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Inhibiting DNA methylation alters olfactory extinction but not acquisition learning in *Apis cerana* and *Apis mellifera*

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ABSTRACT

DNA methylation plays a key role in invertebrate acquisition and extinction memory. Honey bees have excellent olfactory learning, but the role of DNA methylation in memory formation has, to date, only been studied in *Apis mellifera*. We inhibited DNA methylation by inhibiting DNA methyltransferase (DNMT) with zebularine (zeb) and studied the resulting effects upon olfactory acquisition and extinction memory in two honey bee species, *Apis cerana* and *A. mellifera*. We used the proboscis extension reflex (PER) assay to measure memory. We provide the first demonstration that DNA methylation is also important in the olfactory extinction learning of *A. cerana*. DNMT did not reduce acquisition learning in either species. However, zeb bidirectionally and differentially altered extinction learning in both species. In particular, zeb provided 1 h before acquisition learning improved extinction memory retention in *A. mellifera*, but reduced extinction memory retention in *A. cerana*. The reasons for these differences are unclear, but provide a basis for future studies to explore species-specific differences in the effects of methylation on memory formation.

1. Introduction

Honeybees are an important and useful invertebrate model for studying learning and memory (Menzel, 1999, 2001, 2012) because they exhibit excellent learning and memory of colors, patterns, landmarks, time, and odor (Menzel et al., 1996; Srinivasan et al., 1998). For example, honeybees can easily learn to associate an odorant (conditioned stimuli, CS) with a sugar reward (unconditioned stimuli, US) during the olfactory conditioning of the proboscis extension reflex (PER) (Bitterman et al., 1983; Menzel and Giurfa, 2006). After one to three trials of olfactory reward training, short term memory is formed, followed by the formation of long term memory, which, unlike short term memory, depends upon protein synthesis (Giurfa, 2007; Menzel and Muller, 1996; Grunbaum and Muller, 1998; Muller, 1996, 2000; Friedrich et al., 2004).

In insects, such as honey bees, DNA methyltransferase activity (Wang et al., 2006) is crucial for memory formation (Biergans et al., 2012, 2015). DNA methylation also plays a role in the preservation of long term vertebrate memories. Miller et al. (2010) showed that rat long term memory (30 days after training) was disrupted by zeb. In invertebrates, such epigenetic mechanisms are also necessary for learning and memory (Lockett et al., 2010; Biergans et al., 2012, 2015). Thus, DNA methylation is likely an ancestral and conserved mechanism of memory formation.

Acquisition (Greggers and Menzel, 1993) and extinction memory (Bouton and Moody, 2004; Couvillon and Bitterman, 1980; Menzel, 1968) are both key for honey bee foragers, which must learn the locations and characteristics of ephemeral food resources, but do not need to retain these memories once other food resources become available. In *Apis mellifera*, Lockett et al. (2010) showed that DNMT inhibition could alter the rate of extinction learning, depending upon the timing of chemical inhibition relative to memory formation. DNMT inhibition, applied at different time points, bidirectionally affected extinction memory formation during extinction learning.

Although *A. mellifera* is the most common model for bee learning, a sister species, *Apis cerana* is an emerging model for studying learning (Qin et al., 2012; Wang and Tan, 2014; Zhang et al., 2014). *Apis cerana* is an important Asian species that plays a key role in the pollination of crops and native plants (Partap and Verma, 1993, 1994; Kremen et al., 2004), an ecosystem service in which learning plays a role because bees using their highly developed memory to learn which plants offer rewarding food (Giurfa,

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2007). Both bee species share common genetic and physiological traits and *A. cerana* diverged from *A. mellifera* approximately 2–3 million years ago (Oldroyd and Wongsiri, 2006; Ruttner, 1988). Comparisons between these species are valuable because they facilitate understanding the evolutionary history of learning and memory in honeybees. For example, *A. cerana* workers exhibit better visual learning than *A. mellifera* (Qin et al., 2012), but *A. cerana* has poorer olfactory learning than *A. mellifera* (Wang and Tan, 2014). To date, however, no studies have investigated the role of DNA methylation in *A. cerana* or compared the role of DNA methylation between both species. Our goal was thus to determine if DNA methylation has different effects upon the formation and extinction of olfactory memory in these species. Because of the sensitivity of learning and memory studies to experimental conditions (Menzel and Muller, 1996), we compared both species in tests run under identical laboratory conditions.

2. Materials and methods

2.1. Study site and colonies

We used three colonies of *A. cerana* and three colonies of *A. mellifera* at the apiaries of the Apicultural Research Institute, Yunnan Academy of Agricultural Sciences, Yunnan, China. Experiments were conducted from April to September 2015 and February to 2016. We used five treatments and a control for each of these five treatments (sample sizes in Figs. 1 and 2). Thus, we used a total of 459 *A. cerana* and 489 *A. mellifera* foragers. Detailed sample sizes are given in Table 1.

2.2. Conditioning procedure

We based our protocol upon Lockett et al. (2010), but used absolute conditioning instead of differential conditioning (Giurfa, 2007) and provided bees with separate olfactory and reward (sucrose solution) stimuli, rather than odor in the sucrose reward. Absolute conditioning tests the ability of bees to associate a novel odor with a food reward, but differential conditioning tests the ability of bees to discriminate between odors. We therefore examined a different type of learning than Lockett et al. (2010) in order to expand our understanding of the role of DNA methylation in honey bees.

We captured returning foragers from bee colony entrances and anesthetized them on ice for 5 min until bee movement significantly diminished. We then harnessed bees in 0.5 ml plastic centrifuge tubes that had the holes cut out of the tips to fit the different head sizes of *A. cerana* and *A. mellifera*. Each restrained bee could still move its head and proboscis. We tested olfactory learning and memory with a proboscis extension response (PER) assay as previous described (Bitterman et al., 1983). Unlike Lockett et al. (2010) who used natural vanilla odor in their rewarding stimulus (Maleszka et al., 2000), we used the odor of hexane as the rewarded stimulus (Sigma-Aldrich, Co. St. Louis, USA). We placed 5 µl of hexane onto a filter paper (1 cm × 1 cm) into a syringe. During the test, bees were exposed to a continuous air flow of 0.5 L/min, but hexane was only supplied as a conditioned stimulus (CS), as described below. A fan placed 12 cm behind the test bee exhausted all odors. During acquisition training, the CS (hexane) was paired with the unconditioned stimulus (US: 30% w/w pure unscented sucrose solution in a pipette tip) as a reward. We lightly tapped one antenna with the US to elicit PER and then allowed the bee to feed. The US elicits a proboscis extension response (the unconditioned response). Once the bee is classically conditioned, the CS (odor) alone will elicit PER (Bitterman et al., 1983).

The US was presented 3 s after CS and overlapped with the CS for 2 s. If a bee exhibited learning, it would extend its proboscis during the presentation of the CS only. The subsequently pairing of CS + US reinforces this olfactory learning. However, not reinforcing this learning with the sugar reward (presenting the CS only)

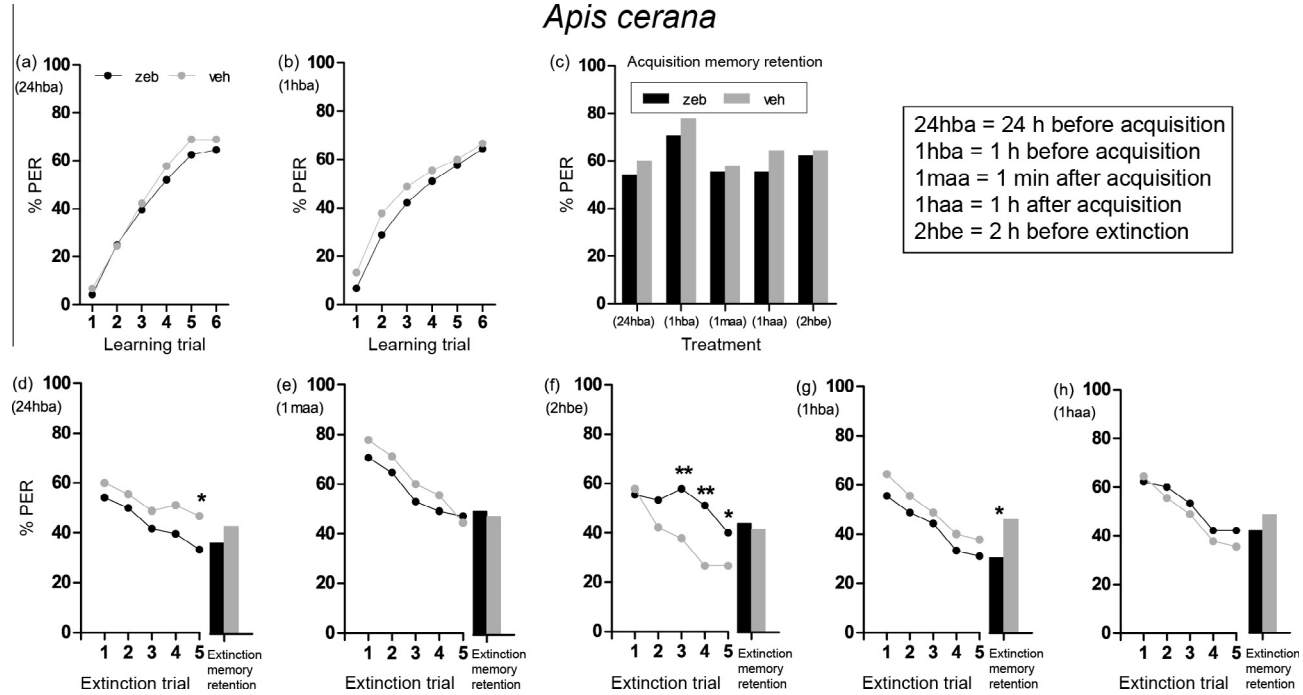

Fig. 1. In *A. cerana*, the effects of DNA methyltransferase inhibition (zeb) on (a, b) acquisition learning, (c) acquisition memory retention (tested 24 h after the last acquisition trial), and (d–h) extinction learning and extinction memory retention for the five different zeb treatments (see figure legend). Zeb did not significantly alter (a, b) acquisition learning or (c) acquisition memory retention for any of the five tested treatments. However, there are significant effects of some zeb treatments upon (d–h) extinction learning and extinction memory retention. Asterisks above each trial show significant differences (Chi-square test: $^{*}P < 0.05$, $^{**}P < 0.01$). The extinction memory results (d–h) are organized to show the bidirectional modulation of extinction memory. Sample sizes are shown in Table 1.

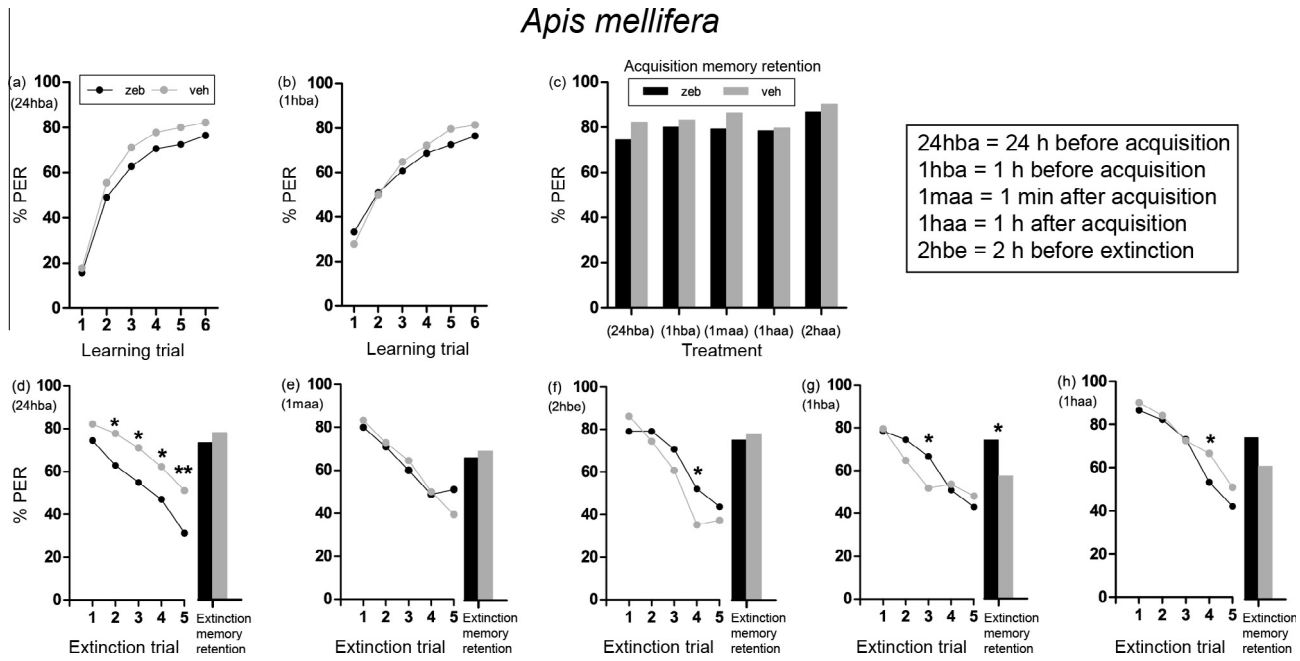

Fig. 2. In *A. mellifera*, the effects of DNA methyltransferase inhibition on (a, b) acquisition learning, (c) acquisition memory retention (tested 24 h after the last acquisition trial), and (d–h) extinction learning and extinction memory retention for the five different zeb treatments (see figure legend). Zeb did not significantly alter (a, b) acquisition learning or (c) acquisition memory retention for any of the five tested treatments. However, there are significant effects of some zeb treatments upon (d–h) extinction learning and extinction memory retention. Asterisks above each trial show significant differences (Chi-square test: $^{*}P < 0.05$, $^{**}P < 0.01$). The extinction memory results (d–h) are organized to show the bidirectional modulation of extinction memory. Sample sizes are shown in Table 1.

Table 1
The number of *Apis cerana* (*Ac*) and *Apis mellifera* (*Am*) foragers used in at each treatment time point from each colony (veh = DMF solvent vehicle, zeb = zebularine).

	Colony		Treatment time point									
			24 hba		1 maa		2 hbe		1 hba		1 haa	
			veh	zeb	veh	zeb	veh	zeb	veh	zeb	veh	zeb
Bee species		C1	15	16	15	17	15	15	15	15	15	15
	Ac	C2	15	16	15	17	15	15	15	15	15	15
		C3	15	16	15	17	15	15	15	15	15	15
	Total bees per group		45	48	45	51	45	45	45	45	45	45
		C1	15	17	16	15	17	16	18	17	17	15
	Am	C2	15	17	16	15	17	16	18	17	17	15
		C3	15	17	16	15	17	16	18	17	17	15
	Total bees per group		45	51	48	45	51	48	54	51	51	45

will lead to extinction memory because the bee is no longer being rewarded for proboscis extension (Deisig et al., 2001; Johannes et al., 2012; Giurfa and Sandoz, 2012; Eisenhardt, 2012). Each bee was trained six times, with an inter-trial interval of 10 min. This inter-trial interval facilitates honeybee olfactory learning (Menzel et al., 2001). The increased number of reinforcement trials is another difference between our protocol and that of Lockett et al. (2010), who used three trials. We used six trials to test the effect of stronger acquisition learning.

Like Lockett et al. (2010), we then waited 24 h after the sixth acquisition training trial to test acquisition memory retention by presenting the CS only with no US. Immediately after this retention test, we began extinction training. In these extinction trials, we continued to present bees with CS only and used an inter-trial interval of 2 min for five extinction trials. As a final measure of extinction memory retention, we waited 5 h after the fifth extinction trial and tested the bees' responses to the CS only.

We used five treatment groups consisting of applying zeb (1 μl of 2 mM zeb in DMF solvent per bee) topically to bee thoraces at different time points relative to memory formation (Lockett et al., 2010). Zeb can form a covalent complex with all DNA methyltransferases and thereby inhibits multiple DNMT's (Zhou et al., 2002). We used the following treatments: (24hba) 24 h before acquisition training, (1hba) 1 h before acquisition training, (1maa) 1 min after acquisition training, (1haa) 1 h after acquisition training, and (2hbe) 2 h before extinction training.

Control treatments consisted of a separate group of control bees receiving 1 μl of the DMF solvent vehicle (veh) at the same time points. The topical thoracic application of DMF as a vehicle for a neuroactive compound is a standard procedure (Barron et al., 2007; Lockett et al., 2010). The control treatment and all other treatments were run simultaneously in each trial.

2.3. Statistics

We analyzed our response variable, PER, with repeated-measures ANOVA, using bee identity as the repeated-measure, trials as the within-subject effect, and treatment (zeb or veh) or bee species (*A. cerana* or *A. mellifera*) as fixed effects. We used Chi-square tests to perform post hoc pairwise comparisons. All

calculations were performed with SPSS Statistics 17.0 (www.spss-china.com) statistical software.

3. Results

3.1. No effect of DNMT inhibition on A. cerana (Ac) and A. mellifera (Am) acquisition learning

Foragers of both bee species exhibited short term learning in the 24 hba treatment (trial effect: *Ac*: $F_{5,455} = 46.51$, $P < 0.0001$; *Am*: $F_{5,470} = 46.80$, $P < 0.0001$, Figs. 1a and 2a) and the 1 hba treatment (*Ac*: $F_{5,440} = 26.76$, $P < 0.0001$; *Am*: $F_{5,515} = 30.93$, $P < 0.0001$, Figs. 1b and 2b).

However, there was no significant effect of treatment in either species when zeb was administered 24 h (*Ac*: $F_{1,91} = 0.28$, $P = 0.60$, Fig. 1a; *Am*: $F_{1,94} = 0.94$, $P = 0.335$, Fig. 2a) or 1 h (*Ac*: $F_{1,88} = 0.59$, $P = 0.44$, Fig. 1 b; *Am*: $F_{1,103} = 0.11$, $P = 0.74$, Fig. 2b) before acquisition learning. There were also no significant treatment∗trial interactions for the 24 hba (*Ac*: $F_{5,455} = 0.13$, $P = 0.99$; *Am*: $F_{5,470} = 0.10$, $P = 0.99$) or at 1 hba (*Ac*: $F_{5,440} = 0.12$, $P = 0.99$; *Am*: $F_{5,515} = 0.49$, $P = 0.78$) treatments.

For all treatments, there was no effect of zeb on acquisition memory retention (*Ac:* 24hba: $X^2_{(1)} = 0.73$, $P = 0.39$; 1hba: $X^2_{(1)} = 1.02$, $P = 0.25$; 1maa: $X^2_{(1)} = 1.29$, $P = 0.26$; 1haa: $X^2_{(1)} = 0.09$, $P = 0.77$; 2hbe: $X^2_{(1)} = 0.08$, $P = 0.78$, Fig. 1c. *Am*: 24hba: $X^2_{(1)} = 1.45$, $P = 0.23$; 1hba: $X^2_{(1)} = 0.12$, $P = 0.73$; 1maa: $X^2_{(1)} = 0.30$, $P = 0.59$; 1haa: $X^2_{(1)} = 0.44$, $P = 0.51$; 2hbe: $X^2_{(1)} = 1.70$, $P = 0.19$, Fig. 2c).

Between species, *Am* exhibited higher PER than *Ac* for every treatment (on average, 80% vs. 60%, respectively, Figs. 1a, b and 2a, b) and acquisition learning was significantly different for every treatment (24hba: $F_{(1,97)} = 6.43$, $P = 0.01$; 1hba: $F_{(1,94)} = 7.89$, $P = 0.006$; 1maa: $F_{(1,94)} = 4.70$, $P = 0.03$; 1haa: $F_{(1,88)} = 23.75$, $P < 0.0001$; 2hbe: $F_{(1,91)} = 8.79$, $P = 0.004$).

Am exhibited significantly higher acquisition memory retention than *Ac* for every treatment, too (24hba: $X^2_{(1)} = 10.147$, $P = 0.01$; 1hba: $X^2_{(1)} = 16.817$, $P < 0.0001$; 1maa: $X^2_{(1)} = 3.947$, $P = 0.047$; 1haa: $X^2_{(1)} = 16.044$, $P < 0.0001$; 2hbe: $X^2_{(1)} = 14.483$, $P < 0.0001$).

3.2. DNMT inhibition altered extinction learning in Am and Ac

Zeb significantly altered extinction learning and extinction retention for some treatments. In *Ac*, extinction learning was significantly altered by zebularine treatment 24 h before acquisition (trial 5: $X^2_{(1)} = 4.08$, $P = 0.04$) and 2 h before extinction (trial 3: $X^2_{(1)} = 8.01$, $P = 0.005$; trial 4: $X^2_{(1)} = 12.11$, $P = 0.001$, and trial 5: $X^2_{(1)} = 5.88$, $P = 0.02$), as compared to controls (Fig. 1).

In *Am*, extinction learning was significantly altered by zeb treatment 24 h before acquisition (trial 2: $X^2_{(1)} = 5.41$, $P = 0.02$; trial 3: $X^2_{(1)} = 5.49$, $P = 0.02$; trial 4: $X^2_{(1)} = 4.54$, $P = 0.03$; trial 5: $X^2_{(1)} = 8.27$, $P = 0.004$), 2 h before extinction (trial 4: $X^2_{(1)} = 5.879$, $P = 0.02$), 1 h before acquisition (trial 3: $X^2_{(1)} = 4.67$, $P = 0.03$), and 1 h after acquisition (trial 4: $X^2_{(1)} = 4.08$, $P = 0.04$, Fig. 2).

There were species-specific differences in how zeb altered extinction memory. In *Ac*, zeb treatment 1 h before acquisition ($X^2_{(1)} = 5.38$, $P = 0.02$, Fig. 1g) resulted in significantly lower extinction memory PER than the veh treatment. However, in *Am*, zeb treatment provided 1 h before acquisition ($X^2_{(1)} = 4.60$, $P = 0.03$, Fig. 2g) resulted in significantly higher extinction memory PER as compared to the veh treatment.

3.3. DNMT inhibition resulted in bidirectional modulation of extinction learning

In both species, zeb bidirectionally modulated extinction learning. In *Ac*, zeb significantly increased extinction learning when given 24 h before acquisition (trial 5: $X^2_{(1)} = 4.08$, $P = 0.04$), but significantly decreased it when given 2 h before extinction (trial 3: $X^2_{(1)} = 8.01$, $P = 0.005$; trial 4: $X^2_{(1)} = 12.12$, $P = 0.001$, Fig. 1d, f). Likewise, in *Am*, zeb significantly increased extinction learning when given 24 h before acquisition (trial 2: $X^2_{(1)} = 5.41$, $P = 0.02$; trial 3: $X^2_{(1)} = 5.49$, $P = 0.02$; trial 4: $X^2_{(1)} = 4.54$, $P = 0.03$; trial 5: $X^2_{(1)} = 8.27$, $P = 0.004$), but significantly decreased it when given 2 h before extinction (trial 4: $X^2_{(1)} = 5.88$, $P = 0.02$. Fig. 1d, f).

4. Discussion

These data provide the first evidence for a role of DNMT in *A. cerana* olfactory extinction memory and a comparison of these effects between *A. cerana* and *A. mellifera*. Overall, DNMT inhibition altered learning and extinction in similar ways in both species: zeb did not alter acquisition learning or acquisition memory retention, but did alter extinction learning and extinction memory retention. These results support the hypothesis that DNMT is more important in honey bee extinction memory than acquisition memory. Interestingly, our results are overall similar to those of Lockett et al. (2010). These similarities suggest that the regulatory role of DNMTs in honey bee extinction learning is largely independent of species and methodological differences in training paradigms (differential vs. absolute), number of trials, and CS-US presentations.

In both species, zeb exhibited a bidirectional modulatory effect upon extinction memory: the timing of DNMT inhibition either increased or decreased extinction memory. There were species-specific differences the effects of the treatments on extinction memory. Two of the treatments altered *A. cerana* extinction learning, whereas four of treatments altered *A. mellifera* extinction learning (Figs. 1 and 2). *Apis cerana* may be more sensitive to the timing of DNMT inhibition than *A. mellifera*. However, it is also possible that zeb is active for different periods of time in *A. cerana* as compared to *A. mellifera*. More experiments are needed.

Interestingly, zeb treatments provided 1 h before acquisition learning (1hba) had opposing effects on extinction memory retention in the different species. The reasons for these opposite effects are unclear, but may reflect species differences, such as differences in the spontaneous recovery of olfactory memories (Myers and Davis, 2002) after extinction training.

4.1. Dosage

The average masses of *A. cerana* and *A. mellifera* foragers were respectively 62.1 ± 4.9 mg and 86.5 ± 6.5 mg (measured from 10 bees from each of the three colonies of *A. cerana* and *A. mellifera* that we used, total of 60 bees). We chose to give both species the same dose. As a result, we used a 39% higher dose of zeb per mg of bee body mass for *A. cerana* than for *A. mellifera*. However, although *A. cerana* received a higher dose per mg of body mass, zeb had a lower influence on *A. cerana* learning than on *A. mellifera* learning (Figs. 1 and 2).

4.2. Acquisition memory

Apis mellifera exhibits better PER olfactory learning acquisition than *A. cerana* (Wang and Tan, 2014). In our study, we likewise found that *A. mellifera* exhibited better acquisition learning than *Apis cerana* (on average, 80% vs. 60% PER, respectively, Figs. 1a, b and 2a, b, $P \leqslant 0.03$). *Am* exhibited significantly higher acquisition memory retention than *Ac* for every treatment, too (Figs. 1c and 2c, $P \leqslant 0.047$). Compared to Lockett et al. (2010), we obtained higher PER learning for *A. mellifera,* perhaps because we used foragers, which exhibit better olfactory learning (Steve and Ben, 1997) than the younger bees used by Lockett et al. (2010).

In addition, learning was likely better in our trials because we used twice as many acquisition learning trials as Lockett et al. (2010). We made these methodological changes because *A. mellifera* is known to exhibit higher levels of PER learning than *A. cerana* (Wang and Tan, 2014), and we wished to compare the species in an assay in which olfactory learning was maximal.

4.3. Extinction memory

In some cases, there was spontaneous recovery of olfactory memories, shown when the extinction memory retention test (conducted 5 h after the last extinction trial) was compared with the last extinction trial (Figs. 1d–h and 2d–h). Bitterman et al. (1983) also found a similar spontaneous recovery in *A. mellifera* (rising from 10% to 70%). Such spontaneous recovery of olfactory memories has also been shown in other animals (Myers and Davis, 2002).

We found evidence for bidirectional modulation of extinction learning and show that the effects of zeb are broadly similar in both species. In *A. cerana*, this is the first demonstration of bidirectional modulation of extinction learning. Our results are consistent with the idea that consolidated memories can return to a more labile state in which they are subject to modification. (Nader, 2003). In *A. cerana*, zeb increased extinction learning (lower PER during extinction trials) for 24hba and decreased it for 2hbe, agreeing with the results of Lockett et al. (2010) for *A. mellifera*.

In *A. mellifera*, zeb increased extinction learning for some treatments (24hba and 1haa) and slightly decreased extinction learning in other treatments (2hbe and 1hba). The strongest effect occurred for zeb administered 24 h before acquisition (Fig. 2d). Using differential conditioning, Lockett et al. (2010) also found bidirectional modulation of extinction learning in *A. mellifera*, with zeb also increasing extinction learning in the 24hba treatment and decreasing extinction learning in the 2hbe treatment. These results match ours. However, there are also differences between our results. Lockett et al. (2010) found that zeb increased extinction memory in the 1maa treatment and did not find a decrease in extinction in the 1hba treatment.

The main difference between bee species arose for two treatments. For extinction trials in the 1hba treatment, extinction memory increased for *A. mellifera*, but decreased for *A. cerana*. This phenomenon is also illustrated by the final 1hba extinction memory trial, in which DNMT inhibited *A. mellifera* and *A. cerana* respectively exhibited significantly lower and higher memory retention (Figs. 1g and 2g). The cause of this difference is unclear, but it may arise from differences in the role of methylation during extinction memory in the two species. It is possible that the lower PER levels shown by *A. cerana* as compared to *A. mellifera* could also affect extinction memory. However, we expected that this difference in PER responsiveness would reduce the magnitude of responses, not invert the effects of 1hba DNMT inhibition between species.

It would be valuable to determine the mechanistic bases for these differences in future studies. Honey bee acquisition learning and extinction learning are regulated by different mechanisms. These different mechanisms may include histone acetylation/deacetylation (Lockett et al., 2014) and dimethylation of specific lysine residues on histone proteins (Martinowich et al., 2003; Kramer et al., 2011). Finally, it would be beneficial to compare stimulus-specific acquisition and extinction memory in *A. cerana* and *A. mellifera*.

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