

Chapter 3:

Sleep in honeybees: its role in learning and memory

Summary:

Sleep-like behavior has been studied in honeybees before but the possibility of sleep related memory processes had not been explored. We here describe a new technique which allowed us to not only investigate sleep parameters but also to manipulate bees to perform learning tasks. Restrained bees were placed under a web camera and their antennal activities, which we used as indicators of sleep, were monitored. We found that bees sleep more during dark cycle and less during the light cycle. We observed two distinct patterns of antennal activities. The symmetrical activities were more prominent during night and asymmetrical activities were more common during the day. In next set of experiments we subjected the bees to various conditioning stimuli and observed their sleep thereafter. We showed that bees subjected to odor and reward, using classical conditioning of the proboscis extension response (PER) showed lowered sleep compared to bees that were subjected to odor alone, reward alone or air alone. Sleep depriving with light interference after conditioning did not affect duration of sleep and learning was similar to the non-deprived group. Also, sleep deprivation with shaking did not have any effect on acquisition memory. In another set of experiments, we tried to block the extinction memory by subjecting the bees to acquisition learning on day-1, two extinction trials on day-2 followed by sleep deprivation with shaking and extinction memory retrieval on day-3. Sleep deprivation after two extinction trials showed reduced extinction memory compared to non-deprived group on day-3 retrieval tests. This showed that sleep deprivation does not affect all forms of learning.

Introduction:

Aristotle's theory (350 B.C) for sleep was that, a person is awake from sleep when digestion is complete. Several wild theories have been put forward since then to explain sleep, but Freud's (1900) interpretation that dreams could be our subconscious memories and emotions was a revelation considering the fact that this theory could still be true. Philosophers thought that brain was quiet during sleep, but Caton in 1875 showed the electrical properties of brain with the help galvanometer in dogs and apes. In 1913, Henry Peiron's book on sleep "Le probleme physiologique du sommeil" kick started the modern sleep research. German psychiatrist Johannes Berger, the inventor of Electro-Encephalo-Graph (EEG), showed the difference between sleep and wake brain in humans in 1929. It was only in 1953 that Rapid Eye Movement (REM) sleep was discovered by Nathaniel Kleitman and his student Eugene Aserinsky. Soon a relationship between REM and sleep was established (Dement, 1958).

RW Hoffman of Göttingen in his paper "Der Insektenschlaf als reflektorische Immobilisation" in 1937 describes some characteristics of insect sleep. But since then very little has been reported about sleep in insects. Sleep has been shown in invertebrates like honeybees (Kaiser et al, 1983 & 1988; Schuppe H, 1995; Sauer et al, 2003 & 2004), fruit flies (Hendriks et al, 2000; Shaw et al, 2000, Greenspan, 2001), solitary bees (Kaiser, 1995), cockroach (Tobler, 1983), pacific beetle cockroach (Stephenson et al, 2007), moths (Anderson FO, 1968), paper wasps (Withgott J, 2002), locusts (Schuppe et al, 1996), Scorpion (Tobler et al, 1988), Jellyfish (Seymour et al, 2004) and crayfish (Ramon et al, 2004). Despite early studies on moths (1968), honeybees (1983) etc, sleep research in insects was dormant until the discovery of sleep in drosophila (Shaw et al., 2000) which spurred renewed interest in insect sleep research. There is much known about drosophila sleep now than any other insect because of its genetic tractibility and general popularity. Studies in honeybees have shown that, they sleep like other diurnal insects both in hives (Kaiser W, 1988) and in lab conditions (Sauer et al, 2003), and their sleep is controlled by circadian rhythms. Like in mammals, sleep depriving bees makes them to sleep longer the following night, a phenomenon called "sleep rebound". This

shows that sleep in honeybees is regulated by homeostatic mechanisms (Sauer et al, 2004).

Considering that we humans spend 1/3rd of our lifetime sleeping, “Why do we sleep?” is the most sought after question in sleep research. Most researchers are now focusing on the functions of sleep and are particularly interested in finding correlates with learning and memory. Recent studies on human subjects (Born et al, 2006; Daurat et al, 2007; Gais et al, 2006) show that slow-wave-sleep or SWS (a part of non-REM) is important for declarative memory and REM sleep is important for procedural memory. Additionally, declarative but not procedural memory could be boosted when subjects were stimulated with electrical potentials during the first 45 minutes of SWS sleep (Marshall et al, 2006). Another study (Rasch et al, 2007) showed boost in memories when subjects were exposed to odors (used as context during learning) during SWS sleep. All these data show improvement of hippocampus-dependent (declarative) memories and not of hippocampus independent (procedural) memories.

In insects, there are very few studies that link sleep with learning and memory. However, in drosophila there is sufficient evidence now that sleep is regulated by mushroom bodies (Joiner et al, 2006) and plays a dynamic role in promoting sleep (Pitman et al, 2006). One study (Bushey et al, 2007) shows that drosophila hyperkinetic mutants have reduced sleep and poor memory and another study (Ganguly-Fitzgerald et al., 2006) shows how long-term courtship memories in flies at 48h after training could be abolished by sleep deprivation within 24h after training. Sleep deprivation after 24h of training had no effect on memory.

To study sleep in honeybees, we monitored the movements of the antennae. It has been previously shown that antennal immobility is a good indicator of sleep (Sauer et al, 2003 & 2004). Our focus therefore was not only to study sleep, but to understand its relationship with learning and memory. We started out by studying antennal immobility in restrained bees which allowed us to combine sleep experiments with the well established proboscis extension reflex (PER) paradigm (Bitterman et al, 1983) for learning. Therefore, in a single experimental setup bees could be conditioned, monitored for sleep and tested for memory. We first checked if our sleep results confirm with other studies and if there were any special patterns in sleeps. We then asked if learning affected

sleep by studying sleeps in conditioned bees and unconditioned bees. And finally, we sought the question, is sleep necessary for learning? We therefore sleep deprived bees to see if previously learnt information was retained post-deprivation.

Materials and methods:

Preparation of bees:

Foraging honeybees (*Apis mellifera carnica*) were caught from the entrance of the outdoor or indoor hives 1 day prior to an experiment and were cold-anesthetized on ice. Anesthetized bees were fixed inside plastic restraining tubes such that only the mandibles, proboscis, and antennae could move freely (Bitterman et al. 1983). The scapes of the antennae were fixed onto the head using eicosane (Sigma-Aldrich) such that only the flagellum could move. Bees were fed 30% sugar solution until satiation and were kept under 12h light and 12h dark cycle at approx. 25-27°C.

Training procedure:

Bees were checked for PER (unconditioned response: UR) by lightly touching the antennae with 30% sucrose solution 10 minutes before training. Only bees which demonstrated the UR were trained (<5% were discarded). 2-octanol, limonene and peppermint were used as odor CSs and 30% sucrose solution was used as an appetitive reinforcer (US). Olfactometer (a computer-driven device), blowing continuous stream of air over the bee's antennae, was used to deliver the odors to the bees (Galizia et al. 1997, Komischke et al. 2002). 4 µl of odor was pipetted onto a half sq. inch filter paper, placed inside 1-ml syringe and was fitted into a hole of the olfactometer (Galizia et al. 1997). For each experiment four syringes were used; three syringes contained odors and one syringe contained odorless filter paper (Air control). During the training each bee was placed in front of the olfactometer with its antennae facing the air-stream for 60 seconds. During this period either CS or US or both were presented to the bee and an exhaust system behind removed the odors.

Sleep-box setup for antennal activity recording:

All antennal recordings were done in a dark room. An individual bee was placed inside a cubical plastic box which had 5 sides closed and one side open (Fig. 3.1). A web camera (Philips ToUcam Pro II) was fixed on top of the box such that the antennae of the bee were clearly visible as black structures against a white background. An infra-red light

was fixed alongside the camera for recording videos in the dark. Bees were kept under 12hr dark and 12h light cycle. A bright white/yellow light was kept on in front of the box during the day (07:00 – 19:00 hrs) and during the night (19:00 – 07:00 hrs) the light was kept off. Temperature probe was kept inside the box to monitor day and night temperatures and a filter paper soaked in water was kept inside the box to keep the interior humid. The web camera was connected to a PC via USB 2.0 and a camera software (Active webcam v6.2) was used to record non-stop videos for 19 hours at 5-10 frames per second (fps). The videos were stored in a hard-disk for online or offline analysis. Note: Since antennae were fixed, 5-10 fps recordings were sufficient for antennal detections. Higher frame rates (15fps and 25fps) did not improve antennal detection.

Antennal activity detection:

Antenna tracking program (Fig. 3.2), a custom-made computer program written in C++ language (by Tim Landgraf), was used to track the angular movements of the antennae.

After the bees were placed under the web camera, a video recording software was started and adjusted to obtain dark antennae against the bright background. Simultaneously, antenna tracking program was started. In this program, user had to interactively define the coordinates of the antennae on a frame of video. Right and left antennal positions were defined by drawing lines over them and arcs were drawn around each antenna to define its area of movement. Angles of the antennae were measured against an imaginary line between the two pedicles of right and left antennae. After the coordinates were defined for one frame, the program automatically tracked the angular movements of the antennae for all subsequent frames. The data containing angles of both antennae along with time of day was saved to a text file. This text file was analyzed online or offline using MATLAB.

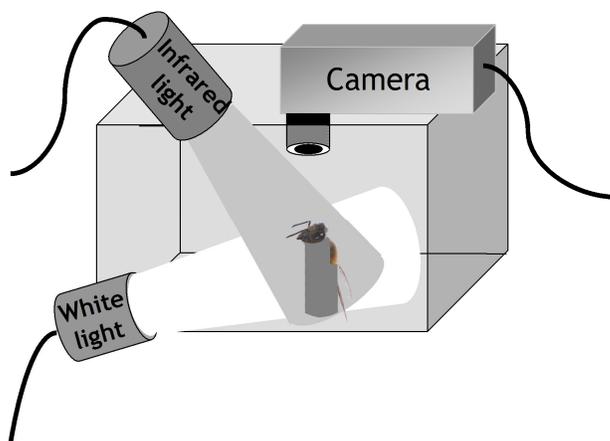


Fig. 3.1: Sleep-box- Bee was placed inside a 5-sided box under a web camera such that dark antennae were visible against the white background. Infra-red light was used for recording in dark. White light was turned on during the day and was also used for sleep deprivation experiments.

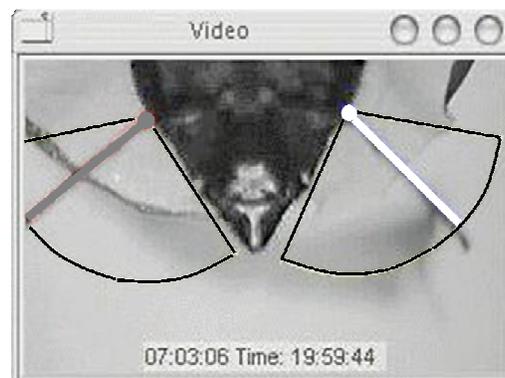


Fig. 3.2: Antenna tracking program- The antennae of the bee were monitored using this program. After the user defined the left (white) and right (grey) antenna, the program automatically detected their movements and reported their positions in angles.

Sleep deprivation:

- 1) Light: The white light (Fig. 3.1) that was used during the day was also used for sleep deprivation. Bees were subjected to 5 minutes of white light and 5 minutes of no light (Dark) during the 12h dark cycle.
- 2) Shaking: Bees were placed on a vortex (Fig. 3.3) and shaken at 80-120rpm for 5 minutes and rested for 5 minutes during the entire light and dark cycle.

Multi-antennal activity:

To analyze more than one pairs of antennae, a bigger sleep-box setup was used. About 8 bees were placed inside the sleep-box and all antennae were simultaneously video recorded with a web camera at 10 fps and monitored for antennal activities. Since the resulting videos were different from single bee videos, a new computer program (by Tim Landgraf) was designed to analyze the antennal activities of all bees simultaneously. The program allowed user to define the bee heads individually and it automatically used the non-selected areas as reference or background noise. This allowed efficient removal of fluctuations in lighting conditions and the values returned were true representations of antennal activities. The program analyzed the pixel changes from one frame to another and this pixel change was reported as absolute multi-antennal activity. The relative multi-

antennal activity was calculated as the ratio between antennal activities vs. background noise. The unit of multi-antennal activity was pixel change per hour (Δ pixels/ hr). If the antennae stay in the same position then there will be no pixel change and therefore relative multi-antennal activity would be 1. The antennal activities of day-1 and day-3 were plotted and compared.

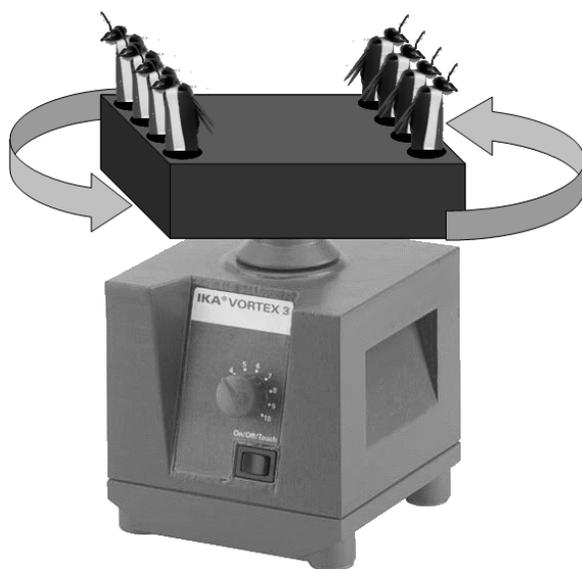


Fig. 3.3: Sleep deprivation by shaking- Bees were placed on the vortex immediately after training and shaken at 80-120 rpm until the following day.

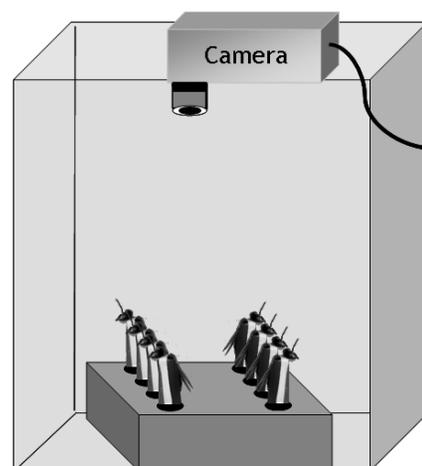


Fig. 3.4: Multi-antennal activity- To monitor many antennae, a bigger sleep-box setup was used. Up to 8 bees were placed inside the sleep-box and all antennae were simultaneously video recorded with web camera at 10 fps and monitored for antennal activities.

Analysis:

Matlab software was used to analyze and plot the antennal positions from the stored data. Specific Matlab functions were written (by Tim Landgraf) to automatically detect specific patterns of antennal movements. Plotting and statistics were done using Microsoft Excel, Matlab and R (statistical and programming language). Paired t-tests and Wilcoxon tests were used for determining statistical significance.

Experiments:***Experiment 1: Antennal activity***

In this experiment, individual bee was fed with 4-6 drops of 30% sucrose solution and placed under the web camera for video recording. Antenna tracking procedure was performed on the video and resulting data was analyzed with Matlab.

Based on previous studies (Shaw et al, 2000), sleep was defined as immobility or inactivity of both antennae lasting 5 minutes or more. The amount of sleep (sleep duration- minutes/hour) and number of sleeps (sleep bout- bouts/hour) was calculated for every hour. Cumulative sleep for the entire day (19hrs) was also calculated. Additionally, user identified antennal patterns were detected and their activities were calculated per hour basis.

Experiment 2: Olfactory conditioning and antennal activity

In this experiment bees were subjected to olfactory conditioning before observing antennal activity. Bees were randomly allotted to one of the 4 groups, viz. Group-1: CS+US, Group-2: US only, Group-3: CS only and Group-4: Air only.

Training protocol:

Individual bees were placed in front of the olfactometer facing the air stream. Each training trial was 60 seconds; After 27 seconds of air, one of the following stimuli were presented which was followed by air until end of 60 seconds.

Group-1: 4 seconds of CS and 3 seconds of US with 1 sec overlap.

Group-2: 4 seconds of CS only.

Group-3: 4 seconds of Air only.

Group-4: 3 seconds of US only.

Each bee was trained 5 times with inter-trial interval (ITI) of 10 minutes and one hour after the training all bees were fed with 4-6 drops of 30% sucrose solution. Each bee was placed inside the sleep-box under a web camera and their antennal activities were recorded. The bees were kept on 12h light/12h dark cycle at room temperature. Next day the antennal activities for each group were analyzed and their memories were tested by presenting them with CS.

Experiment 3: Sleep-deprivation with light

This experiment was similar to experiment 2 except that every group was interfered with white light during the 12h dark cycle and the memory was tested the following day. Bees were randomly allotted to one of the 4 groups, viz. Group-1: CS+US, Group-2: US only, Group-3: CS only and Group-4: Air only.

Training protocol:

Individual bees were placed in front of the olfactometer facing the air stream. Each training trial was 60 seconds; After 27 seconds of air, one of the following stimuli were presented which was followed by air until end of 60 seconds.

Group-1: 4 seconds of CS and 3 seconds of US with 1 sec overlap.

Group-2: 4 seconds of CS only.

Group-3: 4 seconds of Air only.

Group-4: 3 seconds of US only.

Each bee was trained 5 times with ITI of 10 minutes and one hour after the training all bees were fed with 4-6 drops of 30% sucrose solution. Each bee was placed inside the sleep-box under a web camera and their antennal activities were recorded. During the 12h dark cycle, bees were interfered with white light every 5 minutes. Bees were undisturbed during the light cycle. Next day the antennal activities for each group were analyzed. Also, animals were tested for memory by presenting them with CS. Since these experiments were done in parallel with experiment 2, it was possible to compare them.

Experiment 4: Sleep-deprivation by shaking**Experiment 4.1: Absolute conditioning and shaking**

In this experiment bees were conditioned with 3 trials of CS+US and divided into two groups. Immediately after training Group-1 bees were placed on a vortex and shaken at 100-120rpm every 5 minutes until the next day under 12h light/12h dark cycle. Group-2 bees were left undisturbed and placed under 12h light/12h dark cycle. On the second day both groups were tested for memory retrieval by presenting them with the same CS that was used during conditioning.

Experiment 4.2: Extinction block experiment and shaking

Day-1 of this experiment was similar to that of previous experiment, i.e. bees were first absolute conditioned with 3 trials of CS+US with 10 minute ITI and divided into two groups.

On day-2, Group-1 bees were placed in front of the olfactometer and presented with 2 trials of CS alone (extinction trials) and Group-2 bees were placed in front of the olfactometer but were not presented with any CS (no extinction trials). Group-1 and Group-2 bees were further divided into two groups each. Group-1a and Group-2a bees were placed on a vortex and shaken at 100-120rpm every 5 minutes until the next day under 12h light/12h dark cycle. Group-1b and Group-2b bees were left undisturbed and placed under 12h light/12h dark cycle.

On day-3 all bees were tested for memory by presenting them with the same CS used for conditioning on day-1. Few bees were used for experiment 5.

Experiment 5: Multi- antennal activity

In this experiment, a subset of bees from experiment 4.2 was used for multi-antennal activity. The behavior protocol was similar to experiment 4.2. On day-1, after 3-trial absolute conditioning with 10 minute ITI, bees were divided into two groups. Each group was placed under a web camera such that all pairs of antennae could be clearly seen. Their antennal activities were video recorded overnight.

On day-2, bees were subjected to 2 extinction trials and Group-1a bees were shaken at 100-120rpm every 5 minutes until the next day under 12h light/12h dark cycle and Group-1b bees were left undisturbed and placed under 12h light/12h dark cycle.

On day-3, both groups were subjected to retrieval tests and again placed under their respective web cameras and for antennal activity recordings. Next day, the antennal activities of both groups were compared.

Results:

Experiment 1: Antennal activity

The videos of the antennal activities were analyzed and angles of both antennae were plotted on the y-axis against time of day on the x-axis (Fig. 3.5). These plots showed that, the position of the right antenna (RA) was between 0 degrees (extension) and 100 degrees (flexion) and activity of the left antenna (LA) was between 80 degrees (flexion) and 180 degrees (extension) to the scapus. Any antennal angle less than 0 degree and more than 180 degrees was considered an artifact and ignored for analysis. More than 85% of the bees rested their antennae in flexed positions (RA-100 and LA-80). Based on previous studies on drosophila (*Shaw et al., 2000*), sleep was defined as antennal immobility lasting 5 minutes or more. Bees slept more during the dark cycle than the light cycle. After the onset of dark cycle the hourly average of sleep (sleep duration-Fig.3.6) increased from <10 minutes during the light cycle and peaked about 27 minutes at 7th hour ($P < 0.01$). This data conformed to previous studies (*Sauer et al, 2005*). The number of sleep bouts (Fig. 3.7) also increased ($P < 0.05$) during the dark cycle as compared to light cycle. The sleep was not continuous, but was interrupted by distinct patterns of antennal movements.

The activities (Fig. 3.5) of right (grey color) and left antenna (white color) were not completely random, but had some signs of synchronous movements. Apart from the several random patterns of antennal movements, two patterns viz. Symmetrical activity and Asymmetrical activity occurred in almost all bees. Symmetrical activity (Fig. 3.8) was the movement where both the antennae moved in opposite directions to each other. It was either antennae moving away from each other (right antenna moving right and left antenna moving left) or moving towards each other (right antenna moving left and left antenna moving right). In asymmetrical activity (Fig. 3.9), both antennae moved together in the same direction simultaneously. Analysis showed that the symmetrical activity was significantly higher ($p < 0.01$) during the dark cycle (peak: 6.13/hr) than the light cycle (peak: 3.42/hr), while asymmetrical activity appeared higher during the light cycle (peak: 6.64/hr) than the dark (peak: 4.67/hr) cycle but this effect was not statistically significant.

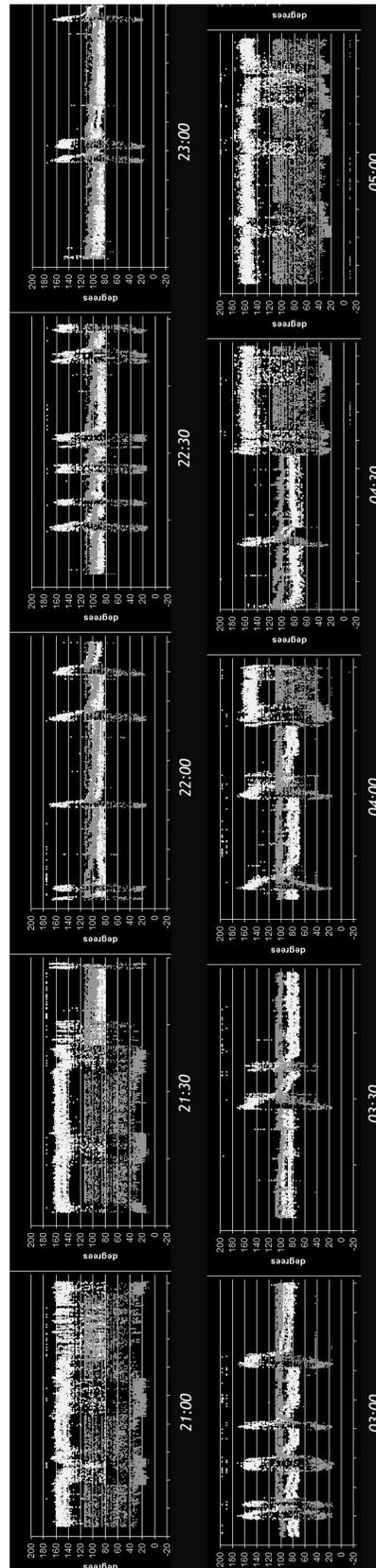
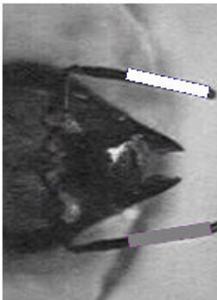


Fig. 3.5: Antennal activity: Plot showing angles of the left (white) and right (grey) antenna on y-axis against time of day on the x-axis. The antennae were active until around 22:00 hrs when suddenly the antennae shift to flexed position resembling sleep like posture. At this point left antenna was 80 degrees and right antenna was 100 degrees.. There were occasional bursts of activity during the night. After about 04:30 hrs the antennae return to normal activity which was observed commonly during the day.

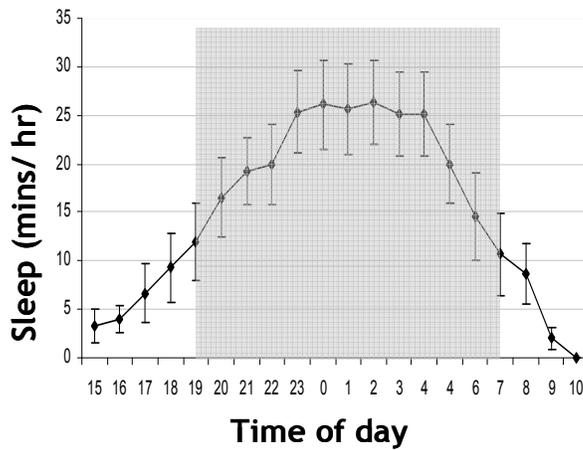


Fig. 3.6: Sleep duration: sleep was defined as immobility of antenna lasting 5 minutes or more. Based on this hourly sleep duration was calculated. Plot shows increasing sleep after the onset of dark cycle (gray shaded area). Peak sleep was about 27 mins/hour at 7th hour after dark cycle onset. Sleep decreased towards the end of dark cycle.

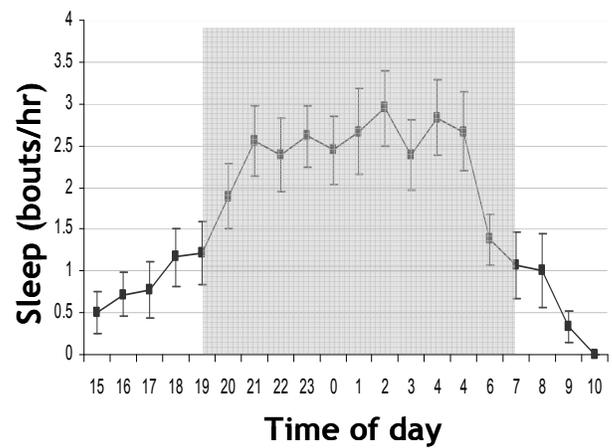


Fig. 3.7: Sleep bout: When bees slept, the sleep was not continuous, but there were interruptions such as symmetrical and asymmetrical movements. Sleep bouts there was the number of sleeps after such interruptions. Since bees did not sleep very often during the day sleep bouts were less too. After onset of dark cycle sleep bouts increased but decreased again towards the end of dark cycle.

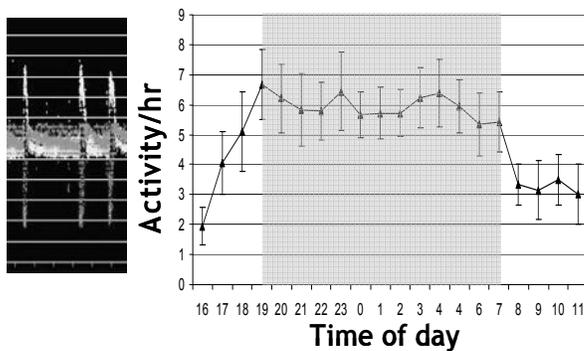


Fig. 3.8: Symmetrical activity: The activity in which both the antennae moved in opposite directions to each other. It was either antennae moving away from each other (right antenna moving right and left antenna moving left) or moving towards each other (right antenna moving left and left antenna moving right). Symmetrical activity was significantly higher ($p < 0.01$) during the dark cycle compared to light cycle.

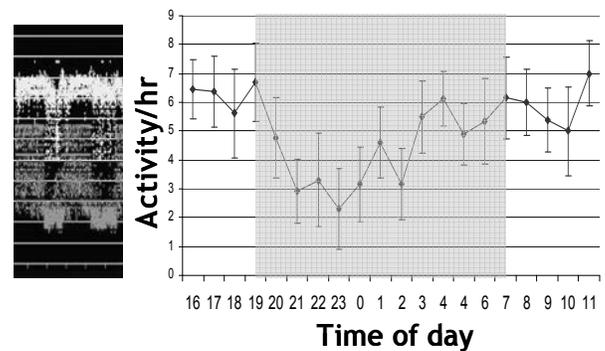


Fig. 3.9: Asymmetrical activity: The activity in which both antennae moved together in the same direction simultaneously. Asymmetrical activity appeared higher during the light cycle compared to dark cycle but was not statistically significant.

Experiment 2: Olfactory conditioning and sleep

After training the bees with 4 different conditions; CS+US, CS alone, Air alone and US alone and the cumulative sleep differed considerably (Fig. 3.10). The cumulative sleep was 337.5 minutes for Group-1, 407.44 minutes for Group-2, 436.4 minutes for Group-3 and 558.2 minutes for Group-4. Group-1 bees which were trained with both CS and US slept significantly lesser than Group-4 ($p < 0.01$) that was trained with US only and Group-3 ($p < 0.05$) that was trained with air only. Bees trained with CS only (Group-2) showed a longer sleeping trend compared to Group-1 but this was not statistically significant.

During the retrieval tests on second day, 65% of Group-1 bees showed a response to the learnt CS (Data not shown) while Groups 2, 3 and 4 showed a PER of 17%, 10% and 10% respectively towards CS.

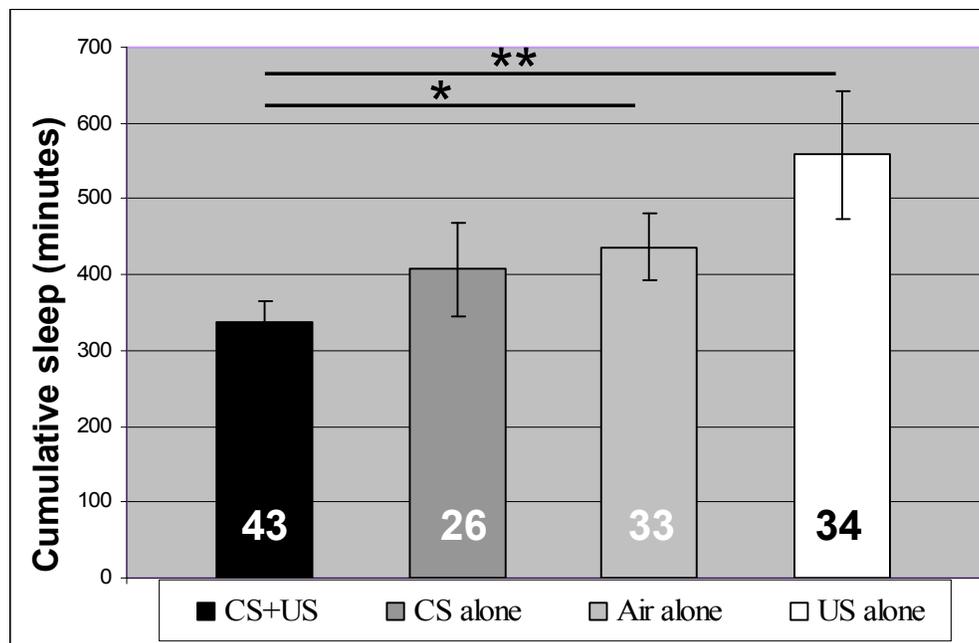


Fig. 3.10: Cumulative sleep after conditioning: The bees conditioned with CS+US showed significantly lower sleeps compared with Air alone ($P < 0.05$) and US alone ($P < 0.01$) groups. There was reduced but no significant difference in sleeps of CS+US group compared with CS alone group.

Experiment 3: Sleep-deprivation with light

After interfering the Groups 1-4 with white light, the cumulative sleep of Group-1 was significantly different ($P < 0.05$) compared to Group-1 of experiment 2 (Fig. 3.11). But there was no difference in cumulative sleeps between Groups 2-4 of experiments 2 and 3.

Group-1 bees slept lesser (232.7694) compared to Group-2 (417.966), Group-3 (476.995) and Group-4 bees (590.7124).

Retrieval tests on second day showed that 67% of the Group-1 bees responded to CS (Fig. 3.12), which was not significantly different from Group-1 of experiment 2. Therefore, interfering sleep with light did not affect memory retrieval. Responses of the Groups 2-4 were similar to experiment 2 (data not shown).

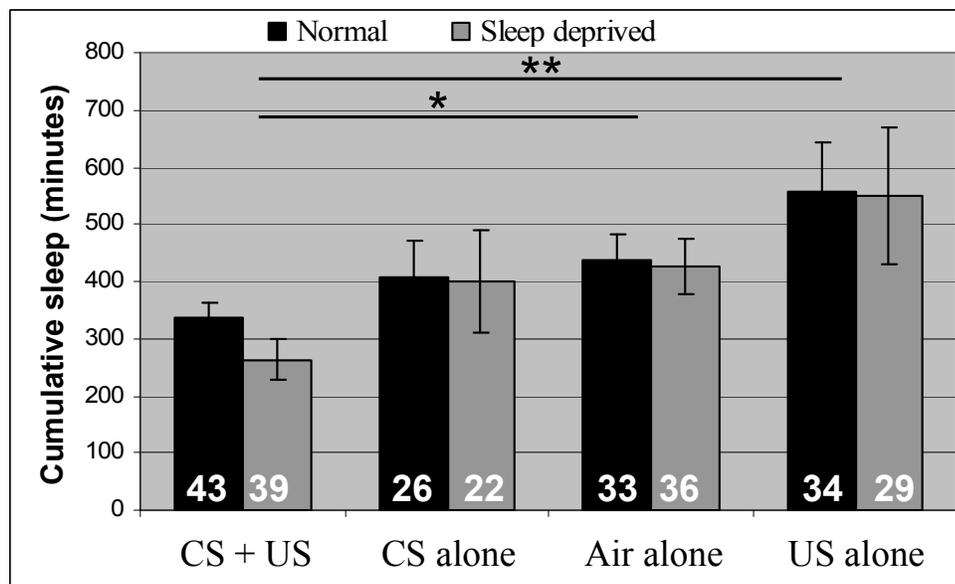


Fig. 3.11: Sleep deprivation with light: Similar to Fig. 3.10, the group, CS + US showed least cumulative sleep compared to other groups. Additionally, the CS + US group that was sleep interfered with light showed further decrease in sleep compared to other groups.

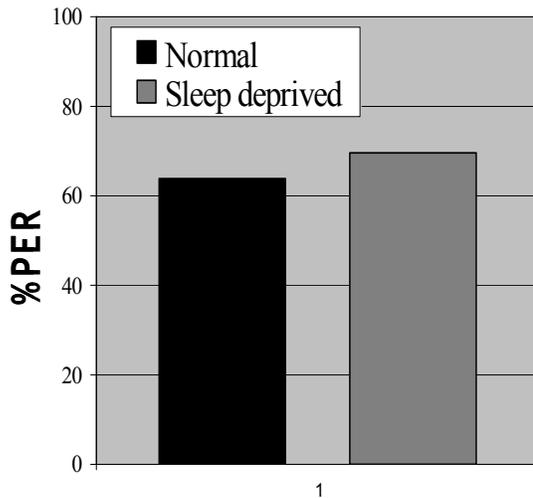


Fig. 3.12: Sleep deprivation and learning: The PER response of the sleep deprived group and normal group was not significantly different during the retrieval

Experiment 4: Sleep-deprivation by shaking

Experiment 4.1: Absolute conditioning and shaking

After 3-trial absolute conditioning, Group-1 bees were subjected to shake-deprivation during sleep and Group-2 bees were left undisturbed. Retrieval tests on the second day showed no significant difference (data not shown) between the Group-1 (64.3%) and Group-2 (61.7%).

Experiment 4.2: Extinction block experiment and shaking

On day-1, all bees were subjected to 3-trial absolute conditioning. After 3 trials, the responses of Group 1a, 1b, 2a and 2b bees were 61.4, 62.4, 57.9 and 64.6 respectively (Figs. 3.13 and 3.14).

On day-2, Group-1 bees were presented with 2 extinction trials of CS while Group-2 bees were not presented with any CS (serving as control). During the 2 extinction trials, the responses of Group-1a bees (49.2 & 40.4) compared to Group-1b bees (55.7 & 46.9) were not significantly different.

On day-3, the 4 groups treated with different conditions were as follows:

Group-1a – extinction trials followed by shaking,

Group-1b – extinction trials followed by no-shaking,

Group-2a – no extinction followed by shaking and

Group-2b – no extinction and no shaking.

All groups were subjected to retrieval tests by presenting them with one trial of CS. About 50.6% of Group-1a bees responded to CS compared to 31.8 % Group-1b bees and this effect was statistically significant ($p < 0.01$). The responses of Group-2a (47.7%) and Group-2b (53.3%) bees were no statistically different.

Also, the responses of the Groups 1a and 1b bees used in experiment 5 were not different from Groups 1a and 1b bees of this experiment.

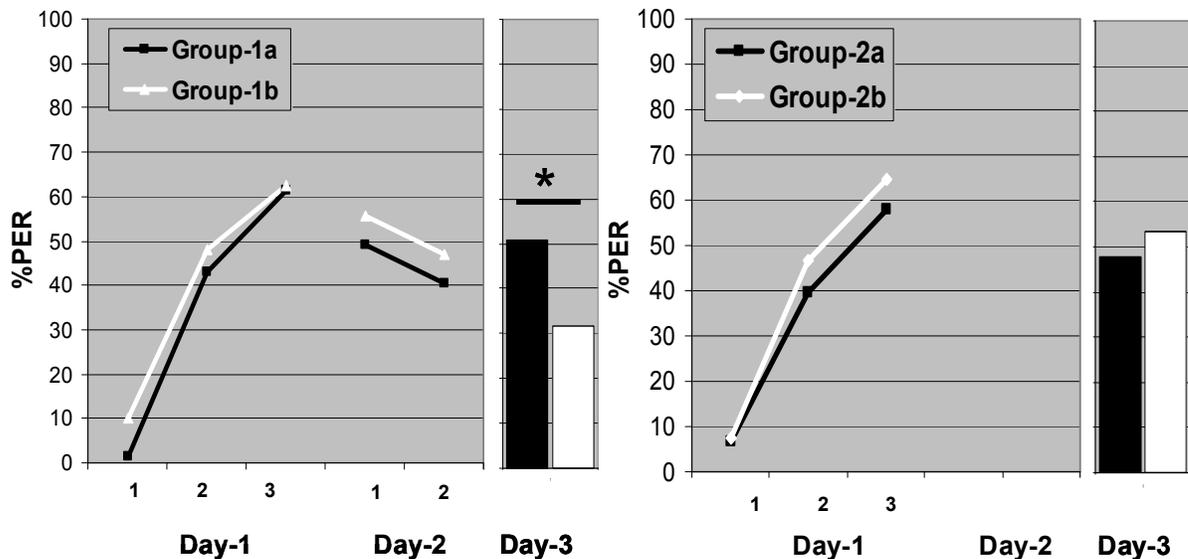


Fig. 3.13: Sleep deprivation after extinction: On day-1 after 3 trial conditioning Group-1a and Group-1b showed up to 60% learning. On day-2 after 2 extinction trials both groups showed similar extinction. After extinction trial, Group-1a bees were sleep deprived by shaking. On day-3 the sleep deprived (shaken) group showed significantly higher response compared to Group-1b which showed normal extinction learning.

Fig. 3.14: Sleep deprivation without extinction: The PER responses of groups 2a and 2b after 3 trial conditioning showed up to 65% learning. On day-2 both groups were not subjected to extinction trials, but Group-2a bees were sleep deprived by shaking. On day-3, both groups showed similar responses.

Experiment 5: Multi- antennal activity

After the 3-trial absolute conditioning on day-1, all bees were placed under the camera for recording multi-antennal activities. Analysis showed (Fig. 3.15) that multi-antennal activities (Δ pixels/hr) of Groups 1a and 1b were higher during the light cycle (2.04 and 1.80) compared to dark cycle (1.44 and 1.35). Between groups, there was no significant difference.

The day-3 multi-antennal activities (Δ pixels/hr) of Group-1a bees were lower (1.49) than Group-1b bees (1.80) before the onset of dark cycle. During dark cycle, both groups had similar activities (1.31 and 1.40). After the onset of light cycle, the activities of Group-1a bees were significantly ($P < 0.01$) lower (1.34) than Group-1b bees (1.93) for up to 4 hours. There was a general trend of sleep even before onset of dark cycle but the most obvious difference was observed after onset of light cycle when sleep deprived bees continued to sleep.

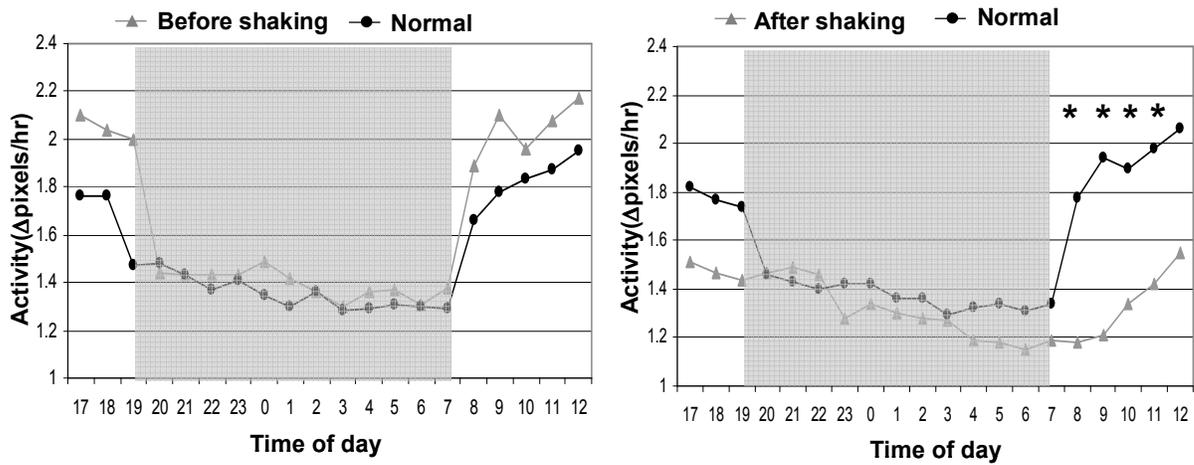


Fig. 3.15: Multi-antennal activities: On day-1 (before sleep deprivation) all groups had reduced activity during dark cycle and during light cycle the antennal activities were normal. The analysis of day-3 the antennal activities showed that, Group-1a bees, which were sleep-deprived the previous night showed reduced activity or more sleep compared to Group-1b which were not sleep-deprived. The reduced activity was significantly different at hours 08:00 to 11:00.

Discussion:

Sleep in honeybees has been shown before (Kaiser, 1988; Sauer et al., 2003), but we had to confirm bee sleep under our setup which allowed both sleep monitoring and PER learning.

The first experiment showed that bees sleep longer during the dark cycles compared to light cycle. The longest duration of sleep occurred 7 hours after the onset of dark cycle. These results corroborated nicely with previous studies by Sauer et al.

The flexed positions of antennae (left antenna- 80 degrees and right antenna- 100 degrees) were more during dark cycle and indicated sleep-like behavior. The patterns observed; symmetrical and asymmetrical activities were novel findings in our study. The symmetrical activities appeared most often during the peak of sleep duration which suggests that these could be involuntary movements of antenna. While the asymmetrical activities mostly occurred during the day when sleep duration was low.

To study the effect of learning on sleep, we trained the bees with different stimuli and observed its sleep. The experiment 2 showed that in Group-1, pairing sucrose reward (US) with an odor (CS) reduced the duration of sleep compared to groups 'Air alone' (Group-3) and 'US alone' (Group-4). During the retrieval tests, Group-1 bees showed higher PERs compared to Groups 2, 3 and 4. This indicated that the differences in sleep durations might be because of CS+US pairings which leads to higher PER on the second day.

It has been shown previously that US activates the octopaminergic system via VUMmx pathway in bees (Hammer & Menzel, 1998). And in another study it has been shown that OA injections increase the antennal scannings in honeybees (Pribbenow et al., 1996). Therefore, there is a strong possibility that conditioning bees with CS+US activates the octopaminergic pathway leading to increased activity of antennae and reducing sleep. Hammer et al. have shown that OA when injected without CS is not as effective as a reinforcer and in Pribbenow et al.'s experiment OA works best in operant conditioning protocol. Therefore, it is plausible that US alone is not sufficient to activate octopaminergic system for a longer duration and hence does not increase antennal activity and reduce sleep. Bees presented with Air alone slept normally because of no

possible arousal mechanism, while CS alone bees had a low sleeping trend (not significant). Although all bees were fed with 30% sucrose 1 hour after the conditioning experiment, it seems very unlikely that CS only bees can associate CS with 1-hour delayed feeding. From experiment 2 we conclude that, shorter sleep duration in bees conditioned to CS+US might be due to higher level of OA that increases antennal activity.

In addition to previous theory, a more convincing theory for reduced sleep in conditioned bees is based on the following findings: (a) In honeybees, injecting dopamine in alpha lobes of MBs has shown reduced PER learning. (Blenau et al, 1998). (b) In *Drosophila* long-sleepers have 3 times more dopamine in their brain compared to short-sleepers (Indrani Ganguly-Fitzgerald et al., 2006). It is therefore possible that conditioning leads to reduction in dopamine levels which leads to reduced sleep and in unconditioned bees dopamine levels are intact and does not affect sleep. In short, conditioned bees → lowered dopamine → lowered sleep and normal bees → normal dopamine → normal sleep. This trend is consistent with dopamine regulation in mammalian sleep; For eg. rats depleted with dopamine, have a suppressed slow-wave-sleep and REM sleep (Dzirasa et al., 2006) and in humans, dopamine agonists enhance sleep (Plowman et al., 2005).

Light is an important zeitgeber which resets circadian rhythm and alters sleep cycle. It has been shown in birds and humans that light exposure during dark cycle can disrupt sleep (Berger et al., 1994 and Daurat et al., 1997). To check if light played any role in disrupting sleep we performed experiment 3 which was similar to experiment 2, except that all groups of bees were interrupted with 5min light/5min dark during sleep. Sleep deprivation with light had very little or no effect on sleep durations of bees. Also, learning scores were not affected after deprivation with light. Since the sleep deprivation effects with light interference were too small, we did not further pursue learning related experiments.

To study the effect of sleep on learning we shifted our focus to a more robust sleep deprivation technique. Deprivation by shaking has shown to be effective in honeybees (Sauer et al, 2004) and causes sleep rebound the following day. In experiment 4, we adopted a similar technique to study the effect of sleep deprivation on learning.

After 3-trial absolute conditioning, all bees showed similar acquisition scores (60-65%). After sleep deprivation, Group-1 (shaken) and Group-2 (normal) bees showed no differences in learning scores during the retrieval tests on the following day. Therefore, absolute conditioning could not be disrupted by sleep deprivation by shaking.

From sleep studies in mammals it is known that sleep deprivation does not affect all kinds of memory. Therefore, we chose to target another form of memory called extinction memory. When a previously rewarded odor (CS) is presented without any reward (US), a new memory, that CS predicts absence of reward, is formed. Therefore, extinction memory is formed when animals stop responding to the CS. A study by Stollhoff et al (2005) showed that this memory can be blocked by injecting emetine (a protein synthesis inhibitor) after extinction trials. We attempted a similar experiment to disrupt memory, but with sleep deprivation. We subjected two groups (1a and 1b) of bees to 3-trial learning on first day and 2 extinction trials on second day. As expected both groups showed reduced learning after extinction trials, hence showing extinction memory. But after subjecting one group to sleep-deprivation by shaking, they failed to show reduced response on second day, while the group that was not sleep-deprived showed normal extinction (reduced response). We used control groups (2a and 2b) to show that shaking per se does not influence decrease or increase in PER response.

After sleep deprivation animals normally show increased sleeping the following day, this is termed sleep rebound and has been shown in honeybees (Sauer et al., 2004) too. In our sleep deprivation experiments it was very unlikely that bees could sleep during the alternate 5 minute period when they were shaken at 100-120 rpm, but to confirm that bees were indeed sleep deprived, in experiment 5 we observed the multi-antennal activities of sleep deprived and normal bees. Analysis showed that on day-1, after the 3-trial conditioning, sleep was normal in all bees. But the night after sleep deprivation, sleep duration was longer in sleep deprived bees compared to normal bees.

Our results therefore show that in bees, sleep deprivation does not affect acquisition learning but affects extinction learning. In honeybees it has been shown previously that mechanisms of antennal lobe and mushroom bodies are not similar (Hammer et al., 1998) and also studies have shown that elementary learning (differential conditioning) does not require mushroom bodies (Scheiner et al., 2000; Malun et al.,

2002). It is therefore possible that in our experiments the absolute conditioning (which is more elementary than differential conditioning) is not affected during sleep deprivation while extinction learning is affected.

It is worthwhile to note that in the previous study by Stollhoff et al., both acquisition and extinction memories were affected by injecting emetine (a protein synthesis inhibitor), while our sleep deprivation experiment affected only extinction memory by sparing acquisition memory. It is not exactly clear what molecular changes are brought about after sleep deprivation, therefore it is reasonable to argue that sleep deprivation might be acting in a different way compared to emetine. Also, some studies and reviews show that acquisition learning and extinction learning are different (Bouton, 1993; Wagner, 1981; Myers et al., 2002; Gottfried et al., 2004, Eisenhardt et al., 2006) and it could well be possible that sleep deprivation affects only extinction learning. In fact, it is known in rats that disruption of AMPA receptor selectively impairs extinction but not acquisition (Dalton et al., 2007). While another study in rats has shown that REM sleep deprivation affects extinction alone by sparing retention of memory (Silvestri, 2005; Fu et al., 2006) and Silvestri et al. have subsequently shown that NMDA might be partially mediating this mechanism. In any case, molecular mechanisms involved in learning was beyond the scope of our experiments and but are necessary to show how sleep deprivation affects acquisition and extinction learning.

However, from our current set of experiments we know that acquisition learning is a robust phenomenon in honeybees and can lead to long lasting memories. But extinction memory is weaker compared to acquisition memory. Our experiments showed that after 3-trial conditioning the PER increased from ~10% to ~60% (50% change) while after 2 extinction trials the PER decreased from ~55% to ~45% (10% change). Therefore, the weak extinction effect could be easily disrupted by sleep deprivation while the robust acquisition learning could not be disrupted by sleep deprivation.

Our final experiments therefore showed that sleep deprivation affected only extinction memory while acquisition memory was left intact.