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Comparative analysis of olfactory learning of *Apis cerana* and *Apis mellifera*

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Abstract – The Eastern honeybee, *Apis cerana*, is indigenous to Asia and is an important pollinator for Asian ecosystems; the Western honeybee, *Apis mellifera*, has been introduced to Asia because of its high honey yields. These two species are now sympatric and share a similar environment. Whether learning in *A. cerana* can be studied using the proboscis extension response paradigm, as developed for *A. mellifera*, is still unexplored. Here, we investigate *A. cerana*'s associative olfactory learning with three different odors (hexanal, nonanal, and geraniol) and compared it with the learning performances of *A. mellifera*. After an acquisition phase, the bees were tested to determine whether they discriminated between the experienced odors (an odor paired with a reward during acquisition) from non-experienced odors (an odor not paired with a reward during acquisition). During the acquisition phase, *A. mellifera* showed higher learning scores than *A. cerana*. However, there was no statistical difference between the two species in the retention phase. Both species discriminated an experienced odor from a non-experienced odor. Our results suggested that *A. cerana* is equally amenable to the study of learning using the proboscis extension response paradigm supporting the behavioral evidence for future olfactory learning research with *A. cerana*.

Apis cerana / olfactory learning / proboscis extension response

1. INTRODUCTION

The Western honeybee, *Apis mellifera*, is well established as a model for studying olfactory learning using a classical association assay. This assay is based on the powerful Pavlovian conditioning protocol, the proboscis extension response (PER; Bitterman et al. 1983; Giurfa and Sandoz 2012; Matsumoto et al. 2012). In PER conditioning, a hungry bee is first exposed to a conditioned stimulus (CS; such as an odor, color, or texture) followed by gently touching the bee's antennae with unconditioned stimulus (US) that elicits proboscis extension response; US was then delivered to the proboscis.

If the bee responds with proboscis extension when presented with CS, this suggests that the bee has learned of the association (Bitterman et al. 1983).

Recently, PER assays have been used to investigate the learning ability of other bee species. Some have proved amenable to the use of PER, such as *Vespula* wasps (Vorel and Pitts-Singer 2010) and the red dwarf honey bee *A. florea* (Kaspi and Shafir 2013). Conversely, PER has proved to be an ineffective tool for some species such as the stingless bee *Scaptotrigona depilis* (Mc Cabe et al. 2007) and solitary species of megachilids (*Osmia lingnaria*, *Megachile rotundata*, and *Megachile pugnata*; Vorel and Pitts-Singer 2010).

A. mellifera and *Apis cerana* have evolved in distinct ecologies; their social organization as well as mating behavior has been successfully shaped by their respective ecosystems. *A. mellifera* was

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introduced in China in the 1920s for its high productivity (Yang 2005) and now live sympatric with *A. cerana* in Asia. *A. cerana* was smaller in body size than *A. mellifera* and also showed stronger merits in resisting bee mites (Peng et al. 1987), hornets (Tan et al. 2012a, b), and extreme climates (Tan et al. 2012a, b). Studies utilizing color and grating patterns to investigate learning differences between these two species suggested that *A. cerana* perform as well as *A. mellifera* (Qin et al. 2012). However, the role of olfaction in learning in *A. cerana* is still unknown. Here, we aim to (a) determine whether *A. cerana* workers can be used to study learning applying the classic PER conditioning paradigm, (b) investigate the olfactory learning ability of *A. cerana*, and (c) compare this ability with olfactory associative learning of *A. mellifera*.

2. MATERIALS AND METHODS

Three *A. cerana* and three *A. mellifera* test colonies were set up at an apiary of the Eastern Bee Research Institute, Yunnan Agricultural University, Kunming, China. All colonies were queenright and housed in standard Langstroth hives. Before we started our experiments, we equalized the colonies such that each contained four frames covered with adult workers and at least two frames of brood and two frames of honey and pollen.

Olfactory conditioning experiments were conducted between May and July 2012. We blocked the entrances to the hives and collected 20 returning foragers (nectar foragers but not pollen foragers) from each colony per trial. These bees were transferred to the laboratory and narcotized on ice. The bees were harnessed in 0.5-ml plastic centrifuge tubes which had the bottoms cut out according to the head sizes of *A. cerana* and *A. mellifera*, respectively (Figure 1). Thus, the bees were restrained in such a way that they were still able to move their heads and proboscis. The bees were fed 10 μ L of 2 M sucrose solution and placed in an incubator (20 °C and 65 % RH) overnight. The next morning, we trained all surviving bees to associate an odor with a sucrose reward using the PER procedure (Bitterman et al. 1983). Prior to training, we tested the bees and discarded any that showed no PER to sugar solution (unconditioned stimulus) or showed PER to air flow carrying a test odor.

Odors were delivered to the bees from three parallel 20-mL syringes mounted on a training station. One of the syringes contained only filter paper to deliver air flow as continuous stream. In the other two syringes, 2 μ L of each pure odor, hexanal (98 %; Sigma-Aldrich, Co., St. Louis, USA), nonanal (98 %; Sigma-Aldrich), and geraniol (96 %; Sigma-Aldrich, Co) was placed on a strip of filter paper (5 $\times$ 5 mm) inside each syringe. The tip of the syringe was placed 1.5 cm from the bees' head, while a valve was attached to the other end along with a Y-shaped tube, silicon tube, and air pump. An air stream exhaust system was formed by a 10-cm diameter tube that was placed 12 cm behind the bee.

For proboscis extension response experiments, bees were exposed to a constant flow of clean air for 35 s, and then to a flow containing a particular odor for 6 s (CS). Three seconds after the odor stimulus onset, we touched the test bee's antennae with a toothpick soaked in sucrose solution for 3 s. Bees showing proboscis extension in the first 3 s after exposure to the odors were recorded as 1; those that did not were recorded as 0. In the acquisition phase, each bee was trained for six conditioning trials sequentially at an inter-trial interval of 10 min. We performed retention tests 24 h after the acquisition phase by presenting only the particular odor. The response of each bee was tested with a pair of odors; the first was the one experienced during the acquisition phase (represented by a CS), followed by a novel odor (represented by non-experienced odors (NOD); Matsumoto et al. 2012), which were tested for bees' CS-specific memory. The following CS/NOD pairs were established: CS_{hexanal}/NOD_{nonanal}, CS_{nonanal}/NOD_{geraniol}, CS_{geraniol}/NOD_{hexanal}. The procedure was repeated with hexanal, nonanal, and geraniol as CSs to test for any odor bias in both *A. cerana* and *A. mellifera*. The number of tested bees for hexanal, nonanal, and geraniol odor of *A. cerana* were 39, 43, and 53, respectively, while the number of tested bees of *A. mellifera* were 57, 45, and 48, respectively.

To determine if the learning abilities of *A. cerana* and *A. mellifera* was affected by their hunger motivation, the gustatory responsiveness was measured in each individual of *A. cerana* ($n=64$) and *A. mellifera* ($n=64$). One hour after mounting in the tube, each individual was first stimulated with a droplet of water and then with the following sucrose solutions offered in ascending order: 0.1, 0.3, 1, 3, 5,

Figure 1. Tethered bees in plastic tube so that their heads and probosces could move freely.

10, and 30 % to test its PER. The interstimulus interval was about 2 min to exclude sensitization effects (modified from Scheiner et al. (2005)).

2.1. Statistics

Data were recorded as the proportion of bees exhibiting PER of the total number of bees for each species and odor. This data were analyzed with a repeated measures ANOVA, using trials as the within-subject effect and bee species (*A. cerana* and *A. mellifera*) and CSs (hexanal, nonanal, and geraniol) as the between-subject effect, respectively. We performed Mauchly's test for sphericity to ensure that the assumption of sphericity was not violated. We used post hoc tests (least significant differences) to determine if there were differences between the two species.

Paired *T* tests were used to determine if there were differences of the retention scores between *A. cerana* and *A. mellifera* for experienced odors (CS pairing with reward, CS_{hexanal}, CS_{nonanal}, and CS_{geraniol}) and NOD, which pairing without any reward during acquisition phase (NOD_{hexanal}, NOD_{nonanal} and NOD_{geraniol}, respectively).

The different gustatory responsiveness was analyzed with chi-square tests between *A. cerana* and *A. mellifera* to every different sucrose concentrations. All calculations were performed with SPSS Statistics 17.0 (www.spss-china.com).

3. RESULTS

In *A. cerana*, bees are able to associate an odor with reward. PER scores rose from 0 % to nearly 60 % for hexanal, a significant increase (repeated measures ANOVA, $F=36.314$, $df=5$, $P<0.001$). For nonanal and geraniol, learning scores also increased significantly with number of trials (repeated measures ANOVA, $F=3.503$, $df=10$, $P<0.001$). There were no significant differences in the abilities of bees to learn a conditioned response to each of the three different odors (CSs effect: $F=0.92$, $df=2$, $P=0.401$; Figure 2).

However, there were some differences of bees in odor response in the first three trials, while after six training trials, about 60 % of bees responded to all of the odors (trial 2: $F=9.09$, $df=2$, $P<0.001$; trial 3: $F=5.59$, $df=2$, $P=0.005$; trial 4: $F=0.39$, $df=2$, $P=0.675$; trial 5: $F=0.75$, $df=2$, $F=0.472$; trial 6: $F=0.17$, $df=2$, $P=0.841$; Figure 2).

Retention tests showed that for *A. cerana*, responses to experienced odors were significantly higher than for non-experienced odors (CS_{hexanal}/NOD_{nonanal}: $T=4.42$, $df=38$, $P<0.001$; CS_{nonanal}/NOD_{geraniol}: $T=6.04$, $df=42$, $P<0.001$; CS_{geraniol}/NOD_{hexanal}: $T=5.21$, $df=52$, $P<0.001$). No statistical difference was found for hexanal, nonanal, and geraniol in either the experienced ($F=0.6$, $df=2$, $P=0.55$) or the non-experienced group ($F=1.57$, $df=2$, $P=0.213$).

Figure 2. Acquisition of olfactory conditioning in *A. cerana* and *A. mellifera* in response to hexanal, nonanal, and geraniol, respectively. The purple, dark blue, and the light blue lines represent *A. mellifera*'s acquisition function for hexanal ($n=57$), nonanal ($n=45$), and geraniol ($n=48$), with a sucrose reward, respectively. The green, yellow, and orange lines represent the same for *A. cerana*'s for hexanal ($n=39$), nonanal ($n=43$), and geraniol ($n=53$) with sucrose reward respectively.

A. mellifera also showed a significant increase in learning scores during the acquisition phase ($F=95.792$, $df=5$, $P<0.001$). However, *A. mellifera* showed significantly higher learning scores than *A. cerana* did ($F=39.635$, $df=1$, $P<0.001$; Figure 2, Table I).

Both *A. cerana* and *A. mellifera* showed high retention scores (up to 60 %) after 24 h of training. There were no significant difference between these two bee species to CS_{hexanal}/NOD_{nonanal} pair (hexanal: $F=0.91$, $df=1$, $P=0.343$; nonanal: $F=0.37$, $df=1$, $P=0.546$). Similar results were obtained for CS_{nonanal}/NOD_{geraniol} pair (nonanal: $F=0.36$, $df=1$, $P=0.551$; geraniol: $F=0.45$, $df=1$, $P=0.505$) and in CS_{geraniol}/NOD_{hexanal} (geraniol: $F=1.39$, $df=1$, $P=0.242$; hexanal: $F=0.26$, $df=1$, $P=0.873$; Figure 3). There was an increase in response to the last conditioning trial and the retention test in both species; however, there was no statistic significant in nonanal and geraniol. There was a decline even been found when *A. mellifera* learnt of geraniol. That is may be because geraniol was the component of Nasonov pheromone (Table II).

A. mellifera bees were more responsive to low sucrose concentrations (0.3 %: $\chi^2=6.81$, $P=0.009$; 1 %: $\chi^2=8.003$, $P=0.005$ and 3 %: $\chi^2=6.817$, $P=0.009$) than *A. cerana* bees, while there were no significantly statistical differences in water, 0.1 % sucrose group and 5 % and other two high concentration groups (Figure 4).

4. DISCUSSION

Our results show that the classical PER conditioning can be applied to *A. cerana*. Twenty-four-hour retention scores of *A. cerana* were similar to those of *A. mellifera*. However, *A. cerana*'s PER acquisition scores were lower than those of *A. mellifera* after six trials (Figure 2). Several other bee species are known to be amenable to the PER paradigm such as the stingless bees *Melipona quadrifasciata* (Vorel and Pitts-Singer 2010) and eusocial *Vespula* wasps (Vorel and Pitts-Singer 2010). Among *Apis*, learning performance was only well-studied in different subspecies of *A. mellifera*, with only one study performed on *A. florea* (Kaspi and Shafir 2013). Our findings suggest

Table 1. Repeated measures ANOVA on the effect of species (*A. cerana* and *A. mellifera*) during acquisition of three odors (hexanal, nonanal, and geraniol), respectively, and on conditioned PER.

	<i>df</i>	MS	F	<i>P</i>
Between subjects				
Species	1	24.077	39.635	<0.001
CSs	2	0.978	1.609	0.202
Species × CSs	2	0.001	0.002	0.998
Error	279	0.607		
Within subjects				
Trials	3.466	24.135	170.115	<0.001
Trials × species	3.466	1.605	11.310	<0.001
Trials × CSs	6.932	0.312	2.196	0.033
Trials × species × CSs	6.932	0.368	2.593	0.012
Error	967.075	0.142		

The assumption of sphericity was violated (Mauchly's test: $W=0.353$, $\chi^2=288.286$, $df=14$, $P<0.001$), so the results are reported with the Greenhouse–Geisser correction to degrees of freedom

Figure 3. Mean retention scores ($\pm$ SD) of *A. cerana* and *A. mellifera* for experienced and non-experienced odors. To eliminate odor bias, hexanal paired with a reward during the acquisition phase (CS_{hexanal}) was compared with the non-experienced odor (NOD_{nonanal}). The CS_{nonanal} group was also compared with NOD_{geraniol} group, while the CS_{geraniol} group compared with NOD_{hexanal}. Odors paired with the reward were termed as experienced odors (brown), while those not paired with the reward were termed as non-experienced odors (dark yellow).

Table II. The retention scores between last training trial and the retention trail in both *A. mellifera* and *A. cerana*.

	<i>A. mellifera</i>				<i>A. cerana</i>			
	Last trial	Retention	χ^2	<i>P</i>	Last trial	Retention	χ^2	<i>P</i>
Hexanal	70.18	82.46	3.947	0.047	59	74.36	5.05	0.025
Nonanal	68.89	71.11	0.095	0.758	60.5	65.12	0.455	0.5
Geraniol	81.25	75	1.049	0.306	54.7	64.15	1.681	0.195

that *A. cerana* is also amenable to PER conditioning. Thus, *A. cerana* may also be of use as a model organism for physiological and neurobiological research.

The paradigm we have used to compare learning and memory in these two *Apis* species relies on the fact that a hungry bee reflexively extends its proboscis in response to sucrose stimulation of the antennae and associates an odor stimulus with the sucrose reward. Associative learning under these conditions is usually rapid with successful acquisition within few trials of forward pairing (Bitterman et al. 1983). Memory is formed in a trial- and time-dependent manner indicating processes of memory consolidation that can be traced to particular regions of the bee brain and to molecular and cellular processes (Menzel 1999, 2012; Giurfa

2007; Giurfa and Sandoz 2012). Acquisition and retention depend on many parameters, e.g., the state of satiation reflecting the motivation of the animal and the reward value of a particular concentration of sucrose solution (Erber et al. 1980). Here, we still cannot exclude the possibility that rather unspecific parameters could have also influenced our experiments. For example, the two species may have had differing abilities to adapt to the confined test conditions or may have required different amounts of food in order to achieve satiation meaning that the reward value of the sucrose solution could have been different for each species.

A. cerana had lower PER scores than *A. mellifera* in the first three trials of the acquisition phase, suggesting that it is a slow learner. This may be because the strain of *A. mellifera*

Figure 4. Gustatory responsiveness between *A. cerana* (blue line) and *A. mellifera* (pink line). The x-axis shows the water stimulation (0) and the logarithm of the sucrose concentrations tested. The y-axis gives the percentage of bees showing the proboscis extension response (PER). Star marks (***P*<0.01) indicated significant differences, ns presented no significant difference.

that was introduced in China has been subjected to intense artificial selection to be more docile, while the *A. cerana* we utilized were managed in a half wild situation. Thus, these two bee species could have different reactions to harnessing. Yang (2009) reported even though there are overlaps on its niche between *A. cerana* and *A. mellifera*, these two species have its own properties to reduce the food competition. *A. cerana* is adept in collecting sporadic nectar flowers, while *A. mellifera* exploits large flower fields. In dearth period, we always can see that *A. mellifera* colonies are shorter of nectar than *A. cerana* colonies when they are kept in a constant place. This may indicate that *A. cerana* can better use the little patches near the hives than *A. mellifera* do. *A. cerana* always meet the lower selective pressure to learn associations between odors and rewards. This may be one reason why *A. cerana* performed worse than *A. mellifera* during their acquisition phase.

After acquisition, there was no significant difference between these two sibling species in memory retention of different odors. Both species discriminated between the experienced odor and non-experienced odor. These results are consistent with previous studies on odor learning and odor discrimination in bees (Bitterman et al. 1983; Menzel 1999).

We conclude that *A. cerana* is amenable to be studied using classic olfactory PER assay. However, further work is required to unveil the physiology of the olfactory learning mechanism based on this behavioral study.

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Analyse comparative de l'apprentissage olfactif d'*Apis cerana* et d'*Apis mellifera*

Apis cerana / apprentissage / olfaction / REP / réflexe d'extension du proboscis

Vergleich des olfaktorischen Lernens von *Apis cerana* und *Apis mellifera*

Apis cerana / *Apis mellifera* / Duftlernen / Rüsselreflex

REFERENCES

- Bitterman, M., Menzel, R., Fietz, A., Schäfer, S. (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**(2), 107–119
- Erber, J., Masuhr, T., Menzel, R. (1980) Localization of short term memory in the brain of the bee, *Apis mellifera*. *Physiol. Entomol.* **5**(4), 343–358
- Giurfa, M. (2007) Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* **193**, 801–824
- Giurfa, M., Sandoz, J.C. (2012) Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* **19**(2), 54–66
- Kaspi, R., Shafir, S. (2013) Associative olfactory learning of the red dwarf honey bee *Apis florea*. *Apidologie* **44**(1), 100–109
- Matsumoto, Y., Menzel, R., Sandoz, J.C., Giurfa, M. (2012) Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step towards standardized procedures. *J. Neurosci. Methods* **211**, 159–167
- McCabe, S., Hartfelder, K., Santana, W., Farina, W. (2007) Odor discrimination in classical conditioning of proboscis extension in two stingless bee species in comparison to Africanized honeybees. *J. Comp. Physiology A: Neuroethol. Sens. Neural. Behav. Physiol.* **193**(11), 1089–1099
- Menzel, R. (1999) Memory dynamics in the honeybee. *J. Comp. Physiology A: Neuroethol. Sens. Neural. Behav. Physiol.* **185**(4), 323–340
- Menzel, R. (2012) The honeybee as a model for understanding the basis of cognition. *Nature Rev. Neurosci.* **13**, 758–768
- Peng, Y.-S., Fang, Y., Xu, S., Ge, L. (1987) The resistance mechanism of the Asian honey bee, *Apis cerana* Fabr., to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. *J. Invertebr. Pathol.* **49**(1), 54–60

- Qin, Q.H., He, X.J., Tian, L.Q., Zhang, S.W., Zeng, Z.J. (2012) Comparison of learning and memory of *Apis cerana* and *Apis mellifera*. J. Comp. Physiol. A: Neuroethol. Sens. Neural. Behav. Physiol. **198**(10), 777–8
- Scheiner, R., Kuritz-Kaiser, A., Menzel, R., Erber, J. (2005) Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees. Learn. Mem. **12**(6), 626–635
- Tan, K., Wang, Z., Li, H., Yang, S., Hu, Z., Kastberger, G., Oldroyd, B.P. (2012a) An ‘I see you’ prey–predator signal between the Asian honeybee, *Apis cerana*, and the hornet, *Vespa velutina*. Anim. Behav. **83**(4), 879–882
- Tan, K., Yang, S., Wang, Z.W., Radloff, S.E., Oldroyd, B.P. (2012b) Differences in foraging and broodnest temperature in the honey bees *Apis cerana* and *A. mellifera*. Apidologie **43**(6), 618–623
- Vorel, C.A., Pitts-Singer, T.L. (2010) The proboscis extension reflex not elicited in megachilid bees. J. Kansas Entomol. Soc. **83**(1), 80–83
- Yang, G.H. (2005) Harm of introducing the western honeybee *Apis mellifera* L. to the Chinese honeybee *Apis cerana* F. and its ecological impact. Acta Entomol. Sin. **48**, 401–406 (In Chinese)
- Yang, G.H. (2009) Chinese honeybee *Apis cerana* F. influence on Chinese forestry ecological system. Apic. China **60**, 5–10 (In Chinese)