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VISUAL MODULATION OF OLFACTORY LEARNING IN HONEYBEES

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Summary

We use classical conditioning of the honeybee (*Apis mellifera*) proboscis extension reflex with a visual (A) and an olfactory (X) conditioned stimulus in a blocking paradigm. Typically, learning about one element (X) of a compound (AX) is decreased (blocked) if the other component (A) has previously been rewarded alone. Our results show that visual pretraining did not produce blocking in honeybees: instead, forward pairings of A with a reward increased subsequent learning about X relative to a backward pairing control. This finding violates the independence assumption, which holds that elements of inter-modal compound stimuli change associative strength independently of each other. Furthermore, it is at odds with common theories of conditioning that predict blocking and assume that the elements of a compound stimulus rely on one common internal reinforcing signal. Taking the functional anatomy of the honeybee brain into account, we suggest that vision and olfaction may not rely on the same internal reinforcing signal; compound interactions might thus reflect the wiring of the honeybee nervous system and the biological significance of different sensory modalities during natural behaviour.

Key words: blocking, vision, olfaction, classical conditioning, reinforcement processing, reward processing, learning, memory, honeybee, *Apis mellifera*.

Introduction

The phenomenon of ‘blocking’ (Kamin, 1968) remains a central issue in associative learning research. It demonstrates that pairing (contiguity) of a stimulus with reinforcement is not sufficient to support associative learning: training to a compound AX after a pretraining phase to one of its elements (A) produces fewer conditioned responses to X than in controls that have not received pretraining with A. This finding was instrumental to the introduction of most current models of associative learning (Mackintosh, 1975; Rescorla and Wagner, 1972; Sutton and Barto, 1981; Wagner, 1981). Although differing in a number of respects, these models share three important features with regard to the present analysis. First, stimuli retain their elemental integrity within compound stimuli. Second, a reward stimulus has only one internal representation. Third, the elements of compound stimuli compete for the effects of a learning trial; that is, changes in associative strength of one element are dependent on the associative strength of the other element.

In the honeybee, blocking between the elements of binary odorant mixtures has been demonstrated (Smith and Cobey, 1994) using classical conditioning of the proboscis extension reflex (PER), a paradigm in which restrained honeybees learn to associate odorants with a sucrose reward. More recently, Couvillon *et al.* (1997) also demonstrated blocking for freely flying honeybees for intra-modal (vision–vision and olfaction–olfaction) compound stimuli, but not for inter-modal (vision–olfaction) compounds. These results were taken as support for the ‘independence’ assumption (Bitterman, 1996), which holds that elements of inter-modal compound stimuli change in associative strength independently of each other. This would suggest that honeybee learning differs fundamentally from that of vertebrates. Therefore, an inter-modal analysis of blocking for classical conditioning in the PER paradigm is required to test the independence assumption. We use visual and olfactory stimuli in a blocking study of PER conditioning. Such an extension of inter-modal blocking studies to the PER procedure will enable a physiological analysis of independence in these processing pathways (Joerges *et al.* 1997; Menzel and Müller, 1996), in particular with respect to an identified neurone that carries an internal reinforcement signal (Hammer, 1997).

Materials and methods

Worker honeybees (*Apis mellifera* L.) were caught, harnessed and fed (2 mol l^{-1} sucrose solution) according to standard methods (Bitterman *et al.* 1983). On the following day, subjects were evaluated for unconditioned responses by touching one antenna with 2 mol l^{-1} sucrose solution. Only subjects that extended their proboscis were used in the training procedure. They were then placed in position on a wheel, 1 m

in diameter, on which ten subjects could be mounted simultaneously. Training began 30 min later. Rotation of the wheel, which placed subjects at the training site, and stimulus delivery were controlled by a computer. The timing of manual sucrose delivery was signalled acoustically.

Geraniol and 1-hexanol were used as olfactory stimuli (termed stimulus 'X'). Odour cartridges were freshly prepared daily by placing 3 ml of odorant onto a filter paper loaded into a 1 ml glass syringe. An aquarium pump delivered a continuous flow of air through a Teflon tubing system. Odour was delivered using computer-controlled valves to shunt odour for 4 s into that air stream. Odour was immediately removed by an exhaust system.

The visual stimulus (termed stimulus 'A') was delivered using a standard laboratory lamp 20 cm above the experimental site. Background illumination was ultraviolet-free fluorescent light. Light was filtered through a high-pass glass filter with a cut-off wavelength of 455 nm (human yellow; Edmund Scientific Companies, Barrington, NJ, USA). Stimulus duration was 4 s; the stimulus was visible only to the experimental subject. No thermal component of this stimulus was detectable to the human observer.

The 2 mol l^{-1} sucrose reward was delivered using a precision syringe (Gilmont Instruments, Barrington, IL, USA). A 2 µl droplet was used to touch one antenna, and the subject was then allowed to feed from that droplet, reward delivery lasting for a total of 3 s.

Each trial lasted for 1 min; 26 s after a subject had been rotated to the training site, the respective stimulation protocol (see below) started. After stimulation, subjects were left untreated until a full minute had passed.

During training trials, the reward and the respective training stimulus overlapped for 1 s. The reward typically began 3 s after training stimulus onset (FORWARD trials, inter-stimulus interval, ISI +3 s); only in the BACKWARD group (see below) was this sequence reversed (ISI −2 s). The reward was omitted during test trials.

Using an inter-trial interval (ITI) of 10 min, all groups received two training phases (six trials each) and a final test (Table 1). For all experimental groups, the second (compound) training phase consisted of rewarded trials using a compound stimulus with a visual (A) and an olfactory (X) component (AX+). Groups differed in terms of visual pretraining during the first (pretraining) phase (see below). The critical question is whether groups respond equally to the olfactory stimulus X during a final test.

In the pretraining phase, the FORWARD group received forward trials (A+) whereas the BACKWARD group received A and the reward in the reverse order (+A). The UNPAIRED group experienced separate trials with either A or the reward (pseudorandom sequence: +, A, A, +, A, +, +, A, +, A, A, +), and the PLACEMENT group was exposed to the training site without the presentation of a reward or a training stimulus. Unpaired presentations of A and + require twice the number of trials as other forms of training; thus, placement trials were pseudorandomly interspersed for all other groups during the pretraining phase. This procedure follows that used by Smith and Cobey (1994).

Table 1. *Summary of the experimental paradigm*

Group	Pretraining	Compound training	Final test
PLACEMENT		AX → +	X
FORWARD	A → +	AX → +	X
FORWARD^{LONG}	A ⟶ +	AX → +	X
UNPAIRED	A ↔ +	AX → +	X
BACKWARD	+ → A	AX → +	X

A is the visual stimulus and X is the olfactory stimulus.

Forward and backward pairing with the reward are indicated (→ + and + →, respectively). For the FORWARD^{LONG} group, ⟶ indicates a longer duration of A. For the UNPAIRED group, A ↔ + indicates that A and the reward are presented on separate trials.

All animals received six pseudorandomly interspersed placement trials in the pretraining phase to equilibrate all groups for exposure to the conditioning site. After the experiment had been completed, all honeybees were also tested for their responses to A and were checked for intact reflexes.

Since, during foraging, visual cues might be far distant cues (see Discussion), a FORWARD^{LONG} group was included. During pretraining, this group received a prolonged stimulation with A of 11 s duration (ISI +10 s).

After the final test with X, we also tested the visual stimulus (A) alone, and all animals were tested for intact reflexes.

Data are presented as the percentage of honeybees showing proboscis extension. Additionally, subjects' test responses were video-taped at 30 frames s^{-1} to determine the response latency and duration (Smith, 1997) and the number of protractions and retractions of the proboscis. If the proboscis was already extended at stimulus onset, that subject was excluded from the video-tape analysis. For ranked statistical analyses, non-responders were assigned durations of 0 s, latencies of 10 s (much longer than measured latencies) and zero proboscis extensions. Data on response duration are represented by box plots (see Fig. 2) to acknowledge the skewed distribution. Data were analysed with Mann–Whitney *U*-test, the Kruskal–Wallis test or the χ^2-test, as appropriate.

Results

Given that all groups received identical training with respect to stimulus X and the reward, all groups should, according to the independence assumption, show equal response levels to the final test with X. Therefore, any pattern of significant differences in the response levels to X among our treatment groups (Table 1) would indicate an interaction between visual and olfactory stimuli and would thus lead to rejection of the independence assumption.

All response measures yielded significant differences among groups in test performance towards X. This was true for response probability (Fig. 1, $P<0.05$, $\chi^2=14.74$, d.f.=4),

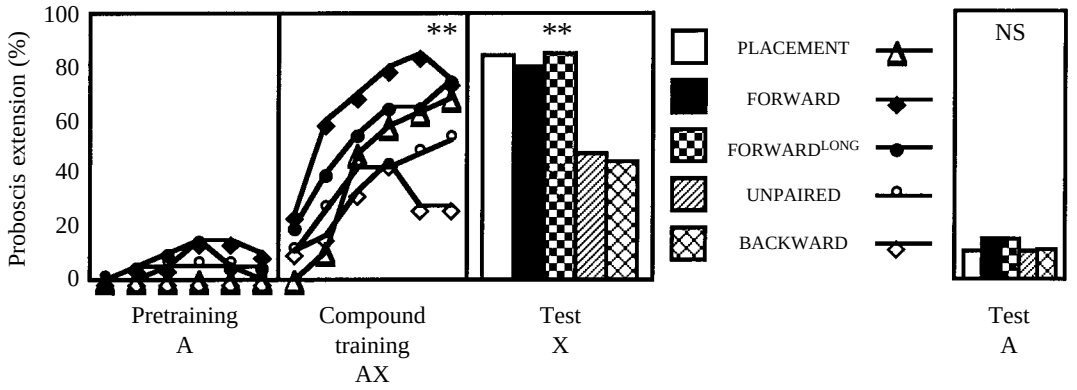

Fig. 1. Percentage of honeybees showing proboscis extension during each trial of the pretraining and compound training phases and during the final test with the olfactory stimulus X. A is the visual stimulus. Groups differ in their experience with A during pretraining. Labelling of groups refers to their treatment in the pretraining phase (see Table 1). Sample sizes are PLACEMENT, N=19; FORWARD, N=20; FORWARD^{LONG}, N=20; UNPAIRED, N=19; BACKWARD, N=18. The panel on the right shows the performance of animals in a test performed subsequently using A alone. Asterisks indicate significant (**P<0.01; NS, P>0.05) differences among all groups. For two-group comparisons, see text.

duration (Fig. 2, P<0.05, H=12.05, d.f.=4), latency (results not shown; P<0.05, H=10.55, d.f.=4) and the number of proboscis protractions during a response (results not shown; P<0.005, H=16.87, d.f.=4).

To specify the nature of these differences, data from the FORWARD and BACKWARD groups were used in pairwise comparisons: these groups had equivalent exposures to A and the reward and equivalent overlap of A and the reward but differed with respect to the predictive value of A. In test performance towards X, animals in the FORWARD group showed higher response probabilities (Fig. 1, P<0.05, χ^2=5.14, d.f.=1), longer durations (Fig. 2, P<0.05, U=64), shorter latencies (results not shown; P= 0.05, U=70) and more extensions of the proboscis during a response (results not shown; P<0.05, U=69) than did animals in the BACKWARD group. In addition, a similar pattern emerged when the FORWARD and UNPAIRED groups were compared (response probabilities, Fig. 1, P<0.05, χ^2=4.50, d.f.=1; duration, Fig. 2, P>0.05, U=109; latency, results not shown, P>0.05, U=94; number of proboscis extensions, results not shown, P<0.05, U=77.5). 'Blocking' would have been indicated by the reverse pattern of results: a lower response probability, a shorter duration, a longer latency and fewer extensions of the proboscis in the FORWARD compared with either the BACKWARD or the UNPAIRED group.

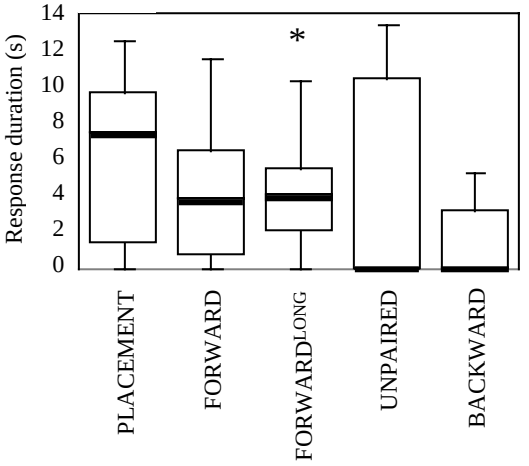

Fig. 2. Response durations to the olfactory stimulus X during the final test. Labelling of groups refers to their treatment in the pretraining phase (see Table 1). In these box plots, the median is the bold line, the boxes indicate the 25% and 75% quartiles, respectively, and the small horizontal bars are the 10% and 90% values. Sample sizes are PLACEMENT, N=18; FORWARD, N=17; FORWARD^{LONG}, N=18; UNPAIRED, N=14; BACKWARD, N=15. The asterisk indicates a significant (P<0.05) difference among all groups. For two-group comparisons, see text.

The trend for longer response durations in the PLACEMENT than in the FORWARD group was not significant (Fig. 2, P>0.05, U=111). The same was true for the differences between these two groups for response probability (Fig. 1, P>0.05, χ^2=0.11, d.f.=1), latency (results not shown; P>0.05, U=119) and the number of proboscis extensions during a response (results not shown; P>0.05, U=117).

Finally, we compared response levels to the visual stimulus A (Fig. 1). There were no significant effects of training history (Fig. 1, P>0.05, χ^2=0.39, d.f.=4). This absence of a conditioned response, however, does not imply that subjects did not establish memories for the visual stimulus, as is demonstrated indirectly by the interactions with subsequent odour learning (test responses, see above) and by the significant effect of visual pretraining on performance during the compound training phase (Fig. 1, P<0.01, H=13.9, d.f.=4).

Discussion

We have demonstrated behaviourally an interaction between visual and olfactory learning in PER conditioning (Figs 1, 2). First, this shows that harnessed honeybees do learn

associatively about visual stimuli. Second, the modulating effect that these visual memories can exert violates predictions of the 'independence' assumption, which proposes that there is no interaction between visual and olfactory stimuli (Bitterman, 1996; Couvillon *et al.* 1997; Funayama *et al.* 1995).

What is the nature of these interactions and where might they occur in the honeybee brain? Clearly, the effect of these interactions is quite different from that observed using intra-modality compound stimuli (Couvillon *et al.* 1997; Smith and Cobey, 1994). For binary odorant mixtures, pretraining with odorant Y blocks learning about odorant Z during subsequent training to the YZ mixture (Smith and Cobey, 1994). In that study, response probabilities to Z in the FORWARD group were lower than in the BACKWARD and UNPAIRED groups. The same result has been reported for olfactory mixture learning in the terrestrial slug *Limax maximus* (Sahley *et al.* 1981) and is indeed predicted by most associative learning theories (e.g. Sutton and Barto, 1981; Wagner, 1981). In the present study, using a visual stimulus (A) and an olfactory stimulus (X), however, the pattern of results is reversed (Figs 1, 2): response levels to X in the FORWARD group are higher than in the BACKWARD and UNPAIRED groups. Thus, visual stimuli seem to enhance, rather than to block, olfactory learning.

In terms of learning theory, the lack of an enhancing effect in the BACKWARD and UNPAIRED groups might be due to inhibitory learning during pretraining. Such inhibitory learning has been demonstrated for olfactory PER conditioning (Hellstern *et al.* 1998; Menzel, 1990). This inhibition could carry over to the olfactory stimulus during compound training, potentially *via* within-compound associations, which have been demonstrated between visual and olfactory stimuli in freely flying honeybees (Couvillon and Bitterman, 1982).

In physiological terms, the nervous system might set up separate internal reinforcing systems for vision and olfaction. The identified neurone VUM_{mx1} carries an internal reinforcement signal for olfactory PER conditioning (Hammer, 1997). VUM_{mx1} converges with olfactory processing in the primary olfactory neuropile (antennal lobes), in premotor centres (lateral protocerebrum) and in the mushroom bodies, which are multisensory centres intimately related to memory function (Menzel and Müller, 1996). VUM_{mx1} innervates two of the three mushroom body input regions, the lip and basal ring, but not the collar. Olfactory projection neurones also innervate the lip, but not to the collar; visual projection neurones, however, project in a complementary way: to the collar but not to the lip (Bicker *et al.* 1993; Gronenberg, 1986; Mobbs, 1985; in these studies, there is mixed evidence about the inputs to the basal ring). Thus, within the mushroom body, visual processing cannot rely on the same internal reinforcement signal as olfactory processing. Instead, the compartmentalization of the mushroom body inputs suggests a separation of their access to internal reinforcement as carried by VUM_{mx1}. Most associative learning theories, however, assume a common internal reinforcement signal for the elements of compound stimuli and thus predict blocking to occur (Dickinson, 1980). Therefore, they might apply within but not between sensory modalities, suggesting that blocking might occur within but not between modalities (Couvillon *et al.* 1997). This does not mean that visual and olfactory processing do not interact at all. Indeed, we have provided behavioural evidence for such an interaction; possible loci of interaction include the mushroom bodies downstream from their input region (Homberg, 1984, found that mushroom body output neurones often respond to visual and olfactory stimuli) and the lateral protocerebrum (Maronde, 1991).

Separated reinforcement systems might reflect separated biological roles for visual and olfactory stimuli. They inform honeybees about different and complementary aspects of flowers (location, blooming status, nectar availability and rate, species) and might be relevant to different motor programs (flight, orientation, choice, landing, PER) (Greggers and Mauelshagen, 1998; Mauelshagen and Greggers, 1993; Menzel and Müller, 1996). Their 'meaning' might therefore be complementary rather than redundant; hence, vision might speed up, rather than block, olfactory learning. The separation of their biological roles might also be the reason why visual stimuli can modulate olfactory learning (Figs 1, 2) but can only poorly release the PER (Fig. 1; Masuhr and Menzel, 1972; see also Greggers and Mauelshagen, 1998).

Thus, compound interactions might reflect the wiring of the honeybee's nervous system and the biological roles of the stimuli. The PER paradigm will now allow a physiological analysis of how different sensory modalities, and possibly separated internal reinforcement systems, jointly organize behaviour. This might contribute to the more general question raised in the vertebrate literature (Dickinson, 1977; Mirenowicz and Schultz, 1996) of how many internal reinforcement systems exist and how they interact.

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