons of the cockroach terminal ganglion (Hue et al., 1989) . If the nicotinic system in honeybees is down-regulated by transmission along the muscarinic pathway, then treatment with an acetylcholinesterase blocker would only mimic the expected agonistic effect on the muscarinic pathway, with the agonistic effect on the nicotinic pathway being masked by its down-regulation by the muscarinic pathway at the presynaptic level.

Cano Lozano et al. (2001) have shown that micro injection of a nicotinic antagonist (mecamylamine) in the mushroom body calyces blocked acquisition of olfactory conditioning of the PER, whereas micro injection of both muscarinic (scopolamine) and nicotinic antagonist (mecamylamine) in the α lobes (vertical lobes) blocked recall. (Scopolamine injection in the calyces had no effect on acquisition or recall.) These results would suggest, if our previous hypothesis is correct, that our presynaptic muscarinic autoreceptors should be found in the mushroom body calyces. Nonetheless, one could also hypothesise that the absence of a MeP effect on acquisition is due to the fact that MeP has not yet reached the target site for acquisition (here the calyces), whereas it has already reached critical concentration in the structure responsible for recall (here the α lobes). This scenario is unlikely since the α lobe is further away from the MeP application point on the thorax than the mushroom body calyces (for 3D reconstruction of the honeybee brain see Haddad et al. (2004); Brandt et al. (2005)). Nevertheless, it is evident that further work using agonist and antagonists of nicotinic and muscarinic receptors and various methods of administration and timing is required to fully elucidate the role and relationship between these two cholinergic systems.

From an ecotoxicological point of view the results presented here are quite interesting. Although MeP (an organo-phosphate) is now banned in a lot of countries, insecticide such as carbamates (e.g. aldicarb, furadan and carbaryl) and other organophosphate (e.g. coumaphos, Malathion) are also acetylcholinesterase inhibitors. Thus, it is possible to consider that the exposure of honeybees to these pesticides may result in essentially the same sub-lethal effects on recall that were observed with the use of MeP.

If such was the case one can foresee two main consequences of honeybee exposure. Firstly, if bees have learnt to forage in a certain area and that this area has become contaminated, the bees are likely to persist visiting the contaminated area longer. They would therefore be more likely to come into contact with more pesticides and thus may accumulate lethal concentrations. Secondly, it is obviously important for the survival of the honeybee colony that the supply of food to the hive be maintained at an optimum. Therefore it is necessary for the foragers to be able to learn new food sources when old ones become depleted. If bees have become contaminated with acetylcholinesterase inhibitors it is more likely that they would persist in visiting the depleted food source for longer. This would be likely to induce sub-optimal foraging. Sub-optimal foraging may have various negative consequences for the hive in terms of nectar and pollen collection and the pollination of the plants visited may be less efficient as a result. Equally importantly, persisting with a particular food source may decrease the variety of food sources visited by the bees, which may represent a significant problem as it has been recently shown that a restricted variety in honeybee diet can be responsible for a decrease in their immune function (Alaux et al., 2010). Therefore, more studies evaluating and modelling the sub-lethal effects of these compounds on honeybees are necessary in order to evaluate their possible impact upon honeybee behaviour.

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Figure Legends

Figure 1. Plan view of Y-maze (transparent plexiglass top, sides and bottom). Each stimulus is presented in the vertical plane on the end wall of one arm. The feeder is placed in the reward box, R, behind the positive stimulus. A similar box, carrying no reward, is placed behind the negative stimulus.

Figure 2. Experimental protocol for training odour discrimination using the proboscis extension reflex.

Figure 3. Effect of Methyl Parathion on the acquisition of a visual orientation discrimination task, where the 135°grating is the positive stimulus. The results reveal that MeP has no effect on the acquisition of this task. The results represent data pooled from 3 experiments, using a total of 48 individually marked bees. In this figure and in Figures 2-7, the number against each data point represents the total number of choices analysed at that particular condition. The numbers of choices analysed under different conditions are represented by different fonts, as shown. The dashed line represents the level of random choice (50%).

Figure 4. Effect of Methyl Parathion on the reacquisition of a visual orientation discrimination task, where the 135°grating is the positive stimulus. The results show that MeP enhances reacquisition when administered at the beginning of Day 4. The results represent data pooled from 4 experiments, using a total of 77 individually marked bees.

Figure 5. Effect of Methyl Parathion on the acquisition of a visual colour discrimination task (yellow versus blue) where blue is the positive stimulus, after being trained on a visual orientation discrimination task. MeP does not enhance the acquisition of the new (colour discrimination) task. The results represent data pooled from 3 experiments, using a total of 62 individually marked bees.

Figure 6. Effect of Methyl Parathion on long-term memory recall after learning a visual orientation discrimination task. The results show that MeP enhances recall 3 days after training, but not after 5 days. The results represent data pooled from 4 experiments, using a total of 171 individually marked bees.

Figure 7. Effects of Methyl Parathion on performance in a one trial olfactory discrimination task, as a function of the time at which the treatment was applied (before acquisition, during consolidation, or post consolidation (recall)). For each case, the figure shows data pooled across at least 4 independent experiments. Significant differences are obtained only in the recall experiment, and are indicated by corresponding labels (a,b) over bars. Kruskal-Wallis statistical test =16.872, df=2, $p< 0.001$, and Mann-Whitney pairwise comparisons give a (U=5710.5, df=1), b (U=2676, df=1) $p< 0.001$ in each case.

Table 1. Olfactory discrimination task. Results of statistical tests comparing response levels (% correct responses) before and after training.