

# Current Biology

## Context Odor Presentation during Sleep Enhances Memory in Honeybees

### Highlights

- Deep-sleep phases in honeybees have the potential to prompt memory consolidation
- Re-exposure to a learning context in deep sleep leads to transition from STM to LTM
- Sleep has similar effects on memory consolidation in mammals and insects
- Sleep most likely has a conserved role in memory processes

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### In Brief

Zwaka et al. investigate whether sleep's role in memory processing is similar in evolutionarily distant species and demonstrate that a context trigger improves memory in invertebrates, as it does in humans. They show that in honeybees, exposure to an odor during deep sleep that has been present during learning improves memory performance.



# Context Odor Presentation during Sleep Enhances Memory in Honeybees

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## SUMMARY

Sleep plays an important role in stabilizing new memory traces after learning [1–3]. Here we investigate whether sleep's role in memory processing is similar in evolutionarily distant species and demonstrate that a context trigger during deep-sleep phases improves memory in invertebrates, as it does in humans. We show that in honeybees (*Apis mellifera*), exposure to an odor during deep sleep that has been present during learning improves memory performance the following day. Presentation of the context odor during wake phases or novel odors during sleep does not enhance memory. In humans, memory consolidation can be triggered by presentation of a context odor during slow-wave sleep that had been present during learning [3–5]. Our results reveal that deep-sleep phases in honeybees have the potential to prompt memory consolidation, just as they do in humans. This study provides strong evidence for a conserved role of sleep—and how it affects memory processes—from insects to mammals.

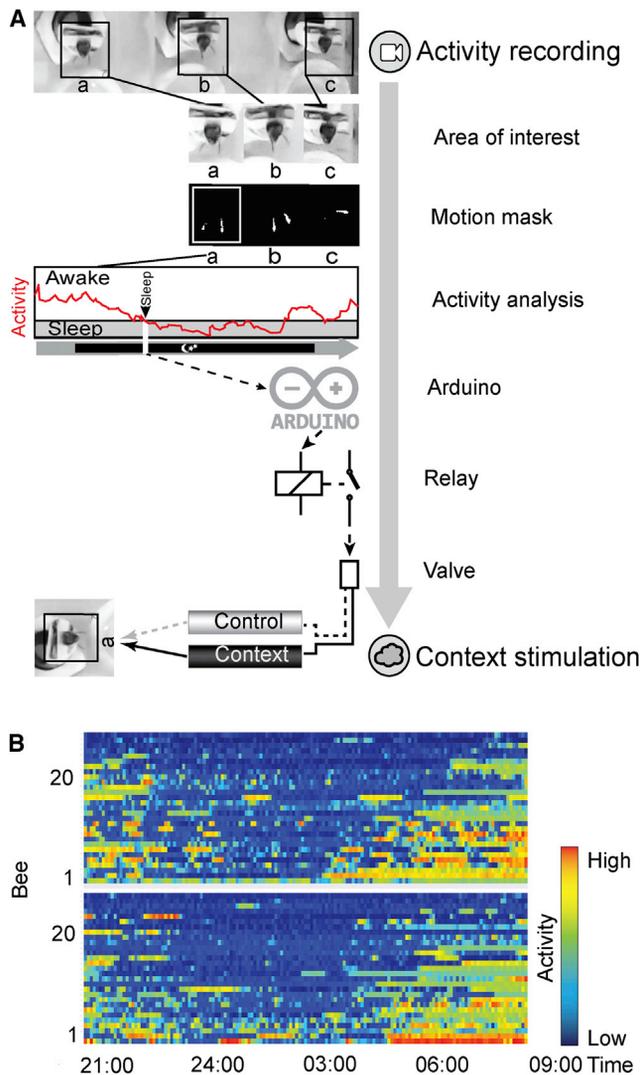
## RESULTS AND DISCUSSION

### Context Odor Presentation in Identified Sleep Phases Improves Retention

Honeybees are a well-established model for learning and memory. Classical conditioning of the proboscis extension response (PER) can be used to monitor learning and memory. By pairing of a conditioned stimulus with the unconditioned stimulus of a sugar reward, acquisition can be quantified at the level of the individual animal, and retention tests provide information about the strength of the memory trace [6–8]. Multiple stimuli can be used as conditioned stimuli, e.g., olfactory, thermal, and mechanical stimuli [9]. Furthermore, conditioning can be made dependent on a specific context [10–13]. Such training paradigms allow us to address the question of whether the re-exposure to a learned context condition during sleep improves memory consolidation. We examined whether the presentation of a context odor during deep-sleep phases leads to increased memory on the next day. All bees were conditioned five times to a thermal-sucrose pairing in the presence of a context odor.

After the last training trial, animals were randomly placed into either a test group or a control group. Bees were subsequently monitored during the night, and deep-sleep phases were identified by tracking of antennal movements. Since physiological measures like slow waves as indicators of deep sleep [14] are lacking in invertebrates, defining sleep and deep-sleep in bees relies on behavioral criteria. Honeybees behaviorally display three different sleep stages that can be distinguished, of which the deep-sleep stage is defined by antennal immobility, while they also display the highest reaction thresholds [15–18]. During five deep-sleep phases, when a bee's antennae become immobile, each bee in the test group was stimulated with the context odor and those in the control group with solvent paraffin oil, which is not perceived as an odor by the bee [19]. During the night, the distribution of high and low antennal activity was similar for bees in the control and test group (Figure 1B).

Repeated presentations of the context odor in deep-sleep phases resulted in a better performance in retention tests in the test group the following day, with the retention score acting as a measure for memory (Figure 2A). The control group shows the well-documented effect of reduced (or sometimes not changed) performance scores after 24 hr [9]. The improved retention scores of the test animals in relation to the control animals reveal that memory consolidation can be manipulated during sleep in insects, as well. But is the memory-enhancing effect selective for context odor stimulation during sleep? If any odor during deep-sleep phases has the potential to increase memory, we would expect no discernible differences in results from a group that received a novel odor and a group that received the context odor. We therefore compared memory retention in bees that had received either the context odor or a novel odor during deep sleep, adding two more extinction trials the following day to test resistance to extinction as a measure of the strength of the memory (Figure 2B). In our series of experiments, the retention score of the group of bees subjected to the context odor was significantly higher than that of the insects in the novel odor control group. Differences between the two groups remained stable over all three extinction trials. We can therefore conclude that improved retention in the experimental group depended on stimulation with the specific context odor, although we still do not know whether memory improvement would result from a repetition of the context odor at any time or whether it relies specifically on a presentation during sleep phases.



**Figure 1. Experimental Setup and Antennae Activity during the Night** (A) A video camera recorded antennae movement in 20 bees simultaneously (activity recording). Specified areas of interest that were monitored included antennal movement in each bee. A motion mask of every pixel that was in motion between two pictures was extracted. These pixels were counted and used as a measure for activity over time (single-bee activity). The bee received the odor based on the criterion that antennal activity was lower than a threshold for this individual bee for at least 1 min. A signal was sent to an Arduino microprocessor, which was connected to a relay that controls a magnetic valve. Each valve was connected to one pair of syringes related to one bee's chamber. When the magnetic valve was switched by the relay, a constant airflow switched to another syringe, and the context odor or control odor flowed into the chamber. The air was sucked out of each chamber separately. (B) Antennal activity in 58 bees from three consecutive nights receiving either the context odor or a control (animals included in the experiment in Figure 4). The heatmap displays the individual's antennal activity calculated using the rolling mean with a 5 min window size. The scale indicates activity from low to high in each bee.

### Presentation of the Context Odor in Wake Phases Does Not Improve Retention

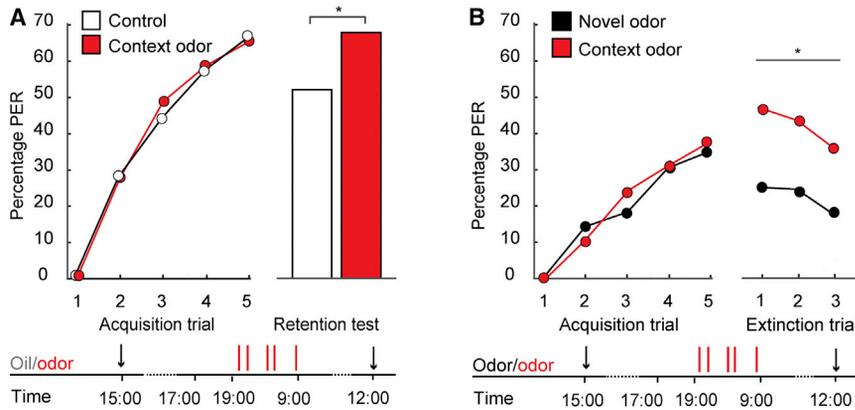
Using the same training procedure as in the previous experiment, we then added a group that received the context odor

when the bees were awake in order to test whether the context odor needs to be presented during sleep phases to improve memory. Bees were divided into two groups: animals that were stimulated during five identified waking phases (indicated by active movements of the antennae; Figure 3A) and animals that were stimulated during deep-sleep phases, similar to the experiments described above (Figure 3B). Each of the two groups consisted of a test group and a control group. Animals in the test group were exposed to the context odor, whereas animals in the control group were exposed to the solvent paraffin oil. A repeated presentation of the context odor during waking phases did not improve retention (Figure 3A), whereas the same odor presented during sleep phases did (Figure 3B). However, at this point, there was still no evidence that context odor presentation specifically during deep-sleep phases is instrumental to increased retention. Could it also be caused simply by the presentation of a context odor during rest periods at night? We therefore addressed the question of whether the presentation of a context odor at any time during the night leads to an improvement of retention scores independent of the sleep status of the animal. To do so, we presented the context odor at fixed points in time, either independently of wake/sleep phases (see Figures S1A and S1B) or constantly (see Figure S1C) throughout the night. Because bees differ in sleep-bout distribution (see Figure 1B), the presentation of context odor at fixed points in time for each individual bee appears to be random in relation to sleep status. All bees were conditioned as they had been in the previous experiments.

We tested three conditions: five stimulations with the context odor and a paraffin oil control (see Figure S1A), five stimulations with the context odor and a novel odor as a control (see Figure S1B), and constant stimulation with the context odor and paraffin oil during the night (see Figure S1C). None of the treated groups differed significantly from their respective control groups. This implies that the context odor improves memory only when it is presented in deep-sleep phases.

### Presentation of the Context Odor in Deep Sleep Improves Retention after Single-Trial Learning

In honeybees—when compared to the memory induced by multiple-trial learning—single-trial conditioning induces a low-level, 24 hr memory. Stable, long-term memory apparently requires more than one stimulus-reward pairing. Single-trial conditioning is known to lack a translation- and transcription-dependent form of memory consolidation, leading to the greatly reduced retention after 24 hr [20]. Re-exposure to a context odor can potentially lead to a transition from short-term to long-term memory, and thus to the initiation of cellular processes involved in long-term consolidation. After pairing thermal stimulus and sucrose reward only once during training, we subsequently asked whether the presentation of a context odor during deep-sleep phases also leads to increased retention of a conditioned stimulus on the following day. To establish this, we treated the bees again as we did during our initial experiment, with the exception that two of the three groups of animals were conditioned just once to the thermal-sucrose pairing in the presence of the context odor. During the night, the animals received either the context odor or paraffin oil as a control. Retention scores the following day were significantly higher in the test and five-trial



**Figure 2. Repeated Presentation of the Context Odor in Identified Sleep Phases Improves Retention**

All groups of animals learned to associate a thermal stimulus with a sugar reward in the presence of a context odor during appetitive conditioning in five acquisition trials. During the night, either the context odor or a control (paraffin oil, A; or a novel odor, B) was presented for 1 min in five separate phases of deep sleep, as indicated by the graphic.

(A) In the retention test the following day, the group stimulated with the context odor during sleep phases showed a statistically significant higher retention score than the control group ( $n_{\text{control}} = 89$ ,  $n_{\text{context}} = 94$ ; repeated measures ANOVA [rANOVA],  $F_{(4,724)} = 2.7$ ,  $p = 0.029$ ; Fisher's least significant difference [LSD],  $p = 0.02$ ).

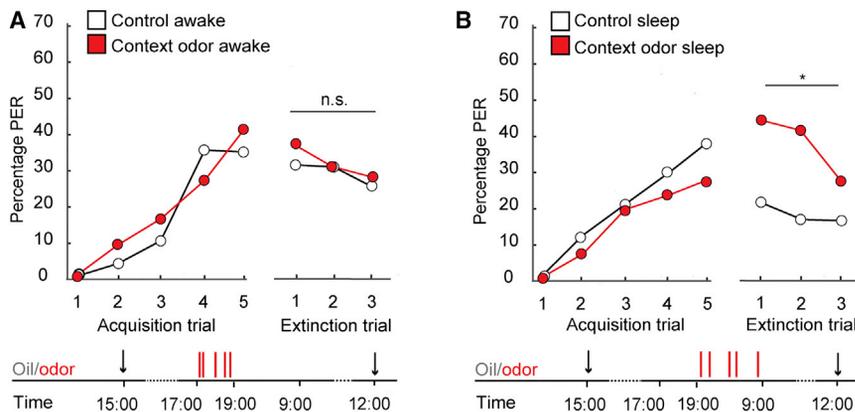
(B) Retention scores were higher in the group that received the context odor during sleep phases compared to the group that received a novel odor. The retention score remained high over all three extinction trials ( $n_{\text{novel odor}} = 63$ ,  $n_{\text{context}} = 88$ ; rANOVA,  $F_{(6,894)} = 3.9$ ,  $p = 0.0008$ ; Fisher's LSD,  $p_{(1\text{st extinction trial})} = 0.001$ ,  $p_{(2\text{nd extinction trial})} = 0.008$ ,  $p_{(3\text{rd extinction trial})} = 0.012$ ).

groups than in the control group (Figure 4). This increased memory after single-trial learning in our experiments further confirms that repeated presentations of a learned context during deep-sleep phases consolidate the new memory. Further experiments will have to ask whether transition to stable long-term memory after multiple learning trials and after a single learning trial plus context stimulation during deep-sleep phases are mechanistically related and whether these mechanisms reflect a replay of neural activity indicative of the learning effect.

Our results demonstrate that the repeated presentation of the odor cue in deep-sleep phases resulted in a better memory performance the following day. Bees also showed an increased memory performance the next day when they were trained with only a single trial and received the context odor in deep-sleep phases. Memory consolidation in mammals during sleep is suggested to function via reactivation [21–23]. In humans, presentation of the odor cue during slow wave sleep (SWS) activates the hippocampus [5]. Olfactory and—in different ex-

periments—also auditory context stimuli can reactivate newly encoded memories during sleep and thereby strengthen the processes underlying consolidation of these newly acquired memories [4, 5, 24]. Sleep supports consolidation of memory in insects [14, 25–27], but whether reactivation of the memory trace is essential is not yet known.

The first proof that insect species sleep was discovered in honeybees around 30 years ago [28]. Since then, the list of other invertebrate model organisms in sleep research has grown, and it now includes *Drosophila melanogaster*, scorpions, cockroaches, and *Caenorhabditis elegans* [29–34]. Several findings suggest a relation between sleep and memory in insects. In honeybees, retention scores improve if sleep is not disturbed, whereas memory formation after extinction learning—but not after acquisition learning—is selectively reduced if animals are prevented from sleeping [27]. In sleep-deprived honeybees, waggle-dance precision is impaired [35], and newly acquired navigation memory is compromised when night sleep is interrupted [25]. Similar effects



**Figure 3. Repeated Presentation of the Context Odor in Wake Phases Does Not Improve Retention**

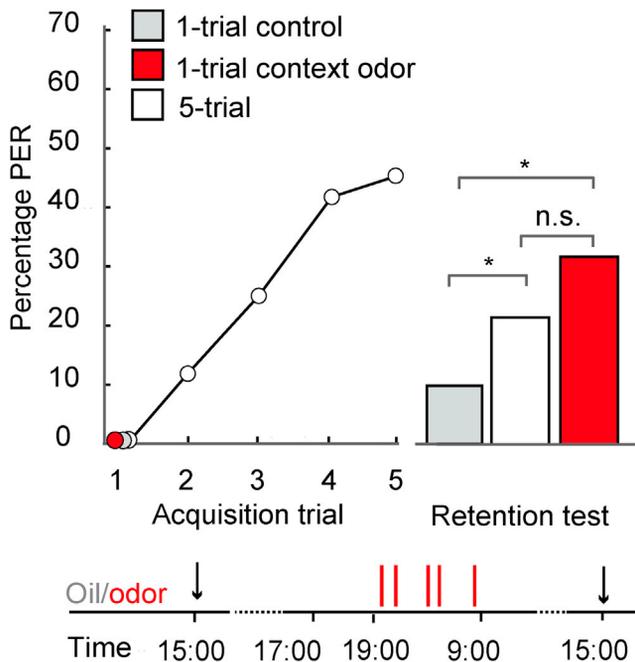
Both groups learned to associate thermal stimulus-sugar pairings in the presence of a context odor during appetitive conditioning.

(A) Bees were subjected to either the context odor or paraffin oil during five phases of lasting antennae activity for 1 min during the early evening, as indicated by the graphic. In three extinction trials the next day, retention scores were similar in both groups that received the context odor in wake phases and the control group ( $n_{\text{control}} = 29$ ,  $n_{\text{context}} = 29$ ; rANOVA,  $F_{(6,336)} = 0.3$ ,  $p = 0.9$ ).

(B) During the night, either the context odor or a control (paraffin oil) was presented for 1 min in five phases of deep sleep. In a retention test the next

day, the group that received the context odor during sleep phases exhibited a statistically significant higher retention score than the control group. The retention score remained high over three extinction trials ( $n_{\text{control}} = 23$ ,  $n_{\text{context}} = 38$ ; rANOVA,  $F_{(6,354)} = 2.6$ ,  $p = 0.01$ ; Fisher's LSD,  $p_{(1\text{st extinction trial})} = 0.004$ ,  $p_{(2\text{nd extinction trial})} = 0.003$ ,  $p_{(3\text{rd extinction trial})} = 0.3$ ).

(A) and (B) were conducted in parallel. See also Figure S1.



**Figure 4. Repeated Presentation of the Context Odor in Identified Sleep Phases Improves Retention after Single-Trial Learning**

Two groups of animals received one pairing of a thermal stimulus with a sugar reward in the presence of a context odor during appetitive conditioning, a third group (five-trial) received five pairings in the presence of a context odor. During the night, the test group was subjected to the context odor and the control group to the solvent paraffin oil for 1 min during five phases of deep sleep. The third group did not receive any treatment. In the retention test the following day, the test group showed a statistically significant higher retention score than the control ( $n_{\text{control}} = 75$ ,  $n_{\text{context}} = 77$ ;  $p = 0.001$ , two-tailed Fisher's exact test) as did the five-trial group ( $n_{\text{control}} = 75$ ,  $n_{\text{five-trial}} = 73$ ;  $p = 0.042$ , two-tailed Fisher's exact test).

involving spatial memory are known in mammals [22, 36]. Data in *Drosophila* also support the hypothesis that sleep and neuronal activity may be interdependent. Experience-dependent changes in sleep require among others a particular subset of long-term memory genes [37]. In addition, induction of sleep in *Drosophila* facilitates the formation of long-term memory. Sleep shortly after learning has been shown to consistently lead to an increase in retention in long-term courtship memory [26].

Tononi and Cirelli suggested that sleep might affect memory acquisition, consolidation, and integration via activity-dependent down-selection of synapses [38]. New findings support the interpretation that newly formed and not yet stabilized memory may be converted to more stable memory during sleep. This hypothesis was suggested by the finding that increased sleep led to a decrease in ongoing dopaminergic activity and an increase of memory retention [39]. It will be important to ask in future experiments how these cellular mechanisms relate to reactivation processes, as suggested here. Increased memory after single-trial learning in our experiments suggests that repeated presentation of the learned context during phases of deep sleep thus may act similarly to repeated learning trials by some form of reactivation of new memory. Single-trial conditioning is known to lack a translation- and transcription-dependent

form of memory consolidation, leading to greatly reduced retention 24 hr after learning [20]. Re-exposure to the context odor might lead to the initiation of cellular processes involved in long-term consolidation, as multiple learning trials do.

Several findings indicate an evolutionarily conserved process of memory consolidation during sleep [14]. Other studies have discovered that similar signaling pathways regulate sleep and wakefulness in mammals and insects [40, 41]. The molecular response to sleep deprivation also appears to be conserved: similarities have been found between rats, mice, birds, *Drosophila*, and other insects [42], although the behavior to sleep deprivation might differ [31, 43, 44]. *D. melanogaster* and *C. elegans* especially have the potential to help elucidate the molecular mechanisms of sleep [45–49]. However, paradigms applied in human learning tasks like those reported by Rasch and colleagues [5] have not yet been reported in these two species.

Although we found that deep-sleep phases in honeybees share the same potential to prompt memory consolidation with human deep-sleep phases (SWS), we do not know the neural processes involved. Still, sleep in insects is more similar to human sleep than was previously known. Our results not only suggest that closely related neural processes regulate sleep [42], but also strengthen the argument that sleep itself has similar effects on memory consolidation in mammals and insects.

## EXPERIMENTAL PROCEDURES

### Learning Experiments

The temperature/sugar conditioning procedure followed [9, 10]. A constant odor stream was placed in front of the animal. One conditioning trial consisted of placement of the bee in front of an exhaust where the context odor was continuously present during the whole trial: the conditioned stimulus (CS) was a thermal stimulus provided by a warm soldering iron and presented close to the animal's head (no direct contact). Presented for 5 s, the thermal stimulus was followed by the presentation of a 30% sucrose solution lasting for 4 s (unconditioned stimulus, US) with an overlap of 2 s. If the bee responded solely to the thermal stimulus by extending its proboscis (PER), this was noted as a conditioned response. Conditioning consisted of five trials (CS-US pairings) in the presence of the context odor. The next day, the bees were tested for retention.

### Odor Cues in Phases of Sleep or Waking State

Animals were placed in a setup where they individually received the context or control odor in phases of sleep or waking. When sleep was detected, the bee received the context odor, paraffin oil, or a novel odor for 1 min. Sleep was defined by a period of 1 min of little to no movement of the respective bee's antennae. For detection of deep-sleep phases, a threshold was set to 15% of the bee's specific average awake activity. No physiological measures like local field potentials are yet available as indicators for deep sleep in bees. There are three sleep stages reported in honeybees. Phases (1) with swaying motions of antennae, (2) with minute twitching of antennae, or (3) with antennae immobile [18]. The third stage in bees is described as "deep sleep," with an increase of reaction thresholds and decrease in muscle tone [16]. 15% activity resembled no antennae movement like swaying, twitches, or random movement, which are common when bees are awake or in other than deep-sleep stages. When the activity was lower than this threshold for at least 1 min—thereby excluding phases with minute antenna twitching—the particular bee was considered to be in a deep-sleep phase. For odor triggering in wake phases, the bee's antennal movement needed to be above 50% of the initial threshold for at least 15 s. The value was chosen because it represented a wake state without indication of a higher state of arousal.

Each bee received a maximum number of five stimulations during the night. The minimum time interval between two consecutive stimuli was set to 1 hr.

For presentation of the context odor independently of the wake/sleep phases of a particular animal, it was presented to all animals at the same

time, with fixed intervals between the repeated presentations. Sleep bout distribution differs among individual bees (Figure 1B). Therefore, presentations of the context odor at fixed points in time meant for each individual bee that it appeared randomly in terms of sleep status. Three conditions were tested: (1) stimulation with the context odor, (2) stimulation with a novel odor, and (3) stimulation with paraffin oil.

To test for the effect of a permanent odor cue overnight, bees were kept in two dark plastic boxes overnight with either 4  $\mu$ l of the context odor or 4  $\mu$ l paraffin oil on a filter paper in a Petri dish.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2015.09.069>.

### AUTHOR CONTRIBUTIONS

H.Z. and R.B. designed the study, performed data analysis, carried out experiments, and wrote the manuscript. J.G. designed the study and the software for sleep analysis and odor trigger and performed data analysis. V.F., A.C., and M.G. carried out experiments. R.M. designed the study and revised the manuscript.

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