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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)

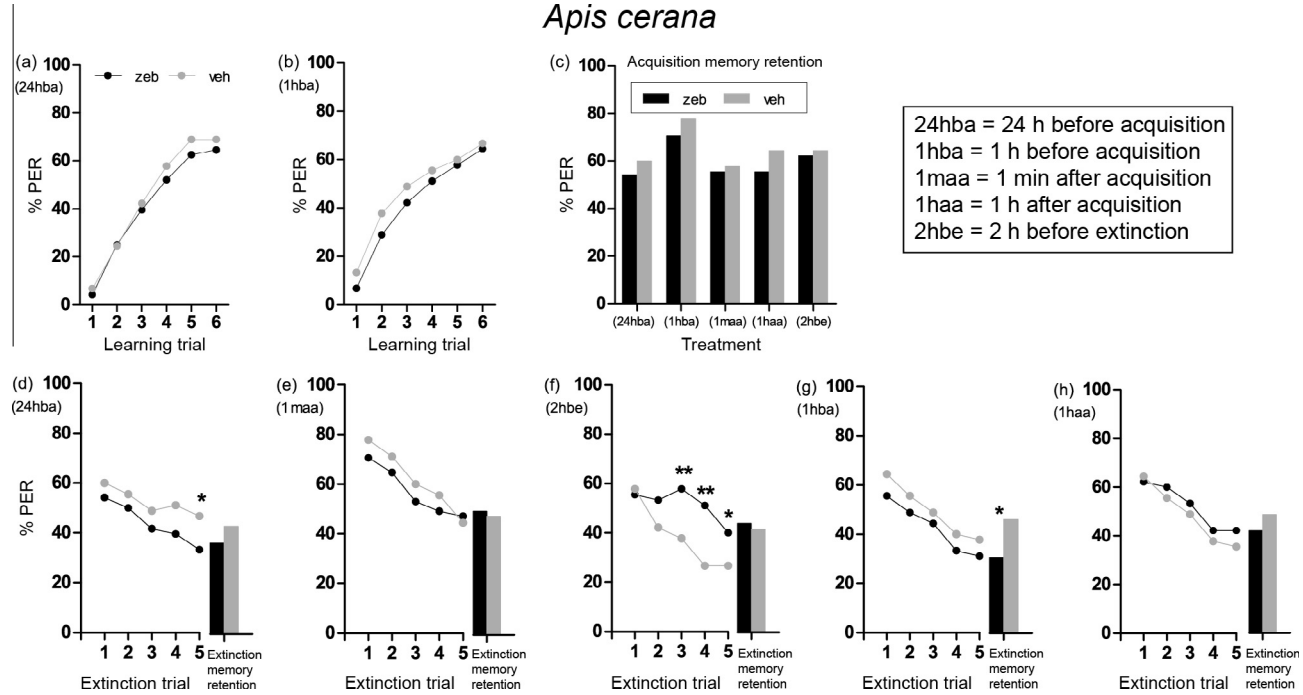

Fig. 1. In *A. cerana*, the effects of DNA methyltransferase inhibition (zeb) on (a, b) acquisition learning, (c) acquisition memory retention (tested 24 h after the last acquisition trial), and (d–h) extinction learning and extinction memory retention for the five different zeb treatments (see figure 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.