

## Spatial learning in the restrained American cockroach *Periplaneta americana*

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### Summary

**Spatial learning abilities were tested in restrained cockroaches by observing antennal projection responses towards the positions of a learned visual cue perceived monocularly by one eye in the context of a second stimulus provided to the contralateral eye. Memory of the position of the conditioning stimulus relative to the contralateral reference stimulus was tested by altering the relative positions of the two stimuli. Memory of the conditioning stimulus is retained if the angle between the conditioning**

**stimulus and the contralateral reference stimulus is maintained. The results suggest that during learning the insect recognizes spatial relationships between the conditioning stimulus and the contralateral reference stimulus. Possible mechanisms, such as retinotopic matching versus angular matching, are discussed.**

Key words: place memory, cockroach, *Periplaneta americana*, antennal movement, behavior.

### Introduction

The use of landmarks for navigation has been demonstrated to employ retinotopic matching, in which a series of previously memorized images are retinotopically matched with current scenes (Cartwright and Collett, 1982; Dill et al., 1993; Judd and Collett, 1998). Place learning in mammals is evidenced by the activity of hippocampal neurons that fire only when the animal is at a specific position in its learned environment (Barnes et al., 1997; Poe et al., 2000). There is evidence that cockroaches have comparable abilities because they can learn to relate distant visual cues with hidden targets (Mizunami et al., 1998) in a manner that is similar to place memory behavior of mammals (Morris, 1984). However, little is known about the underlying neural mechanisms and brain regions that support such spatial learning abilities in insects. This is in part due to the fact that few behavioral paradigms are available for studying spatial learning in an immobilized animal that might then be subjected to intracellular recording, as is done on rodents.

The antennal motor system of insects can be used to develop novel behavioral paradigms for studying associative memory (Lent and Kwon, 2004) and, by extension, spatial memory. Antennal motor actions can be elicited by different modalities, including olfactory, tactile and visual stimuli (Erber et al., 1993; Erber and Pribbenow, 1997). Antennal movements elicited by visual stimuli demonstrate active sensory exploration, such as by restrained honey bees that move their antennae towards the direction of a moving grating (Erber and Pribbenow, 1997). Visual inputs have also been shown to elicit

antennal movements in crickets (Honegger, 1981). Such behaviors have been utilized by experiments in which animals were operantly conditioned to extend their antennae towards a target in order to receive a reward (Erber et al., 1993; Kisch and Erber, 1999). In nature, directed antennal movements that are elicited by a sensory stimulus (here termed antennal projection responses or APRs) may be employed to locate an olfactory stimulant, such as the odor of food, a conspecific or a predator (Bell, 1981). As shown previously, APRs can be conditioned to point to a visual cue after its learned association with a food odor (Lent and Kwon, 2004).

The present study describes a novel visual association paradigm to demonstrate spatial learning on restrained cockroaches, again exploiting antennal movements as an indicator of learning. The present results show that restrained insects can learn to recognize spatial relationships between distant cues. Because this can be demonstrated on an immobilized animal, the results provide a crucial step towards investigating place memory at the level of defined circuitry.

### Materials and methods

#### *Animals*

Male American cockroaches (*Periplaneta americana* L.) raised in a laboratory colony maintained on water and cat food (IAMS, Dayton, Ohio) were used in the behavioral experiments. Cockroaches were kept at 25±1°C on a 12 h:12 h light:dark cycle. Test animals were isolated from colonies.

They were maintained individually in small plastic cages and starved for 24 h prior to behavioral experiments. Cockroaches were restrained in plastic tubes, as described previously (Lent and Kwon, 2004). The test insect was positioned in the center of an arena, the walls of which were decorated with a series of green and white light-emitting diodes (LEDs), as schematized in Fig. 1A. Experiments were run after spontaneous antennal movements began to occur 10–30 min after the animal was placed in the arena and after body struggling movements abated. Individuals showing no antennal movements to odor stimulation during training trials were rejected.

#### Arena and stimuli

As described previously, experiments were conducted in an arena enclosed within a visually uniform chamber illuminated with an infrared lamp (Fig. 1A). A restrained cockroach was positioned in the middle of the arena and aligned with respect to five green LEDs on the arena wall positioned at 15° intervals to the right of the insect (Fig. 1A). The distance from the insect's head to the position of these cues was 15 cm. Each diode was given a number from 1 to 5. Four white LEDs (E1000; Gilway Technical Lamp Co., Woburn, MA, USA) were positioned on the wall of the arena to the left of the insect. These contralateral reference stimuli (ConRS) were also spaced at 15° intervals with respect to the cockroach and named A–D.

Food odors controlled by a solenoid valve were presented through an odor delivery system positioned at green diode 1. The duration of the odor stimulation was 1 s. A ventilation system was placed above the arena to remove odor after each trial (see Lent and Kwon, 2004 for details).

#### Monitoring and video recording of antennal movements

Antennal movements were captured with either an 8 mm Camcorder (Sony) or a digital video camera (Panasonic) and recorded on tape. Digitized images provided the raw data used to analyze antennal movements in each trial. Antennal movements recorded for 10 s after stimulation were digitized by the Motus program (Peak Performance Technologies, Inc., Englewood, CO, USA), which captured images every 167 ms, producing ~60 images per trial. From these digitized images, the tip and base of the right antenna of each test cockroach and the position of the green light cue were recorded to obtain angle data with respect to the midline of the head. These antennal angles thus quantified antennal movements with respect to different positions of the green LED.

#### Training procedures

The white diode at position A was switched on during the pre-training and training trials. One pre-training trial was followed by five training trials. These were succeeded by 3–8 test trials, depending on the experiment. These protocols are summarized in Fig. 1B.

During the training trials, peanut butter odor was emitted under solenoid control at a position coincident with green LED 1 to provide the unconditioned stimulus (US). The green LED at position 1 served as the conditioned stimulus (CS). A Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA) controlled the sequence of the US and CS. As described by Lent and Kwon (2004), the US was given 1 s after CS onset, thus providing a simultaneous conditioning protocol. In all experiments, except test 1 (see below), pre-training consisted of a 2 s presentation of the green LED at position 1, without

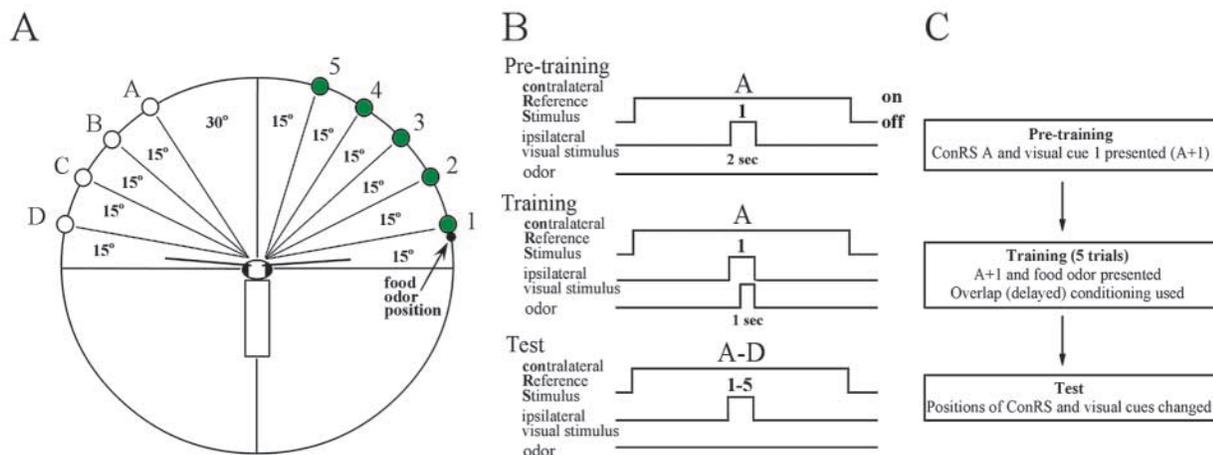


Fig. 1. Spatial learning paradigm. (A) The restrained cockroach is placed in the center of the arena. White light-emitting diodes (LEDs; A–D) and green LEDs (1–5) subtend its left and right visual fields, respectively. During trials (except in test 1, see text), one of the white LEDs serves as the contralateral reference stimulus (ConRS) and is illuminated throughout that trial. Green LEDs are positioned at 15° intervals with respect to the midline of the cockroach's head. Emission of the food odor is at green LED position 1. (B,C) Experimental procedures. Pre-training consisted of one trial in which the positions of the ConRS and green light were fixed at A and 1, respectively (referred to in the text as A+1). The ConRS was illuminated for the whole trial, during which the green LED was illuminated for 2 s. No odorants were presented in these initial tests. Training consisted of five presentations during which ConRS A was illuminated throughout and the green LED (CS) illuminated for 2 s, with the odor present during the second half of the CS. During tests, only visual cues were provided. The positions of the ConRSs and green LEDs could be variously changed. The CS was present for 1–5 s during continuous illumination of the ConRS. Projection responses of the ipsilateral antenna (APRs) to illuminated green LEDs were scored in each trial.

an odor cue, and during continuous illumination by the white diode (ConRS) at contralateral position A (referred to as A+1). Pre-training measured a cockroach's innate response in the presence of the A+1 configuration. In the training trials, the green LED at position 1 was coupled with the food odor. The CS and US were presented in the context of continuous contralateral reference illumination by the white LED at position A (A+1+odor) for five trials, with an inter-trial interval (ITI) of 1 min. Post-training tests began 5 min after the last training trial and each lasted 1 min, with a 3 min ITI between each test. For the duration of each test, one of the white contralateral LEDs at one of positions A–D was illuminated. Then, one of the green LEDs at one of positions 1–5 was presented for 2 s. Between each test, the animal was covered with a black box (15 cm×15 cm×20 cm) while a white LED was switched on at a new position (A–D), after which the box was removed. After 40 s, the animal's APR was tested by illuminating the ipsilateral eye with a green LED at a new position for 2 s. Because of the time required to change the positions of the contralateral visual cues (Fig. 1B) 3 min ITIs were maintained during test trials.

#### Testing procedures

Testing procedures determine whether an APR can be elicited when the CS alone is displaced from its original position (test 1), when the CS is displayed with a ConRS (test 2), when the CS is displaced but the ConRS is not (test 3), when the ConRS is displaced but the CS is not (test 4) or when both CS and ConRS are displaced in various combinations (test 5).

#### Scoring APRs and statistics

An APR for the trial was scored as '1' if there were antennal movements towards the green LED ( $\pm 2.5^\circ$ ) during the 10 s that followed stimulation. A score of '0' was assigned if there were no antennal projection movements or if there was only antennal tremor but no projections towards the green LED. APRs are shown as percentages of '1' responses during a given trial, as assessed by video observation. Non-parametric analytical tests were performed to compare APRs during pre-training and testing. The Friedman test was used to compare APRs within subjects. Once a significant difference was shown, a Wilcoxon signed-rank test ( $Z$  statistic) was performed in parallel to compare each value of every trial. The Kruskal–Wallis test ( $H$  statistic) was performed to compare the antennal responses between groups. Mann–Whitney  $U$  tests ( $U$  statistic) were used to test the differences between two groups. Values shown depict the responses '0' or '1' in percentages. Statistical analysis was carried out using Statistica 5.5 for Windows and results were regarded as 'not significant' if  $P > 0.05$ .

## Results

### Test 1: CS displaced

APRs can be conditioned to a visual cue when the visual cue is coupled with a food odor using simultaneous conditioning

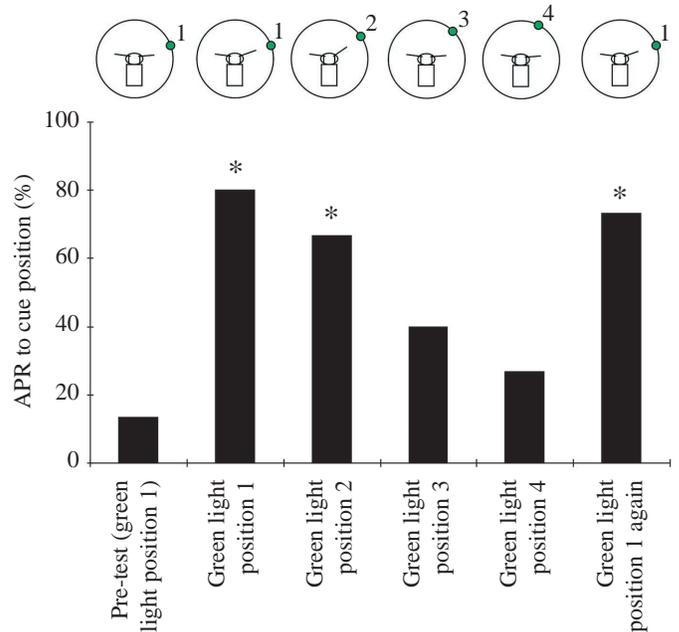


Fig. 2. Antennal projection responses (APRs) to different positions of green light-emitting diodes (LEDs) without a contralateral reference after animals were trained to LED position 1. APRs to LED positions 1 and 2 were significantly increased from pre-training ( $P < 0.05$ ). APRs to LED positions 3 and 4 were not different from those at pre-training ( $P > 0.1$ ). APRs to green LEDs at 'position 1 again' (the last post-training trial) were significantly increased from APRs during pre-training ( $P < 0.05$ ). The diagram above each column represents the relevant test. The right antenna pointing towards the position of the green LED indicates a positive response.

(Lent and Kwon, 2004). The question arises of whether the insect will project its antenna towards the visual cue if the position of the cue is subsequently changed. In these first experiments, test trials changed the positions of the green LED from position 1 to positions 2, 3 or 4 (Fig. 2). This experiment is the only experiment to omit a contralateral reference stimulus and examines whether a learned response is spatially generalized (Fig. 2). APRs towards the changed position of the green LEDs 1, 2, 3, 4 and 'position 1 again' showed significant differences (Friedman test,  $\chi^2 = 17.8$ , d.f. = 5,  $P < 0.004$ ). APRs to the green LED at position 1 during pre-training were below 20%. After training, APRs to the green LED at position 1 were significantly greater than during pre-training (Wilcoxon signed-rank test,  $N = 15$ ,  $Z = 2.80$ ,  $P < 0.01$ ). APRs tested to the green LED at position 2, which was displaced  $15^\circ$  from position 1, were also significantly increased compared with the pre-training percentages ( $Z = 2.07$ ,  $P < 0.04$ ). However, APRs to the green LED at positions 3 or 4, which were displaced  $30^\circ$  and  $45^\circ$ , respectively, from position 1, showed no difference from APRs in pre-training. Additionally, they were not significantly different (Wilcoxon signed-rank test,  $Z = 1.33$ ,  $1.68$ ,  $0.733$ ,  $1.21$ ;  $P > 0.09$ ) from the first two trials (positions 1 and 2). APRs towards the green 'position 1 again' at the final test were significantly increased compared with pre-training

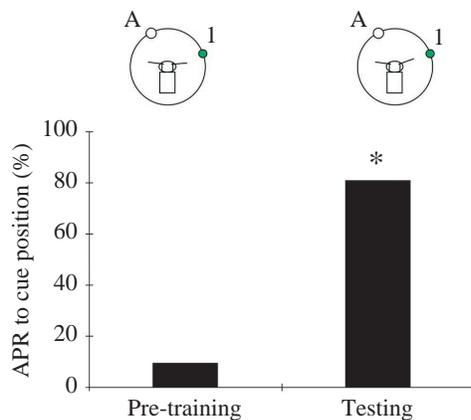


Fig. 3. Antennal projection responses (APRs) to green LED position 1 with the ConRS at A (A+1) before and after training. APRs elicited by post-training tests were significantly increased from those at pre-training ( $P < 0.05$ ). The diagram above each column schematizes the behavioral response. The right antenna pointing towards the green LED indicates a positive response.

APRs ( $Z = 2.67$ ,  $P < 0.008$ ). These results, which provide insight into the animal's recognition of the CS when it is moved from its learned position, are discussed later.

#### Test 2: CS and ConRS fixed

This experiment determines whether a cockroach can project its right antenna towards the unchanged position of the green light cues during the simultaneous presentation of ConRS at a fixed position 'A'. The positions of the ConRS and the CS at position 1 (A+1) were maintained throughout this experiment. APRs to the CS at position 1 were scored (Fig. 3). APRs of the right antenna towards the green light position after training were significantly increased compared with pre-training (Fig. 3; Wilcoxon signed-rank test,  $Z = 2.37$ ,  $N = 7$ ,  $P < 0.02$ ), showing that cockroaches learn the CS in the presence of additional visual information. These results do not show how or if the animal is using this additional sensory information.

#### Test 3: CS displaced and ConRS fixed

If APRs can be elicited by the changed position of the CS (as described in test 1), does this still occur when the CS is displaced but ConRS is maintained at A throughout? During pre-training, APRs to the green LED at position 1 were tested in the absence of odor cues but in the presence of the ConRS at position A. Training of APRs was to the CS+US in the presence of the ConRS. Insects were then tested with altered positions of the CS while maintaining the position of the ConRS. The spatial configurations to which the insects were tested are thus A+1, A+2, A+3 and A+4 (Fig. 4).

Throughout tests, APRs to different green light positions showed significant differences (Fig. 4; Friedman test,  $N = 18$ ,  $\chi^2 = 33.82$ , d.f. = 5,  $P < 0.0001$ ). The first test in response to A+1 revealed a significant increase in APRs compared with pre-training (Fig. 4; Wilcoxon signed-rank test,  $N = 18$ ,  $Z = 2.95$ ,

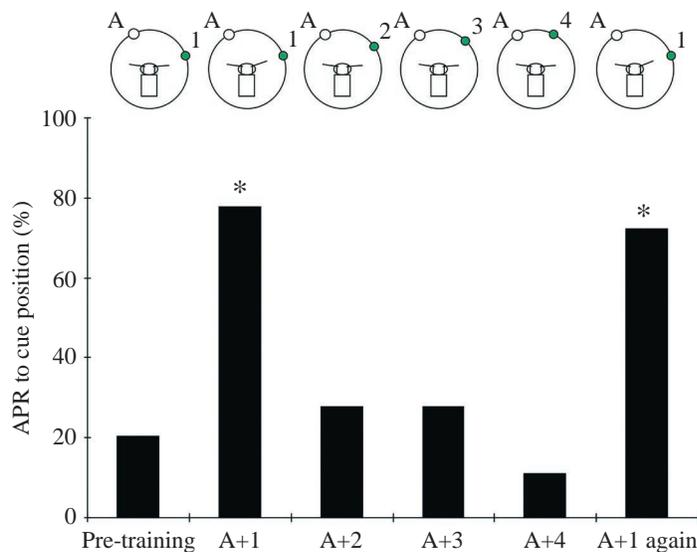


Fig. 4. Antennal projection responses (APRs) to green LED positions in which the green LED positions were changed after training trials and the ConRS position remained the same. APRs to green LED position 1 during the ConRS at position A, A+1 and 'A+1 again' were significantly increased from APRs to LEDs at pre-training and positions 2, 3 and 4 ( $P < 0.05$ ). The diagram above each column schematizes the behavioral response for each LED and ConRS position. The right antenna pointing towards the green LED indicates a positive response.

$P < 0.005$ ). APRs to A+2, A+3 and A+4 were not significantly different from those during pre-training (Wilcoxon signed-rank test,  $Z = 1.83$ , 1.46, 0.534, respectively,  $N = 18$ ,  $P > 0.1$ ). To control for sensitization or arousal, APRs to A+1 were tested again ('A+1 again'); the tests showed a significant difference compared with tests using A+2, A+3 and A+4 (Wilcoxon signed-rank test,  $N = 18$ ,  $Z = 2.04$ , 2.04, 2.69, respectively,  $P < 0.05$ ). These results are evidence that the ConRS plays a role in place recognition of the CS and may be used in relation to the CS during learning. In contrast to test 1 above, the animal no longer points to the green LED if the LED position is shifted from that learned during training.

#### Test 4: CS fixed and ConRS displaced

After training, tests were made with the ConRS positions changed from A to B, C or D, with the final test made with the ConRS position returned to A again. Throughout these tests, the position of the CS remained at position 1. The spatial configurations that tested APRs were thus A+1, B+1, C+1, D+1 and 'A+1 again'. The sequence of changed positions of the ConRS was randomized, but the first and last trials were always A+1 (Fig. 5).

APRs to the CS at position 1 coupled with the altered positions of the ConRS were significantly different throughout the tests (Friedman test,  $N = 17$ ,  $\chi^2 = 38.2$ , d.f. = 5,  $P < 0.0001$ ). APRs towards A+1 and 'A+1 again' were significantly increased compared with those of pre-training (Wilcoxon signed-rank test,  $N = 17$ ,  $Z = 2.69$ , 3.06, respectively,  $P < 0.01$ ).

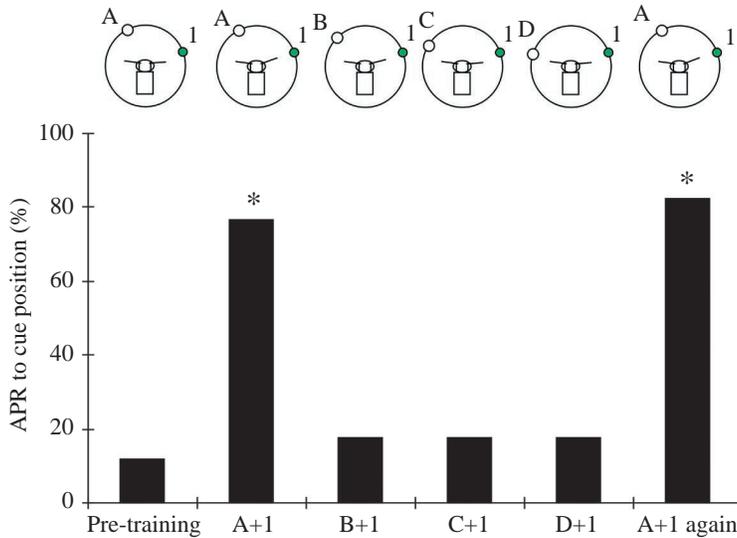


Fig. 5. Antennal projection responses (APRs) to the green LED at position 1 and the ConRS at position A. APRs to position 1 at A+1 and to position 1 at 'A+1 again' were significantly increased from those in pre-training ( $P < 0.05$ ). APRs to the green LED at position 1 and the ConRS at positions B, C or D, named B+1, C+1 and D+1, respectively, did not differ from those in pre-training ( $P > 0.5$ ). The right antenna pointing towards the position of the green LED indicates a positive response.

However, APRs elicited by B+1, C+1 and D+1 were not significantly different from APRs in pre-training (Wilcoxon signed-rank test,  $N=17$ ,  $Z=0.535$ ,  $0.535$ ,  $0.535$ , respectively,  $P > 0.5$ ). This experiment provides further support that the animal is using the relationship between the CS and ConRS during learning.

#### Test 5: CS and ConRS displaced

Finally, we investigated whether APRs could be elicited to a changed position of the CS if the ConRS position was correspondingly changed. We compared tests in which the angular relationships between the CS and ConRS were the same as the original trained angular relationship of the ConRS at position A and the CS at position 1 (see Fig. 1A). In these tests, the original angular relationships were preserved when A was shown with 1 (A+1), B with 2 (B+2), C with 3 (C+3) and D with 4 (D+4). Random sequences were tested, after which APRs were tested using different angular relationships, such as B+3, C+4 and D+5. The final test was for APRs towards 'A+1 again' (Fig. 6).

APRs towards A+1, B+2, C+3 and D+4, which all had the same angular relationships, showed significant differences from pre-training APRs (Fig. 6A; Wilcoxon signed-rank test,  $N=17$ ,  $Z=3.18$ ,  $3.18$ ,  $2.93$ ,  $2.37$ , respectively,  $P < 0.02$ ). However, APRs towards B+3 ( $N=14$ ), C+4 ( $N=16$ ) and D+5 ( $N=14$ ), all of which had angular relationships that differed from the combination of the training stimulus A+1, were not significantly different from APRs during pre-training (Fig. 6A; Wilcoxon signed-rank test,  $Z=1.47$ ,  $0.53$ ,  $0.91$ , respectively,  $P > 0.14$ ). Within APRs to the same angular relationships,

responses to D+4 were significantly different from those towards A+1 and B+2 (Wilcoxon signed-rank test,  $N=17$ ,  $Z=2.20$ ,  $2.20$ , respectively,  $P < 0.03$ ) but not C+3 (Wilcoxon signed-rank test,  $N=17$ ,  $Z=1.47$ ,  $P > 0.14$ ). APRs towards B+3 (angle mismatch) showed no difference from C+3 (Wilcoxon signed-rank test,  $N=14$ ,  $Z=1.82$ ,  $P > 0.06$ ) and D+4 (Wilcoxon signed-rank test,  $N=14$ ,  $Z=0.73$ ,  $P > 0.4$ ). APRs towards 'A+1 again' increased significantly in the last trial of the tests and were significantly different from APRs during pre-training (Wilcoxon signed-rank test,  $N=7$ ,  $Z=2.36$ ,  $P < 0.03$ ).

APRs towards the same and discrepant angular relationships of the green light and ConRS were pooled to compare overall behaviors. Fig. 6B elaborates on the patterns of APRs towards the same and discrepant angular relationships compared with during pre-training. APRs to the green light were significantly influenced by the ConRS, being generally maintained when the angles between the green light and ConRS were maintained (Kruskal–Wallis test,  $N=53$ ,  $68$  and  $44$  for pre-training, same and different angular relationships, respectively,  $H=40.95$ ,  $P < 0.0001$ ). The average APRs to green light positions when the angular relationships between the ConRS and green light positions were maintained were  $\sim 70\%$  (Fig. 6B). This was significantly different from APRs in response to the green light positions when the angle between these and ConRS were altered (Mann–Whitney  $U$  test,  $U=746$ ,  $P < 0.0001$ ). Also, compared with APRs towards A+1 in the tests ( $N=21$ ) in which positions of visual cues were not changed throughout (CS and ConRS fixed; Fig. 3), APRs towards green light positions having the same angular relationships ( $N=68$ ) showed no significant difference (Mann–Whitney  $U$  test,  $U=640$ ,  $P=0.47$ ). Pre-training APRs showed no difference from APRs towards green light positions having angular relationships to ConRS that were different from A+1 (Mann–Whitney  $U$  test,  $U=1162.5$ ,  $P > 0.9$ ).

## Discussion

### Retinotopic matching versus spatial relationship matching

Comparing the results of tests 1 and 2 shows that a CS displaced  $15^\circ$  from its original position at training will elicit a strong APR in the absence of the ConRS. When the CS is rotated more than  $15^\circ$  from its original position, APRs diminish. This suggests that in an environment devoid of additional reference cues the position of the CS is, within a given receptive field, ambiguous. Subsequent experiments demonstrate that in the presence of additional visual cues, a shift of the CS across the retina ceases to be ambiguous when the ConRS is held at the same location or *vice versa*. Thus, when a reference cue is stabilized on the contralateral retina, APRs towards a CS shifted by more than  $15^\circ$  across the ipsilateral retina (A+2 in Fig. 4) showed no difference from pre-training APRs. This indicates that the spatial reference

stimuli in the contralateral visual fields provide precision to associative learning of the original position of the CS

compared with when only the CS is present (Fig. 2). In the presence of the contralateral stimulus, shifts in the position of the CS render it meaningless.

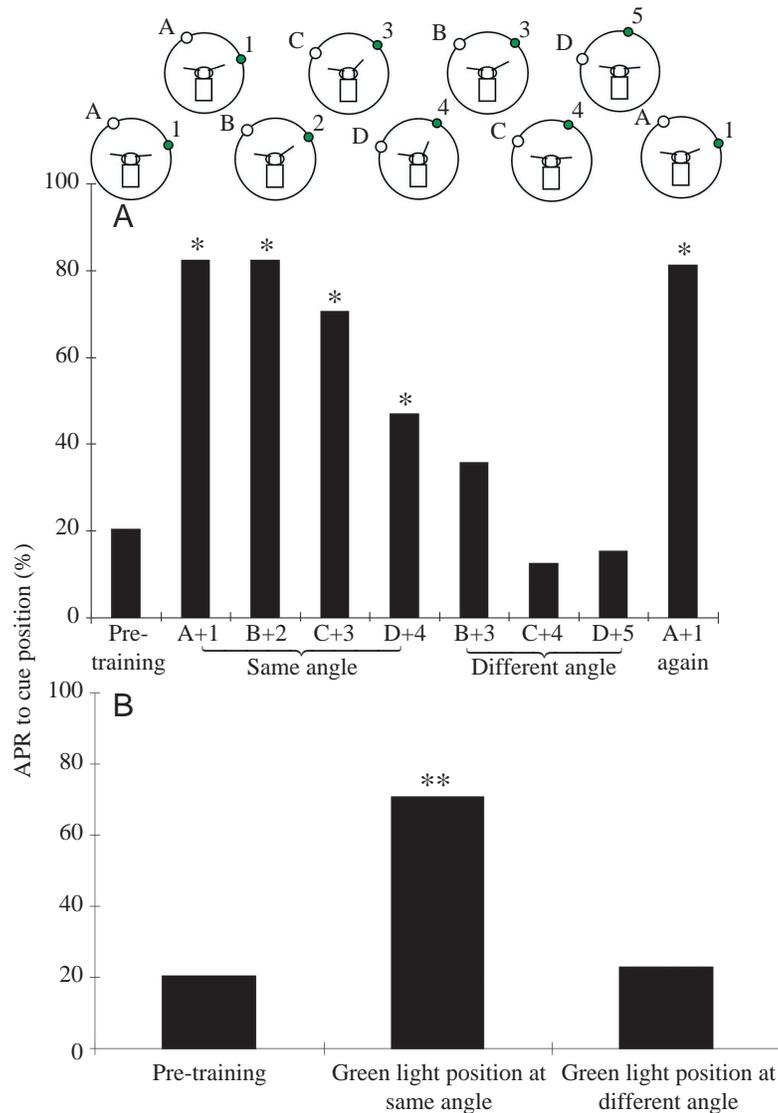


Fig. 6. Antennal projection responses (APRs) to positions of the green LED in which the positions of the LEDs and the ConRS were changed while either maintaining or altering their angular relationships. Angles between B and 2, C and 3, and D and 4 were the same as between A and 1, whereas the angles between B and 3, C and 4, and D and 5 were smaller (see Fig. 1A). (A) APRs to the green LED positions having the same angular relationships showed a significant difference from those at pre-training ( $P < 0.05$ ), whereas APRs to green LED positions having different angular relationships with the ConRS [i.e. B+3 ( $N=14$ ), C+4 ( $N=16$ ) and D+5 ( $N=14$ )] showed no difference from APRs at pre-training ( $P > 0.1$ ). APRs to green LED position 1 in the final trials ('A+1 again') were increased compared with those at pre-training ( $P < 0.05$ ). The right antenna pointing towards the position of the green LED indicates a positive response. Asterisks indicate tests that are significantly increased compared with pre-training. (B) APRs pooled with respect to pre-training ( $N=53$ ), same angular relationships ( $N=68$ ) and different angular relationships ( $N=44$ ). APRs to green LED positions having the same angular relationship to the ConRS as during training showed a significant increase compared with those of pre-training and tests when the different angular relationship between the green LED and the ConRS was smaller than during training (\*\* $P < 0.05$ ).

If the recognition of the learned CS is a function of point-to-point retinotopic matching, does this mean that retinotopic matching itself requires two discrete retinal stimuli? Experiments in which the position of the CS and ConRS are changed suggest that retinotopic matching also involves the retinotopic subtense of the two stimuli. When the learned spatial configuration [the arc distances (angle)] of the two stimuli is maintained, the animal projects its antenna to the new location of the CS (Fig. 6A). But if the arc distances are altered from that provided during training (e.g. B+3, C+4 and D+5 in Fig. 6A), the antenna is not projected to the new position of the CS. Therefore, APRs to the CS in the context of a second visual cue rely on the recognition of angular matching rather than retinotopic matching.

Does this occur in nature? Rust et al. (1976) showed that cockroaches turn their heads towards a pheromone source to facilitate antennal scanning in the direction of the odor plume. This behavior indicates that head movements follow antennal movements and that by realigning their antennae insects achieve greater precision of information about an odor source (Murlis, 1992). However, as far as we are aware, the role of visual cues in such olfactory-driven behaviors has not been investigated. Here, we have provided evidence that animals are able to localize food sources in the absence of information provided by an odor plume given that they have previously learned to associate the food source with visual cues. We suggest that external reference cues provide increased precision in localizing odor sources.

#### *Behavioral and neural correlates of spatial learning*

Where and how memory templates of sensory scenes are formed, stored and compared in the insect brain is still unknown. A requirement for investigating underlying mechanisms, using electrophysiological methods, is to have behavioral paradigms for spatial learning. This and our previous study (Lent and Kwon, 2004) demonstrate spatial learning abilities in a restrained insect using antennal movement patterns to provide reliable behavioral indicators. Evidence from lesion studies suggests that the mushroom bodies play a crucial role in visual associative and spatial learning (Mizunami et al., 1998). When adapted for intracellular recordings, the present behavioral paradigm should provide new insight into mechanisms of spatial learning in insects.

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