Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees

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Abstract

Pesticides are important agricultural tools often used in combination to avoid resistance in target pest species, but there is growing concern that their widespread use contributes to the decline of pollinator populations. Pollinators perform sophisticated behaviours while foraging that require them to learn and remember floral traits associated with food, but we know relatively little about the way that combined exposure to multiple pesticides affects neural function and behaviour. The experiments reported here show that prolonged exposure to field-realistic concentrations of the neonicotinoid, imidacloprid, and the organophosphate acetylcholinesterase inhibitor, coumaphos, and their combination impairs olfactory learning and memory formation in the honeybee. Using a method for classical conditioning of proboscis extension, honeybees were trained in either a massed or spaced conditioning protocol to examine how these pesticides affected performance during learning and short- and long-term memory tasks. We found that bees exposed to imidacloprid, coumaphos, or a combination of these compounds, were less likely to express conditioned proboscis extension towards an odor associated with reward. Bees exposed to imidacloprid were less likely to form a long-term memory, whereas bees exposed to coumaphos were only less likely to respond during the short-term memory test after massed conditioning. Both imidacloprid, coumaphos and a combination of the two compounds impaired the bees' ability to differentiate the conditioned odour from a novel odour during the memory test. Our results demonstrate that exposure to sublethal doses of combined cholinergic pesticides significantly impairs important behaviors involved in foraging, implying that pollinator population decline could be the result of a failure of neural function of bees exposed to pesticides in agricultural landscapes.

Keywords: pesticides, honeybees, long-term memory, pollinator decline, olfactory learn

Introduction

In the last 20 years, pesticide use has shifted away from organophosphates and carbamates towards neonicotinoid compounds that are agonists of insect nicotinic acetylcholine receptors (Buckingham et al., 1997; Elbert et al., 2008; Ihara et al., 2006). Unfortunately, because they are systemic insecticides that persist in plants throughout the growing season, they affect non-target organisms such as pollinators. For example, pollen and nectar that are collected and eaten by pollinators often contains these pesticides, even when the plant was only exposed to neonicotinoids as a seed treatment (Halm et al., 2006; Rortais et al., 2005). The extent to which neonicotinoids are implicated in pollinator population decline, however, is controversial (Maxim and van der Sluijs, 2010); some pollinators, such as honeybees, also experience stress from infestation with parasites and pathogens such as *Varroa destructor* and *Nosema* spp. (Dainat et al., 2011; Le Conte et al., 2010).

Neonicotinoids often affect non-target organisms through prolonged sub-lethal exposure (Halm et al., 2006) and may have even larger effects on survival when combined with exposure to other agrochemicals (Wu et al., 2011) or other forms of stress. Honeybees are likely to be exposed to additional potentially harmful chemicals during treatment for the mite, *Varroa destructor*. For example, mite treatments are often themselves potent pesticides like the organophosphate, coumaphos (Mullin et al., 2010; Rosenkranz et al., 2010). This particular combination is of interest due to the potential for additive effects when both compounds are administered simultaneously, as both neonicotinoids and coumaphos target cholinergic signalling. The target of neonicotinoid pesticides, nicotinic acetylcholine receptors (nAChRs), play an important role in honey bee learning and memory processes vital to successful foraging behaviour (Gauthier, 2010). Both acute and chronic administration of the neonicotinoid, imidacloprid, impairs olfactory learning and memory (Decourtye et al., 2004a; Decourtye et al., 2004b) probably as a result of a change in the way that neurons in the honeybee's mushroom bodies function (Gauthier, 2010). The organophosphate acetylcholinesterase (AChE) inhibitor, coumaphos (commercially known as Checkmite) is used as a miticide in honeybee colonies but could potentially harm bees as well as their parasites (Hawthorne and Dively, 2011). The combination of two pesticides could be more toxic and have stronger effects on behaviour than exposure to a single compound because the same mechanisms are used to detoxify both, notably the p-glycoprotein xenobiotic efflux transporters and the cytochrome P450 monooxygenase enzymes (Johnson et al., 2009). Whether or not prolonged exposure to imidacloprid or other pesticides and their combinations has a stronger effect on learning and memory in bees or other pollinators is unknown (Biernaskie et al., 2009).

Efficient foraging by bees depends on their ability to rapidly learn, remember, and communicate the identity and location of flowers offering nectar and pollen rewards (Biernaskie et al., 2009; Lihoreau et al., 2011). Substances such as cholinergic pesticides, could have a profound

influence on the bee's ability to forage successfully via their effects on learning and memory. A previous study of learning in bees demonstrated that bees subjected to spaced conditioning (intervals of 3 min or longer between trials) were more likely to form long-term olfactory memories than bees subjected to conditioning with shorter intervals (Menzel et al., 2001). In *Drosophila*, olfactory learning and memory acquired during spaced learning arises from different molecular mechanisms than that produced by massed conditioning (Isabel et al., 2004; Pagani et al., 2009). Whether or not cholinergic pesticides affect massed and spaced learning differently has not yet been tested.

Based on results from previous studies (Decourtye et al., 2004a; Decourtye et al., 2004b), we predicted that learning and memory would be impaired in honeybees subjected to prolonged exposure to sublethal doses of cholinergic pesticides and that the combination of substances that targetted cholinergic signalling would have a stronger effect than either substance alone. We used imidacloprid, a systemic neonicotinoid found in pollen and nectar and the mite treatment coumaphos, an organophosphate AChE inhibitor that accumulates in hive wax and food stores treated with this compound (Mullin et al., 2010; Rortais et al., 2005). We identified a range of sub-lethal doses that were also relevant to field exposure levels (Decourtye et al., 2004b; Mullin et al., 2010; Rortais et al., 2005). Using a classical conditioning assay for olfactory learning (Bitterman et al., 1983), we specifically compared performance during both massed and spaced learning assays with the aim of testing how disruption of cholinergic signalling affected performance during acquisition and during short-to-mid-term memory (STM) and early-long-term memory recall tests (LTM) (Menzel et al., 2001).

Methods

Honeybees

Foraging adult worker honeybee colonies (*Apis mellifera mellifera)* were originally obtained from stock of the National Bee Unit (York, UK) and maintained at Newcastle University. Bees were collected in plastic vials at the colony entrance and placed on ice; when they stopped moving, they were immediately transferred to small plastic boxes where they were treated with pesticides as described below.

Pesticides

Imidacloprid and coumaphos were obtained in dry powder form (>99% purity, Sigma-Aldrich). Solutions of imidacloprid, coumaphos, and a combination of the two drugs, were made to concentrations 1µM, 100 nM and 10 nM. Imidacloprid was directly dissolved in 1M sucrose solution; however, coumaphos was first dissolved in DMSO to make a stock solution with a concentration of

10 mM and then diluted with 1 M sucrose. We used a concentration 0.001% DMSO after pilot studies indicated that concentrations less than 0.1% did not influence olfactory learning and memory. Fresh solutions were prepared weekly from frozen aliquots of the stock solutions.

Exposure to pesticides

Exposure to pesticides prior to the behavioral experiments was accomplished by adding pesticides to 1 M sucrose solution and feeding it to adult workers *ad libitum* for 4 days prior to learning and memory experiments. Oral exposure was chosen to allow continuous, measurable exposure over 4 days; and although topical exposure to coumaphos may be more representative of its use as a mite treatment, both imidacloprid and coumaphos have been found in within hive food stores, making oral administration a field realistic exposure route (Mullin et al. 2010; Wu et al. 2011). After capture from the colony, cohorts of 20 honeybees were placed in plastic boxes (16.5cm x 11cm x 6.5cm) which had ventilation holes in the lid, and 4 holes in the sides to allow insertion of feeding tubes. Feeding tubes were made from 2 ml microfuge tubes with four ~2 mm holes drilled along one side to allow the bees to insert their mouthparts into the feeding solution. The solution in each feeding tube was replenished daily. Control bees were fed 1 M sucrose; pesticide treatment groups were fed 1 M sucrose containing imidacloprid, coumaphos, or a combination of the two (see information about concentrations above). The bees were retained in the feeding boxes for 3 days prior to experimentation. On the 4th day, the entire cohort in each box was cold anesthetized and each bee was transferred to a restraining harness as described in Wright et al. (2009). Each bee was allowed to recover for 1 h, fed 25 µl of the solution it experienced for the previous 3 days, and left in a humidified plastic box at room temperature overnight. For the 'reversal' experiments, bees were fed the combined 100 nM imidacloprid and 100 nM coumaphos solution for 3 days; but for an additional 3 days afterwards, bees were fed 1 M sucrose containing no pesticides, and were also fed 25 µl of uncontaminated sucrose solution after harnessing and training.

All treatments were administered to cohorts of 15-25 bees each week, and the surviving bees from all treatment groups were trained and tested in parallel. This process was repeated weekly until N ≥ 25 conditioned bees was reached for each treatment group. We ran these experiments in parallel in order to distribute the variation caused by environmental conditions or other factors across all experimental conditions equally.

Determining consumption rates and sub-lethal dosage

Preliminary experiments were performed to determine sub-lethal doses. The concentrations used were 10 nM, 100 nM and 1 µM. Bees were kept as described above, and the number of bees surviving each day recorded. Mortality rates were compared at the stage of the experiment where the bees had consumed pesticide solutions *ad libitum* for 3 days, and were to be harnessed ready for the

learning and memory experiments (Figure 1). On the basis of this, 100nM and 10nM doses of all treatments were found to be sub-lethal, and so were used in the subsequent learning and memory experiments (see results and Fig. 1 for details).

During this phase of the experiment, food consumption was measured by weighing the feeding tubes before and after the bees had fed for 24 h, and average consumption per bee per day calculated. There were no differences in daily food consumption between the control group and treatment groups fed 100 nM concentrations of the pesticide solutions (Kruskal-Wallis, $\chi_3^2 = 1.51$, $P = 0.680$). Mean consumption of sucrose syrup across all treatments was 143.95 (+/- 3.55) mg per bee per day.

Based on the amount of sucrose syrup consumed (3 days consuming ~144 mg per bee per day, plus 27.5 mg on 2 subsequent days, totaling 487 mg over the whole experiment), we estimated that each bee fed the 10 nM pesticide solution consumed ~1.3 ng of imidacloprid and/or 1.8 ng coumaphos over the 6-day experimental protocol. This amount of imidacloprid is within the range consumed by foraging bees feeding on imidacloprid contaminated nectar (Rortais et al., 2005). Bees fed the 100 nM pesticide solutions consumed imidacloprid at a concentration of 23.3 µg/kg of sucrose syrup, and coumaphos at a concentration of 33 µg/kg. This imidacloprid concentration is within the range of previously published studies (Decourtye et al., 2004b). The coumaphos concentration we used was 6-60 fold lower than that found in a previous study that measured coumaphos within colony stores (180 ppb, (Mullin et al., 2010).

Learning and memory experiments

Honeybees were trained using a procedure for olfactory conditioning of the proboscis extension reflex (Bitterman *et al*. 1983). The conditioned (CS) and unconditioned stimuli (US) were presented on a massed (30 s inter-trial interval) or a spaced schedule (10 min inter-trial interval) as described in Menzel et al. (2001). The conditioned stimulus was the odour, 1-hexanol, presented for 4 s duration, and the unconditioned stimulus was a reward of 0.2 µl of 1 M sucrose solution. The odour stimulus arose from a 3 µl aliquot applied to a strip of filter paper placed within a glass tube and attached to controlled air supply (the arena and training apparatus are previously described in Wright et al. (2008). Each subject received 6 conditioning trials. Bees that responded to the conditioned stimulus alone before training were excluded from conditioning. Bees that failed to respond to the odour during any of the six conditioning trials (even if they continued to exhibit PER in response to antennal stimulation) were defined as "non-responders;" these data were analyzed separately. After conditioning, each bee was tested with the conditioned stimulus and a novel odour (2-octanone) at 10 min and 24 h. The order of presentation of the test odours was randomized across subjects, and each test was presented with a 3-5 min interval between each test. The 10 min test was performed to assess short-term memory (STM) and the 24 h test was performed to test early long-term memory (eLTM) (Menzel et al., 2001). To measure memory, we compared the responses during the last acquisition

trial to those during both recall tests within each treatment group: STM was assessed in terms of whether the response to the CS at the 10 min memory test was significantly less than on the 6th training trial, and eLTM was assessed in terms of whether the response to the CS at the 24 h memory test was less than that at the STM test. Memory specificity measured by comparing the response to the CS with the response to the novel odour, during the 10 min and 24 h memory tests.(We did not test beyond 24 h because most of the pesticide-treated bees died within 72 h after harnessing.).

Statistical analysis

Consumption and mortality data was analyzed using a Kruskal-Wallis test. Comparisons between the proportion of 'responders' and 'non-responders' were analyzed using logistic regression. Data for bees that responded during conditioning were analyzed separately. The response of each subject to the odour stimulus during conditioning and testing was scored as a binary response (full proboscis extension or not) and analyzed using binary logistic regression (lreg) (Generalized Linear Model) in statistics program, SPSS. For logistic regression analysis of the acquisition data, the first training trial was excluded from the analysis to facilitate model fit (all responses at this point were 0). Mean values for the probability of response, and standard errors of the means, are reported for each treatment, dose, and odour presentation. Least squares post-hoc tests (lsc) were performed for pair-wise comparisons.

The specificity of olfactory memory was tested in our experiments by presenting both a novel odour and the CS. To compare the relative response rate of our subjects, we calculated a 'discrimination index' (DI), represented as:

$$D.I. = \frac{\text{(number of responses to the CS – number of responses to the novel odour)}}{\text{Total number of responses to test odours}}$$

Results

Identification of sub-lethal doses of imidacloprid and coumaphos

Preliminary experiments were performed using 3 different concentrations of each pesticide to identify sub-lethal concentrations for use in the learning and memory experiments (Figure 1). Unsurprisingly, all the compounds tested had some effect on mortality (lreg, imidacloprid, $\chi_3^2 = 25.5$, $P < 0.001$; coumaphos, $\chi_3^2 = 12.9$, $P = 0.005$; imidacloprid plus coumaphos, $\chi_3^2 = 25.6$, $P < 0.001$). However, by comparing the different doses of each compound, it was found that only the 1 µM concentration significantly increased mortality compared to the controls (imidacloprid, $P < 0.001$; coumaphos, $P = 0.004$; imidacloprid plus coumaphos, $P < 0.001$). The 10nM and 100nM concentrations of all treatments were found to cause no increase in mortality relative to the controls

(imidacloprid, 10nM $P = 0.607$, 100nM $P = 0.603$; coumaphos, 10nM $P = 0.814$, 100nM $P = 0.625$; imidacloprid plus coumaphos, 10nM $P = 0.680$, 100nM $P = 0.634$).

Learning performance is impaired when bees are exposed to imidacloprid and coumaphos

The proportion of non-responding bees in each treatment group with pesticides was compared (Table 1). Pesticide exposure increased the proportion of non-responding bees in both the massed and spaced conditioning assays (Table 1, imidacloprid: lreg: $\chi_2^2 = 6.10$, $P = 0.047$; coumaphos: lreg, $\chi_2^2 = 7.66$, $P = 0.022$; imidacloprid plus coumaphos: lreg: $\chi_3^2 = 12.7$, $P = 0.005$). For honeybees allowed to recover for 3 days after combined pesticide exposure, failure to respond during conditioning was not significantly different from the level exhibited by the control group during both types of conditioning assay (massed: lsc, $P = 0.220$, spaced: lsc, $P = 0.639$).

Prolonged exposure to imidocloprid and coumaphos reduces the rate of olfactory learning

In the population of bees that exhibited olfactory learning, we found that 100 nM doses of all compounds and their combinations affected the rate of olfactory learning in both massed and spaced conditioning. Each drug produced a slightly different effect on the acquisition curve in both learning assays (Figure 2, Table 2). Exposure to imidacloprid influenced the rate of learning for bees trained with both massed (Fig 2A, lreg, $\chi_2^2 = 16.8$, $P < 0.001$) and spaced (Fig 2D, lreg, $\chi_2^2 = 19.8$, $P < 0.001$) conditioning protocols: the rate of acquisition was slower, as exhibited by the lower probability of responding during the first 3 trials, and the population reached a lower asymptote (trials 4-6). Imidacloprid had a stronger effect on spaced conditioning than on massed conditioning: both doses reduced acquisition during spaced conditioning (10nM: lsc, $P = 0.004$; 100nM: lsc, $P < 0.001$), whereas only the 100 nM dose reduced the rate of learning during massed conditioning (lsc, $P < 0.001$).

Coumaphos also impaired learning during both massed (Fig. 2B, lreg, $\chi_2^2 = 11.3$, P = 0.003) and spaced conditioning (Fig. 2E, lreg, $\chi_2^2 = 14.7$, $P = 0.001$), but the effects on massed learning were greater than those seen during spaced learning. During massed conditioning, the effect on acquisition was strikingly different to that produced by imidacloprid (Fig. 1A). Initially, coumaphos treated bees responded as well as the control bees, but the number of animals responding to the CS began to decrease during the course of training, and by trial 6, significantly fewer animals responded to the CS (lsc, trial 6: 10nM: $P = 0.003$; 100nM: $P = 0.001$). This effect was not seen during the spaced conditioning protocol, which qualitatively resembled the curves produced by imidacloprid, where the rate of learning was slightly lower during the first 3 trials with the highest dose (lsc, $P < 0.001$).

The effect of combined exposure to imidacloprid and coumaphos on the rate of acquisition during learning resembled both the strong effect of coumaphos on massed learning (Fig 2C, lreg, $\chi_3^2 =$

18.3, $P < 0.001$) and the impact of imidacloprid on the rate of acquisition during spaced conditioning at the highest doses (Fig 2F, lreg, $\chi_3^2 = 30.9$, $P < 0.001$). However, both the 10nM and the 100nM treatment reduced the proportion of bees which responded on the 6th trial of massed learning (lsc, 10nM: $P = 0.016$; 100nM: $P < 0.001$) in a manner observed when bees were exposed to coumaphos alone. Bees that were fed the 'reversal' treatment did not perform differently from control animals during massed conditioning for most trials, but they also exhibited the decline on the last two trials of the bees subjected to the combined exposure. Their responses during spaced conditioning were not significantly different from the control (lsc, $P = 0.071$).

Exposure to imidacloprid impairs memory formation

We measured how exposure to the imidacloprid, coumaphos, and their combination influenced short/mid-term (STM) and early long-term (eLTM) memory by testing bees at 10 min and 24 h after conditioning (Fig. 3). The pesticides altered the way that bees responded during the STM and eLTM tests after both kinds of conditioning. Imidacloprid exposure impaired STM after massed but not spaced conditioning (Fig 3A and D, massed: lreg, $\chi_2^2 = 8.13$, $P = 0.017$; spaced: lreg, $\chi_2^2 = 4.44$, $P = 0.327$). However, it reduced eLTM after conditioning in both assays (massed: lreg, $\chi_2^2 = 6.54$, $P = 0.038$; spaced: lreg, $\chi_2^2 = 11.5$, $P = 0.003$). Prolonged coumaphos exposure also reduced the average rate of response of the massed conditioned bees on the 6th conditioning trial and during both of the recall tests (Fig. 3 B and E, lreg, $\chi_2^2 = 9.95$, $P = 0.007$).

While the responses of the bees subjected to prolonged coumaphos exposure were less than those of the control group, the average rate of response of these bees did not change from the 6th trial to the 10 min and 24 h tests (10 nM: lreg, $\chi_2^2 = 0.137$, $P = 0.934$; 100 nM: lreg, $\chi_2^2 = 1.14$, $P = 0.565$). This is especially apparent when the responses of the bees subjected to spaced conditioning were compared to the massed-conditioned bees: the responses of spaced conditioned bees during the recall test were unaffected by coumaphos exposure (lreg, $\chi_2^2 = 2.12$, $P = 0.344$).

Exposure to the combination of imidacloprid and coumaphos caused effects most similar to that of coumaphos after massed conditioning. Response rates were lower at all timepoints (lreg, $\chi_2^2 = 14.2$, $P = 0.001$) although no notable decrease in response rate equivalent to memory impairment was seen between the last acquisition trial and the memory tests (see Table 3). This effect was not reversed in bees which had been allowed to recover from combined pesticide exposure: response rates were still much lower than controls (lreg, $\chi_1^2 = 18.1$, $P < 0.001$).

After spaced conditioning, combined imidacloprid and coumaphos treatment also had an effect (lreg, $\chi_2^2 = 9.18$, $P = 0.010$), and in this case a true memory impairment was observed, with

response rates at the 10 minute memory test being lower than on the last acquisition trial ($P = 0.011$). This effect was reversed in bees allowed to recover from the pesticide treatment, which did not respond differently to the controls (lreg, $\chi_1^2 = 1.18$, $P = 0.277$).

Olfactory memory specificity is reduced after prolonged exposure to pesticides

To compare the responses of the bees during the tests for STM and eLTM to the responses to the novel odour, we calculated a 'discrimination index' that reflected the proportion from each treatment that responded to the CS in preference to the novel odour during each test (Fig. 4, Table 4; data for the comparison of the CS and the novel odour are in Figure A1). All pesticide treatments affected the specificity of the responses during the recall test. It is notable that 100nM imidacloprid treated bees were as likely to respond to the CS as the novel odour at 24 h (the discrimination index in this case was less than 0, Fig 4 A, D). Treatment with 100nM coumaphos was also detrimental to the specificity of the test response; less than 10% of the bees preferentially responded to the CS (Fig 4 B, E). For the combined pesticide treatment at the 100nM concentration, the bees retained some specificity in the test response at 10 min after conditioning; however, when tested 24 h later, they failed to respond preferentially to the CS, even though their response rates to the test odours were still relatively high (Fig 3 C, F).

Discussion

Combinations of sub-lethal doses of modern pesticides often produce additive or even synergistic effects on mortality and behaviour of animals (Laetz et al., 2009). In our experiments, we combined a neonicotinoid pesticide, imidacloprid, with an AChE inhibitor, coumaphos, to simulate the situation where honeybees are exposed to pesticides in food and miticides applied within the colony. We found that each of the cholinergic pesticides we examined had specific effects on learning and memory that were reflected in the responses of bees given the combination, and that these effects on learning were additive. Combined pesticide exposure also strongly reduced the specificity of the response during the 24 h test. The influence of the pesticides on memory, however, was more complex and depended on pesticide exposure. Furthermore, bees allowed to recover for 3 days after pesticide exposure exhibited performance during conditioning that indicated they were still affected by exposure, but their responses during testing were not different from the control.

Because cholinergic signalling plays a key role in olfactory learning and memory, it is reasonable to assume that impairment in cholinergic signalling caused by prolonged exposure to nAChR agonists or AChE inhibitors should also lead to deficits in acquisition and, therefore, memory formation. In this study, disruption of cholinergic pathways by chronic exposure to imidacloprid or

coumaphos affected performance during both massed and spaced learning. This may have been due to direct impairment of the neural circuits involved in olfaction or gustation, or to a disruption of the mechanisms of associative learning. Interestingly, in bees that could perform associative learning, prolonged exposure to imidacloprid, produced different effects on learning and memory to those produced by coumaphos, although both compounds target cholinergic signalling pathways. However, it is possible that the partial agonist imidacloprid could in fact decrease cholinergic signalling, by competing with the full agonist ACh for the receptor binding sites (Deglise et al., 2002); whereas coumaphos will increase ACh signalling initially, via both nicotinic and muscarinic receptors, until either receptor desensitisation of neuronal death occur (Fukuto, 1990; Pohanka, 2011). .

Although we observed a modest impairment in acquisition in coumaphos-treated bees during spaced conditioning, the main effect on acquisition was expressed as a decline in PER in the last three conditioning trials of massed conditioning. This precipitous decline during the last three trials was also observed in the honeybees that had been exposed to the combination of the pesticides. These animals continued to respond to the US, but rather slowly, and their head and proboscis shook in a way that suggested that perhaps some additional effects on motor function may also be involved; although it should be noted that this was never seen with coumaphos-treated animals during spaced training. Organophosphate pesticides are known to affect motor function in many different animals including flies, fish and rodents: observed effects include tremors, uncoordinated movement, and transient paralysis (Miller and Kennedy, 1972; Moser, 1995; Patil and David, 2010). We have also observed altered motor behaviour in coumaphos treated bees, including episodes of paralysis and decreased co-ordination (Williamson et al., 2013). Acute application of AChE inhibitors results in an acceleration of olfactory learning in honeybees, presumably because inactivation of the enzyme leads to a transient elevation of ACh during sensory stimulation (Guez et al., 2010). In our experiments, bees were continually exposed to low levels of an irreversible AChE inhibitor; this would result in an elevation of ACh in the synaptic cleft that would also lead to eventual desensitization of the over-stimulated cholinergic neurons rather than an increase in excitation (Chen, 2012; Hartmann et al., 2007). The fact that bees simply didn't respond during the last few trials of conditioning even though they were able to learn during the first few trials strongly suggests that the olfactory system and other circuits that rely on cholinergic signalling cannot cope with the high levels of ACh released during the rapid stimulation that occurs during massed conditioning. This would result in an inability of the neurons to detect and respond to the ACh produced by each episode of synaptic transmission resulting from olfactory stimulation, and hence, lead to a failure in expression of the learned behaviour. This effect is observed in humans that have been poisoned with AChE inhibitors; an accumulation of Ach in the synaptic cleft leads to paralysis and death (Pope et al., 2005).

Imidacloprid impaired LTM after both massed and spaced conditioning, whereas coumaphos did not influence LTM. The response rates during our test periods for the control subjects were very

high when compared to previous studies of massed and spaced conditioning in bees during memory recall tests (Menzel et al., 2001). This is likely due to the fact that the bees in our experiments were highly motivated as a result of the feeding regime. In light of this, the fact that we observe a drop in the response after imidacloprid exposure strongly suggests that it influences LTM consolidation. In contrast, the responses of the coumphos-treated bees indicated that they did not forget that the odour was associated with reward; but the response rate was equally high to the incorrect odour stimulus. Perhaps the most striking effect of prolonged imidacloprid and coumaphos exposure found in this study is the inability of treated bees to correctly select the conditioned odour rather than a novel odour during the memory tests. Both imidacloprid and coumaphos administered alone reduced the bees' ability to differentiate the olfactory stimuli during the tests, an effect which has previously been reported for coumaphos, but not for imidacloprid (Weick and Thorn, 2002). The combination of the two compounds impaired olfactory discrimination after massed training, but only the higher dose impaired discrimination after spaced training. This effect was neither additive nor synergistic, which is in contrast to the effects seen on acquisition, where a small additive effect on learning impairment was observed. It is not clear whether the impairment of olfactory discrimination was caused by a true deficit in learning and memory consolidation (ie. the bees did not learn the correct odour) or arose from a deficit in olfactory perception (ie. the bees could not detect which odour was correct). Cholinergic signalling plays a key role in the antennal lobes, where odour information from the antennae is initially processed, in addition to its importance in the mushroom bodies where olfactory information is integrated and learning and memory processes occur (Gauthier, 2010).

The contrasting effects of the two pesticides on memory and general responsiveness may be explained by the involvement of distinct sub-types of nAChRs in different aspects of the memory formation and retrieval process (Dacher et al., 2005; Gauthier et al., 1992). Previous studies using antagonists to block nAChR function have shown learning and memory impairments very similar to the ones we describe here (Gauthier et al., 2006; Lozano et al., 1996). Mecamylamine, a broad spectrum antagonist which blocks all nAChRs, impaired learning and responsiveness to the CS during and immediately after olfactory conditioning, but did not affect LTM (Lozano et al., 1996). This is reminiscent of the effects we report for coumaphos, which by raising ACh levels throughout the brain will also affect all nAChRs. In another study, □-bungarotoxin, a specific antagonist of a particular nAChR subtype, also impaired learning, but had much more dramatic effects on LTM (Gauthier et al., 2006). This effect is very similar to our observed effects of imidacloprid, and it is known that imidacloprid acts on □-bungarotoxin-sensitive receptors (Deglise et al., 2002; Jepson et al., 2006).

Our data clearly show that bees have difficulty performing simple learning and memory tasks when they have experience prolonged exposure to combinations of pesticides as adult foragers. Foraging for food is a demanding task that requires bees not only to learn, but also to optimise their foraging strategies by accurately learning and remembering which flowers offer the best rewards

(Biernaskie et al., 2009; Lihoreau et al., 2011). Comparisons of laboratory learning tests and foraging in the field suggest that learning ability is a good predictor of foraging ability at the colony level (Raine and Chittka, 2008). Our data, in combination with other studies that have revealed foraging and communication impairments in bees exposed to imidacloprid or other neonicotinoid pesticides (Eiri and Nieh, 2012; Henry et al., 2012; Whitehorn et al., 2012) implies that commonly used pesticides are a strong culprit for the observed declines in pollinator populations, and that the exposure to multiple pesticides simultaneously additively amplifies this effect on important behaviours.

List of symbols and abbreviations: ACh: acetylcholine; AChE: acetylcholinesterase; nAChR: nicotinic acetycholine receptor; CS: conditioned stimulus; STM: short-term memory; LTM: long-term memory; PER: proboscis extension response

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Figure legends

Figure 1. Survival data for bees fed different concentrations of pesticide treatments over a 3 day period prior to conditioning. (A) Imidacloprid, (B) Coumaphos, (C) Imidacloprid and coumaphos. For each treatment, $N \geq 3$ replicates using cohorts of $N \geq 15$ bees. For all treatments, the 1 μM concentration caused greater mortality than the control. The 100nM and 10nM concentrations did not increase mortality, so were selected for use in the subsequent learning and memory experiments.

Figure 2. Rates of acquisition during olfactory conditioning (massed conditioning A-C, spaced conditioning (D –F) are affected by pesticide treatments. (A) 100 nM imidacloprid and 10 nM and 100 nM coumaphos (B) reduced the rate of acquisition. (C) Imidacloprid and coumaphos had similar effects on acquisition to that produced by 100 nM coumaphos. Bees allowed to recover from the 100nM treatment did not differ from the control. (D) During spaced conditioning, (D) 10 nM and 100 nM imidacloprid and (E) 100nM coumaphos reduced the rate of acquisition. (F) The 100 nM dose of imidacloprid and coumaphos strongly reduced the rate of acquisition; bees allowed to recover from the 100nM treatment did not differ from the control. Sample sizes and pairwise comparison statistics for all treatments and doses are shown in Table 2. Note: the control group is the same in A-C and the same in D-F.

Figure 3. Pre-exposure with imidacloprid affects long-term memory formation. After massed conditioning, bees exposed to 100 nM imidacloprid (A) had poor performance during the short- and long-term memory tests. (B-C) Exposure to coumaphos and the mixture of imidacloprid and coumaphos did not influence recall. After spaced conditioning, imidacloprid (D) affected recall at 24 h but not at 10 min. (E-F) Exposure to coumaphos and the mixture of imidacloprid and coumaphos did not influence recall. Note: the control group is the same in A-C and the same in D-F.

Figure 4. Pesticides affect odour discrimination during olfactory recall tests. A discrimination index was calculated to measure when bees responded to the CS at a greater rate than a novel odour during the 10 min and 24 h recall tests. Values greater than '0' reflect a preference for the CS over the novel odour; negative values reflect a preference for the novel odour. (A-C) Bees tested after massed conditioning; (D-F) bees tested after spaced conditioning. Sample sizes and pairwise comparison statistics for all treatments and doses are shown in Table 4; recall response rates to the CS and the novel odour are reported in Figure A1.

Figure 1

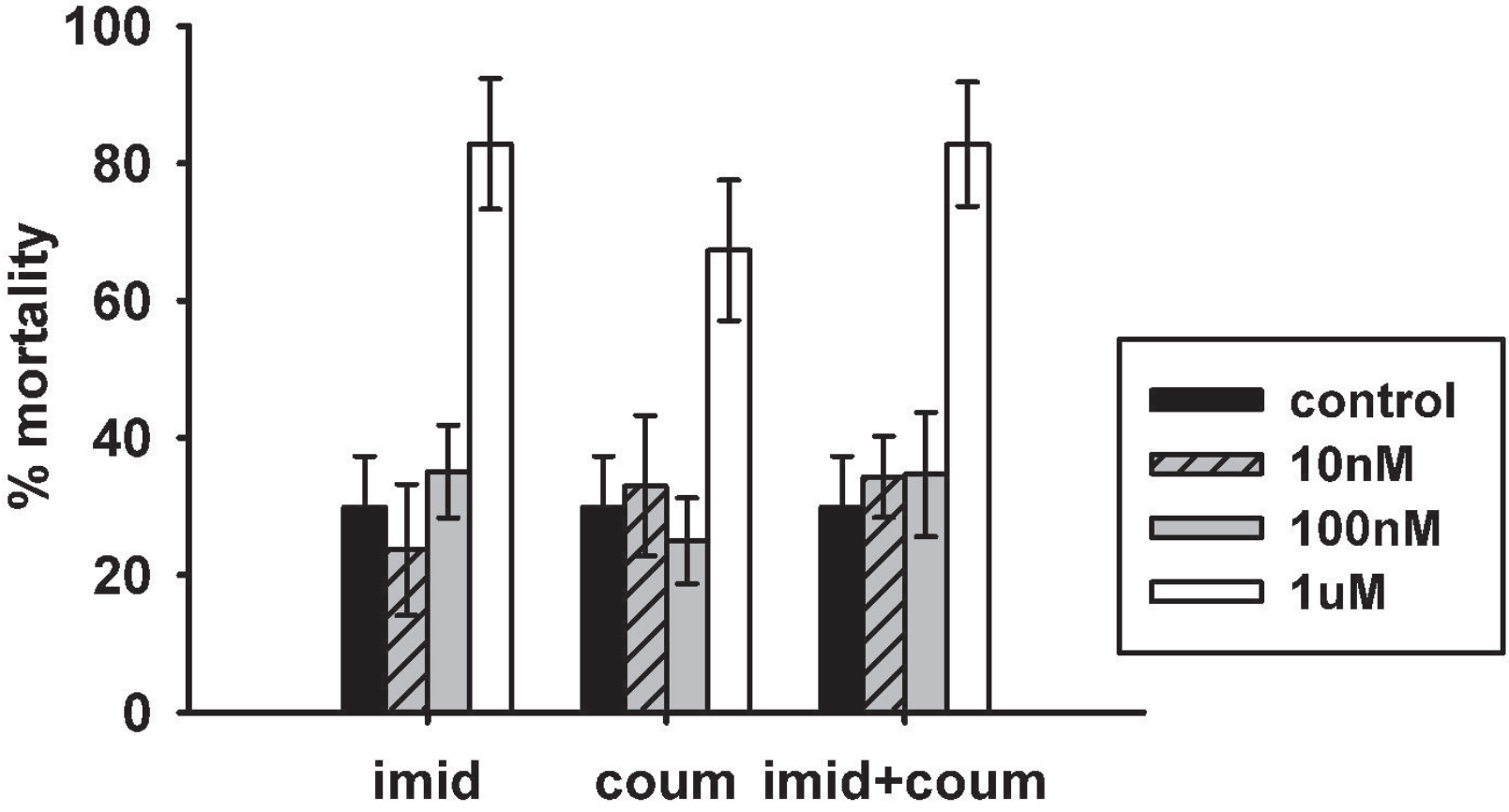
100
80
60
40
20
0
% mortality
imid
coum
imid+coum
control
10nM
100nM
1uM

Figure 2

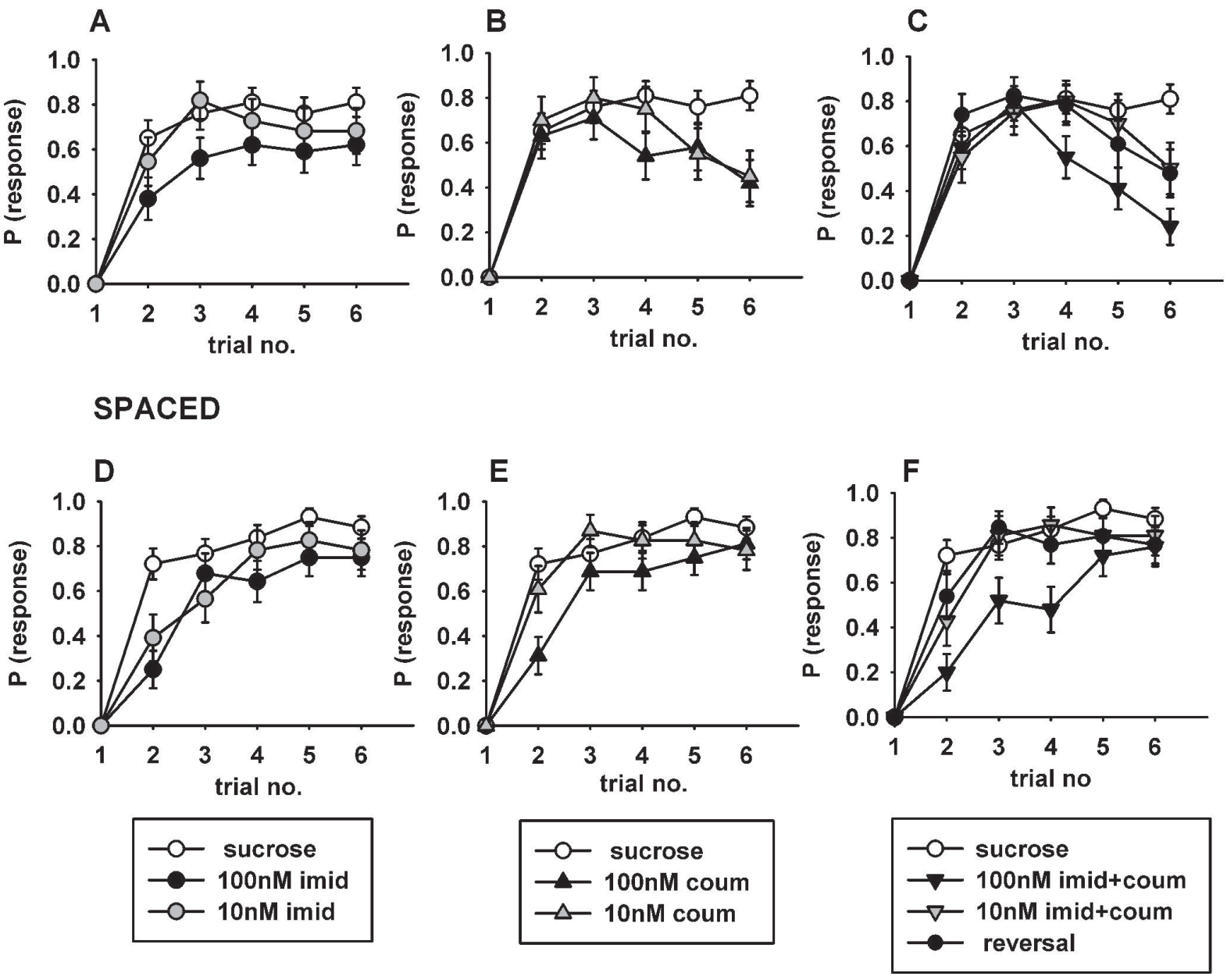
MASSED
A
B
C
P (response)
trial no.
SPACED
D
E
F
trial no
sucrose
100nM imid
10nM imid
100nM coum
10nM coum
100nM imid+coum
10nM imid+coum
reversal

Figure 3

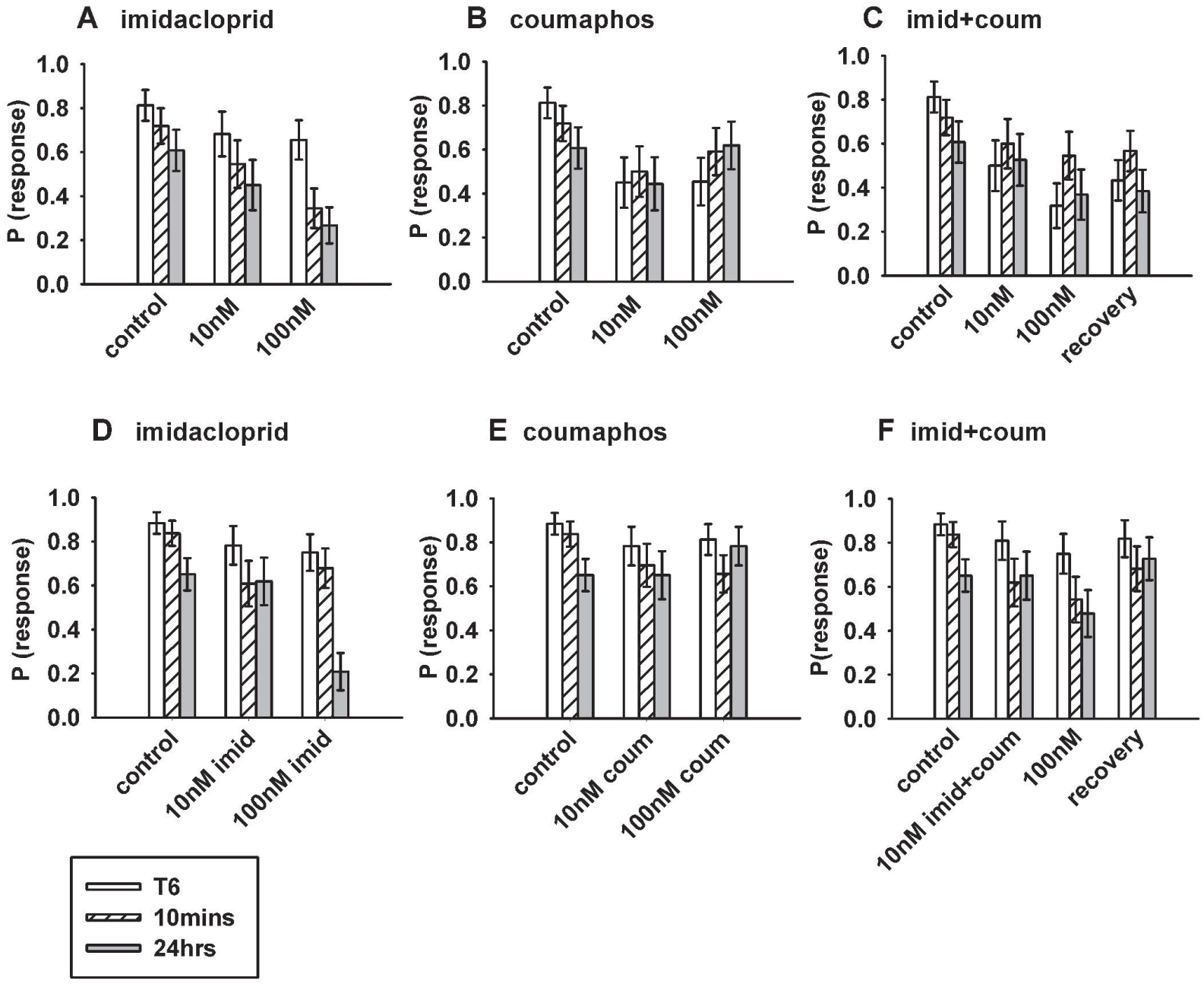
A imidacloprid
B coumaphos
C imid+coum
D imidacloprid
E coumaphos
F imid+coum
P (response)
P(response)
1.0
0.8
0.6
0.4
0.2
0.0
control
10nM
100nM
recovery
10nM imid
100nM imid
10nM coum
100nM coum
10nM imid+coum
T6
10mins
24hrs

Figure 4

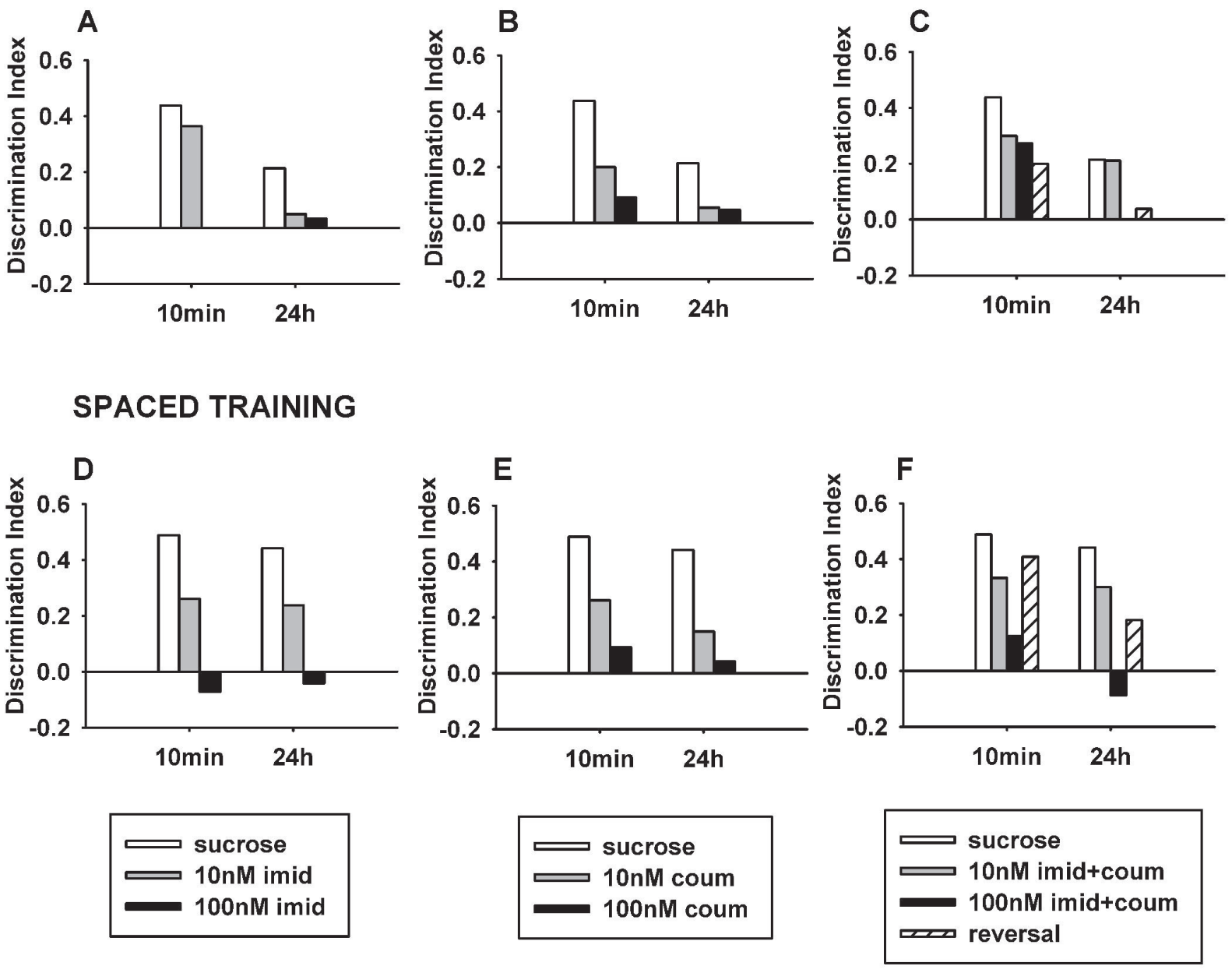
MASSED TRAINING
A
B
C
Discrimination Index
0.6
0.4
0.2
0.0
-0.2
10min
24h
SPACED TRAINING
D
E
F
sucrose
10nM imid
100nM imid
sucrose
10nM coum
100nM coum
sucrose
10nM imid+coum
100nM imid+coum
reversal

Table 1. Comparison of proportion of honeybees from each treatment group that failed to respond to the odour stimulus at any point during the training regime (non-responders). Figures in bold indicate values which differ significantly from the control group. The column 'recall' indicates that the number of subjects from the 'non-responders' group that responded to the CS during the 24 h recall test. I = imidacloprid, C = coumaphos

Treatment	Massed conditioning			Spaced conditioning		
	Non-responders	Total	Recall	Non-responders	Total	Recall
Control	1	33	0	0	45	0
10 nM I	5	27	0	2	25	0
100 nM I	7	34	0	6	34	0
10 nM C	5	25	1	2	25	1
100 nM C	13	30	2	5	37	3
10 nM I+100 nM C	5	26	0	4	26	0
100 nM I + 100 nM C	9	28	0	11	36	2
3 days recovery	3	26	0	2	28	0

Table 2. Sample sizes and post-hoc pairwise comparison statistics for the acquisition data (Figure 2). Figure in bold denote significant differences of treatment groups compared to control (sucrose). Post-hoc pairwise comparisons (lsc) were performed for responses on the 6th training trial. For control data, N = 43 for spaced training and N = 32 for massed training.

Figure	Treatments	conditioning	Sample size	P-value
2A	10nM I	massed	22	0.253
2A	100nM I	massed	28	**<0.001**
2B	10nM C	massed	20	**0.003**
2B	100nM C	massed	21	**0.001**
2C	10nM I + C	massed	20	0.117
2C	100nM I + C	massed	22	**<0.001**
2C	3 days recovery	massed	23	0.070
2D	10nM I	spaced	23	**0.004**
2D	100nM I	spaced	28	**<0.001**
2E	10nM C	spaced	23	0.307
2E	100nM C	spaced	32	0.400
2F	10nM I + C	spaced	21	0.411
2F	100nM I + C	spaced	25	0.209
2F	3 days recovery	spaced	26	0.233

Table 3. Sample sizes and post-hoc pairwise comparison statistics for the recall test (Figure 3). Figures in bold show values significantly different to the controls. STM = comparison of T6 to 10 min test; LTM = comparison of 10 min to 24h test.

Figure	Treatments	Conditioning	Sample size			STM P-value	LTM P-value
			T6	10 min	24 h		
3A-C	Control	massed	32	32	28	0.373	0.360
3A	10nM I	massed	22	22	20	0.191	0.277
3A	100nM I	massed	28	28	28	**0.002**	**0.005**
3B	10nM C	massed	20	20	18	0.111	0.275
3B	100nM C	massed	21	21	20	0.331	0.932
3C	10nM I + C	massed	20	20	19	0.380	0.583
3C	100nM I + C	massed	22	22	19	0.191	0.098
3C	3 days recovery	massed	23	23	21	0.207	0.094
3D-F	Control	spaced	43	43	43	0.533	0.063
3D	10nM I	spaced	23	23	21	0.307	0.803
3D	100nM I	spaced	28	28	24	0.367	**<0.001**
3E	10nM C	spaced	23	23	20	0.203	0.993
3E	100nM C	spaced	32	32	23	0.073	0.243
3F	10nM I + C	spaced	21	21	20	0.069	0.993
3F	100nM I + C	spaced	25	22	21	**0.011**	0.173
3F	3 days recovery	spaced	26	26	24	0.173	0.524

Table 4. Multiple comparisons for the discrimination index (Figure 4 and Supplementary Figure A1). Figures in bold indicate where treated bees performed differently to the controls.

Figure	Treatments	Conditioning	Number of bees		STM P-value	LTM P-value
			10 min	24 h		
4A-C	control	massed	32	28	**>0.001**	0.101
4A	10nM I	massed	22	20	0.070	0.749
4A	100nM I	massed	28	28	1.00	0.765
4B	10nM C	massed	20	18	0.187	0.735
4B	100nM C	massed	21	20	0.543	0.753
4C	10nM I + C	massed	20	19	0.061	0.179
4C	100nM I + C	massed	22	19	0.056	1.00
4C	3 days recovery	massed	23	21	0.113	0.773
4D-F	control	spaced	43	43	**>0.001**	**>0.001**
4D	10nM I	spaced	23	21	0.070	1.00
4D	100nM I	spaced	28	24	0.112	0.731
4E	10nM C	spaced	23	20	0.064	0.320
4E	100nM C	spaced	32	23	0.440	0.729
4F	10nM I + C	spaced	21	20	**0.021**	**0.047**
4F	100nM I + C	spaced	22	21	0.382	0.553
4F	3 days recovery	spaced	26	24	**0.003**	0.202