

## RESEARCH ARTICLE

# First demonstration of olfactory learning and long-term memory in honey bee queens

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## ABSTRACT

As the primary source of colony reproduction, social insect queens play a vital role. However, the cognitive abilities of queens are not well understood, although queen learning and memory are essential in multiple species such as honey bees, in which virgin queens must leave the nest and then successfully learn to navigate back over repeated nuptial flights. Honey bee queen learning has never been previously demonstrated. We therefore tested olfactory learning in queens and workers and examined the role of DNA methylation, which plays a key role in long-term memory formation. We provide the first evidence that honey bee queens have excellent learning and memory. The proportion of honey bee queens that exhibited learning was 5-fold higher than that of workers at every tested age and, for memory, 4-fold higher than that of workers at a very young age. DNA methylation may play a key role in this queen memory because queens exhibiting remote memory had a more consistent elevation in *Dnmt3* gene expression as compared with workers. Both castes also showed excellent remote memory (7 day memory), which was reduced by 14–20% by the DNA methylation inhibitor zebularine. Given that queens live approximately 10-fold longer than workers, these results suggest that queens can serve as an excellent long-term reservoir of colony memory.

**KEY WORDS:** Queen learning, Worker learning, DNA methylation, Remote memory

## INTRODUCTION

Cognition plays a key role in animal ecology. All central place foragers face a common task of learning and remembering the locations of their nest sites and, in many cases, rewarding food sites (Gordon and Pearson, 1979; Collett et al., 2013). In social animals, such as social insects, the study of learning and memory has been especially productive because multiple aspects of sociality and how animals interact with their environment – colony defense, foraging and even communication – rely upon sophisticated learning and memory (Dukas, 2008). In eusocial insects, these cognitive abilities have almost exclusively been studied in the worker caste (Menzel and Muller, 1996; Graham et al., 2010; Richter, 2000), but have their evolutionary beginnings in solitary ancestors. For example, Tinbergen (1935) elegantly demonstrated that solitary beewolf

wasps use learning to find their nest sites, a trait that is likely ancestral because it is needed by most nesting animals. Such nest site learning is equally valuable to queens of social insects such as bumble bees (Goulson, 2010) and social hornets and wasps (Richter, 2000) that must found and initially forage for their own colonies. The evolution of swarming reproduction largely eliminated the necessity of queen foraging but maintained a crucial need for queens to mate and return to their colonies (Michener, 1974). Queen learning is therefore indispensable.

In honey bees, virgin queens leave the nest to mate and can make up to five mating flights over multiple days (Winston, 1987). Although workers provide Nasanov pheromone to help guide the queen when she is close to the nest (Winston, 1987), young queens must learn the landmarks and, perhaps, other olfactory cues and signals that mark the nest. Because an *Apis mellifera* queen generally has no ability to survive for a prolonged period outside her colony, virgin queen loss has strong fitness consequences for the nest (Winston, 1987). The colony's ability to reproduce via swarming or to re-queen itself would be impaired (Winston, 1987). There should be strong selection for excellent queen learning, particularly at an early age, given that virgin queens mate almost immediately (5–13 days) after emergence (Oertel, 1940; Winston, 1987).

To date, no studies have demonstrated that honey bee queen learning exists or examined it in detail. Dreier et al. (2007) showed that *Pachycondyla* spp. founding ant queens can remember chemical cues that individually identify other queens and remember this information for at least 24–42 h. Similarly, paper wasp (*Polistes fuscatus*) foundresses can remember identity of other queens that they have interacted with based upon their facial patterns (Sheehan and Tibbetts, 2008). However, the details of this memory formation and its molecular bases were not explored. In bumble bees, *Bombus terrestris*, queens and workers learned to choose a rewarding flower color (Evans and Raine, 2014). Queens exhibited significantly better learning than workers, but took longer to make decisions, perhaps exhibiting more caution (Evans and Raine, 2014).

In honey bees, worker learning, particularly olfactory learning, has been extensively explored (Menzel and Muller, 1996), but the maximum duration of bee memory is unclear. As in other animals, workers learn and form short-term memories that are consolidated and transformed into stable, long-term memories (Menzel and Muller, 1996). Worker learning ability depends upon what is needed, improving with age (Ichikawa and Sasaki, 2003), because workers leave the nest and begin foraging at approximately 20 days of adult age (Winston, 1987). The longest duration of these memories is unclear, but they could persist for an individual's lifetime. Adult honey bee workers live for approximately 33–45 days (Winston, 1987), and Lindauer (1960) reported a case in which foragers trained to a feeder seemed to remember and waggle dance for it >30 days later. Chittka (1998) demonstrated that bumble bee workers can retain memories for over 3 weeks, and long-term

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memory in honey bee foragers can last over winter or throughout their lifespan (Lindauer, 1963; Menzel, 1968).

The longevity of long-term memory depends upon protein synthesis (Menzel, 2001) and DNA methylation, an epigenetic mechanism that regulates gene transcription (Wang et al., 2006). There are three known DNA methyltransferases (Dnmts) in honey bees (Wang et al., 2006). Dnmt3 is responsible for methylating hemimethylated and unmethylated DNA in mammals (Okano et al., 1999), and may function similarly in bees. Inhibiting Dnmts with the DNA methyltransferase inhibitor zebularine (zeb) affected memory extinction in *A. mellifera* (Lockett et al., 2010) and *Apis cerana* (Gong et al., 2016). Biergans et al. (2012, 2015, 2016, 2017) showed that Dnmt inhibition (with zeb or RG108) altered stimulus-specific memories and relearning without altering stimulus perception in *A. mellifera*.

We therefore tested learning in *A. mellifera* queens because honey bees have a major ecological role as pollinators in multiple ecosystems (Aebi et al., 2012) and because honey bee worker learning has been relatively well studied (Giurfa and Sandoz, 2012) and provides a rich data source for comparison with the unexplored phenomenon of honey bee queen learning. We focused on olfactory learning because queens, like workers, likely have good olfactory abilities and olfactory learning has been studied in more detail than any other form of honey bee learning (Giurfa and Sandoz, 2012). We compared queen and worker olfactory learning at different ages, using classical conditioning of the proboscis extension reflex (PER), examined long-term memory, and tested the role of DNA methylation in learning and memory by using zeb (Biergans et al., 2012, 2015, 2016, 2017, Lockett et al., 2010) and measuring *Dnmt3* gene activation.

## MATERIALS AND METHODS

For each experiment, we used six *A. mellifera* Linnaeus 1758 colonies (three for breeding workers and three for rearing queens) maintained at the apiaries of the Apicultural Research Institute, Yunnan Academy of Agricultural Sciences, Yunnan, China, and the Eastern Bee Institute of Yunnan Agricultural University, Yunnan, China. Experiments 1 and 2 were conducted August–October 2014, experiment 3 was conducted April–October 2015 and experiment 4 was conducted March–October 2016. During these months, colonies were in strong, healthy condition and engaged in natural foraging. Workers were obtained from colonies with four frames of comb: two combs of honey and pollen and two brood combs. Queens were bred in colonies with six frames of comb: three combs of honey and pollen and three brood combs. Throughout this paper, all ages are given as days after adult emergence. Samples sizes for each experiment are provided in Table S1.

### Worker breeding

We reared workers of known ages from three colonies by placing a clean and empty comb into a colony, allowing the queen to lay eggs and then moving this comb, 5 h later, to a section of the colony from which the queen was excluded. When adult bees were ready to emerge 21 days later, we placed this comb in a nuc box in an incubator (33°C, 70% relative humidity), collected the newly emerged bees, marked them with a paint pen, returned them to their colonies and allowed them to live in their colonies until they had reached the ages needed for the experiments.

### Queen breeding

We bred queens of known ages from three colonies, using standard techniques (Dietemann et al., 2013). Each queen breeding colony

consisted of two stacked hive boxes. The primary queen was kept in the bottom box with a queen excluder, while we bred queens in the upper box (Dietemann et al., 2013). To breed queens, we first allowed the primary queen to lay eggs into an empty comb placed into the lower box. After 5 h, we placed this comb into the upper box and waited for the eggs to hatch into 1-day-old larvae. We used beeswax to make standard artificial queen cells (50 cells per frame, Fig. S1A) and placed this frame into the colony for 5 h to allow bees to clean it and for it to acquire colony odors. We then removed both frames and grafted a single 1-day-old larvae into each queen cell. Once the pupae matured (10 days later), we transferred each queen cell into a separate cage (Fig. S1B,C) and returned these cages to the colony. After 3 days, adult queens would emerge. Queens were returned to their individual cages inside their colonies when not being used.

### Classical olfactory conditioning

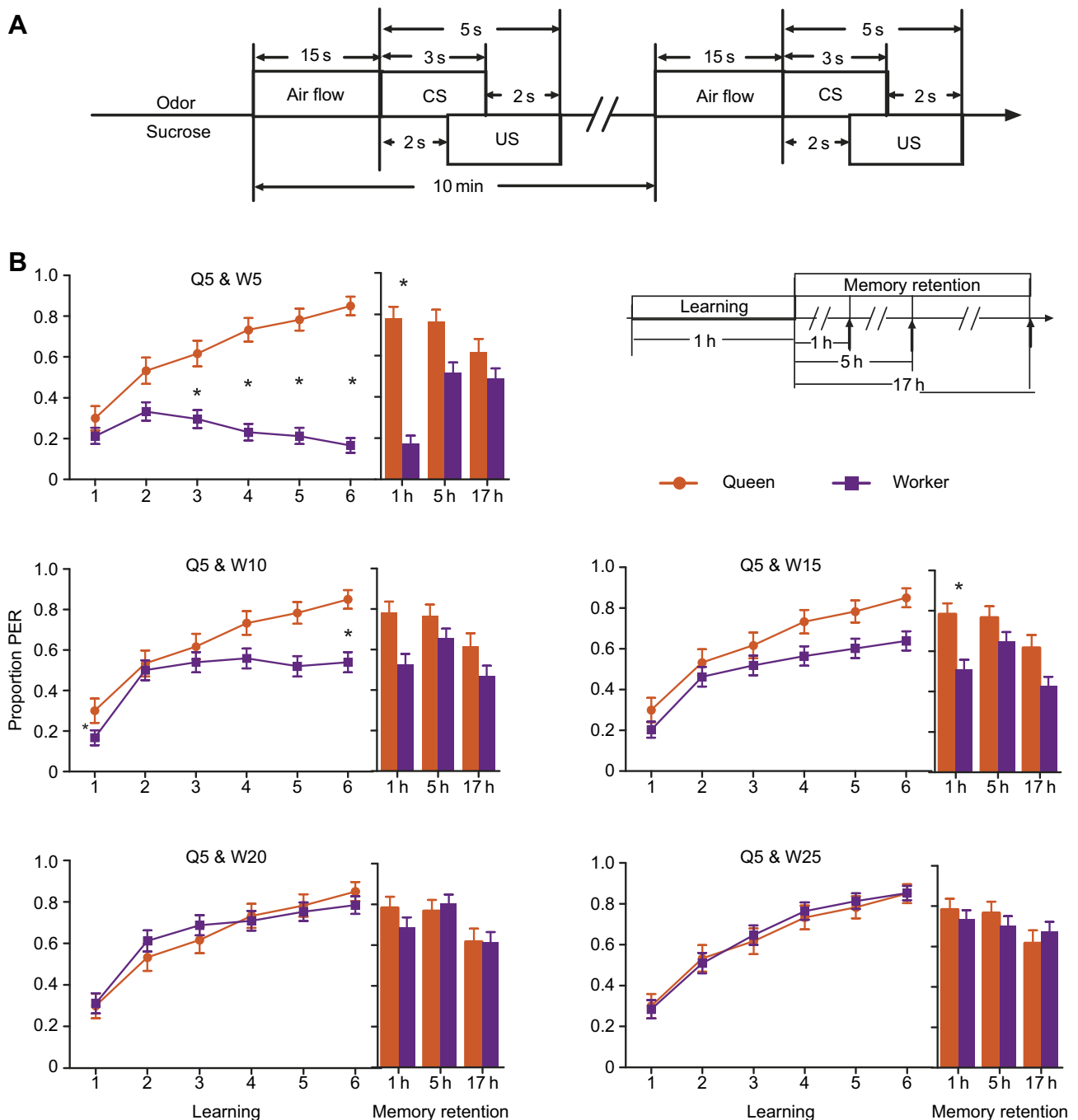
Following standard protocols (Giurfa and Sandoz, 2012), all bees were starved overnight to facilitate successful conditioning the next day. To prepare bees for the PER experiments, we placed each bee in a clean glass vial on ice for approximately 5 min until bee movement significantly diminished. We then placed bees in 0.5 ml plastic centrifuge tubes that had the holes cut out of the tips (Gong et al., 2016). Bees were still able to move their heads and proboscises and were then trained 5 h later. Olfactory learning and memory was tested with a PER absolute conditioning assay (Bitterman et al., 1983). During each trial, bees were exposed to a continuous air flow of 0.5 l min<sup>-1</sup>. The olfactory conditioned stimulus (CS) was 5 µl of hexane (Sigma-Aldrich, St Louis, MO, USA) dispensed onto a filter paper (1×1 cm) inside a syringe. 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 (Fig. 1A). We lightly tapped one antenna with the US to elicit PER and then allowed the bee to feed. The US elicited a PER (the unconditioned response). The US was presented 3 s after CS and overlapped with the CS for 1 s (Fig. 1A). If a bee exhibited learning, it would extend its proboscis during the presentation of the CS only (scored as 1). A fan placed 12 cm behind the bee exhausted all odors through a window. In all experiments, each bee was conditioned six times with an inter-trial interval of 10 min, which facilitates honeybee olfactory learning (Menzel, 2001). During the memory tests, we exposed trained bees to the CS alone without providing any sugar reward (methods of Lockett et al., 2010; Menzel, 1999). Additional memory testing with an unrewarded odor (a control) is preferred in current protocols (Giurfa and Sandoz, 2012), but this was unfortunately not a part of our design.

### DNA methyltransferase inhibition with zebularine

In experiments 2 and 4, we inhibited DNA methyltransferases with 1 µl of 2 mmol l<sup>-1</sup> zeb (Tocris Bioscience, Bristol, UK) delivered topically in the solvent dimethylformamide (DMF) applied to the dorsal thorax (Gong et al., 2016). Topical thoracic application in DMF is an efficient and standard delivery method for delivering neuroactive compounds to the honey bee brain (Zhou et al., 2002; Barron et al., 2007; Lockett et al., 2010). Control bees were treated with 1 µl of DMF only.

### Experiment 1: learning and memory on age-5 queens and different age of workers

We tested the hypothesis that queens would exhibit superior olfactory learning and memory as compared with workers. In preliminary



**Fig. 1. Comparisons between the proportions of queens and workers that exhibited learning (experiment 1).** (A) Design of the olfactory training apparatus and overall timing of the classical conditioning experiments. CS, conditioned stimulus; US, unconditioned stimulus. (B) Results of experiment 1, showing that 5-day-old queens (Q5) had better learning than 5- and 10-day-old workers (W5 and W10) and better memory than 15-day-old workers (W15). PER, proboscis extension reflex. Asterisks indicate significant pairwise differences (Tukey HSD test, \* $P < 0.05$ ). In the diagram, vertical black arrows indicate memory test time points. Plots show means  $\pm$  1 s.e.m. Sample sizes are in Table S1.

experiments, we found that 5 days was the youngest age at which we could reliably begin to condition workers. Queens can begin to make mating flights, and therefore require learning and memory, when they are only 5 days old (Winston, 1987). In a separate experiment (experiment 3) we observed that the learning in queens older than 5 days of age did not substantially improve. However, it is known that worker olfactory learning improves with age (Ichikawa and Sasaki, 2003). Therefore, we used 5-day-old queens and workers aged 5, 10, 15, 20 and 25 days (Table S1). Memory retention was tested 1 and

17 h after the last memory acquisition trial. We based the 17 h time point on Tan et al. (2015), which also tested olfactory learning in *A. cerana*. No zeb was used in this experiment.

#### Experiment 2: learning and memory on age-5 queens and workers treated with zebularine

In experiment 1, we found that 5-day-old queens had excellent olfactory learning. To determine the role of DNA methylation in queens and workers of the same age, we next compared the effects

of zeb on 5-day-old queens and 5-day-old workers. Bees were treated with zeb 2 h before training because there were minimal effects of zeb on memory when it was given 1 h before training (retention tested 24 h after acquisition by Lockett et al., 2010). Based upon prior studies (Lockett et al., 2010; Biergans et al., 2012; Gong et al., 2016), we hypothesized that a time period of 2 h would reveal an effect. As in experiment 1, we tested memory 1, 5 and 17 h after the last memory acquisition trial.

### Experiment 3: testing the existence of remote memory in workers and queens

We chose to use the term ‘remote memory’ based upon studies on rats and mice, which define very long-term memory lasting  $\geq 30$  days as ‘remote memory’, approximately 3.3% of a rat’s lifespan (Miller et al., 2010; Frankland et al., 2004; Squire and Bayley, 2007). Worker honey bees live an average of 45 days (Winston, 1987). Thus, the comparable 3.3% time point is 1.5 days and has already been studied (Giurfa and Sandoz, 2012). We wished to extend our understanding of honey bee memory to a longer, more remote period from memory formation (7 days after acquisition) and therefore use the term ‘remote memory’.

Before testing the effects of zeb on remote memory, which we defined as bee memory persisting for 7 days, we first needed to establish the existence of such long-term memory. In honey bees, prior memory tests occurred no later than 4 days after the last learning trial and were often conducted within 24 h after the last learning trial (Ichikawa and Sasaki, 2003; Lockett et al., 2010; Biergans et al., 2012, 2015). We therefore chose to test remote memory (7 days after memory acquisition, nearly double the maximum period in which such memory has been previously studied). To show that bees have remote memory, we compared the memory of two groups of bees of each caste, those tested at 7 days with those tested at 17 h. We chose bee ages with strong memory abilities: 5-day-old queens (Figs 1 and 2) and foragers (bees collected at the hive entrance after returning from foraging) because foragers have the best worker olfactory learning (Ichikawa and Sasaki, 2003).

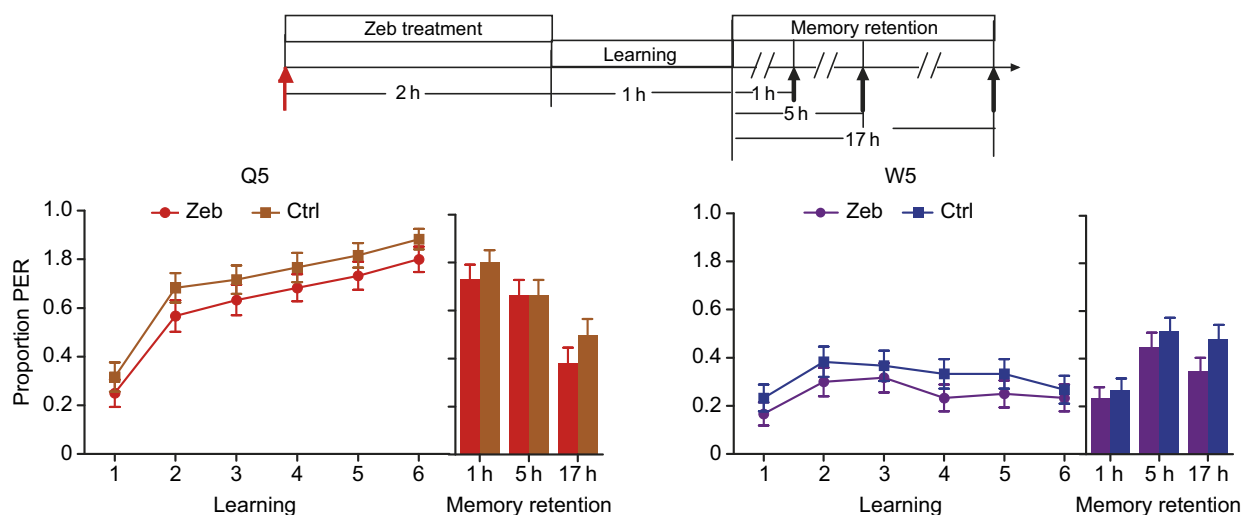
### Experiment 4: remote memory of queens and workers treated by zebularine

In this experiment, we tested the hypothesis that DNA methylation plays a role in remote memory, a form of long-term memory (Miller et al., 2010). To test remote memory, we measured memory retention 7 days (168 h) after the last learning trial. To examine these effects in greater detail, we tested queens and workers of the same ages (1, 5, 10 and 15 days old). In experiment 2, we did not find strong effects of zeb provided 2 h before learning training, but preliminary trials suggested that zeb provided 5 h before training would reveal effects. In experiment 4, we therefore treated bees with zeb 5 h before training. We removed bees from their harnesses after their last learning trial and placed them in cages (30 bees per cage) in an incubator (33°C, 70% relative humidity) and provided them with *ad libitum* sucrose (30% w/v). Prior to the 7 day memory test, we harnessed bees as described above. We only used bees that were healthy, exhibited good learning and showed normal activity inside their cages.

### Measuring *Dnmt3* gene expression

We measured *Dnmt3* gene expression in this experiment by collecting samples from five different groups: (1) control bees treated with DMF that did not exhibit remote memory (ctrl-0), (2) control bees treated with DMF that exhibited remote memory (ctrl-1), (3) bees treated with zeb that did not show remote memory (zeb-0), (4) bees treated with zeb that showed remote memory (zeb-1) and (5) blank control bees that received no treatment (no DMF and no zeb). After the 7 day memory test, we waited 30 min, basing this protocol upon Lockett et al. (2010), who measured gene expression 30 min after training, and placed the bee in liquid nitrogen to immediately halt and preserve gene expression. Heads were removed and stored at  $-80^{\circ}\text{C}$  for later RNA extraction.

We extracted total RNA with TRIzol reagent (Takara Bio, Inc., Kusatsu, Japan). We confirmed the quality and concentration of our extractions by measuring the OD<sub>260/280</sub> values (1.8–2.1). We then diluted our RNA to a concentration of  $1\text{ }\mu\text{g ml}^{-1}$ . Reverse



**Fig. 2. Effects of the DNA methylation inhibitor zebularine (zeb) on learning of 5-day-old queens (Q5) and workers (W5) (experiment 2).** Control bees (ctrl) only received the topical delivery agent DMF. Queens exhibited higher learning ( $P=0.0023$ ) and memory ( $P=0.004$ ) than workers, and zeb reduced overall learning ( $P=0.0001$ ) and memory ( $P=0.026$ ) in both castes. However, there were no pairwise differences in any given trial (Tukey HSD test,  $P>0.05$ ). In the diagram, the red vertical arrow indicates the start of zeb treatment and black vertical arrows indicate memory test time points. Plots show means  $\pm$  1 s.e.m. Sample sizes are in Table S1.



transcription was performed with a PrimeScript RT Reagent Kit with gDNA Eraser (catalog no. RR047Q, Takara Bio). We first incubated each sample with the genomic DNA elimination reagent for 2 min at 42°C. We then reverse transcribed the RNA into cDNA at 37°C for 15 min and 85°C for 5 s. We stored the cDNA at –20°C until it was used for real-time qPCR.

We used a C1000 Real Time PCR System (model CFX96, Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analyzed in triplicate. Each amplification was conducted in a 25 µl reaction containing 12.5 µl SYBR Premix Ex Taq™ II (2×), dNTPs, Taq DNA polymerase, 6 mmol l<sup>–1</sup> MgCl<sub>2</sub>, SYBR Green, 6.5 µl of double-distilled RNase free water (Takara Bio), 0.5 µl PCR forward primer (10 µmol l<sup>–1</sup>), 0.5 µl PCR reverse primer (10 µmol l<sup>–1</sup>), 9.5 µl dH<sub>2</sub>O and 2 µl cDNA template. Primer sequences were *Dnmt3*: F-CAGCGATGACCTGCGATCGGCGATA, R-TACAGGGTTTAATTCCGAAC; and ribosomal protein gene *Rps8*: F-ACGAGGTGCGAACTGACTGA, R-GCACTGTCCA-GGTCTACTCGA. PCR conditions consisted of one cycle of denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 10 s, and 62°C for 30 s. The specificity of the amplified product was monitored using its melting curve. Gene expression data were normalized relative to one housekeeping gene, *Rps8*. We then calculated changes in gene expression levels with the 2<sup>–ΔΔC<sub>t</sub></sup> method (Schmittgen and Livak, 2008).

### Statistics

We used JMP Pro v13.0.0 (SAS Institute, Cary, NC, USA) for all statistical analyses and report means±1 s.e.m. We analyzed our learning data with repeated-measures ANOVA, with bee identity as the repeated measure (Matsumoto et al., 2012). As appropriate, our models included colony as a random effect, with all other effects fixed (species, caste, bee age, zeb treatment and experimental group), and with all interactions. We used residuals analysis to ensure that our data met parametric assumptions. For experiment 1, we compared 5-day-old queens with workers of five different ages and therefore created six groups that we ran as a caste group factor: queen 5 days, worker 5 days, worker 10 days, worker 15 days, worker 20 days and worker 25 days. For clarity, our figures show multiple individual plots, one per bee age, that compare queen and worker learning. However, the significant differences shown are based upon a single Tukey honestly significant difference (HSD) test applied to make all pairwise comparisons, corrected for Type I error. For experiments 2 and 3, we also applied a single model and a single Tukey HSD test per experiment. In experiment 4, we ran a single model on the PER results (reported), but, given the complex interactions, ran a separate Tukey HSD test per age group to make pairwise comparisons.

In experiments 3 and 4, to test the effects of zeb treatment on survival to 7 days, we ran an ANOVA with treatment (DMF or zeb), caste and bee age as fixed effects and the mean percent of bees surviving to 7 days as our dependent variable. We included colony as a random effect and used Tukey HSD tests to make pairwise comparisons.

To analyze gene expression in experiment 4, we normalized our data using the 2<sup>–ΔΔC<sub>t</sub></sup> method described by Schmittgen and Livak (2008). *Dnmt3* was the focal gene and *Rps8* was the housekeeping gene: ΔC<sub>t</sub> (under test gene)=[C<sub>t</sub> value of *Dnmt3* – C<sub>t</sub> value of *Rps8*], ΔC<sub>t</sub> (blank control gene)=[C<sub>t</sub> value of *Dnmt3* – C<sub>t</sub> value of *Rps8*], ΔΔC<sub>t</sub>=ΔC<sub>t</sub> (under test gene) – ΔC<sub>t</sub> (blank control gene). We averaged the gene expression levels from five different individuals that received the same treatment and log-transformed this mean value for our ANOVA model (REML algorithm) with colony as a

random variable, all other factors (age, caste, and treatment) fixed, and with all interactions. We then ran a separate Tukey HSD test per age group.

### RESULTS

We conducted standard PER experiments with six learning trials in which bees associated the odor of hexane (conditioned stimulus) with sugar solution (reward), followed by memory retention tests in which only odor was presented (Fig. 1A). Zeb treatment and *Dnmt3* gene expression measurements followed standard protocols. We analyzed our learning data with repeated-measures ANOVA because we measured the learning of the same bee over multiple trials (Bitterman et al., 1983). Sample sizes are given in Table S1.

#### Experiment 1: even young queens have better learning and memory than workers

Queens (age 5 days) and workers (ages 5, 10, 15, 20 and 25 days) exhibited learning (trial  $F_{5,2835}=109.06$ ,  $P<0.0001$ ). A significantly higher proportion of queens exhibited learning than workers (caste group  $F_{5,565}=20.73$ ,  $P<0.0001$ ), leading to a significant interaction of trial×caste ( $F_{25,2835}=7.98$ ,  $P<0.0001$ ). In the sixth learning trial, the proportion of 5-day-old workers that exhibited PER was reduced (–80%) in comparison with 5-day-old queens. The proportion of 10-day-old workers that exhibited learning was reduced (–37%) in comparison with 5-day-old queens (Tukey HSD test,  $P<0.05$ ). Colony accounted for <1% of model variance (Fig. 1B).

In addition, queens (5 days old) and workers (5, 10, 15, 20 and 25 days old) exhibited significant changes in memory over time (trial  $F_{2,1134}=18.81$ ,  $P<0.0001$ ). A significantly higher proportion of queens remembered as compared with workers (caste  $F_{5,565}=14.04$ ,  $P<0.0001$ ), resulting in a significant learning trial×caste interaction ( $F_{10,1134}=5.84$ ,  $P<0.0001$ ). At the 1 h memory test, the proportion of 5-day-old workers that exhibited memory was strongly reduced (–78%) in comparison with that of 5-day-old queens, and 15-day-old workers had reduced memory (–35%) relative to 5-day-old queens (Tukey HSD test,  $P<0.05$ ). Colony accounted for <1% of model variance (Fig. 1B).

In 5-day-old workers, the apparent increase in PER responses at 5 and 17 h as compared with 1 h may have arisen from hunger. In PER experiments, bees are typically allowed to rest for 2–12 h without food to increase their feeding motivation (Giurfa and Sandoz, 2012). Interestingly, queen memory retention did not exhibit the same effect, perhaps because queens are larger (Winston, 1987) and may thus have greater energy reserves than workers.

#### Experiment 2: zeb slightly decreased overall learning and memory in both castes at 5 days of age

Here, we tested the effects of zeb on 5-day-old bees. Overall, both queens and workers learned (trial  $F_{5,1298}=21.60$ ,  $P<0.0001$ ), a significantly higher proportion of queens exhibited learning as compared with workers (caste  $F_{1,4}=47.91$ ,  $P=0.0023$ ; Fig. 2) and there was a significant interaction of caste×trial ( $F_{5,1298}=13.20$ ,  $P<0.0001$ ).

Zeb significantly reduced the overall proportions of bees that showed learning (treatment  $F_{1,1298}=15.14$ ,  $P=0.0001$ ), although there were no significant pairwise differences in any trial (Tukey HSD test,  $P>0.05$ ). No other interactions were significant (caste×treatment  $F_{1,1298}≤0.17$ ,  $P≥0.68$ ; all other interactions  $F_{2,1298}≤0.11$ ,  $P≥0.99$ ). Colony accounted for <1% of model variance.

A higher proportion of queens exhibited memory than workers (caste  $F_{1,4}=37.10$ ,  $P=0.004$ ), and memory changed over time (trial  $F_{2,590}=7.36$ ,  $P=0.0007$ ), though differently in each caste (caste×trial  $F_{2,590}=21.76$ ,  $P<0.0001$ ; Fig. 2). Zeb slightly but significantly reduced overall memory (treatment  $F_{1,590}=5.00$ ,  $P=0.026$ , although no pairwise comparisons at any time point were significantly different, Tukey HSD test,  $P>0.05$ ; Fig. 2). On average, zeb slightly reduced the proportions of queens (−9%) and workers (−18%) that exhibited memory over the three memory trials (1–17 h). No other interactions were significant (caste×treatment  $F_{1,590}\leq 0.07$ ,  $P\geq 0.79$ ; all other interactions  $F_{2,1298}\leq 0.82$ ,  $P\geq 0.44$ ). Colony accounted for <1% of model variance.

### Experiment 3: queens and workers exhibit remote memory

We next tested for the existence of remote memory (7 day memory retention) and compared 17 h and 7 day memory (memory type) in queens and workers. As expected, both castes learned (trial  $F_{5,1590}=164.99$ ,  $P<0.0001$ ), with a higher proportion of queens again showing learning as compared with workers (caste  $F_{1,26}=20.06$ ,  $P=0.0001$ , Fig. 3). There was no difference in learning between queens and workers that were assigned to have their remote memory or long-term memory tested (memory group  $F_{1,316}=0.04$ ,  $P=0.84$ ) because these groups were treated identically during learning. No interactions were significant ( $F_{1,315}\leq 0.30$ ,  $P\geq 0.58$ ;  $F_{5,1590}\leq 0.21$ ,  $P\geq 0.96$ ). Colony accounted for <1% of model variance.

Somewhat surprisingly, the proportion of bees that remembered when tested at 7 days was almost as high as at 17 h (no significant pairwise differences, Tukey HSD test,  $P>0.05$ , overall memory:  $62\pm 6\%$  PER in queens and  $58\pm 4\%$  in workers; Fig. 3). However, there was a slight overall decrease in the proportion of bees that exhibited memory (−17%) at 7 days as compared with 17 h (memory type  $F_{1,316}=4.71$ ,  $P=0.02$ ). There was no significant difference between the memories of queens and workers (caste  $F_{1,12}=1.80$ ,  $P=0.20$ ). The interaction caste×memory type ( $F_{1,316}=0.01$ ,  $P=0.92$ ) was not significant. Colony accounted for <1% of model variance.

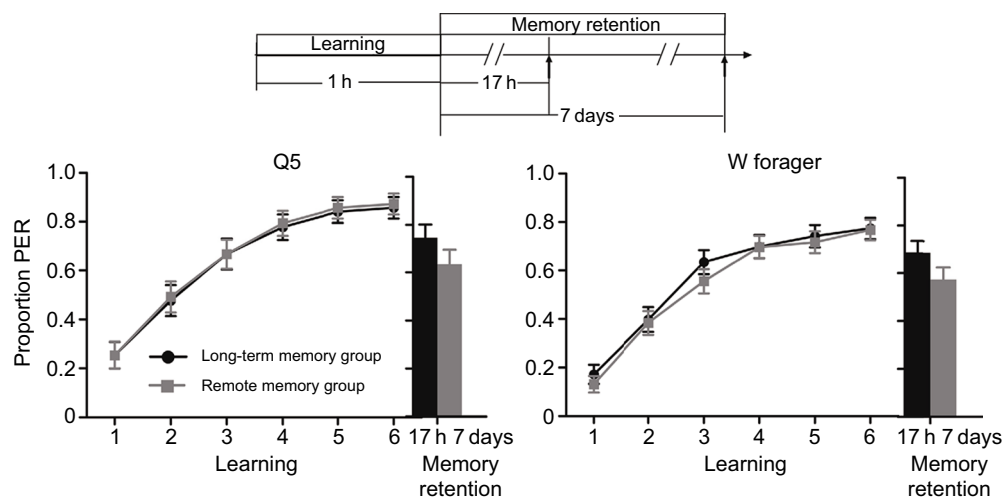
### Experiment 4: zeb reduced queen and worker learning and remote memory

We then tested the effects of caste, bee age, trial and treatment on learning and remote memory. Queens and workers exhibited significant learning (trial  $F_{5,6295}=237.80$ ,  $P<0.0001$ ), and a higher proportion of queens exhibited learning than workers (caste  $F_{1,5}=442.82$ ,  $P<0.0001$ , Fig. 4). There was a significant effect of age ( $F_{3,1256}=13.60$ ,  $P<0.0001$ ).

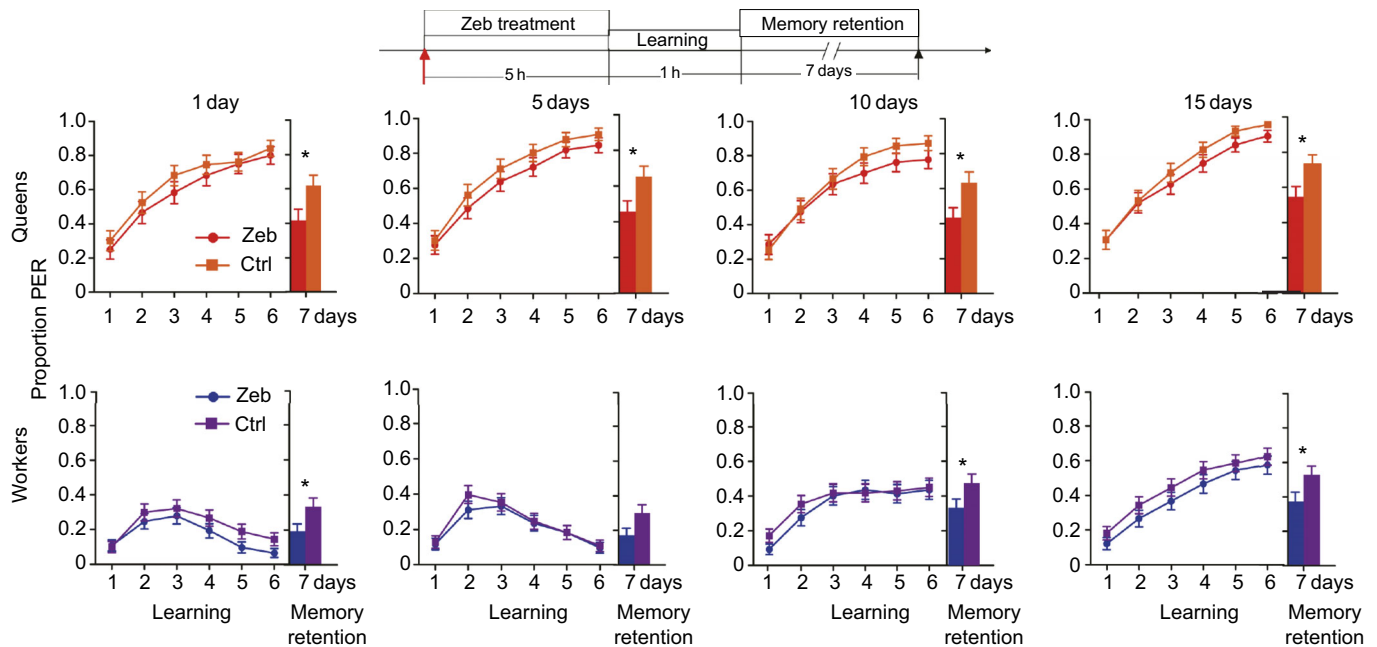
Zeb significantly reduced the proportion of bees that exhibited learning ( $F_{1,1256}=7.90$ ,  $P=0.005$ ). On average, over all ages in learning trials two to six, zeb reduced the proportion of queens that showed learning by 8% and workers by 14%. However, there were no significant effects of zeb at any specific trial (Tukey HSD test,  $P>0.05$ ). There were four significant interactions: caste×trial ( $F_{5,6295}=71.98$ ,  $P<0.0001$ ), caste×age ( $F_{3,1256}=7.36$ ,  $P<0.0001$ ), age×trial ( $F_{15,6295}=10.13$ ,  $P<0.0001$ ) and age×trial×caste ( $F_{15,6295}=6.25$ ,  $P<0.0001$ ). No other interactions were significant ( $F_{1-3,1256}\leq 0.12$ ,  $P\geq 0.73$ ;  $F_{5-15,6295}\leq 0.74$ ,  $P\geq 0.60$ ). Despite this complexity, visual inspection of the data showed that the main differences were consistently higher queen learning at each age (Fig. 4). Colony accounted for <1% of model variance.

A greater proportion of queens exhibited remote memory than workers (caste  $F_{1,4}=62.64$ ,  $P=0.0012$ ), and there was a significant effect of age ( $F_{3,1255}=7.20$ ,  $P<0.0001$ ; Fig. 4). Zeb significantly reduced remote memory on average in queens (−19.7%) and workers (−13.8%) over all ages ( $F_{1,1255}=39.86$ ,  $P<0.0001$ ). No interactions were significant ( $F_{1-3,1256}\leq 2.36$ ,  $P\geq 0.07$ ). Colony accounted for <1% of model variance.

In experiments 3 and 4, 100% of bees survived to the sixth conditioning trial, but there was some mortality 7 days later. There were significant effects of treatment ( $F_{1,38}=79.21$ ,  $P<0.0001$ ), caste ( $F_{1,38}=4.58$ ,  $P=0.0389$ ) and age ( $F_{1,38}=23.76$ ,  $P<0.0001$ ). Colony identity accounted for 2% of model variance. No interactions were significant, although the interaction treatment×caste was nearly significant ( $F_{1,38}\leq 3.95$ ,  $P\geq 0.054$ ). Pairwise comparisons revealed that zeb did not alter queen survival at any age, and only significantly decreased survival in workers aged 5 and 10 days (Tukey HSD test; Fig. S2).



**Fig. 3. Comparisons between long-term memory (17 h) and remote memory (7 days) within queens (proportion remembering at age 5 days) and within workers (proportion remembering at foraging age) (experiment 3).** Foraging-age bees were bees collected at the colony entrance as they returned from foraging. A higher proportion of queens exhibited learning than workers ( $P=0.0001$ ) and a slightly lower proportion of bees in each caste (−17%) exhibited remote memory (tested at 7 days) as compared with long-term memory (tested at 17 h,  $P=0.02$ ). There were no specific pairwise differences in memory in any specific trial (Tukey HSD test,  $P>0.05$ ). In the diagram, vertical black arrows indicate memory test time points. Means±s.e.m. are shown. Sample sizes are in Table S1.



**Fig. 4. Effects of zeb on the proportion of bees showing learning and remote memory (7 days) of queens and workers of different ages (experiment 4).** Zeb slightly but significantly reduced the overall proportions of queens and workers that exhibited learning ( $P=0.005$ ) and likewise decreased the proportion of bees that showed remote memory ( $P<0.0001$ ). There were no significant learning differences in zeb and ctrl treated queens or workers in any individual trial (one Tukey HSD test per age group,  $P>0.05$ ). However, zeb significantly reduced the proportions of bees that exhibited remote memory in all tested queen ages and all tested worker ages, except for 5-day-old workers (one Tukey HSD test per age group,  $*P<0.05$ ). In the diagram, the red vertical arrow indicates the start of zeb treatment and the black vertical arrow indicates the memory test time point. Plots show means  $\pm$  1 s.e.m. Sample sizes are in Table S1.

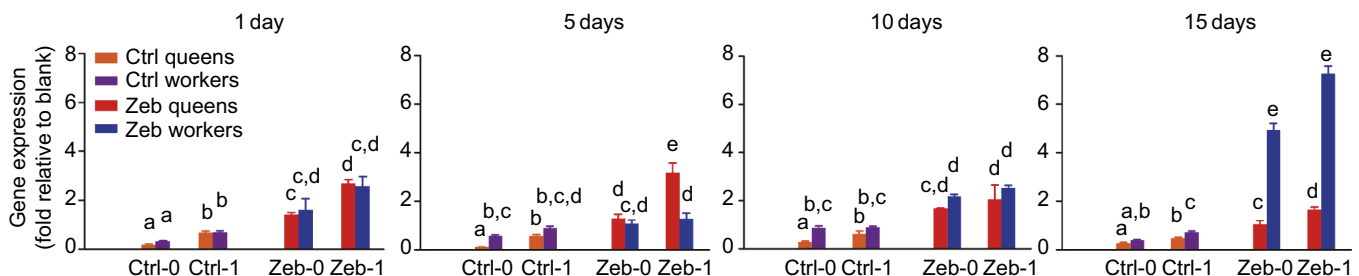
#### Experiment 4: zeb altered queen and worker *Dnmt3* gene expression

Expression of *Dnmt3* increased, as expected, in both queens and workers, because of zeb treatment (treatment  $F_{3,60}=539.40$ ,  $P<0.0001$ ; Fig. 5). Overall, workers showed higher levels of *Dnmt3* expression than queens (caste  $F_{1,4}=51.54$ ,  $P=0.002$ ). There was a significant effect of age ( $F_{3,60}=18.21$ ,  $P<0.0001$ ). All interactions were significant ( $F_{3,60}\geq 8.42$ ,  $P<0.0001$ ). Colony accounted for 10% of model variance.

To analyze these complex effects, we made all pairwise comparisons within each age group and report only significant differences (Tukey HSD tests,  $P<0.05$ , Fig. 5). In general, workers showed higher levels of *Dnmt3* gene expression than queens. *Dnmt3* gene expression was significantly higher in workers as compared with queens in the ctrl-0 group at 5 and 10 days and in the ctrl-1 group at 15 days. *Dnmt3* gene expression

was significantly higher in workers as compared with queens in the zeb-0 group at 15 d and in the zeb-1 group at 5 and 15 days. The strongest effect of age was the markedly higher level of *Dnmt3* gene expression in workers as compared with queens (4.4- to 4.7-fold higher) at 15 days of age.

Finally, we focused on the potential effects of zeb upon remote memory. Zeb treatment significantly increased *Dnmt3* gene expression (zeb versus control) in queens and workers and did not depend upon whether the bees exhibited remote memory. Within the control groups (ctrl-0 versus ctrl-1), remote memory corresponded to significantly increased gene expression in queens (queens versus queens: 1, 5, 10 and 15 days old) and workers (workers versus workers: 1 and 15 days old). Within the zeb groups (zeb-0 versus zeb-1), remote memory corresponded to significantly increased gene expression in queens (queens versus queens: 5, 10 and 15 days old) and in workers (workers versus workers: 15 days old; Fig. 5).



**Fig. 5. *Dnmt3* gene expression in queens and workers of different ages that were tested for remote memory (7 days) and that received different treatments: zeb or the control (dimethylformamide; ctrl) (experiment 4).** In honey bees, there is a negative correlation between DNA methylation and *Dnmt3* gene expression (Biergans et al., 2015). Gene expression levels were measured for bees that did not exhibit remote memory (ctrl-0 and zeb-0) and that exhibited remote memory (ctrl-1 and zeb-1). There were overall significant differences in gene expression between the castes ( $P=0.002$ ) that depended upon bee age ( $P<0.0001$ ). Different letters indicate significant differences within each plot (one Tukey HSD test conducted per age group,  $P<0.05$ ). Plots show means  $\pm$  1 s.e.m. Sample sizes are in Table S1.



## DISCUSSION

No prior studies have demonstrated that honey bee queens can detect odors, though their ability to do so is not surprising given the importance of olfaction for bees (Menzel and Muller, 1996). We expect that queens use olfaction in multiple contexts, yet to be elucidated. For example, colonies often raise multiple queens and newly emerged virgin queens will find and eliminate their rivals even when they are still inside their queen cells (Tarpy et al., 2004). How they find other queens, apart from queen piping (Winston, 1987), is unclear, but detecting queen odors could play a role. Our data also provide the first demonstration that honey bee queens, like workers, have excellent learning and memory. In fact, the proportion of honey bee queens that exhibited olfactory learning markedly exceeded that of workers of the same age, particularly in young bees. At 5 days, an 8-fold higher proportion of queens exhibited learning as compared with workers (sixth trial) and a 4-fold higher fraction of queens likewise showed memory relative to workers (1 h test). This better queen memory likely arose because a higher proportion of queens exhibited learning as compared to the proportion of workers showing learning. Inhibiting DNA methylation with zeb significantly reduced learning and memory in both castes.

Queens and workers were capable of remote memory (a form of long-term memory) at 7 days that, surprisingly, did not exhibit much deterioration in comparison to 17 h memory. This nearly doubles the known long-term memory abilities of honey bees, previously only studied in detail for up to 4 days. Remote memory, in both queens and workers, depended upon DNA methylation. Across all tested ages, queens showed fairly consistent increases in *Dnmt3* gene expression workers when they exhibited remote memory (Fig. 4), even when DNA methylation was inhibited with zeb. This pattern was not as consistent in workers but did hold at some ages.

### Effects of inhibiting DNA methylation

Prior studies found no effects of zeb, a DNA methylation inhibitor (Zhou et al., 2002), on learning in *A. mellifera* (Lockett et al., 2010; Biergans et al., 2012). More recently, Biergans et al. (2016), conducted a meta-analysis of multiple honey bee studies with methylation inhibitors (zeb and RG108), but found no strong overall effect of inhibiting DNA methylation on honey bee learning. Unlike these studies, we found that zeb slightly, but significantly, decreased honey bee learning, although there were no significant pairwise differences at any specific time point. Our finding of a slight zeb effect on learning may have arisen because of our different analysis technique, which used repeated-measures analyses (Matsumoto et al., 2012) to examine the overall effects over all trials and therefore benefits from analyzing differences over the entire arc of learning. Although Gong et al. (2016) found no zeb learning effects with this analysis technique, we used nearly twice the sample size, which may have increased statistical power. Another possible explanation is that the effects of inhibiting DNA methylation are subject to the timing of zeb administration (Lockett et al., 2010). Thus, our timing of zeb administration (2 h before learning in experiment 2 and 5 h before learning in experiment 4, which is different from Gong et al., 2016), may account for the dissimilar results.

Did zeb treatment alter bee health? In our study, we found that zeb decreased the survival of workers aged 5 and 10 days after 7 days of exposure but did not affect the survival of any other worker age groups or any queen age groups. The 7 day memory impairments for the 5- and 10-day-old workers could reflect decreased health owing to zeb treatment. Nonetheless, zeb did not significantly decrease the survival of queens at any tested age or of the 1- and

15-day-old workers, suggesting that the memory impairments observed in these groups (Fig. 4) were not due to poorer health. Queens are generally more robust than workers and have greater longevity (Winston, 1987).

Inhibiting DNA methylation had stronger effects when we tested remote memory (7 days; Fig. 4) as compared with medium-term memory (5 and 17 h; Fig. 2). Zeb significantly decreased the proportion of queens that exhibited remote memory (−13.8%) at all tested ages and significantly reduced the proportion of workers that showed remote memory at ages 1, 10 and 15 days (−19.7%; see pairwise differences in Fig. 4). The effect of zeb on memory was not simply due to reduced learning because the magnitude of zeb impairment of remote memory exceeded the effect of zeb upon learning (Fig. 4). In fact, memory was significantly reduced when tested at the 7 day trial, but was not significantly impaired in any specific learning trial (Fig. 4). Other studies have reported similar effects of zeb upon bee memory (Lockett et al., 2010; Biergans et al., 2012). It would be beneficial for future studies to use other DNA methylation inhibitors that have even greater specificity for knocking down Dnmts. However, multiple studies (reviewed in Biergans et al., 2015) have shown that zeb reduces DNA methylation in honey bees. Because of these prior results (Biergans et al., 2015), we did not test the efficacy of zeb in reducing DNA methylation. However, we recognize that there could be age- and caste-dependent differences in the efficacy of zeb at reducing DNA methylation in queens and workers. Confirming the effects of zeb with different castes at different ages would be useful for future studies.

### Changes in *Dnmt3* gene expression

In honey bees, there is a negative correlation between DNA methylation and *Dnmt* gene expression (Biergans et al., 2015). Worker *Dnmt3* gene expression increased when DNA methyltransferase was inhibited by zeb or RG108, perhaps because of a negative association between DNA methylation and gene expression in memory-associated genes. Biergans et al. (2015) used 10-day-old workers and showed that *Dnmt3* gene expression increased 5 h after learning, but not 24 h after learning. In our 10-day-old bees that showed remote memory, control workers, but not control queens, had increased *Dnmt3* gene expression. For zeb-treated bees, there were no significant differences in gene expression (Fig. 5). Biergans et al. (2015) found that *Dnmt3* expression was only upregulated after treatment with RG108, not zeb. Comparing control bees with zeb-treated bees in the 10-day-old group, we found that zeb treatment increased *Dnmt3* expression within each memory group: workers that did not show remote memory (ctrl-0<sub>worker</sub> versus zeb-0<sub>worker</sub>), workers that showed remote memory (ctrl-1<sub>worker</sub> versus zeb-1<sub>worker</sub>), queens that did not show remote memory (ctrl-0<sub>queen</sub> versus zeb-0<sub>queen</sub>) and queens that showed remote memory (ctrl-1<sub>queen</sub> versus zeb-1<sub>queen</sub>; Fig. 5). It is possible that the differences between our study and Biergans et al. (2015) arise from methodological differences. Our study included a memory test 30 min before the bees were frozen to measure gene expression. This memory test and potential memory reconsolidation may have influenced our results. Another key difference is that we measured *Dnmt3* gene expression at a much later time point (7 days as compared with 1 day; Table S2).

Lockett et al. (2010) did not test the effect of zeb on *Dnmt3* gene expression, but measured *Dnmt3* gene expression 30 min after learning in 7-day-old workers. They found that such learning significantly increased gene expression in the mushroom bodies, but not in other parts of the brain. Our closest comparators would be



measuring *Dnmt3* gene expression in 5- and 10-day-old bees, although we measured gene expression in the entire brain 7 days after learning. In our study, *Dnmt3* gene expression in 5- and 10-day-old workers was significantly elevated 7 days after learning. There was no significant difference for queens of this age (Fig. 5). Because of the multiple differences between our study and Lockett et al. (2010) and Biergans et al. (2015), we should be cautious of comparisons (Table S2). It would be useful for future studies to examine the detailed time course of *Dnmt3* gene expression over 7 days.

In our experiment, workers generally showed higher levels of *Dnmt3* gene expression than queens, with the strongest difference (4-fold to 5-fold higher expression at the group level) at 15 days of age. This may have occurred because foragers require good olfactory learning and a higher proportion therefore exhibit better learning as they approach foraging age (Ichikawa and Sasaki, 2003). Across all age groups, queens exhibited more consistent gene expression elevation when they showed remote memory. Regardless of treatment, queens therefore exhibited a more consistent elevation than workers in *Dnmt3* gene expression when they exhibited remote memory (Fig. 5). These differences between queens and workers align with prior results showing that DNA methylation has significantly different patterns in the brains of queens as compared with the brains of workers (Lyko et al., 2010).

### Queen versus worker learning

Social insect queens generally share similar sensory learning abilities with workers: for example, *Pachycondyla* ant odor learning (Dreier et al., 2007), paper wasp visual learning (Michael et al., 2008) and bumble bee color learning (Evans and Raine, 2014). Males and workers likewise possess visual learning in bumble bees (Wolf and Chittka, 2016; Robert et al., 2017; Lichtenstein et al., 2015) and olfactory learning in honey bees (Bhagavan et al., 1994; Menzel and Muller, 1996). Young queens should require excellent spatial learning to successfully return to the nest after their mating flights.

We studied olfactory learning because such learning has been extensively tested and provides a good foundation to compare workers with queens (Maleszka and Helliwell, 2001). However, honey bee spatial and olfactory learning are known to be closely related, passing through the same phases of short-term to long-term memory from sensory structures to the mushroom bodies (Menzel, 2001). We therefore hypothesized that young queens would have excellent olfactory learning. Confirming our hypothesis, queen learning and memory was already excellent at 5 days of age and did not show much improvement up to 15 days of age (Fig. 4). Worker learning improved with age, as expected (Maleszka and Helliwell, 2001; Ichikawa and Sasaki, 2003).

The proximate reasons why queen learning and memory is superior are unclear, but may relate to the nutrition and compounds in a lifetime of royal jelly that queen larvae and queen adults are fed. In contrast, workers are only fed as larvae with brood food that differs in composition from royal jelly (Haydak, 1970). Royal jelly proteins are found in the mushroom bodies of the honey bee brain, which play a major role in learning and memory (Kucharski et al., 1998; Peixoto et al., 2009). Isolated worker bees fed only sucrose solution had decreased expression of royal jelly protein 1 (Hojo et al., 2010), and isolation was correlated with a decline in learning ability, in a separate experiment (Ichikawa and Sasaki, 2003). Interestingly, Zamani et al. (2012a, 2012b) reported that learning and memory could be rescued by feeding rats royal jelly after impairment with streptozotocin.

### Queen memory and longevity

Colonies depend upon worker learning and memory for foraging, but their fitness also hinges upon the ability of young queens to learn and remember how to return to the colony during their multiple mating flights. Without such successful returns, colonies cannot reproduce or maintain themselves. As hypothesized, queen learning and memory is far better than that of workers at the same young age. This ability may be tied, in part, to DNA methylation and to the royal jelly proteins fed to queens throughout their lives. However, it begs a larger question. What do queens use learning for? Queens can live for 1–2 years, whereas workers typically live for only 35–45 days (Page and Peng, 2001). Workers may be able to retain memory for their lifetime (Lindauer, 1960) and we wonder whether queens can do the same.

This points to a key question: what does the queen remember that her daughters do not, and does this provide a fitness advantage to the colony? For example, seasonal migration and absconding occurs in Asian *A. mellifera*, *A. cerana*, *A. florea*, *A. andreniformis*, *A. dorsata* and *A. laboriosa* and in African *A. mellifera* and are important aspects of the behavioral ecology of these species and their pollination biology (McNally and Schneider, 1992; Hepburn, 2011). Queens move with their colonies and in some cases, make multiple stops, taking as long as 1 month (*A. dorsata*) to reach the final destination (Hepburn, 2011). DNA evidence demonstrates that *A. dorsata* queens can live at least 3 years and return to their original nest sites (Paar et al., 2000) over as much as two annual migrations (Paar et al., 2000). It is unclear how colonies find their way back. Queen guidance is speculative, but, a plausible hypothesis given that memory plays a key role in the long-distance migrations of animals such as fish, turtles and birds (Milner-Gulland et al., 2011).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: G.Z., K.T., J.C.N.; Methodology: G.Z., K.T.; Validation: G.Z., J.C.N.; Formal analysis: G.Z., J.C.N.; Investigation: G.Z., K.T.; Resources: K.T.; Writing - original draft: G.Z., J.C.N.; Writing - review & editing: G.Z., K.T., J.C.N.; Visualization: G.Z., J.C.N.; Supervision: K.T.; Project administration: K.T.; Funding acquisition: K.T.

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### Data availability

All data are available in the Zenodo database (doi: 10.5281/zenodo.1148794).

### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.177303.supplemental>

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## SUPPORTING INFORMATION

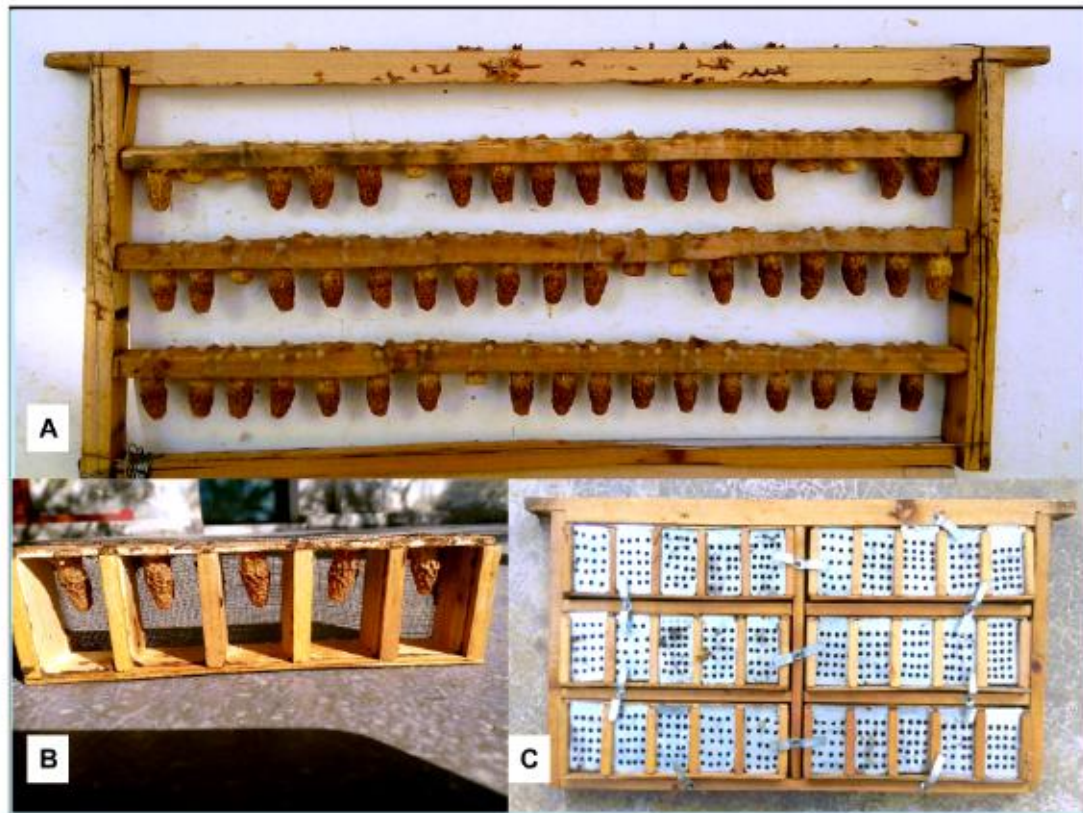
**Table S1.** The number of bees and colonies used in each experiment (LTM=Long Term Memory, DMF= dimethyformamide).

Experiment	Caste	Colony	Adult age		10 d	15 d	20d	25 d	Forager
			1 d	5 d					
EXP. 1	queen	Colony 1		20					
		Colony 2		20					
		Colony 3		20					
	worker	Colony 1		36	34	36	31	34	
		Colony 2		36	34	36	31	34	
		Colony 3		36	34	36	31	34	
EXP. 2	queen	Colony 1		20					
		Colony 2		20					
		Colony 3		20					
	worker	Colony 1		20					
		Colony 2		20					
		Colony 3		20					
EXP. 3	queen	Colony 1 (LTM / RM)		22 / 22					
		Colony 2 (LTM / RM)		22 / 22					
		Colony 3 (LTM / RM)		21 / 21					
	worker	Colony 1 (LTM / RM)							31 / 33
		Colony 2 (LTM / RM)							31 / 33
		Colony 3 (LTM / RM)							31 / 33
EXP. 4	queen	Colony 1 (DMF / Zeb)	21 / 20	22 / 24	21 / 21	25 / 25			
		Colony 2 (DMF / Zeb)	21 / 20	22 / 24	21 / 21	25 / 25			
		Colony 3 (DMF / Zeb)	21 / 20	22 / 24	21 / 21	25 / 25			
	worker	Colony 1 (DMF / Zeb)	30 / 31	31 / 31	31 / 29	33 / 30			33 / 36
		Colony 2 (DMF / Zeb)	30 / 31	31 / 31	31 / 29	33 / 30			33 / 36
		Colony 3 (DMF / Zeb)	30 / 31	31 / 31	31 / 29	33 / 30			33 / 36
	Gene expression	Colony 1	5	5	5	5			
		Colony 2	5	5	5	5			
		Colony 3	5	5	5	5			

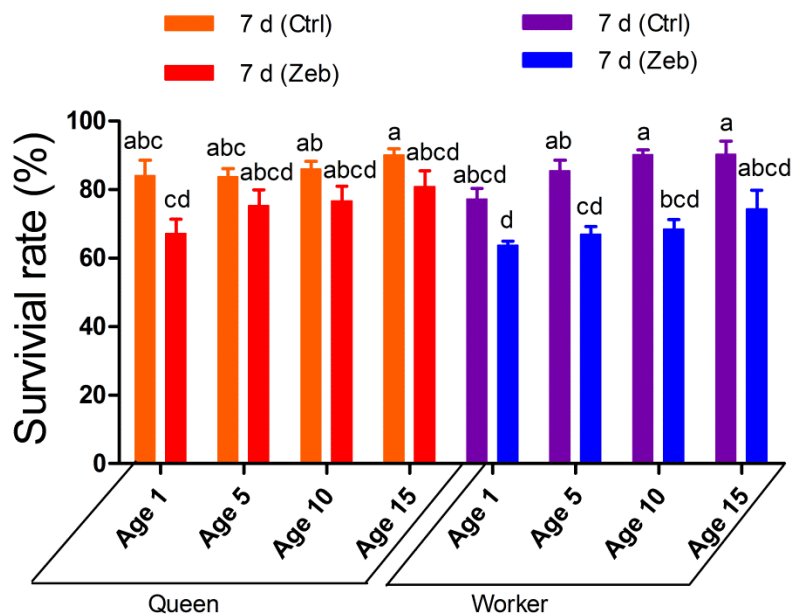
**Table S2.** Comparisons between the methods of our study and two relevant studies (Lockett et al. 2010 and Biergans et al. 2015). This table is not a complete summary of all of the differences between these three studies but is given to highlight why caution is needed in comparisons. For example, our study differed from the others because it included a memory test 30 min before the bees were frozen for gene expression analysis, unlike the other two studies.

	Our study	Lockett et al. (2010)	Biergans et al. (2015)*
Age	1, 5, 10, 15	7	10
Caste	Queens and workers	Workers	Workers
Zeb treatment time point (where applicable)	5 h before training	No zeb treatment	2 h after training
Test time point	Zeb treatment or no zeb treatment, then tested 7 days after training	30 min after training	(1) Zeb treatment and 24 h after training (Fig. 2b*)  (2) 1, 3, 5 and 24 h after training (Fig. 3b*) with no zeb treatment





**Fig. S1.** Queen-rearing setup showing (A) queen cells nearing completion, (B) completed queen cells now separated into (C) individual chambers.



**Fig. S2.** Survival rates in both queens and workers treated with dimethylformamide (DMF, the Ctrl) or zebularine (zeb) at different ages. Survival is based upon the percentage of bees that lived between the last acquisition training trial and the 7 day long term memory test.