

# Cross-modal interaction between visual and olfactory learning in *Apis cerana*

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**Abstract** The power of the small honeybee brain carrying out behavioral and cognitive tasks has been shown repeatedly to be highly impressive. The present study investigates, for the first time, the cross-modal interaction between visual and olfactory learning in *Apis cerana*. To explore the role and molecular mechanisms of cross-modal learning in *A. cerana*, the honeybees were trained and tested in a modified Y-maze with seven visual and five olfactory stimulus, where a robust visual threshold for black/white grating (period of 2.8°–3.8°) and relatively olfactory threshold (concentration of 50–25 %) was obtained. Meanwhile, the expression levels of five genes (*AcCREB*, *Acdop1*, *Acdop2*, *Acdop3*, *Actyr1*) related to learning and memory were analyzed under different training conditions by real-time RT-PCR. The experimental results indicate that *A. cerana* could exhibit cross-modal interactions between visual and olfactory learning by reducing the threshold level of the conditioning stimuli, and that these genes may play important roles in the learning process of honeybees.

**Keywords** *Apis cerana* · Cross-modal learning · Visual stimuli · Olfactory stimuli real-time RT-PCR

## Abbreviations

<i>AcCREB</i>	<i>Apis cerana</i> cAMP response element binding protein gene
<i>Acdop1</i>	<i>Apis cerana</i> dopamine receptor 1 gene
<i>Acdop2</i>	<i>Apis cerana</i> dopamine receptor 2 gene
<i>Acdop3</i>	<i>Apis cerana</i> dopamine receptor 3 gene
<i>A. cerana</i>	<i>Apis cerana</i>
<i>Actyr1</i>	<i>Apis cerana</i> tyramine receptor 1 gene
<i>AmCREB</i>	<i>Apis mellifera</i> cAMP response element binding protein gene
<i>Amdop1</i>	<i>Apis mellifera</i> dopamine receptor 1 gene
<i>AmdOP1</i>	<i>Apis mellifera</i> dopamine receptor 1 protein
<i>Amdop2</i>	<i>Apis mellifera</i> dopamine receptor 2 gene
<i>AmdOP2</i>	<i>Apis mellifera</i> dopamine receptor 2 protein
<i>Amdop3</i>	<i>Apis mellifera</i> dopamine receptor 3 gene
<i>A. mellifera</i>	<i>Apis mellifera</i>
<i>Amyr1</i>	<i>Apis mellifera</i> tyramine receptor 1 gene
CREB	cAMP response element binding protein
DAMB	<i>Drosophila</i> ortholog of <i>AmDOP2</i>
<i>Dmdop1</i>	<i>Drosophila</i> dopamine receptor 1 gene
LTM	Long-term memory

## Introduction

Honeybees are favorable eusocial subjects for neuroethological and ecological studies. Although their nervous systems contain only 950,000 neurons (Witthöft 1967), honeybees have remarkable abilities to learn and remember tasks and objects (Menzel 1990; Martin and Randolf 1995). Previous studies revealed that honeybees are able to learn color (Frisch 1914), odors (Menzel et al. 1996; Laska et al. 1999), shapes, and patterns (Srinivasan 1994). They can form abstract concepts of “sameness” and “difference” (Giurfa et al. 2001), categorize visual objects (Hateren

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et al. 1990; Horridge and Zhang 1994; Avarguès-Weber et al. 2010), generate associative memory (Srinivasan et al. 1998; Zhang et al. 1999), and use context to determine which action to perform and when (Zhang et al. 2006).

Cross-modal perception can allow an organism to extract the logical structure of the world, to react rapidly to related environmental events. In human studies, cross-modal vision facilitates olfactory perception, and the process is mediated by human hippocampus (Gottfried and Dolan 2003). For *Drosophila*, cross-modal interactions can reinforce the memory, facilitate visual or olfactory perception and transfer memory from a conditioned cue to an unconditioned cue (Guo and Guo 2005). In addition, honeybee might also possess cross-modal learning since there is evidence that honeybees can recall a specific color when they encountered a particular scent (Srinivasan et al. 1998). Furthermore, Reinhard et al. (2004) revealed that honeybees could employ associative learning and cross-modal recall to aid navigation back to the site. A recent study has shown that the presence of an olfactory signal can facilitate color learning in *Bombus impatiens* (Leonard et al. 2011). Although the use of cross-modal recall could increase honeybees' fitness in an external environment (Leonard and Masek 2014), little is known about whether honeybees could exhibit cross-modal reinforcement of memory when concurrent visual and olfactory stimulus are marginally effective.

Understanding the molecular mechanisms that regulate cognitive function is important to elucidate the phenomena of learning and memory of animals. Evidences from *Drosophila* (Yin et al. 1995), *Aplysia* (Lee et al. 2008), and rats (Josselyn et al. 2001; Suzuki et al. 2011) show that the cAMP response element binding protein (CREB), a prototypical stimulus-inducible transcription factor, played a central role in the formation and consolidation of memory. Meanwhile, tyramine and dopamine have an important effect on learning and memory of insects (Restifo and White 1990) and exert their effects by binding to G-protein coupled trans-membrane receptors (Blenau and Baumann 2001). With disruption of dopamine or tyramine receptor genes expression, *Drosophila* exhibited negligible learning after conditioning stimuli (Kim et al. 2007; Kutsukake et al. 2000). In honeybees (*A. mellifera*), the *AmCREB* gene that encoded eight splice variants in the brain was the only member of the CREB family of transcription factors (Eisenhardt et al. 2003, 2006). Pharmacological and behavioral studies have demonstrated that dopamine mediate aversive learning (Vergoz et al. 2007), and tyramine increases sucrose responsiveness that has been shown to correlate with associative proboscis extension learning in honeybees (Scheiner et al. 1999, 2002). One tyramine receptor gene (*Amtyr1*) (Blenau et al. 2000) and three dopamine receptor genes (*Amdop1*, *Amdop2*, *Amdop3*) (Blenau et al. 1998;

Humphries et al. 2003; Beggs et al. 2005) have been cloned from the brain of *A. mellifera* and functionally characterized. Unfortunately, the mechanism of how these genes are involved in a specific learning task in honeybees is unclear.

*Apis cerana*, an Eastern honeybee, is a local species of honeybee in Asia and widely distributed in the north, east and middle of china. Compared with the Western honeybee, *A. cerana* is more docile, owing to better searching ability for sparse floral resources and stronger resistance to diseases and parasitic mites (Abrol 2013). Recently, *A. cerana* was reported to have a better sense of smell (Chen 2001) and cognitive abilities on color and orientation learning than *A. mellifera* (Qin et al. 2012). Meanwhile, Wang and Tan (2014) showed that *A. cerana* could discriminate experienced odors from a non-experienced odor as well as *A. mellifera*, using classic olfactory PER assay. Therefore, *A. cerana* could be used as a good model for behavioral and molecular biological research in learning and memory assays.

In this study, visual and olfactory threshold values of *A. cerana* were measured by modulating the magnitude of visual or olfactory stimuli using a modified Y-maze. The aim was to investigate whether *A. cerana* exhibits cross-modal reinforcement of memory with concurrent visual and olfactory stimulus near the threshold level. In addition, real-time RT-PCR was used to detect the mRNA level of the *AcCREB* gene, dopamine receptor genes (*Acdop1*, *Acdop2*, *Acdop3*) and tyramine receptor gene (*Actyr1*) in *A. cerana* under various controlled conditions.

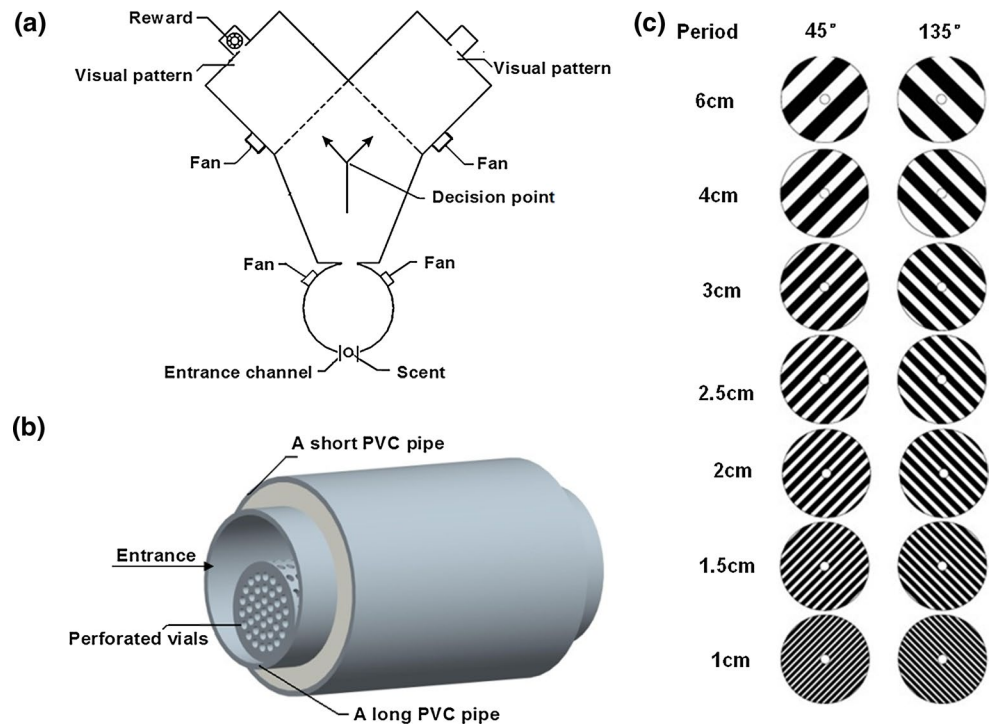
## Materials and methods

### General

The experiments were carried out from March 2013 to October 2013, at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N, 115.49°E). Two Eastern honeybee colonies of *A. c. cerana* were used in this study, and each colony had 4 frames and ~6,000 bees. All experiments were carried out on sunny days.

A modified Y-maze apparatus consisted of vertically oriented cylinders placed in an indoor laboratory, and located close to the windows in the maze learning experiments (Fig. 1a). The first cylinder (diameter 22.5 cm, height 20 cm) had two holes in a line, joining two opposite points on the circumference of the cylinder. One hole (diameter 4 cm) was positioned halfway up the wall of the cylinder and connected with the Y-maze. The other hole is equipped with a channel to provide an entrance into the apparatus for honeybees, and for depositing the olfactory stimuli. This channel consisted of a short PVC

**Fig. 1** The experimental setup and visual patterns. **a** The modified Y-maze setup consisted of a vertically oriented cylinder and usual choice Y-maze, see detailed description in “Materials and methods”. **b** A special channel provided an entrance into the setup for honeybees and for experimentally depositing the scents. **c** Seven pairs of grating pattern (diameter 18 cm) were used in the training and testing procedure. The period of black/white stripes is 6, 4, 3, 2.5, 2, 1.5, 1 cm for each pair. Grating patterns are oriented at 45° “+” versus 135° “-” or 135° “+” versus 45° “-”



pipe (diameter 7 cm, length 10 cm) fixed to the cylinder, a long PVC pipe (diameter 5 cm, length 12 cm), and perforated vials with scents (Fig. 1b). During the training and test, the perforated plastic vials and long pipes were both replaced at the same time to avoid any residual smells when the scent was changed. Two silent computer fans were symmetrically positioned to the left and right of the cylinder to provide a stream of scented air, and remove the residual scents. Additionally, two choice chambers were equipped with a computer fan. The rest of the Y-maze was similar to that described in detail and widely used in honeybee behavioral research (Zhang et al. 1999). The modified Y-maze apparatus was covered with lids of transparent Perspex. Further, a Halogen lamp (240 V, 100 W) was installed above the apparatus for 1 m to provide homogeneous lighting.

The beehives of *A. cerana* were kept outside and housed the healthy honeybees. The distance between the hive and the apparatus was about 50 m. In the experiment, forager bees were guided into the reward box of the apparatus (Fig. 1a) to gain sugar reward, which was described in detail by Guez et al. (2012) and Qin et al. (2012). When honeybees learned to visit a sugar feeder of the reward box, visual or olfactory stimuli were presented. If the honeybee made a correct selection (termed positive stimulus “+”) according to patterns or scents, it could visit the feeder with a sugar reward. On the contrary, if it made an incorrect selection (termed negative stimulus “-”), sugar reward would not be available.

Visual and olfactory stimulus

The visual stimulus consisted of seven pairs of grating patterns (black/white strips) presented on 18-cm-diameter discs. Each disc was printed with black/white strips with spatial period of 6, 4, 3, 2.5, 2, 1.5, and 1, respectively (Fig. 1c). The choice chambers had a central orifice (diameter 2 cm), which allowed honeybees access to a reward box. During the training and test, the patterns were placed vertically at the end of choice chambers with orientation of 45° versus 135° or 135° versus 45° to the vertical. One of two patterns was termed the positive pattern, and the other was negative pattern in each experiment. Differential conditioning was used in the training, as described in Giurfa et al. (1999).

The olfactory stimulus used in the present study was Natural Flavoring Essences (Queen Fine Foods Pty Ltd., Australia): lemon essence or vanilla essence. Each essence was presented by perforated vials containing a piece of filter paper (diameter 9 cm), which was soaked in the above mentioned liquid essence. During the test, the two essences were, respectively, diluted in ultra-pure water to different concentrations (essence: water, 75, 50, 25 and 12.5 % volume/volume).

Training and testing procedures

Five experiments were conducted in the present study. For each experiment, 18–25 honeybees were individually

marked and trained. After 2 days of training, only 10–15 of the residual honeybees were tested (described separately below). Each experiment was repeated three times (three independent biological replicates) using a fresh group of bees without prior experience. During each experiment, unmarked honeybees visiting the feeders were removed, to prevent them from influencing the training of marked bees. In addition, the indoor place was kept quiet, clean, and without specific smell during the experiment.

The total duration of a training block was 20 min, and each stimulus was presented for 10 min (an average of 2–3 rewarded visits per honeybee) in each arm of the Y-maze. During the training and test, the stimuli and sugar feeder were switched regularly between two choice chambers. This operation ensured that the honeybees could learn to associate the sugar reward with the positive stimulus, and not with the arm of the Y-maze. After performing two complete training blocks, honeybees would have a rest for 15–20 min to prevent fatigue.

#### *Series 1: visual stimuli learning and memory*

The aim of the experiment was to test the visual threshold level of *A. cerana*, on black/white gratings. A pair of black/white gratings (period 6 cm) was presented at the choice chamber during the training. In experiment 1, a grating orientated at 45° was set as “positive” with sugar reward, while a grating orientated 135° was set as “negative” without sugar reward, namely 45° “+” versus 135° “–”. The opposite situation, namely 135° “+” versus 45° “–” was presented in experiment 2. During the training and test, the positions of positive and negative patterns were regularly switched every 10 min, so that the honeybees could use these patterns as cues to find the feeder.

Learning tests were carried out for each experiment as follows. When honeybees were trained for about 2 days (including 20–24 blocks), and learned to discriminate the grating (period of 6 cm), six pairs of grating patterns with different periods (4, 3, 2.5, 2, 1.5, and 1) were randomly used during the tests. Each pair, presented in the choice chambers, was tested for the duration of two or three blocks as above to explore the visual threshold of honeybees (*A. cerana*). The performance was quantified by recording the correct or incorrect choices of each honeybee.

#### *Series 2: olfactory stimuli learning and memory*

The aim of the experiment was to investigate the olfactory threshold level of *A. cerana* for the lemon and vanilla essences. Two pure scents held into different perforated vials were alternately presented at the entrance for every 10 min. In experiment 3, honeybees were trained to visit the left chamber to get a food reward when they encountered

the lemon-scent, while on encountering the vanilla-scent, they had to visit the right chamber to access sugar feeder. The opposite situation was presented in experiment 4. Before standard training, honeybees were trained for about 1 h on two feeders containing 67.1 % saturated sugar water with 1 % (volume/volume) lemon or vanilla essence. This operation ensured that the experimental honeybees could quickly and effectively associate the reward with the positive olfactory stimulus.

Learning tests were performed as follows. When the honeybees had trained for about 2 days over 16–20 blocks, five concentrations of lemon/vanilla from 100 to 12.5 % were used during the test. Each concentration of lemon/vanilla at the entrance was tested for two complete blocks as above. To deliver the scents, filter paper (9 cm) soaked with two ml of each scent solution was placed in perforated vials for the tests. During the whole experiment, all fans were running continuously to prevent the mixing of scents.

#### *Series 3: visual-olfactory stimulus learning and memory*

The aim of the experiment was to investigate whether *A. cerana* can exhibit cross-modal reinforcement of memory when simultaneously presented with the threshold level of visual and olfactory cues. In this cross-modal memory experiment, honeybees were trained to choose the positive grating, period 4 cm oriented at 45° with the lemon-scent (100 %) presented at the entrance to get a food reward. After a half day of pre-training, a 3 h break was taken. Next, honeybees were given a half day of pre-training to associate period 4 cm pattern oriented at 135° with vanilla-scent (100 %) for sugar reward. Subsequently, each of above two pairs of scent-pattern was alternated every 20 min in the training. Each block was sustained for 40 min. This repeated training lasted for 1.5 days.

The performance was tested on the third day of the training. The performance using the visual cue (grating 45°, 1.5 cm), together with the olfactory cue (lemon-scent 25 %), and the visual cue (grating 135°, 1.5 cm), together with the olfactory cue (vanilla-scent 25 %) was tested for three blocks in each experiment. In the entire experiment, all fans were running continuously to prevent the mixing of scents.

#### Data collection and analysis

Each type of experiment was performed on three replicates to collect sufficient data. Therefore, each type of test was totally performed about nine times to gather sufficient data. The correct choice frequencies for each block in every experiment was calculated separately for each block as  $N1/(N1 + N2)$ , where  $N1$  and  $N2$  denote the number of correct and incorrect choices across the marked bees,

**Table 1** Gene-specific primers used in real-time quantitative PCR

Gene name	Forward primer	Reverse primer
<i>AcCREB</i>	TGAAAATCCAGTTTGATCATTCGAT	TTCAAATAATCAGCAAATCATGCAC
<i>Actyr1</i>	AATCCAAGGAGTGCGAGGTG	TGCGTGTCAATGGGAAGAAG
<i>Acdop1</i>	CGTGTCGCAAAGTTGTTATG	TCGCTGATGTAGATGATGGAAG
<i>Acdop2</i>	TTGGTTCTCCCTCTCCGA	ACTGTGCGTGTATTGCGTTC
<i>Acdop3</i>	AGAAGGACAAGAAAATGCCG	CCAAGAGGTCACTATGAATGCCG
<i>β-Actin</i>	GGC TCC CGA AGA ACA TCC	TGC GAA ACA CCG TCA CCC
GAPDH	GCTGGTTTCATCGATGGTTT	ACGATTTCCGACCACCGTAAC

respectively (Guez et al. 2012). Kruskal–Wallis (SPSS Statistics Base 17.0) was used to test for homogeneity of data across all blocks distribution among the three biological replicates in each type of experiment (Pankiw and Garza 2012). Based on these tests, the correct choice frequencies for each block were obtained by pooling choices across repeated experiments. The sample size ( $N_1$ ,  $N_2$ ) was the total number of bees in three replicates to ensure that the samples were statistically independent, and the mean values of choice frequency were calculated. Averages are presented as mean  $\pm$  SEM in the text. Chi-square tests were used to determine whether the experimentally measured choice frequencies were significantly different from the random choice level of 50 % (Giurfa et al. 2001). Comparison of learning curves was analyzed using logistic regression.

The visual angle ( $\alpha$ ) of the grating pattern was calculated by  $\alpha = 2 \arctan(D/2L)$ , where  $D$  is the diameter of black/white stripes and  $L$  is the distance between the center of decision chamber and grating (Spaethe and Chittka 2003).

#### Sample preparation for real-time RT-PCR

Samples were collected according to different training conditions. For each control and trained group, about 65–75 bees were obtained from two different colonies. All bees were collected alive, immediately frozen in liquid nitrogen and then stored at  $-80^\circ\text{C}$ . Every 20 brain tissues were pooled (each bee was involved in each block of a given type of training and the similar trend of learning was observed across all bees) as a biological replicate for RNA extraction, and three biological replicates (with three technical replicates for each biological replicate) were performed for each group. Then cDNA was synthesized as previously described (Wang et al. 2014), and the quality of the cDNA was test with  $\beta$ -actin by RT-PCR before subsequent qRT-PCR reactions.

#### The real-time RT-PCR

Primers were designed by Primer 5.0 software based on the cloned mRNA sequences (*AcCREB*: KC814690, *Acdop2*:

KC814691, *Acdop3*: KC814692, *Actyr1*: KC814693) from the brain of *A. c. cerana* and the previously published transcriptome sequences of *A. c. cerana* in the Genebank database (Wang et al. 2012), and were listed in Table 1.

Real-time quantitative PCR (Bio-Rad CFX96, USA) was used for detection of the expression levels of the six specific genes, with GAPDH-1 and  $\beta$ -actin as internal controls. The RT-PCR system was performed in a 10- $\mu\text{l}$  reaction mixture containing 1  $\mu\text{l}$  of diluted cDNA, 5  $\mu\text{l}$  of SYBR Premix Ex Taq (Takara, Japan), 0.4  $\mu\text{l}$  of specific gene primer (10 mM), and 3.2  $\mu\text{l}$  of DEPC-treated water. The reaction system was as follows: preliminary  $95^\circ\text{C}$  for 30 s, 40 cycles including  $95^\circ\text{C}$  for 10 s;  $60^\circ\text{C}$  for 1 min. Finally, melting curves were recorded by increasing the temperature from 50 to  $90^\circ\text{C}$  (Wang et al. 2014).

The Ct values were calculated using the Bio-Rad CFX 2.1 Standard Edition Optical System Software. The PCR amplification efficiency of an individual reaction was calculated using the qpcR package (Spiess and Ritz 2010; Hornik 2011). The mean amplification efficiency of each gene over all samples was used for subsequent calculation. The relative gene expression levels were calculated according to Huang et al. (2012) and then square root transformed to attain normality. Statistical significance between relative expression levels using ANOVA was tested from the Stat View package (SAS Institute, Gary, NC, USA).

## Results

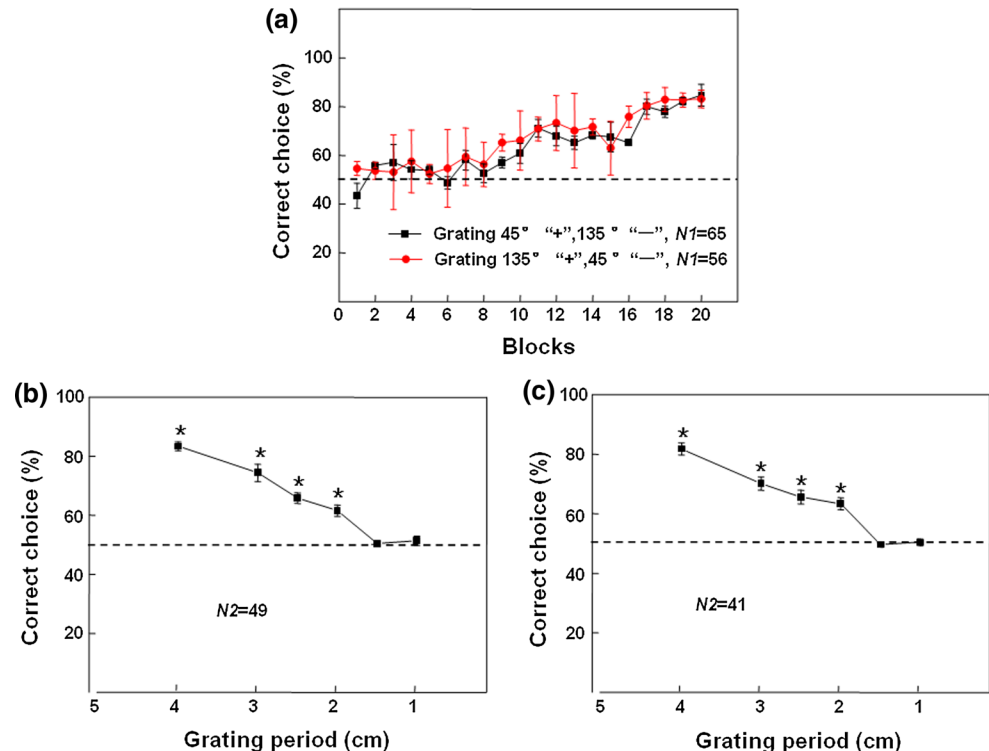
### Results of the behavior

#### *Series 1: effect of visual stimuli learning and memory*

The results of experiments 1 and 2 are shown in Fig. 2. The learning curves showed a significant improvement during the training, and had no significant differences between experiments 1 and 2 (Fig. 2a,  $P = 0.321 > 0.05$ ). In experiments 1 and 2, each of six visual cues (period of grating from 4 to 1 cm) were tested. The total choice frequencies dropped from 62.47 to 50.03 %, when the period of grating reduced from 2 to 1.5 cm, while the total choice



**Fig. 2** Investigating the visual threshold level of *A. cerana* performance. **a** Learning performance curves of *A. cerana* during training on gratings (period 6 cm). **b, c** The total choice frequency of bees during tests on black/white gratings with five visual cues (periods from 4 to 1 cm). **b** Gratings oriented at 45° “+” versus 135° “-” in experiment 1. **c** Gratings oriented 135° “+” versus 45° “-” in experiment 2. *N*1 denotes the total number of bees that were trained and *N*2 denotes the total number of bees that were tested in three replicates of each experiment. Broken horizontal lines denote the random choice level of 50 %. Values are mean  $\pm$  SEM of the data. Asterisks shows that the total choice frequencies are significantly different from random choice (50 %), namely  $p < 0.05$



frequencies were not significantly different from the random choice ( $P > 0.05$ ) (Fig. 2b, c) as the period of grating reduced from 1.5 to 1 cm. Therefore, the visual threshold level of grating ranged from a period of 2–1.5 cm (the visual angle of grating: a period of 2.8° and 3.8° respectively) for *A. cerana* under the present experimental conditions.

#### Series 2: effect of olfactory stimuli learning and memory

The results of experiments 3 and 4 are shown in Fig. 3. The learning curves showed a significant improvement during training, and had no significant differences between experiments 3 and 4 (Fig. 3a,  $P = 0.141 > 0.05$ ). In experiments 3 and 4, five concentrations of lemon/vanilla from 100 to 12.5 % were tested. The results show that the total choice frequencies reduced from 59.83 to 50.6 % when the odor concentration was reduced from 50 to 25 %, and that the choice frequencies of 25 and 12.5 % odor concentration were not significantly different from random choice 50 % ( $P > 0.05$ ) (Fig. 3b, c). Therefore, odor concentration of lemon/vanilla between 50 and 25 % was confirmed to be the olfactory threshold level of *A. cerana* under the present experimental conditions.

#### Series 3: effect of visual-olfactory stimulus learning and memory

In cross-modal learning experiment (Fig. 4), the learning curve showed a significant improvement with training. The total choice frequencies were significantly higher than random

choice of 50 % ( $P < 0.05$ ) during the test. This result indicates that honeybees exhibited cross-modal reinforcement of memory when testing with stimuli below the threshold level.

#### The results of real-time RT-PCR

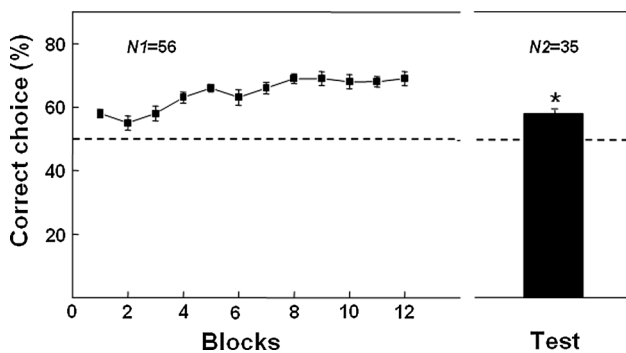
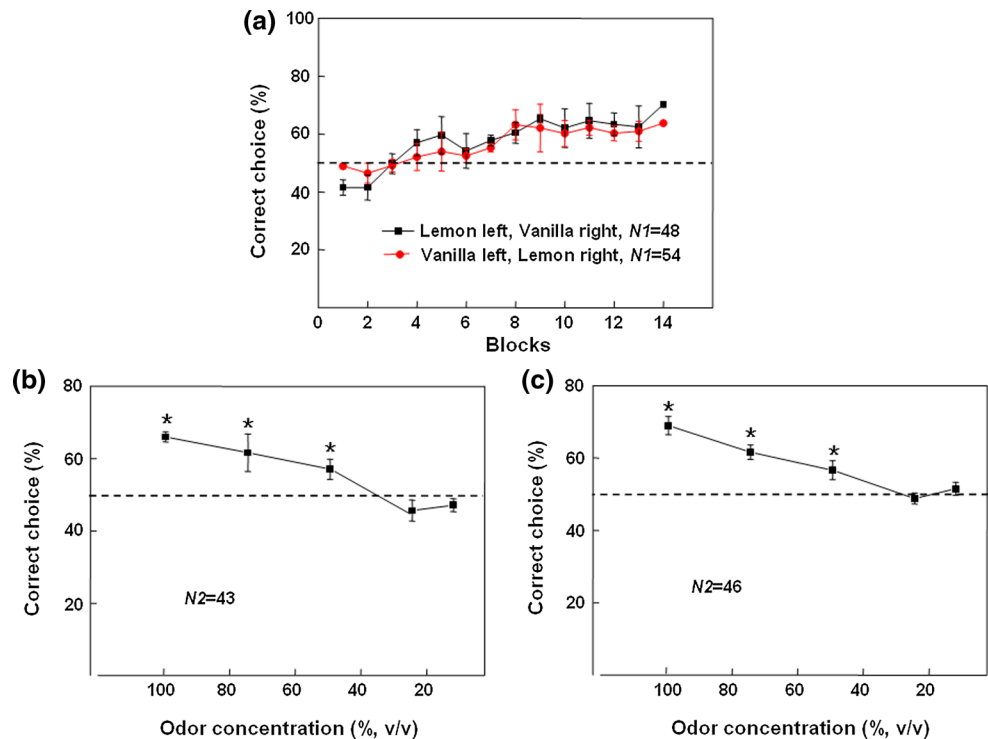
##### Relative gene expression with different visual stimuli

Figure 5 indicates no significant differences ( $P > 0.05$ ) in the relative expression levels of *Acdop3* and *Actyr1* among the three groups. Moreover, *AcCREB*, *Acdop2* showed a much higher expression level ( $P < 0.05$ ) in P1 group, compared to P2 and the control group, but no significant differences were found ( $P > 0.05$ ) between P2 and the control group (the meanings of different groups are described in the figure legends). In addition, both P1 and P2 had a significantly higher relative expression level of *Acdop1* than the control group ( $P < 0.05$ ), but no significant difference existed between P1 and P2.

##### Relative gene expression level with different olfactory stimuli

Figure 6 indicates no significant differences in the relative expression levels of *Acdop2* and *Acdop3* among the three groups ( $P > 0.05$ ). Moreover, *AcCREB*, *Actyr1* showed much higher expression levels ( $P < 0.05$ ) in the S1 group, compared to S2 and the control group. However, there were no significant differences between S2 and the control group (the meanings of different groups are described in the figure legends).

**Fig. 3** Investigating the olfactory threshold level of *A. cerana* performance. **a** Learning performance curves of *A. cerana* during training on pure lemon and vanilla. **b, c**, The total choice frequency performance of honeybees during tests on essence of lemon and vanilla with five concentrations from 100 to 12.5 % v/v. **b** Honeybees were trained to learn to turn *left* for lemon, and turn *right* for vanilla in experiment 3. **c** Honeybees were trained to learn to turn *right* for lemon and turn *left* for vanilla in experiment 4. Others details as in Fig. 2



**Fig. 4** Cross-modal reinforcement of memory. Honeybees were first trained to associate a 45° grating (period 4 cm) with lemon (100 %), and associate 135° grating (period 4 cm) with vanilla (100 %). Tests were commenced after 12 training blocks. During the test, the choice performance was recorded nine times in three replicates of each experiment for one pairing of the visual cue (grating 45°, 1.5 cm), together with the olfactory cue (lemon 25 %), and the other pairing of the visual cue (grating 135°, 1.5 cm) with the olfactory cue (vanilla 25 %). The results indicate statistically significant different from random choice (50 %,  $P < 0.05$ ) in all cases. Others details as in Fig. 2

In addition, the relative expression level of *Acdop1* showed a significant difference among the three groups ( $P < 0.05$ ).

*Relative gene expression level with different visual-olfactory stimulus*

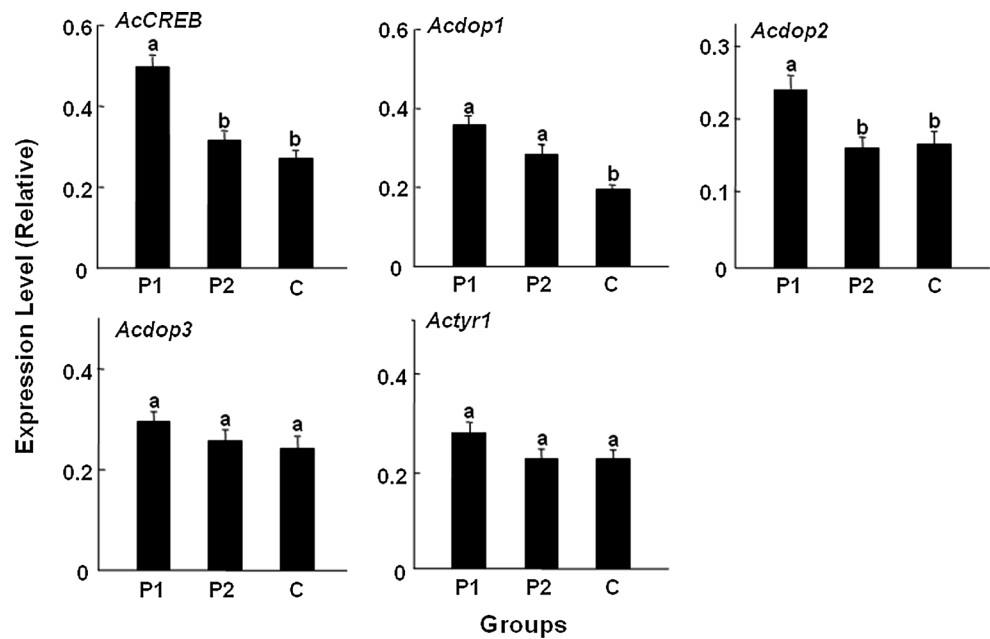
As shown in Fig. 7, the relative expression levels of *AcCREB* and *Acdop1* genes were significantly different

( $P < 0.05$ ) among the three groups (L1, L2, C: the meanings of different groups were described in the figure legends). Furthermore, both L1 and L2 groups had a higher expression level of *Acdop2*, *Acdop3* and *Actyr1* genes than the control group ( $P < 0.05$ ), but the two higher groups were not significantly different from each other ( $P > 0.05$ ).

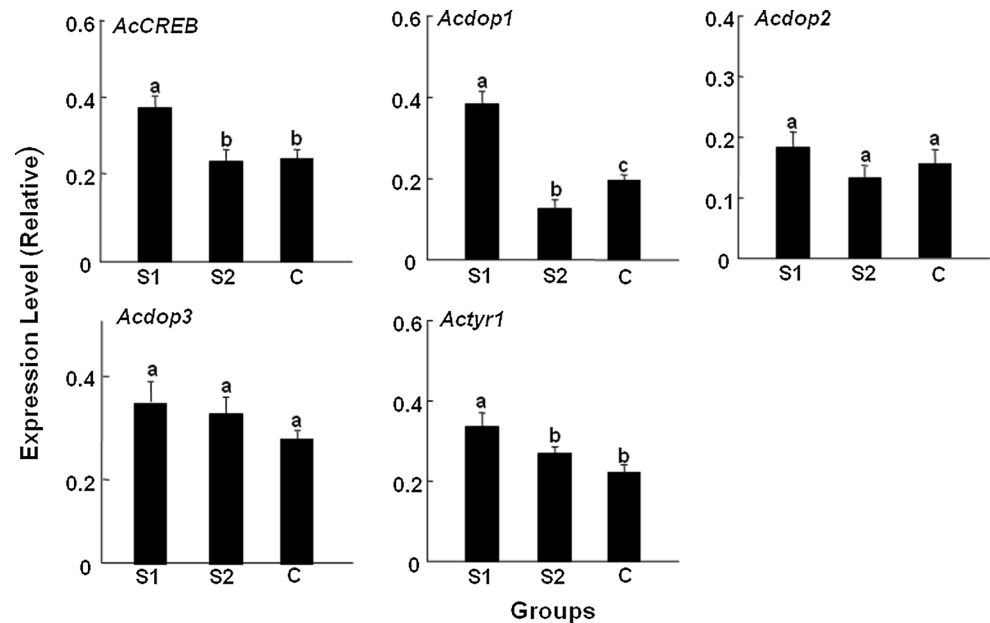
**Discussion**

It is known that the visual acuity of honeybees plays an important role in the identification of food location, navigation and mating. The present study has demonstrated that *A. cerana* can not only distinguish between different orientations of gratings patterns, but also honeybees had no orientation preference (Fig. 2a). This finding is consistent with the results reported by Qin et al. (2012) and Chandra et al. (1998). In addition, the behavioral measurements showed that the minimal visual limit of *A. cerana* for black/white grating was between a period of 2.8° and 3.8°. Srinivasan and Lehrer (1988) found a mean minimum field width near the period of 2.5° with black/white grating in the vertical and horizontal directions in *A. mellifera*. Later, Horridge (2003) further indicated that the visual limit of gratings for worker bees (*A. mellifera*) is near the period providing a visual angle of 2.5° with modulation cues alone, but near 3.5° with an orientation cue. However, for worker bees in *A. cerana*, the compound eyes and body length are smaller than those of *A. mellifera*, and the threshold visual angle is

**Fig. 5** Relative expression levels of the five reference genes for three groups of different training conditions in the visual learning of *A. cerana*. *P1* group: the 4 cm period of black/white grating was used in the training; *P2* group: the 1.5 cm period of black/white grating was used in the training. All grating patterns orientated 45° “+” versus 135° “-”. Control group: honeybees were collected outside the maze and given no acquisition training. Different letters on top of bars indicate significant difference ( $P < 0.05$ ) between the groups. Values are mean  $\pm$  SEM



**Fig. 6** Relative expression levels of the five reference genes for three groups of different training conditions in olfactory learning of *A. cerana*. *S1* group: honeybees were trained using lemon/vanilla (concentration 100 %) as olfactory cues; *S2* group: honeybees were trained using lemon/vanilla (concentration 25 %) as olfactory cues. Honeybees were trained to learn to turn left for lemon, turn right for vanilla. Control group: honeybees were collected outside the maze and given no acquisition training. Different letters above bars indicate significant difference ( $P < 0.05$ ) between the groups. Values are mean  $\pm$  SEM

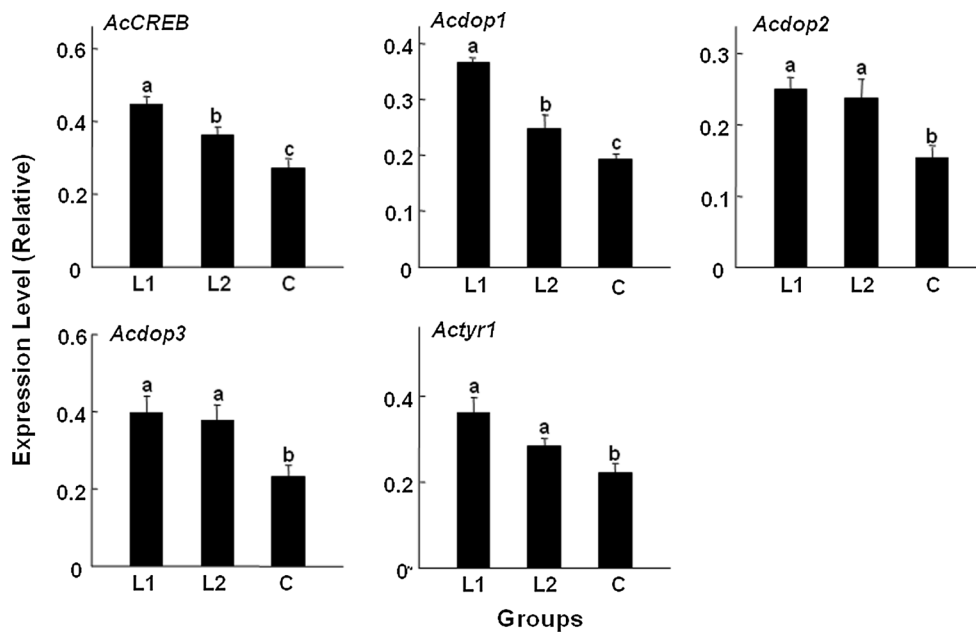


subsequently larger compared to *A. mellifera*. This finding is not consistent with Spaethe and Chittka's (2003) conclusion that larger body size tends to indicate a higher optical quality of the eye, resulting in higher visual resolution. One possible interpretation is that the visual capacity of *A. cerana* is better on grating learning than that of *A. mellifera* under controlled conditions (Qin et al. 2012).

The present study showed that trained honeybees could choose the right or left chamber of the Y-maze to get a sugar reward depending upon which odor they encountered at the apparatus entrance (Fig. 3a). The results indicate that *A.*

*cerana* is able to discriminate different scents (vanilla versus lemon) in a free-flying situation, and can use scent as a cue to detect the correct route through the maze; this finding is consistent with the report of Zhang (2006), who trained *A. mellifera* to learn Lemon and Mango in a Y-maze. Our study revealed that a lower concentration of the scent tends to result in a lower correct choice frequency, suggesting that the odor concentration has an important influence on the memory of honeybees (Pelz et al. 1997). Furthermore, when the concentration of scent is close to, or below, the threshold level, the honeybees cannot make the correct choice. Based





**Fig. 7** Relative expression levels of the five reference genes for three groups of different training conditions in cross-modal learning of *A. cerana*. *L1* group: honeybees were trained with the visual cue (45°, 4 cm) together with olfactory cue (lemon 100 %), and the visual cue (135°, 4 cm) together with the olfactory cue (vanilla 100 %); *L2* group: honeybees were trained with the visual cue (45°, 1.5 cm)

together with the olfactory cue (lemon 25 %), and the visual cue (135°, 1.5 cm) together with the olfactory cue (vanilla 25 %). Control group: honeybees were collected outside the maze and given no acquisition training. Different letters above bars indicate significant difference ( $P < 0.05$ ) between the groups. Values are mean  $\pm$  SEM

on neural circuits in the olfactory learning (Müller 2002), one possible explanation for this phenomenon would be that the olfactory information with a low concentration cannot be received by the chemosensory receptors on the antennae, resulting in the information not being transmitted into the brain of honeybees. The lemon/vanilla essence used in this study consisted of a variety of volatile compounds. However, previous studies demonstrated that the intensity and ratios of volatile compounds affects the ability of honeybees to differentiate among scents (Wright et al. 2005). Meanwhile, Wright and Smith (2004), by recording the responses to three kinds of odor concentrations, suggested that at least two different thresholds exist for perception of pure odors in honeybees. Taking these into account, a relative olfactory threshold is acquired under the present experiment conditions.

Our results indicated that the learning of *A. cerana* exhibits cross-modal reinforcement. This finding is consistent with the cross-modal study of *Drosophila* (Guo and Guo 2005). However, whether *A. cerana* could present cross-modal facilitation of visual and olfactory perception and cross-modal transfer of memory as *Drosophila*, is still unknown, and should be further investigated. In contrast to earlier studies about associative learning of honeybees (Srinivasan et al. 1998; Zhang et al. 1999; Reinhard et al. 2004), the present study indicated, firstly, that honeybees can associate an oblique black/white grating pattern with

an olfactory cue to build a complex picture of the target. Secondly, the capacity of honeybees to exhibit cross-modal learning is quantitatively assessed by modulating features of the visual-olfactory stimulus. Cross-modal learning has been widely reported in vertebrates and invertebrates, but its neural circuits, cellular and molecular mechanisms are not clearly known. Honeybees, which can be easily experimentally manipulated, show quick learning rates, and short generation times can thus be a useful model system for the study of cross-modal learning at the behavioral, cellular and molecular levels.

Analysis of the behavioral and molecular experiment showed that the expression levels of *AcCREB*, *Acdop2* and the correct choice frequency have a significant decline as the period of the visual cue decreased from 4 to 1.5 cm (Figs. 2, 5), that the levels of *AcCREB*, *Acdop1*, *Actyr1* and the correct choice frequency have a significant decline as the concentration of the olfactory cue decreased from 100 to 25 % (Figs. 3, 6), and that the levels of five genes (*AcCREB*, *Acdop1*, *Acdop2*, *Acdop3*, *Actyr1*) are up-regulated after cross-modal learning (Fig. 7). Our results indicate that *AcCREB*, *Acdop2* may be involved in the modulation of visual learning, that *AcCREB*, *Acdop1*, *Actyr1* may affect olfactory learning, and that *AcCREB*, *Acdop1*, *Acdop2*, *Acdop3*, *Actyr1* may be involved in cross-modal learning. Previous studies also have demonstrated that

these genes affect the learning and memory of invertebrates and vertebrates. Prominent examples include the following. The higher expression level of *CREB* gene caused greater synaptic efficacy and contributed to improve memory formation in mice (Zhou et al. 2009). The up-regulation of CREB transcriptional activity could enhance the LTM of transgenic mice (Suzuki et al. 2011). The down-regulation of the tyramine receptor gene caused a defective olfactory response in *Drosophila* (Kutsukake et al. 2000), and *Amyr1* has a wide-spread distribution in the antennal lobes and the mushroom bodies that are essential for olfactory learning (Blenau et al. 2000; Erber et al. 1980). The inhibition of the expression of *Dmdop1* reduced aversive and appetitive learning in *Drosophila* (Kim et al. 2007) and *AmDOP1* was involved in signal processing of visual and olfactory information in *A. mellifera* (Blenau et al. 1998). The defective expression of DAMB, the *Drosophila* ortholog of *AmDOP2*, enhanced memory retention and impaired reversal learning (Berry et al. 2012). Furthermore, the reduced expression of dopamine D3 receptor improved learning performance in memory-impaired rats (Laszy et al. 2005). Our results suggested that some genes might be specifically involved in visual or olfactory learning process. This phenomenon may be related to a specific expression pattern of each gene, and a complex interplay among these genes. So far, few studies have explored whether these genes would up- or down-regulate after learning behavior. Our results indicate that these genes are likely to play a functional role in the cognitive processes of honeybees, providing a basis for further studies of the further molecular mechanisms underlying cross-modal learning.

In conclusion, through the behavioral test, a robust visual threshold for black/white gratings (period 2.8°–3.8°) and relative olfactory threshold (concentration of lemon/vanilla: ranging from 50 to 25 %) were ascertained and cross-modal reinforcement of memory in *A. cerana* was demonstrated. In addition, our results indicate that five genes (*AcCREB*, *Acdop1*, *Acdop2*, *Acdop3*, *Actyr1*) have an important influence on the learning and memory of honeybees. Future research should be aimed at the cross-modal facilitation and transfer of memory, and elucidating the neural circuit of cross-modal learning in *A. cerana* to fully understand the molecular mechanism of the learning and memory of honeybees.

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