Sleep in Honeybees – Searching for a role of sleep in memory consolidation

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"'Hard-eyed' creatures and insects manifestly assume the posture of sleep; but the sleep of all such creatures is of brief duration, so that often it might well baffle one's observation to decide whether they sleep or not."
(Aristoteles, On Sleep and Sleeplessness 350 BC)

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1. Introduction

1.1 The history of sleep research

The mystery of sleep and its functions has fascinated humans for several millennia. The physiological reasons ancient physicians and philosophers saw for sleep seem strange in modern eyes but nevertheless they made some adequate observations about sleep. In the ancient Egypt sleep was considered to be important for healing of diseases at least since the time of Imhotep who lived during the Third Dynasty of Egypt (2780 – 2720 BCE, Ostrin 2002). The Greek philosopher Aristotle (384 – 322 BCE) described sleep as a necessary counterpart to wakefulness, important for conservation and restoring of energy, digestion and growth (Aristotle 350 BCE). The influence of Aristotle can also be seen in the medical work of the Persian physician Avicenna (980 – 1037 CE), who is also known as Ibn Sina. He specifically saw the importance of sleep for health. In general, Aristotle's work on sleep influenced people for over two millennia.

In contrast to Aristotle, who believed the heart to be the driving force of sleep, Galen (129 – 216 CE) located the sleep center in the brain. This interpretation was first confirmed more than 1000 years later in the late 15th and early 16th century by the Italian anatomists Allessandro Achillini (1463 - 1512), Allesandro Benedetti (1452 – 1512) and Niccolò Massa (1489 - 1569) who, even though they still misinterpreted the nature of neuronal networks, discovered that nerve tracts did not originate in the heart but in the brain. The effect of sleep on memory might be mentioned first in the 16th century by the physician Andrew Borde, who described a beneficial effect of moderate sleep on memory (Dannenfeldt, 1986).

The discovery of oxygen and its meaning for human life in the late 18th century led Jakob Fidelis Ackermann (1765 – 1815) to the hypothesis that sleep is due to a deficiency of oxygen in the body organs and especially in the brain (Ackermann 1806). Also others like Alexander von Humboldt (1769 - 1859) and the

physiologist Eduard Friedrich Wilhelm Pflüger (1829 - 1910) believed sleep to be the result of a lack of oxygen uptake in the brain (Dannenfeldt, 1986).

Modern sleep research started first in the early 20th century when Hans Berger developed the electroencephalography (EEG). With this method it has become possible to record the electrical activity of the brain (Berger 1937) and sleep could be defined as a specific electrical attribute of the brain.

Even today, after over 2000 years of research, the functions of sleep are still not fully understood. For a long time it was believed that the main function of sleep was to maintain immobility at times, mostly during the night, when immobility was the optimal survival strategy because foraging for food was inefficient or because predators couldn't been detected fast enough (Meddis 1975). It was further argued that sleep is necessary to restore energy (Roth et al. 2010). In fact many studies point in this direction (van Leeuwen et al. 2010; Walker et al. 1979). In *Drosophila* it is known that the glycogen levels in the brain vary throughout the day and are decreased after sleep deprivation (Zimmermann et al. 2004). And even though it is now widely accepted that sleep is more than a simple lack of activity and occurs in nearly all animal species (Rattenborg et al. 2007), some scientists still argue that real sleep is restricted to homoeothermic vertebrates, especially mammals and birds, while poikilothermic vertebrates only show a resting behavior due to changes in external temperatures (Rial et al. 2007). The fact that also invertebrate animals display signs of sleep has been ignored for a long time (Campbell & Tobler 1984).

One important breakthrough in sleep research was the discovery of different sleep phases. In the late 30ies of the 20th century the group of Alfred Lee Loomis used EEG to observe sleeping humans. They found electrical patterns that differ in amplitude and frequency depending on the deepness of sleep. In drowsy probands the amplitudes were small and the wave frequencies similar to those found in awake probands (ca 10 Hz) whereas the amplitudes were high and the wave frequencies low (0.5 to 3.5 Hz) during the deep sleep phase. (Davis et al.

1937). Sleep phases dominated by these high amplitude low frequency waves, also called δ-waves, have been called Slow Wave Sleep (SWS).

The most important discovery made with the help of EEG is the so called "paradoxical sleep" or Rapid Eye Movement (REM) sleep described by Aserinksy and Kleitman in 1953 (Aserinksy & Kleitman, 1953). They found that humans show jerky and binocularly symmetrical eye movements during a specific sleep phase characterized by irregular spikes of low amplitude in the EEG. The general pattern of activity during REM sleep has been viewed as a "closed system" (Braun et al. 1998) with locally high activity within the brain but little activity in input and output regions.

Both REM sleep (Rasch et al. 2009) and Slow Wave Sleep (SWS) (Marshall et al. 2006) have been proposed to be important for memory formation in humans. REM sleep seems to improve the consolidation of memories in the cortex on a synaptic level, while SWS might coordinate the reactivation of hippocampus-dependent memory (Diekelmann & Born 2010). In mice knockout lines which show a deficit in SWS rebound after sleep deprivation, associative memory was impaired (Bjorness et al. 2009).

While some studies link especially REM sleep to the consolidation of memory (Rasch et al. 2009), others find that REM sleep is required for neurogenesis (Meerlo et al. 2009). Crick and Mitchison proposed that REM sleep is necessary for the reduction of memory overload by reverse learning. This enables brains to be smaller than in species lacking REM sleep like the monotreme Echidna or certain types of dolphins (Crick & Mitchison 1995).

It has been proposed that Slow Wave Sleep (SWS) is important for energy conservation (Zepelin & Rechtschaffen 1974), but recent phylogenetically comparative analyses could not confirm this hypothesis (Lesku et al. 2008).

Sleep or sleep like states have been found in almost all animals. Apart from mammals where sleep has been intensely studied, sleep has also been found

among others in pigeons (Rattenborg et al. 2005), tortoises (Ayala-Guerrero et al. 1988), frogs (Kulikov et al. 1994), zebra fish (Yokogawa et al. 2007), crayfish (Mendoza-Angeles et al. 2007) and even *Caenorhabditis elegans* (Raizen et al., 2008).

Cetaceans such as dolphins and white whales need to swim regularly to the surface for breathing. Nevertheless sleep has also been found in these animals (Lyamin et al. 2007). They can sleep without drowning because their brains evolved the ability to sleep unilaterally, thus just one brain hemisphere at a time (Lyamin et al. 2002). This enables those animals to stay awake while a part of their brain is sleeping. Pinnipeds such as the fur seal which sleep in water and on land show both unilateral and bilateral SWS depending on the sleep location (Lapierre et al. 2007).

Though some studies found ocular movements during active sleep (Ayala-Guerrero et al. 1988) in reptilians and vaguely related EEG patterns in reptilians (Tauber et al. 1967) and crustaceans (Mendoza-Angeles et al. 2007), real REM sleep and SWS seem only to occur in mammals, including monotrema like kangaroo and platypus (Nicol et al. 2000), and birds (Rattenborg et al. 2009). This might be the result of the convergent evolution of a more complex connectivity in mammalian and avian cortical regions, which seems to be necessary for brain wide rhythm generation. This connectivity is absent in the reptilian dorsal cortex (Rattenborg 2006).

For some species sleep mutants are known. Among them are mice (Feil et al 2009), *Drosophila* (Cirelli 2009) and zebra fish (Yokogawa et al. 2007).

1.2 Sleep and memory

Interestingly many sleep mutants also show impaired memory functions. Mice PKA mutants show impaired hippocampus dependent long term memory (Abel et al. 1997) and exhibit non-rapid eye movement (NREM) sleep fragmentation and increased amounts of rapid eye movement (REM) sleep (Hellman et al. 2010). Among the sleep and memory impaired mutants known in *Drosophila* are knockouts for the subunits of a certain type of voltage activated potassium channel. Both a lack of its α-subunit *shaker* (Cirelli et al. 2005) and its β-subunit *hyperkinetic* (Bushey et al. 2007) lead to sleep loss and impaired aversive learning.

In humans a great deal of research has shown that sleep plays a critical role in modulating and regulating memory processes. It is known that learning and memory are dependent on processes of brain plasticity, and sleep-dependent learning and memory consolidation must be mediated by such processes (Walker & Stickgold 2004).

Sleep can be important for learning both before and after a learning episode. Sleep before learning seems to play a role in the initial encoding of certain memories, while sleep after learning is required for subsequent consolidation of numerous forms of memory (Walker 2008). Learning of complex tasks benefits from sleep after an initial training but not from sleep before the initial training (Wagner et al. 2004). Also visual discrimination requires sleep (Stickgold et al. 2000). Motor skill improvement is correlated with the amount of stage 2 NREM sleep in the 4th quarter of the night (Walker et al. 2002). Also motor learning leads to a local increase of slow wave activity (Huber et al. 2004) and spatial memories seem to be strengthened in the hippocampus during SWS (Peigneux et al. 2004).

Emotional memories can be kept alive for years by a brief sleeping period after learning (Wagner et al. 2006). For these memories REM sleep seems to be of special importance (Nishida et al. 2009), which is backed up by the finding that

areas like the amygdaloidal complexes are highly activated during REM sleep (Maquet et al. 1996).

The vast amount of studies finding a correlation between different sleep phases and different forms of memory strongly suggests an active memory consolidation process during sleep. But most of these studies have been done with either complete sleep deprivation or with selective deprivation of different sleep phases. Since sleep deprivation affects daily metabolic and hormonal processes (van Cauter et al. 2008), it could still be argued that memory impairments after sleep deprivation are mainly a side effect of stress and homoeostatic changes. Also not all studies find an effect of selective deprivation of SWS or REM sleep on memory retention (Genzel et al. 2009) and skill memory can even be enhanced after pharmacological REM sleep deprivation. But the hypothesis of an active memory consolidation process during sleep is also supported by studies in humans which show that slow waves and spindles occurring in SWS can be triggered by transcranial magnetic stimulation (Massimini et al. 2007) and that hippocampus dependent declarative memories can be improved through this artificial SWS (Marshall et al. 2006).

1.3 Sleep in insects

1.3.1 Comparing vertebrate and invertebrate sleep

Insect sleep has not been studied as intensively as mammalian sleep. Still, at least within the field of ecology, it has been described long before modern sleep research (Rau & Rau 1917). Due to the different anatomy of vertebrates and invertebrates it is obvious that sleep in insects has to be different from mammalian sleep. Since insects have compound eyes, it is impossible that they show rapid eye movements as the visible characteristic of REM sleep.

Nevertheless, many criteria used to define mammalian sleep are also found in insects. Easily observable signs of sleep are for example the rapid reversibility of sleep, a reduced reaction threshold or sleep rebound after sleep loss (Siegel, 2008). Other described criteria are a reduced muscle tone, specific sleep postures and defined sleep places (Kaiser, 1988). All these criteria also fit insect sleep states.

In addition it has been shown that gene regulation for a couple of genes is sleep dependent (Cirelli et al. 2005). Among these are genes for ion channels, synaptic proteins and neurotransmission as well as clock genes. In most cases a vertebrate homologous gene serves a similar function as in *Drosophila* (Cirelli, 2009). Interestingly, it has been shown that *Drosophila* lacks at least one gene regulating the cellular clock that is present in both mice and honeybees (Rubin et al. 2006).

1.3.2 Sleep in *Drosophila melanogaster*

When dealing with complex biological functions a sensitive first step is often to search for a simple model organism. One problem finding the right model organism is to use a sufficiently simple organism that still shows some characteristics of the function that is to be investigated.

A classical model organism for many questions also within neurobiology is *Drosophila melanogaster* (Sattelle & Buckingham 2006). *Drosophila* sleep has been well characterized (Cirelli & Bushey, 2008). Behavioral studies with *Drosophila* showed that flies have an increased daytime sleep in socially enriched environments and after sleep deprivation. Their courtship memory is decreased when the flies were sleep deprived shortly after training (Ganguly-Fitzgerald et al. 2006).

In *Drosophila* a variety of genes are known which influence sleep behavior. Genes involved in *Drosophila* sleep: *sleepless* is important for recovery sleep after sleep deprivation (Koh et al. 2008). A sleep mutant (*fumin*) with a defective dopamine transporter gene sleeps less than control flies. This indicates that arousal seems to be modulated by dopamine (Kume et al. 2005). Several studies (Joiner et al. 2006; Pitman et al. 2006) show that sleep in *Drosophila* is regulated by the mushroom bodies, thus linking sleep with a brain structure involved in learning.

Drosophila lacks a clock gene that is conserved in other invertebrates like the honeybee as well as in mammals (Rubin et al. 2006). Thus the diurnal rhythm in *Drosophila* has to be regulated slightly differently than in most other species. Though the implications might be minor, it is one additional reason not to restrict invertebrate sleep research solely to this one model organism.

1.3.3 Sleep in Honeybees

In the case of sleep and its possible role in memory formation, the honeybee (*Apis mellifera*) provides us with an insect model that has both a comparatively small brain and the ability to learn rather complex tasks and to store those memories over a long time (Menzel 2001). It has been shown that bees display characteristic sleep signs both in the lab and in the hive (Kaiser 1988). As Kaiser has shown, bees in the hive show signs of sleep, despite a basic constant activity inside the hive. Sleeping bees inside the hive may be recognized by different physiological and behavioral characteristics: Muscle tone and body temperature are decreased, and motility is reduced in general. To sleep, the bees normally retreat into preferred resting places inside the hive and sit in a characteristic posture, which includes immobile antennae. The sleeping bees are comparably insensitive to strong (visual) stimuli (Kaiser 1988). Bees compensate for sleep deprivation by deepening the sleep process (Sauer 2004).

Hive observation showed that all in-hive behaviors are performed at all times of the night and day. The activities of the workers are not synchronized (Moore et al. 1998). Differences in diurnal rhythmicity depend on different levels of *per (period)*-Expression (Bloch et al. 2001, Bloch et al. 2003). While young honeybees don't show a diurnal rhythmicity, they still sleep, but the sleep architecture is different between foragers and young honeybees (Eban-Rothschild & Bloch 2008).

1.4 Studying learning and memory in honey bees

Among invertebrates honeybees outstand for their complex social structure. They need to learn and remember a lot about their environment. Bees are capable of learning and remembering several tasks. New memories don't wipe out old memories (Cheng & Wignall 2006). Most learning paradigms in the lab use classical conditioning, but it has been shown that also harnessed bees are capable of operant learning (Kisch & Erber 1999). The time needed for a good performance is dependent on the difficulty of the tasks (Deisig et al. 2007).

Most work on memory formation in the honeybee is done with foragers, since forager brains are better suited for complex learning tasks than the brains of young bees performing in-hive duties such as nursing (Schulz & Robinson 1999) and foragers show a better performance in learning tasks than their in-hive nestmates (Ben-Shahar & Robinson 2001). The age of foraging onset depends on division of labor in the colony, but in general individual bees start foraging at an age of 14 to 21 days (Ben-Shahar 2003). Foraging honeybees cover a large area on their search for food and are able to reliably return to a known food source over many days and even to communicate the position of food sources (von Frisch 1974). Many studies have shown that they are able to learn and remember different aspects of their environment (Cheng & Wignall 2006). The most complex learning task bees perform is probably orientation learning during their

first orientation flights. These flights occur up to several days before the bees start foraging (Robinson 1987).

In the following paragraphs I will describe three methods of research more in detail.

1.4.1 Classical Conditioning - Proboscis Extension Response

A classical learning paradigm for harnessed honeybees in the lab is the Proboscis Extension Response (PER) conditioning (Kuwabara 1957; Bitterman et al. 1983). This paradigm uses the ability of bees to learn an association between a conditioned stimulus (CS) with an unconditioned stimulus (US) which is usually sugar water. When the main sensory organs of the bee, the antennae, are touched with a sugar soaked toothpick, the bees respond with extension of the proboscis to lick the sugar. Sugar serves as the unconditioned stimulus (US). They do not respond like this when they are presented with a neutral stimulus such as color (Hori et al. 2006), movements (Hori et al. 2007), tactile stimuli (Erber et al. 1998), or odorants, but they can learn to associate those stimuli with the US, when the stimulus is presented just before the US. The excitatory response to a formerly unknown odor increases when the odor is paired with a reward, while the excitatory response to an unpaired odor decreases (Rescorla & Wagner 1972). This form of learning is generally called 'acquisition' of the CS. Over time the memory of this learned odor is consolidated. A single stimulation can lead to memory consolidation, after three trials usually about 90 % of the bees respond to the trained odor (Menzel 2001). The consolidated memory can be extinguished when the bees are exposed to the CS alone. This is called 'extinction' learning and often seen as an active form of learning. During extinction training another form of learning seems to occur which can lead to spontaneous recovery from extinction, thus retention of the original memory which appears a day after the extinction training (Eisenhardt & Menzel 2007). Bees learn better when the olfactory stimulus is given temporally spaced (Menzel et al. 2001).

1.4.2 Radio Frequency Identification

A rather new method to observe bees in their natural environment is Radio Frequency IDentification (RFID). The RFID technology was developed by Harry Stockmann (1948) and first commercial uses came up in the 1960ies, but did not become frequent before the 1980ies (Roberts 2006). In the beginning, RFID technology was used as anti-theft device, in the toll system and for animal tracking. As the tags got smaller and cheaper the usage of RFID technology became more widespread and is today abundant in various aspects. RFID technology has been used to both identify a variety of both domestic and wild animal identity and track their movements, among others mice (Lewejohann et al. 2009) and birds (Fiedler 2009). Specialized RFID-tags have been used to detect bees in the hive (Streit et al. 2003).

A RFID-tag is a microelectronic circuit with transmit- and receive-antenna, with a control-unit and a data- and energy-memory. The RFID-tags consists of an antennal part and a microchip. The antenna is coiled around a small magnet. The detection antennae are 3 or 5 cm copper wire coils. Each tag can be coded with a four digit hexadecimal number. This enables the antennae not only to recognize a chip, but also to individually identify it. When a tag is moved close enough to the detection antenna, the induction coil provides enough energy for the microchip to be read by the receiver. The maximal distance for reading is dependent on the position of the induction coil relative to the receiving antenna.

1.4.3 Electrophysiology

One advantage of doing electrophysiology in insects is the small size of the brain with In honeybees it is possible to record from specific identified neurons like the PE1 neuron (Okada et al. 2007) or the VUMmx1 neuron (Hammer, 1993) in the living bee.

In mammals sleep is characterized by specific electrical properties. These can be visualized using EEG. That is not possible in insects because glands and trachea sacs isolate the brain. Nevertheless local field potentials (LFP) can be measured reliably in *Drosophila melanogaster* (Nitz et al. 2002). Brain activity has been shown to be affected by different sensual stimuli (van Swinderen & Greenspan 2003) and during sleep the brain activity is uncoupled from muscular activity (van Swinderen et al. 2004)

Local field potentials in honeybees are more difficult to characterize because of a strong muscle activity in the head close to the recording site. One of the biggest muscles in the bee head is the proboscis extension muscle, M17 (Rehder 1989). Since it is necessary for PER conditioning it can not be silenced in learning experiments using PER. Rhythmic activity in neurons of the mushroom body alpha has been found in sleeping bees (Schuppe 1995).

1.5 Aim of this work

The purpose of this thesis was to explore the impact sleep might have on memory consolidation in the honeybee. So far most studies on bee sleep have been done in the lab, where the bees could be observed closely. RFID-technology makes it possible to automatically observe individual bees in the hive and the time they spend foraging by monitoring the bees leaving and entering the hive. This allows analyzing the sleep of individual bees in the context of natural behavior.

I performed several experiments with sleep deprived bees to evaluate the effect of sleep loss on memory consolidation. In a first step I performed PER experiments in the lab. I used results from Abid Hussainis PhD thesis to design these sleep experiments. He could show that sleep deprivation impairs extinction memory in harnessed bees (Hussaini et al. 2009). Therefore I investigated the effect of sleep deprivation during different parts of the day. Furthermore I analyzed if already extinction learning was impaired after sleep deprivation.

In humans, the ability to learn new tasks and the improvement of training is strongly dependent on sleep following the learning session (Diekelmann & Born, 2010). If this pattern is consistent also for honeybees, foragers should have a greater need for sleep than hive bees. Lack of sleep would reduce the ability of foragers to forage efficiently.

To test this hypothesis, I deprived the sleep of forager bees and tracked their flights to a feeder they were previously trained to. I also analyzed if their sleep pattern changed after complicated learning tasks and if the formation of memory was impaired by sleep deprivation.

Furthermore, I searched for electrical sleep traces in harnessed bees. I recorded from extrinsic neurons of the mushroom body α-lobe, a region that has been shown to take part in learning processes (Okada et al. 2007).

2. Material and Methods

2.1 Sleep deprivation and olfactory memory

2.1.1 Animals

I worked with the European honeybee, *Apis mellifera*. All experiments were done with forager bees. Since the experiments were performed during late autumn and winter all bees were taken from a temperated flight room with a 12 h light/dark cycle to keep bees flying. The bees were kept in natural sized hives and maintained by the bee keeper of the institute. The colonies were provided with a 30% sugar solution and powdered pollen. For experimental use these bees were captured individually with small glass jars. The bees used for the extinction experiment and those used for the sugar acceptance test were taken from the same hive. The bees for the retention experiment had to be taken from another hive.

2.1.2 Capture and feeding

The bees used to test for the influence of sleep deprivation on extinction learning and the bees used to test for motivation were captured 24h before the start of the experiment. They were cold-anaesthetized on ice and placed into plastic tubes to fix the heads. The bees could still freely move their antennae, the mandibles and the proboscis (Bitterman et al. 1983). After half an hour, when the animals were warmed up to room temperature again, they were fed to satiation with 30 % sugar solution. Bees used to test for the influence of sleep deprivation on retention were captured 8h before the start of the experiment but otherwise treated as the other bees. All bees were fed again 12 h before extinction. The bees

were kept in a humid box, which was lighted for 12 h during the day and dark during the night, mimicking the light/dark cycle the bees were used to from their hive in the glasshouse.

2.1.3 Sleep deprivation

For sleep deprivation of bees by mechanical stimulation, a standard vortex was modified as shown in figure 2.1. A foam rubber block with holes the size of the harnessing tubes was placed on top of the vortex. Harnessed bees were placed into the holes. Up to 12 bees could be treated simultaneously in this way. The bees were shaken for 10 hours at a low frequency either at night or during the day.

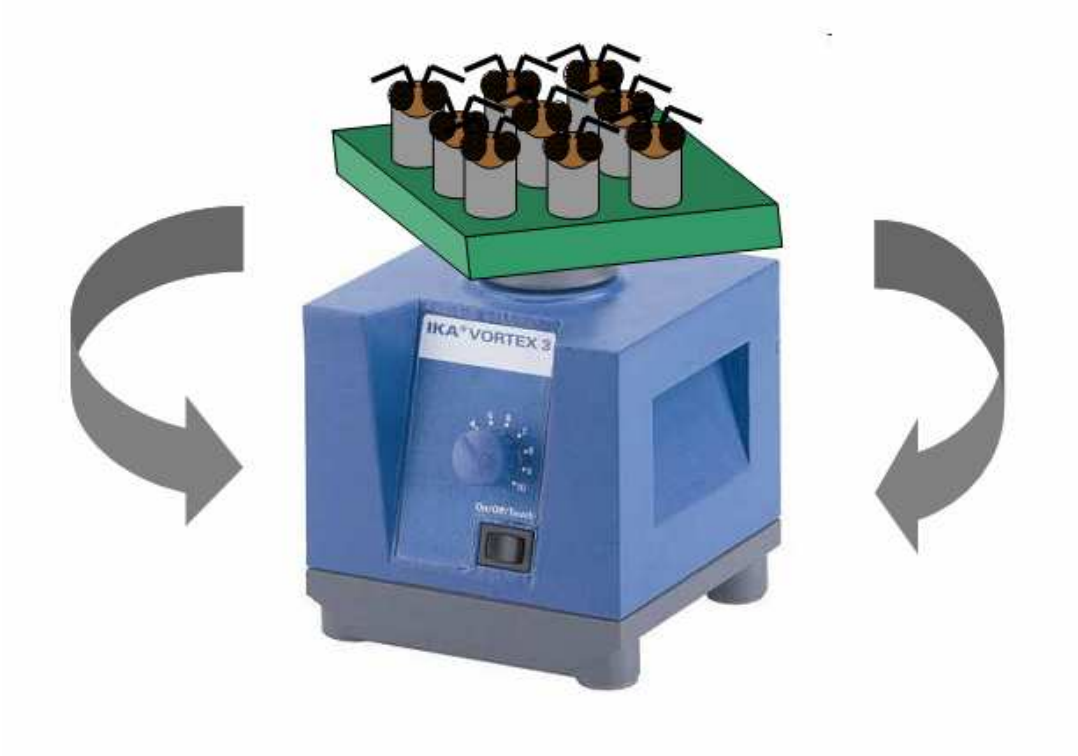

Figure 2.1 mechanical sleep deprivation. The bees were placed on a vortex and gently shaken for 10 hours.

2.1.4 Training setup

To train the bees a computer driven odor device (Galizia et al. 1997) was used. The bees were individually placed in front of this olfactometer which blew a constant air stream over their antennae. Each bee spent 60 sec per trial in front of the olfactometer. The air could be automatically led through different syringes which contained 1 cm^2 sized pieces of filter paper impregnated with 4 µl odorant. Odors were cleared by an exhaust system behind the bee. The olfactometer was triggered by a computer signal and the odor on- and offset as well as the time window for the US stimulation were accompanied by an acoustic stimulus. The sucrose stimulus was applied manually by a moistened toothpick.

2.1.5 PER-conditioning

I used a three trial absolute conditioning protocol. A 30% sugar stimulus given to one antenna served as unconditioned stimulus (US), which automatically elicited PER. In each conditioning session I worked with ten bees. They were trained three times to a single odor (peppermint or limonene) as conditioned stimulus (CS+) with an intertrial interval (ITI) of 10 min (Menzel et al. 2001). Each bee was exposed to the air stream from the olfactometer for 20 sec before odor onset and left there until 20 sec after the stimulus. The odor pulse lasted for 4 sec. Proboscis extension after odor presentation was noted. After 3 sec of the odor presentation one antenna of the bee was touched with a toothpick soaked with 30% sugar solution. The sugar stimulus lasted for 3 sec.

2.1.6 Testing for an effect of sleep deprivation on extinction learning

In this experiment I investigated the effect of sleep deprivation after conditioning on the extinction of olfactory memory consolidation at different times of the day. The training and sleep deprivation protocol is shown in figure 2.2. The training

was done either in the morning starting at 8AM or in the evening starting at 7PM. Extinction training was performed 24 h after the PER acquisition. The first extinction trial served as test for the consolidation of the olfactory memory. The bees were exposed to three CS only trials each one lasting for 4 sec. The ITI was 10 min. After the three trials the bees were tested for responsiveness to the US and non-responsive bees were discarded. 24 h later the surviving bees were tested for retention.

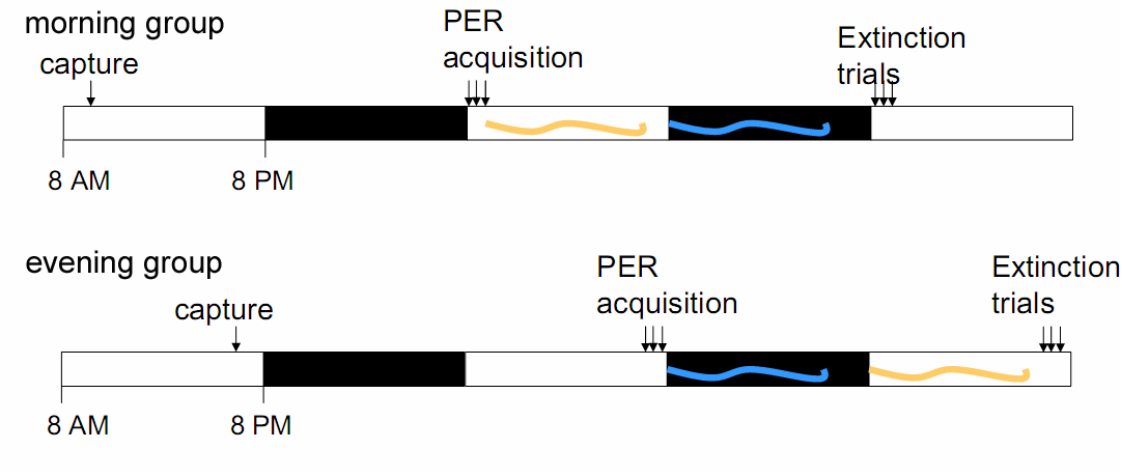

Figure 2.2 extinction experiment sleep deprivation protocol. The experimental day started at 8AM and lasted 12 h (white bars). The experimental night started at 8PM and lasted 12 h (black bars). Two groups of bees were trained. The morning group (upper part) was caught in the morning at 10AM, kept overnight in the lab and the PER was trained in a three trial conditioning (acquisition) at 8AM the following morning. To prevent sleep the bees were shaken for 11h hours. For one subgroup the shaking started directly after the end of the conditioning. For another subgroup the shaking started first at the onset of the dark period. After 24 h the bees were exposed to three extinction trials. A non-shaken group served as control. The evening group (lower part) was treated similarly but both capture and acquisition and extinction training were done in the evening.

2.1.7 Testing for an effect of sleep deprivation on retention

In this experiment I investigated the effect of sleep deprivation before and after three extinction trials on retention and spontaneous recovery of olfactory memory. The training and sleep deprivation protocol is shown in figure 2.3. The training for the retention experiment was done at 4PM. To test the effect of sleep deprivation on extinction learning, I conditioned the bees 8 h after capture. The extinction training was done 24 h later with three CS only trials. I sleep deprived them for 8 h shortly before or shortly after the extinction trials. The retention test was performed 16 h after extinction.

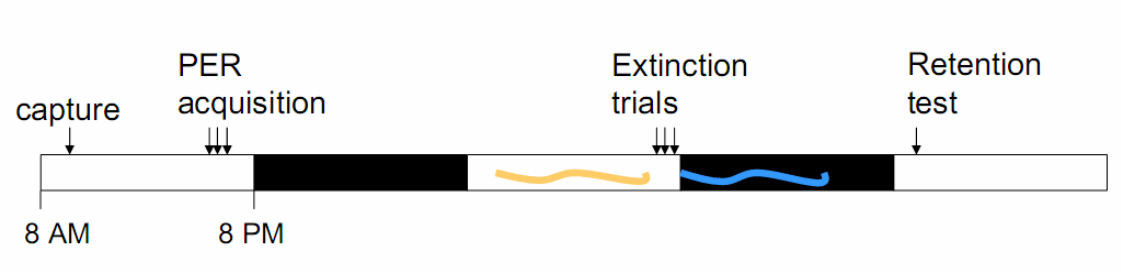

Figure 2.3 protocol for retention experiment. The experimental day started at 8AM and lasted 12 h. The experimental night started at 8PM and lasted 12 h. The bees were caught in the morning at 10AM and trained in the evening with three trial olfactory conditioning (PER acquisition). Directly before and after the extinction trials on the following evening at 7PM the bees were shaken for 8 h to deprive sleep. The bees were then tested for retention the following morning at 8AM.

2.1.8 Testing for the acceptance of sugar to determine motivation

Satiation is known to have a strong influence on acquisition of olfactory memory in forager honeybees (Ben-Shaher 2001). To make sure differences between control and shaken bees were not simply due to different satiation levels I tested the acceptance of ascending sugar concentrations. To validate this approach, I compared bees which had been starved for 5h after capture with bees which had been fed to satiation 1h before the test.

The sleep deprivation and test protocol is shown in figure 2.4. The bees were caught at the same times as the bees used for olfactory training, i.e. at 10AM for the morning group and at 6PM for the evening group, fed unto satiation and kept in the day/night chamber. The morning group was shaken for 11 hours during the night, while the evening group was shaken for 11 hours during the day and both groups were tested for sugar acceptance shortly after the end of the shaking period. Bees that did not respond to a short initial application of 50% sugar solution to the antenna were discarded. The remaining bees were stimulated with increasing sugar concentrations, starting with water. Since the expectance of reward was already triggered with the initial 50% sugar solution, all bees reacted with extension of the proboscis to all concentrations including water. However, most bees did not start sucking at lower sugar concentrations. The lowest concentration that induced sucking was noted as minimal sugar acceptance level.

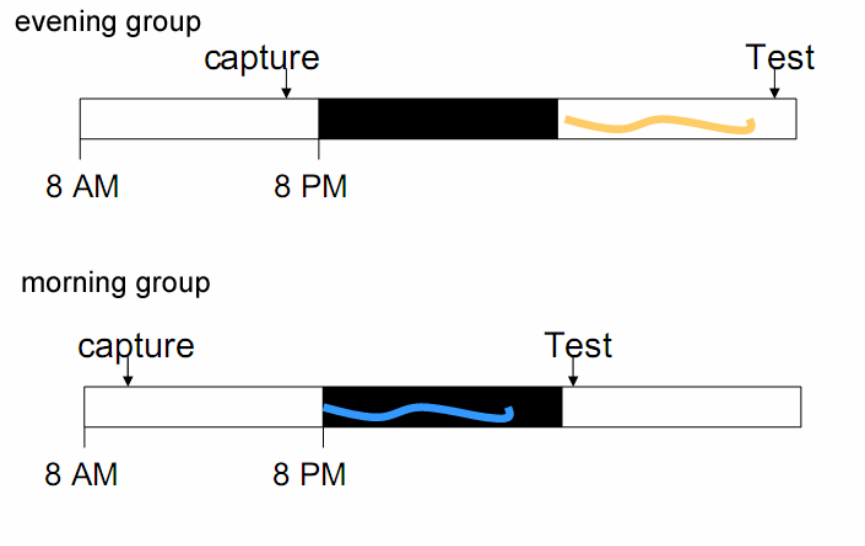

Figure 2.4 protocol for the sugar acceptance test. The bees were caught 24 h before the sugar acceptance test. One group was caught in the evening, another one in the morning. Both groups were sleep deprived (shaken for 11 h before they were tested for sugar acceptance. Bees captured in the evening and in the morning but not sleep deprived served as control.

2.2 Sleep deprivation in free flying bees

2.2.1 Animals

I worked with the European honeybee, *Apis mellifera*. If not stated otherwise all bees I used were foragers.

2.2.2 Observation hive

Experiments with free flying bees were done with bees from a one-frame observation hive. To ensure that the observed bees were detectable inside the hive, the backside of the comb was blocked with a plastic cube fitted into the backside of the frame. To establish the colony around 500 newly hatched or up to 5 days old bees were taken from a hive in the bee garden. A subset of bees flew back to their mother colony, but the majority stayed in the observation hive. The queen was placed into a plastic cage sealed with sugar paste and inserted into the hive. During the time it took the bees to eat their way through the sugar, they got used to the queen and finally accepted her. The back side of the comb was treated against wax moth larvae using *Bacillus thuringiensis* toxin (BTX; B-401, Vita sparm). The bees had to enter and leave the hive through a 30 cm long plastic tube with a diameter of 1.5 cm. This was necessary for the tracking and also had the advantage that the bees could more easily defend their small colony against intruders. Additional sugar was given in a feeding box which was attached to the entrance tube with a Y-shaped piece. To avoid attraction of wasps, bees were fed late in the evening.

2.2.3 Training bees to a feeder

About one week after the founding of the colony, foragers started to leave the hive in search for food. Some of these foragers were trained to a feeder positioned at a distance of 50 m from the hive (figure 2.5). The training procedure was started with a feeder containing very highly concentrated sugar solution directly at the hive entrance. The bees were then trained stepwise to the final position, which took two days. It has been shown that bees exclusively visit a single foraging site when the sugar reward is high enough (Greggers & Menzel 1993).

Figure 2.5 bees at feeding site. The bees were fed with sugar water. After the initial training new bees were recruited by bees foraging at the feeder. The sugar concentration was adjusted to keep the number of bees visiting the feeder constant.

I used sugar concentrations between 10% and 50%. High concentrations were used to initiate foraging in the morning and to trigger dancing which led to the recruitment of new bees. Low concentrations were used to stop recruiting. A well visible blue color cue was placed behind the feeder. Since bees learn visual cues like color patterns while foraging (Backhaus 1992; Menzel & Lieke 1983), this helped orientation of foragers to the feeder. Foragers at the feeder were marked with a red dot to recognize regular visitors for later experiments. Wasps and bees

from other hives than the one observed that searched the feeding site for food, were regularly caught with a forceps and killed with 70% ethanol.

2.2.4 Marking

Apart from some newly hatched bees that served as controls I used only foraging bees for tracking. Bees foraging regularly at the feeder were marked with a red dot on the thorax. To mark bees with RFID-tags regular foragers were captured and placed in an immobilization device, self constructed by using a plastic tube, a metal grid and a foam stamp (figure 2.6). A drop of cyanacrylate glue (Sekundenkleber, Conrad) and a small drop of the necessary activating reagent (Aktivator für Sekundenkleber, Conrad) were used to remove the hairs and some of the wax layer from the thorax. The hardened glue was removed with a forceps and the cuticula roughened with a small grinding pin. A second drop of glue was given onto the thorax and the microchip part of the RFID-tag placed into the drop. The chip and especially the fragile joint between chip and induction coil were coated with glue, before the glue was hardened by another small drop of the activating reagent. An additional thin layer of casein paint (Plaka, Pelikan) helped to protect the microchip. A color coding made it possible to identify the individual bees optically (figure 2.5).

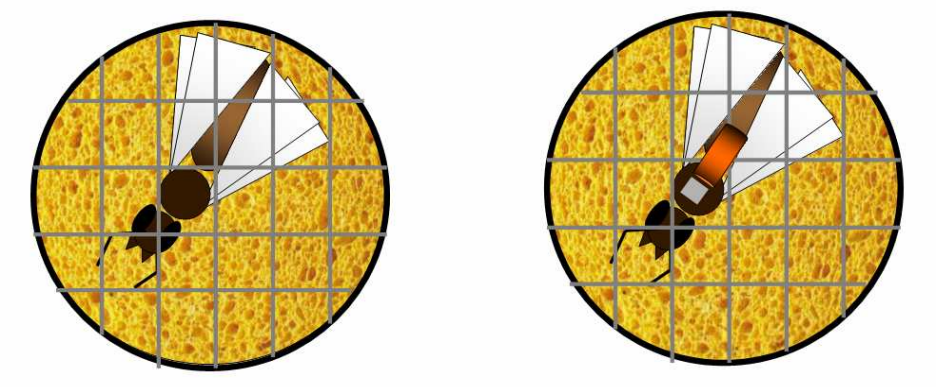

Figure 2.6 mounting of RFID tag. The bees were fixed between a grid and a flexible underground (sponge). The RFID-tag was glued to the thorax. The glue stabilized the joint between the microchip and the induction coil.

2.2.5 Tracking bees to determine foraging time and sleep time

The tags we used were similar to tags generally used in animal identification, but to reduce weight the tags lacked the glass enclosure. In contrast to smaller tags used by other groups (Streit et al. 2003) our tags had the advantage that bees can be detected over distances up to 4 cm centimeters. The tags were 1x6 mm long and weighted 18 mg including the glue used to attach the tag to the bee. This weight made them significantly lighter than the normal nectar load which is about 60 mg (Deng & Waddington 1997) and the average load a forager bee is able to carry, which has been previously shown to be about 70 mg (Nunez 1982).

The bees were detected inside the hive and at the entrance by receiving antennae, each consisting of a RFID sensor coil which sent the incoming data points to a computer. Two receiving antennae were placed at the hive entrance tube. One was placed near the entrance, the other one more near the hive. A distance of twenty centimeters between the antennae made sure that it took the bees a second or longer to get from one antenna to the second. This way, the direction of the bees could be concluded by comparing the data from the two antennae. This was used to determine the duration of foraging trips. Since the bees sometimes spend short time periods at the entrance without flying, only episodes outside the hive that lasted longer than 90 seconds were counted as foraging flights.

To reveal if the flight performance of RFID-tagged bees were impaired by the microchip, I observed RFID-tagged bees and color marked control bees at the feeder. For twelve visits at the feeder I recorded the time between visits.

Inside the hive the positions and movements of the marked bees were tracked with the help of a RFID sensor coil that scanned the hive surface. The coil was led by an adapted XY-plotter (Roland Digital Group DXY-1300) which had been programmed to scan the entire surface once per minute. This resulted in a spatial resolution of app. 1.5 cm. By comparing the position of individual bees between two hive scans, phases of immobility could be detected. Due to the physical properties of the induction powered microchip movements and changes in the

angle of the microchip compared to the receiving antenna, were detected more reliably than actual positions. Phases of immobility lasting for at least one minute were called 'rest' while immobility phases lasting for five minutes or longer were defined as sleeping state as suggested by previous studies (Shaw & Franken 2003).

The first step in this set of experiments was to validate the practicability of the hive scanning method to observe RFID-tagged bees inside the hive. Therefore I first measured the detection density of individual bees. I also tested if the sleep behavior of bees could be detected as prolonged immobility by repeated measurements of bees at one location. In addition I filmed bees inside the hive and compared visible sleep behavior with the plotting data. After validating the plotting method I compared the sleep duration of forager bees and young hive bees during night and day. Especially for the forager bees (the same bees used in the following experiments) I evaluated the sleep distribution throughout 24 h cycles.

2.2.6 Testing for the influence of flight times on sleep

In this analysis I tested if the sleep time of forager bees is dependent on the flight activity of the previous day. For this I compared the overall flight time of RFID-tagged bees with the corresponding overall sleep time during the following night. Additionally I tested if resting behavior inside the hive directly after foraging trips is dependent on the length of the foraging trips. I looked separately at the rest after short and long flights. Short flights were defined as flights lasting less than ten minutes, long flights were defined as flights lasting ten or more minutes.

2.2.7 Testing for the effect of navigational learning on sleep

For the forced navigation experiments the bees were captured with small glass jars at the feeder. To make sure the bees had enough energy to return to the hive, they were not captured before they had stopped feeding.

One subset of bees was displaced three times on one day. Of these bees one group was released three times at the same place 300m away from the hive (figure 2.7C), another one was released three times at different places 300m away from the hive (figure 2.7A) and a third group was released at 400m, 200m and 300m distance from the hive but every time in the same direction (figure 2.7B).

Another subset of bees were displaced on two consecutive days, but only once per day at a distance of 600 m (figure 2.7D).

To test if bees exposed to forced navigation tasks show normal foraging behavior, I analyzed the number of flights per day for free foragers, feeder foragers and feeder foragers exposed to forced navigation tasks. The same was done for the overall flight time of those groups.

To test the influence of the forced navigation tasks on the sleep behavior I measured the total sleep time in the night following the forced navigation tasks as well as one night before and two nights after. I analyzed the sleep time for the first and second part of the night separately.

In a similar experiment I exposed the bees on two consecutive days to one forced navigation task each. The sleep time was analyzed in the night following the tasks.

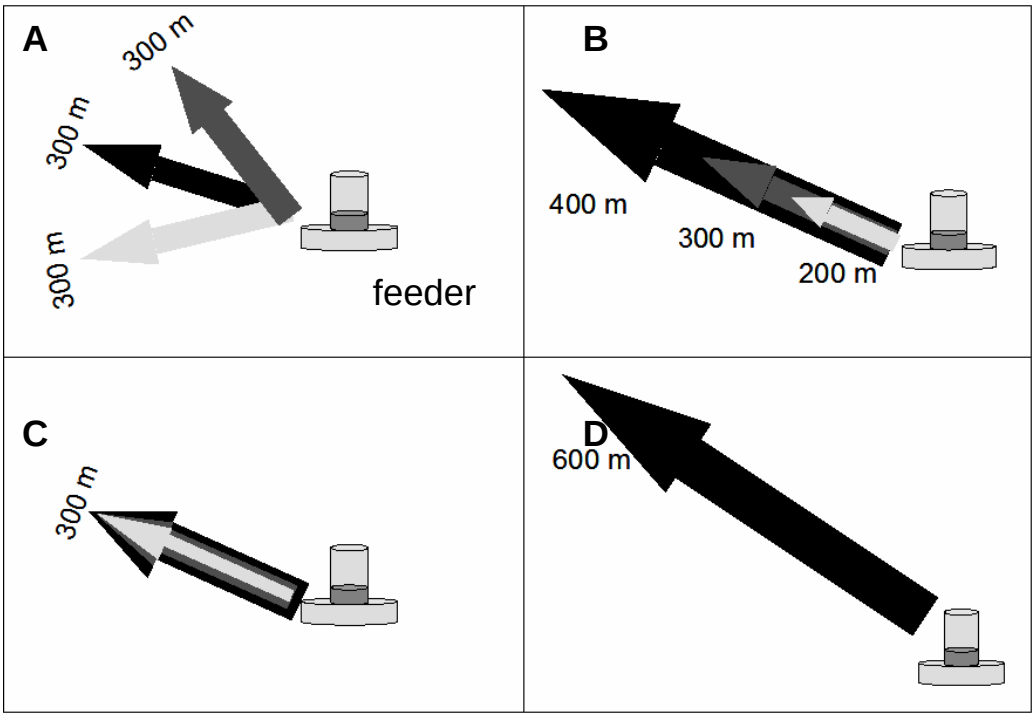

Figure 2.7 Displacement protocol. A-C multiple displacements. (A) Bees were released three times at different sites 300 m away from the feeder. (B) Bees were released three times at different distances to the feeder but in the same direction. (C) Bees were released three times at the same site. (D) Bees were released once at 600 m distance.

2.2.8 Testing for the effect of sleep deprivation on navigational memory

A subset of bees was captured after their return from the displacing and kept in a small cage until evening and fed with sugar paste. Over night the cage with a group of five bees were shaken on a vortex to deprive sleep for 10 h (figure 2.8). In the morning the bees were released into the feeding box and returned actively back into the hive. Bees that did not go back into the hive or did not start foraging regularly on the same day were discarded.

To see if sleep deprivation had an immediate effect on the resting behavior of sleep deprived bees, I measured the resting time of the bees between their return to the hive after sleep deprivation and their first foraging flight. I compared this

resting time to the resting time of control bees. As control served displaced but not sleep deprived bees, which were observed during the same time window. The resting time of these two groups was also measured during daytime.

To see if sleep deprivation had an effect on the consolidation of the navigational memory, I compared the time sleep deprived and not sleep deprived bees needed to return to the hive after the first and the second displacement. Furthermore I analyzed the return rate of sleep deprived and not sleep deprived bees.

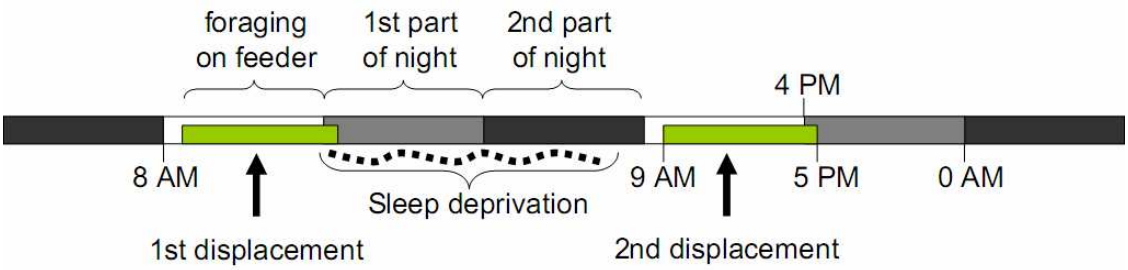

Figure 2.8 Sleep deprivation protocol. A 24h day was divided into three 8h periods. A day phase, lasting from 8AM to 4PM, an early night phase, lasting from 4PM to 0AM and a late night phase, lasting from 0AM to 8AM. The bees were foraging on the feeder between 9 AM and 5PM. During the foraging periods bees which regularly visited the feeder were caught and released at 600 m distance to force navigation learning. A subset of the returning bees were caught and sleep deprived during the night. On the next morning they were placed back into the hive.

2.2.9 Testing the effect of color learning on sleep

Another form of learning I tested in respect to sleep was color learning. For that I trained bees from the observation hive to a feeding site app. 100 m away from the hive. At the feeding site they had to decide between two Styrofoam boxes and enter them through a small hole to find food. Only one of the boxes was rewarded with sugar water. As clue served the color of cardboard that was attached to the front of the boxes. The rewarded box was changed regularly to avoid olfactory cues. I exposed them to color learning tasks of different complexity. The bees had

to choose between grey and blue, two visually easily distinguishable colors for bees; between green and yellow, which are more difficult to distinguish; and between two blue cardboards. In a second step in collaboration with Miguel Eckstein (Eckstein et al. 2004) a black cue was added that was only attached to the rewarded box for 80% of the time. Additionally I changed the colors of the rewarded or the unrewarded box every fifteen minutes, which forced the bees to ignore the unpredictable color pattern and focus on the more reliable black cue.

2.2.10 Data analysis

The RFID data were then analyzed with a computer software program written by Nico Schmidt. It provided the positions where and the times when each bee had been recorded inside the hive as well as the times the bees spend outside the hive. To analyze the sleep time I divided the day into three eight hour periods. The day period started at 8AM and lasted until 4PM. The first part of night started at 4PM and lasted until 0AM and the second part of the night started at 0AM and lasted until 8AM (figure 2.8).

For the statistical analysis of the experiments I used the t-test or the Mann-Whitney u-test. For multiple group analysis I used one way ANOVA. I analyzed dichotomous results with the Fisher exact test or, when the samples were too big for the fisher exact test, the Chi Square independency test. For correlations I used the Chi Square independence test.

2.3 Electrophysiology in sleeping bees

2.3.1 Bee keeping

I worked with the European honeybee, *Apis mellifera*. All bees used in the experiment were foragers. Since the experiments were performed during late autumn and winter all bees were taken from a temperated flight room with a 12 h light/dark cycle to keep bees flying. The bees were kept in natural sized hives and maintained by the bee keeper of the institute. They were fed with a 30% sugar solution and powdered pollen. For experimental use these bees were captured individually with small glass jars.

2.3.2 Setup

I used a custom made electrophysiology setup. The base was an iron table and a metal plate (100 cm x 100 cm x 3 cm) which was mounted on the table. Between them, rubber pads served as shock absorbers. To insulate the setup from electrical noise a metal grid was built in a 1 m^3 cube over the plate, forming a Faraday cage with an opening at the front side. A binocular microscope, an electrode micromanipulator, the olfactometer used for olfactory conditioning, a cold light source, a camera and a red light were placed on the metal plate and grounded. A differential 4-channel amplifier (A-M Systems, USA), an analog-digital converter (1401 micro MKII, CED, UK), an olfactometer-controller, a noise filtering device (Humbug, Digitimer, UK) and a PC were kept inside a metal rack and grounded to a common sink.

2.3.3 Electrodes

The electrodes were built similar to those described by Okada et al. (Okada et al. 2007). Two 14 µm thick polyurethan covered copper wires (Electrisola, Switzerland) served as electrodes. They were glued together with wax and, instead of using a tungsten wire like in the work of Okada (Okada et al. 2007), the copper wires were attached to a rust-proof steel needle (250 µm in diameter). The needle was waxed onto a glass capillary which was then placed into an electrode holder. The loose ends of the copper wires were soldered to the connecting pieces of the electrode holder which connected the electrodes to the differential amplifier (A-M Systems, USA). The electrical resistance of the electrodes was measured before they were placed into the electrophysiological setup and the connections resoldered if the resistance was above 3 MΩ.

2.3.4 Bee preparation

The bees were captured in the afternoon, harnessed and their head capsules waxed to the plastic tube. The scapes of their antennae were waxed to the head capsule in a 90 ° angle from the midline (figure 2.9A). Thus the bee could only move the flagella of its antennae. The Johnston Organ between the scape and the flagellum (Ai et al. 2009) was kept free of wax. To avoid heating up the brain with hot wax, eicosane (melting point 36.7 °C) was used to fix the antennae. After being fed to satiation the bees were kept at a humid place until the next day.

2.3.5 Brain preparation

A 100 µm silver wire was inserted into one eye to serve as ground. The wire was fixed with wax to the eye. The head capsule of a harnessed bee was opened with a small piece of a fine razorblade. To uncover the alpha-lobes of the mushroom bodies, the glands over the brain and the trachea sacs over the alpha-lobes were

carefully removed (figure 2.9B). With small pieces of tissue paper all fluids were removed from the head capsule and the bee was placed under the binocular of the electrophysiological setup. Here the electrodes were inserted ca. 150 µm deep into the output region of the α-lobe. To avoid drying of the brain and to further eliminate movements the brain was sealed with silicon when stable neuronal spikes were recorded.

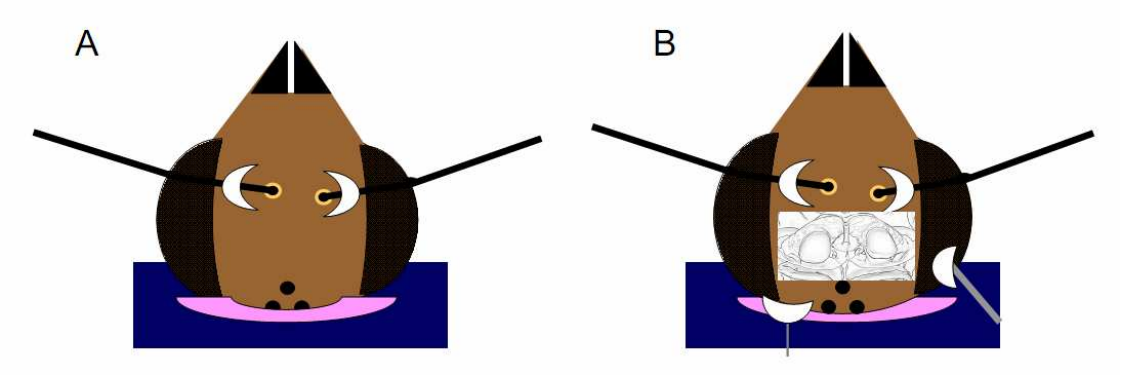

Figure 2.9 brain preparation. (A) fixed bee head. The head capsule has been immobilized with wax and the antennal scapes glued to the head capsule with eicosane. (B) opened head capsule with exposed α-lobes of the mushroom bodies. A silver electrode in the compound eye served as ground. For M17 recording an electrode was inserted into the muscle between the ocelli and the compound eye.

2.3.6 Recordings

Muscle recordings

For the recording of the proboscis extension muscle (M17) a 50 µm silver wire was inserted into the muscle at the back of the head capsule half way between the ocelli and the compound eye and the wire fixed with wax (figure 2.9B). For the

flight muscle recording a 50 µm silver wire was inserted into the thorax close to one wing and fixed with wax.

Neuron recordings

I recorded from mushroom body extrinsic neurons at the output region of the α-lobe (fig. 2.10). After the experiment the recorded neurons were sorted using the spike 2 software.

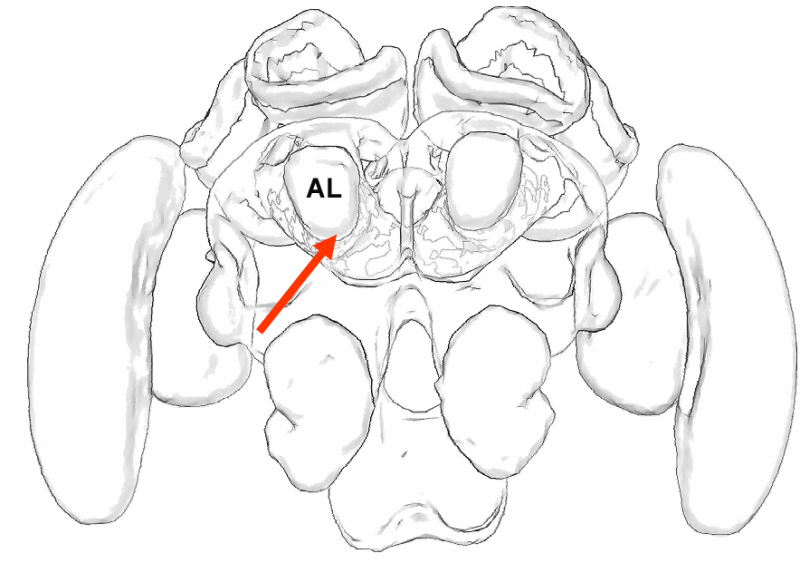

Figure 2.10 electrode insertion site. The electrodes were inserted into the output region of the α-lobe (arrow).

2.3.7 Optical sleep detection

For sleep detection the antennal movements were recorded during the electrophysiological measurement using a camera attached to the binocular (figure 2.11). To enhance the contrast the dark antennae were recorded over a white background (white filter paper on the top of the plastic tube, fixed with

melted wax). The movements were then analyzed by a custom made program written in C++ by Tim Landgraf, which tracked the angular movements of the flagellae. The position of the flagellae was defined with manually drawn lines over one picture of the video. An area of ca. 110° between the mandible and the extension of the scape defined the area of movements. Using these coordinates the program tracked the antennal movements automatically and saved the angular position of each antenna with a frequency of 10 frames per second to a text file. The text files were analyzed offline using MatLab. Lack of antennal movements for more than five minutes was defined as sleep.

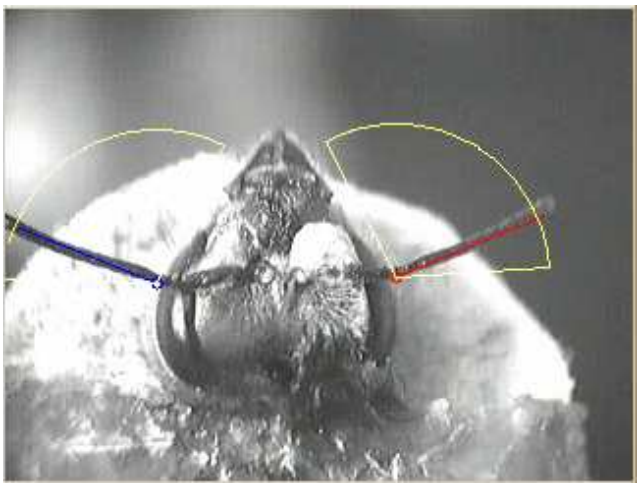

Figure 2.11 antennal tracking. The antennal scape was waxed to the head capsule to reduce the antennal movements to two dimensions. The movements were then recorded over the entire measuring period to detect sleep and activity phases.

3. Results

3.1 Sleep deprivation effects olfactory memory

3.1.1 Sleep deprivation effects extinction learning

To disturb sleep dependent memory consolidation the bees in this experiment were shaken after the acquisition trials, one group during the day and another one during the night. A non shaken group served as control. The bees were trained either in the morning or in the evening. Bees learned the CS+ both when conditioned in the morning and in the evening but bees trained in the evening showed a significantly higher learning rate than bees trained in the morning. More than 80% of the bees trained in the evening showed PER to the CS+ after the third acquisition trial, while only less than 50% of the bees trained in the morning showed the same response (figure 3.1).

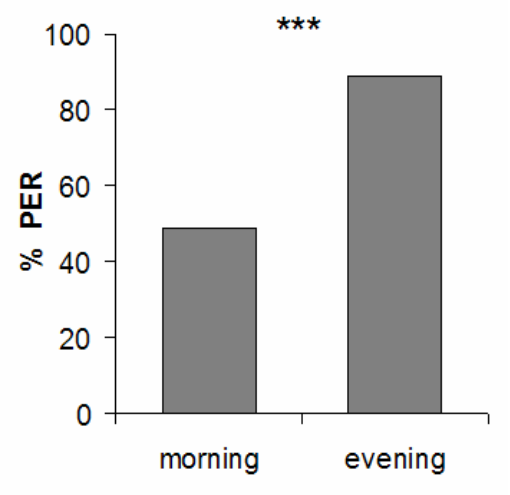

Figure 3.1 PER-Conditioning. Bees trained in the morning showed a significantly lower learning rate than bees trained in the evening. $N_{morning} = 115$; $N_{evening} = 126$. Chi-square test $\chi^2 = 46$, $p < 0.001$

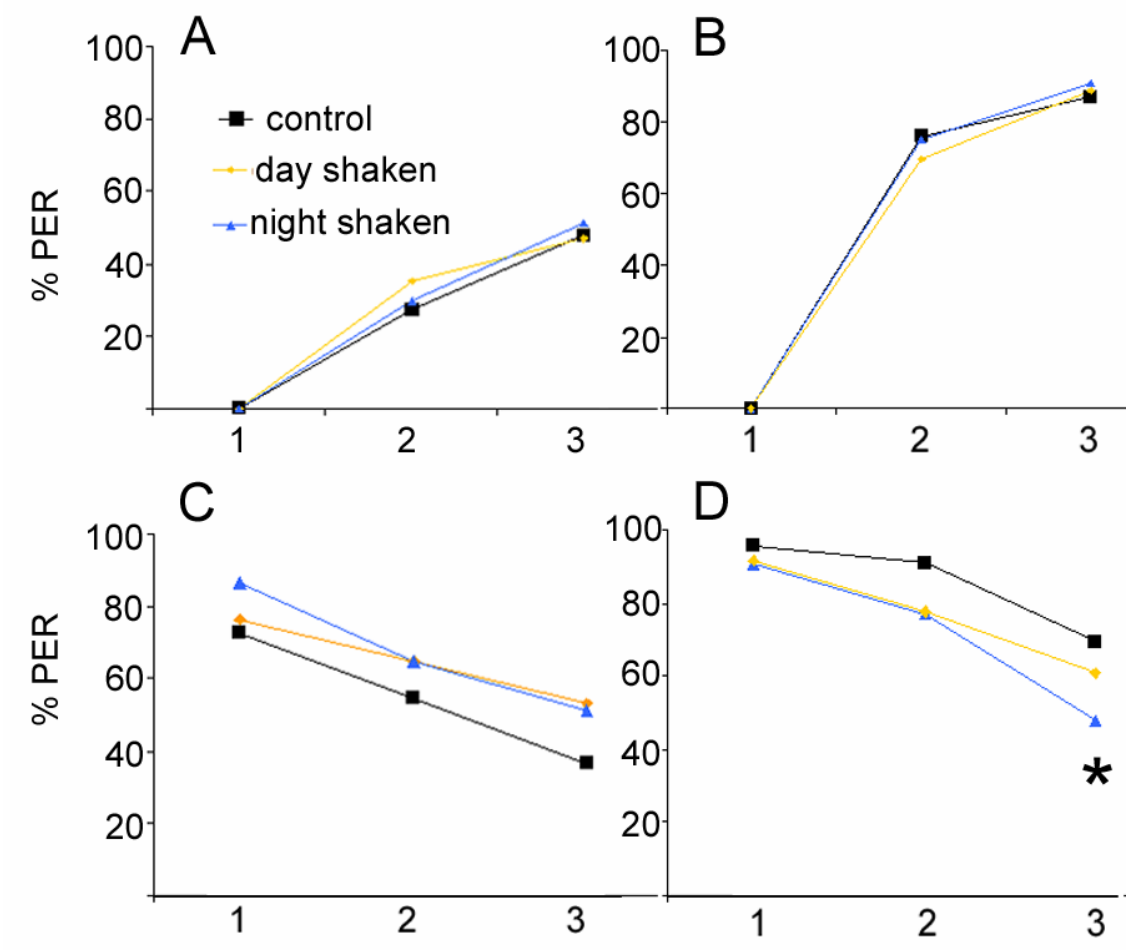

Figure 3.2 PER Acquisition (A, B) and Extinction (C, D). (A) 3 acquisition trials for morning trained bees. (B) 3 acquisition trials for evening trained bees. The acquisition curves differed significantly between morning and evening training. (C) 3 extinction trials in the morning. Morning trained bees show extinction after three CS only trials. No significant differences between the response rates. (D) 3 extinction trials in the evening. Evening trained bees show extinction after three CS only trials. The effect is significantly stronger if the bees were sleep deprived during the night after the acquisition; Fisher exact test $p < 0.05$. Morning bees: $N_{control} = 44$; $N_{day\ shaken} = 34$; $N_{night\ shaken} = 37$. Evening bees: $N_{control} = 46$; $N_{day\ shaken} = 36$; $N_{night\ shaken} = 44$

24 h after the acquisition (figure 3.A+b) the bees trained in the morning or in the evening were exposed to three CS-only trials. There was no significant difference in the response rate in the first CS-only trial. Therefore mechanical disturbance between acquisition and retrieval had neither in morning trained bees nor in evening trained bees a significant effect on memory consolidation compared to

the non shaken control (figure 3.2C+D). Also, since the response rates in the last CS-only trial were the same, the three groups of bees trained in the morning showed no significant differences in the extinction rate on the following CS-only trials. Disturbance during the day or during the night after an undisturbed day had no influence on the extinction (figure 3.2C).

In the evening trained bees there was no significant difference in the PER response in the third CS-only trial for bees which had been disturbed during the day after an undisturbed night compared to the control. Thus sleep disturbance after a night of rest had no effect on the extinction. However, bees disturbed during the night directly after the training showed compared to the control a significantly reduced response rate in the third CS-only trial. This indicates that disturbance during the night facilitates extinction (figure 3.2D).

24 h after extinction the bees were tested for retention. No significant differences were found between the groups neither for morning trained bees nor for evening trained bees.

3.1.2 Sleep deprivation impairs spontaneous recovery

In a second experiment (see 2.1.7) the bees were trained with a three trial olfactory conditioning (figure 3.3A), but they were disturbed directly before or directly after the extinction trials. Disturbance during the day directly before extinction had no significant effect on extinction (figure 3.3B), but only the unshaken control group showed spontaneous recovery when tested the next morning (figure 3.3C), while the performance of the shaken groups was similar to the last extinction trial.

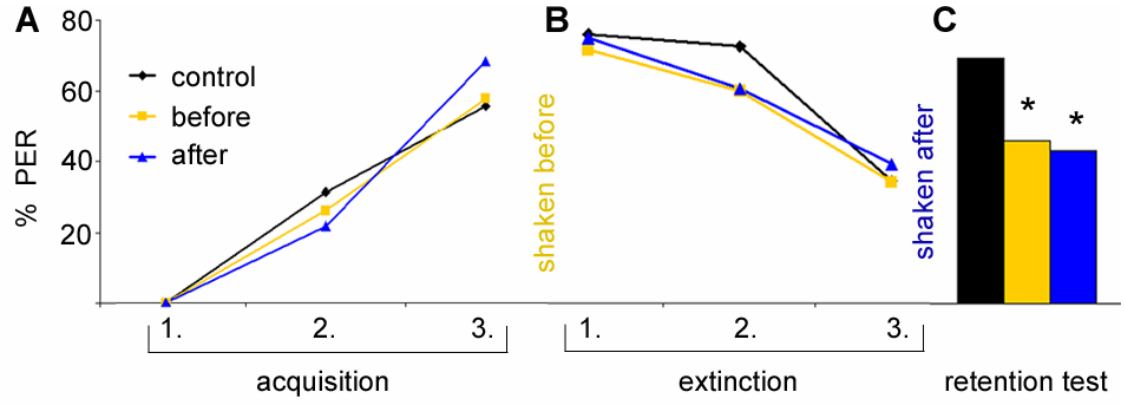

Figure 3.3 PER to conditioned odor in %. The bees were trained in the evening. They were shaken for 10 h either during the day directly before extinction (yellow) or during the night directly after extinction (blue). Acquisition curve (A) and extinction curve (B). The percentage of PER did not differ between shaken and unshaken bees in all three extinction trials. (C) Percentage of bees responding to trained odor during retention test in the morning after extinction learning. Freeman-Tukey test $p < 0.05$; Fisher Exact test shaken before extinction $p< 0.05$, shaken after extinction $p < 0.05$. $N_{control} = 29$; $N_{before} = 35$; $N_{after} = 28$

3.1.3 Sleep deprivation does not alter motivation

A big difference could be seen between satiated and starved bees in the acceptance of different sugar concentrations (figure 3.4A). Satiated bees did not respond to water or low sugar concentrations and only a small percentage responded to higher concentrations. When the bees were starved for 5 h, up to 20% of the bees responded to a solution without any sugar (pure water). With increasing concentration of sugar, the percentage of bees accepting the solution rose in a characteristic manner. At a sugar concentration of 10% the acceptance had reached a maximum, with almost 100% of the bees responding to the solution (figure 3.4A). The response of starved bees to different sugar concentration was independent of the time of testing (figure 3.4B)

The same sugar concentrations were offered to starved but previously disturbed bees. Both in the morning and in the evening the response curves of the disturbed bees were not different from the not disturbed control (figure 3.5A+B).

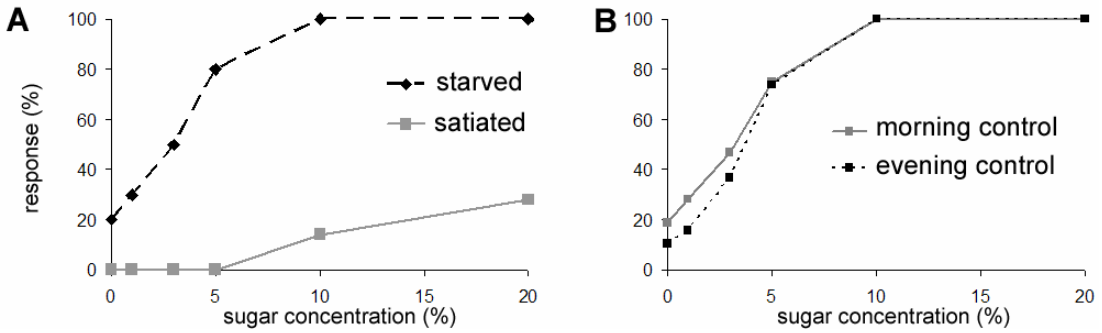

Figure 3.4 Percentage of bees accepting different sugar concentrations. (A) Difference between starved (black diamond) and satiated bees (grey square). Hungry bees accepted lower sugar concentrations, while satiated bees showed only slight responses to even higher sugar concentrations. $N_{starved}$ = 10; $N_{satiated}$ = 7; z = -28; $p < 0.001$; Mann-Whitney u-test. (B) Control bees from the sugar accepting experiment tested in the morning (grey) and in the evening (black) showed responses similar to the 5 h starved bees but were not significantly different from each other. $N_{morning}$ = 32; $N_{evening}$ = 19; z = -0.66; ns; Mann-Whitney u-test.

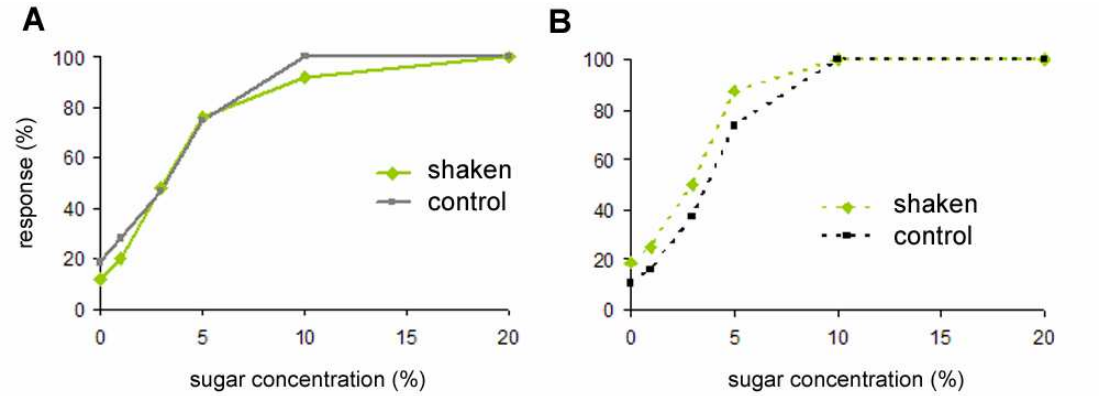

Figure 3.5 Percentage of bees accepting different sugar concentrations. (A) Bees tested in the morning after night shaking; $N_{control}$ = 32; N_{shaken} = 25; z = 0.33; ns; Mann-Whitney u-test. (B) Bees tested in the evening after day shaking. No significant differences between shaken (green diamonds) and unshaken control bees could be shown; $N_{control}$ = 19; N_{shaken} = 16; z = 1.01; ns; Mann-Whitney u-test.

3.2 Sleep deprivation effects the acquisition of navigation memory

3.2.1 Foragers but not young bees sleep more at night than during the day

I analyzed the sleep pattern of individual bees in the hive. Marked bees could be detected most of the time when they were inside the hive. Figure 3.6 shows an example of a single bee during a 24 h period (bee 8007 on July 25th 2008). The times of detection are shown in the lower part. The dark grey color shows the periods when the sun was below the horizon and the light grey color shows the period when the sun was above the horizon. The upper part of figure 3.6 shows the duration of the sleep phases which are more prominent and longer during the dark phases of the day.

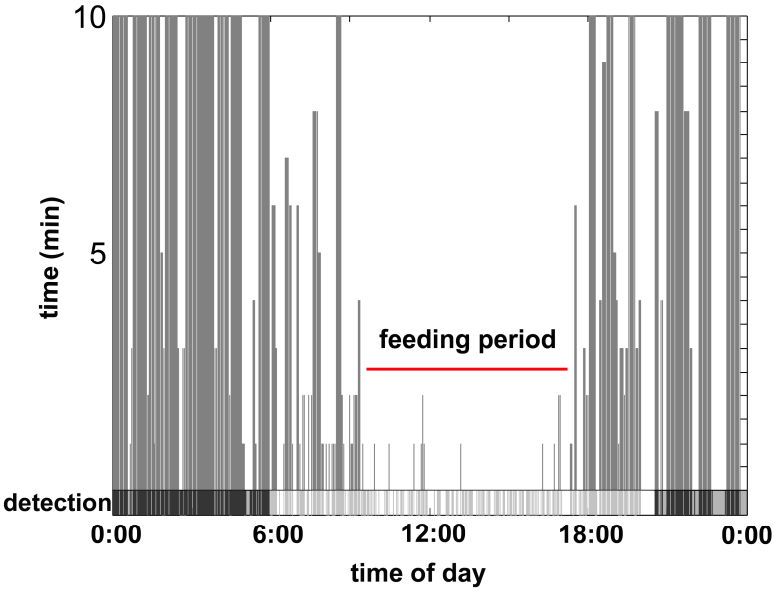

Figure 3.6 Tracking-coverage of a single forager bee over 24 h. Lower part shows all detection events while the upper part shows the occurrence and length of the sleep phases. In the detection part the dark period of the day (the night) is shown in dark grey and the light period (the day) in light grey. Detection was quite complete during the night and less so during the foraging time. Rest phases were much longer and more prominent during the night. The feeding period is indicated by the red bar and lasted from 9: 00 to 17:30. Bee 8007, July 25th 2008

There was a high variability in the distribution of sleep, but within single bees some patterns could be seen. After a forced navigation test the amount and distribution of sleep seemed to be altered (figure 3.7).

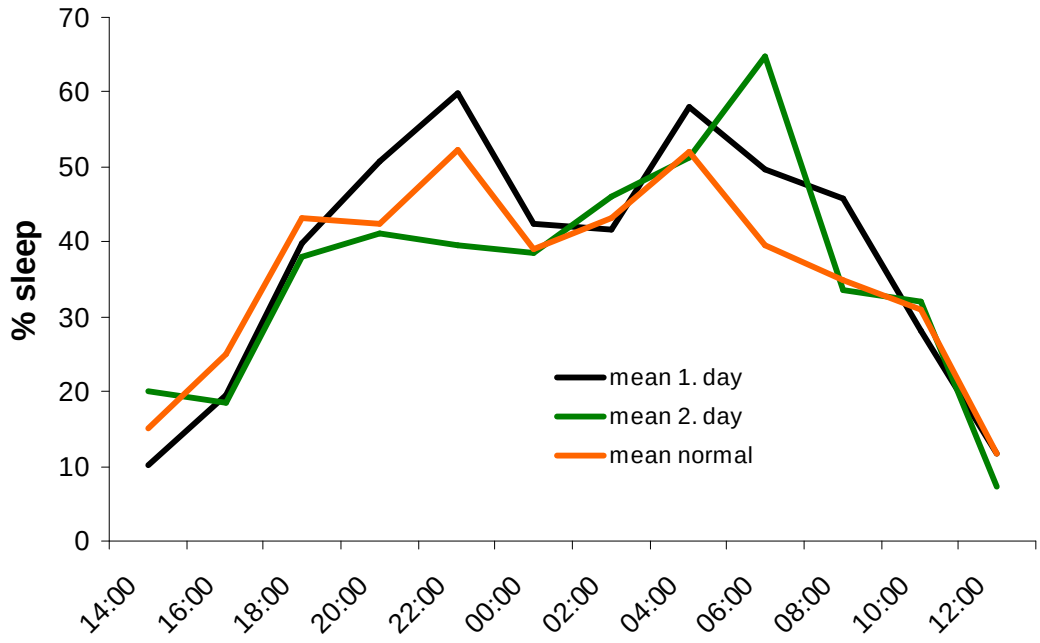

Figure 3.7 Percentage of sleep averaged for a single bee. (Orange) normal sleep distribution. During the night after the first forced navigation task (black) sleep seemed to be enhanced especially during the first part of the night compared to the night after the second forced navigation task (green). Bee 8047 $n_{nights} = 9$.

By evaluating the phases of mobility and immobility, I could compare the activity patterns of individual bees in dependence of their behavioral task and the time of day. I could see a difference in day and night activity in foragers (> 14 days old) which rested much less during the day than during the night (t-test, $p < 0.01$). This pattern was not observed in young (2 – 10 days old) bees, which still spend the whole day inside the hive. Their sleep appeared to be equally distributed over

day and night (figure 3.8). The amount of sleep over 24h was not significantly different between forager bees and young bees.

There was no difference in the sleep time between newly RFID-tagged bees and bees that had carried the tag for several days (data not shown).

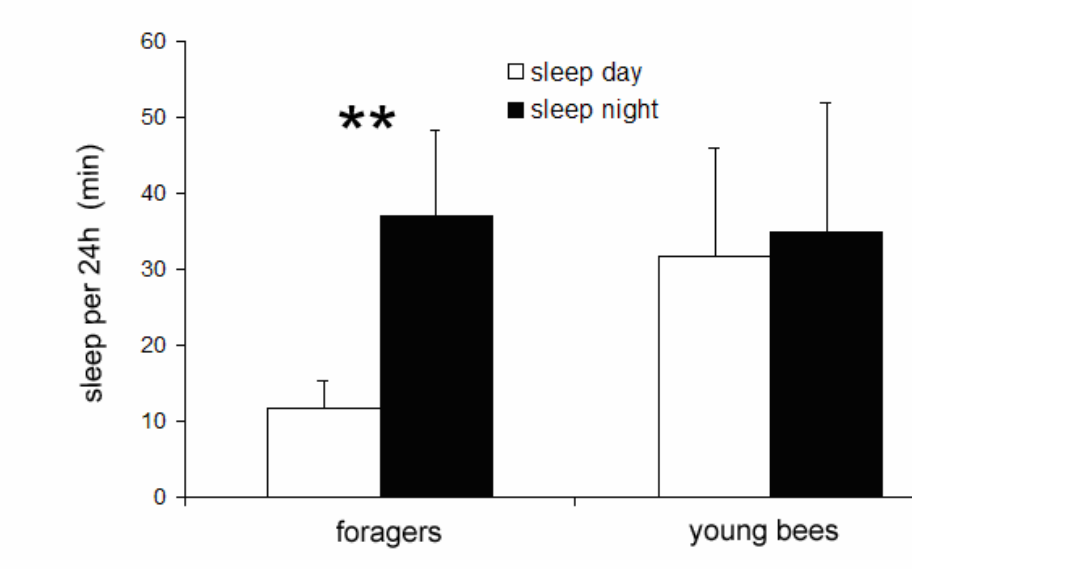

Figure 3.8 Differences in sleep time within 24h between forager bees and young non foraging bees (mean ± SD). Only foragers show an increased sleep time during the night; t-test $p < 0.01$; $N_{foragers} = 10$; $N_{young\ bees} = 10$

The day-night activity pattern of forager bees can also be seen in figure 3.9 and 3.10. Figure 3.9 shows sleep of seven different bees during one 24h period (Sept. 12th 2008). Although sleep phases (light bars) during the day occurred, the great majority of sleep happened during the night. Some variability between the single bees was observed, but the day-night pattern could be seen for all seven bees.

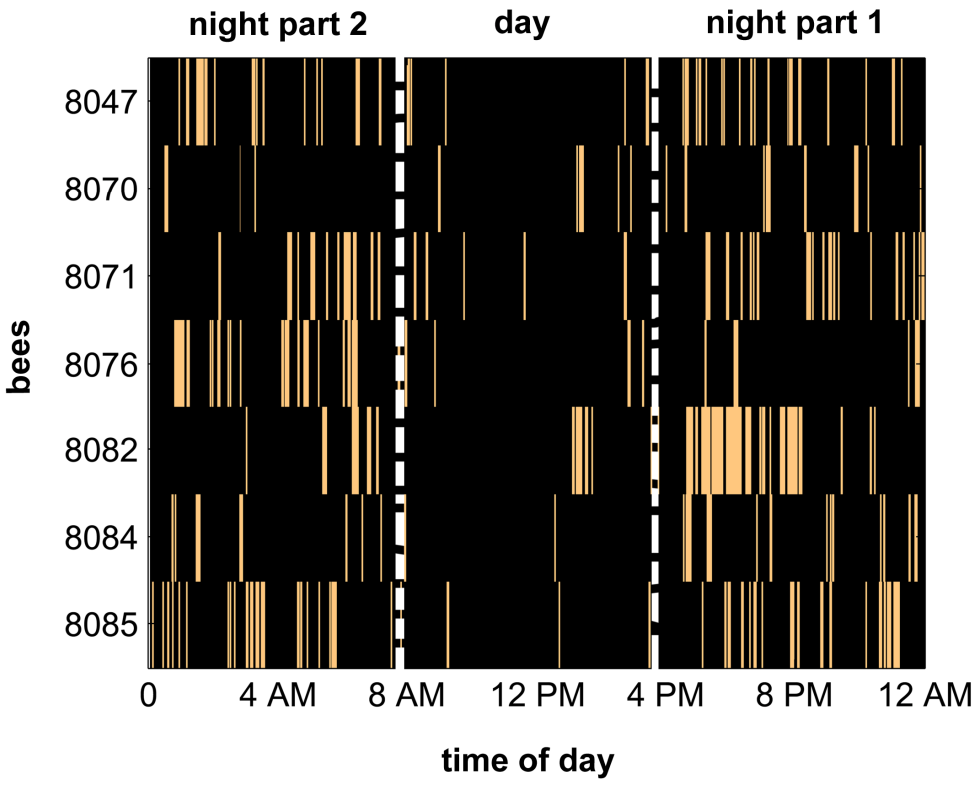

Figure 3.9 sleep pattern of several forager bees during one day (Sept 12th 2008). Phases of sleep are shown in light brown, active phases in black. The first part of the night (4 PM to 12 AM), the second part of the night (12 AM to 8 AM) and the day were analyzed separately.

The sleep pattern of a single bee over several consecutive days is shown in figure 3.10. The observation period started in the afternoon of July 14th 2008 when the bee was marked with the RFID-tag and lasted until July 22nd. Black color indicates no sleep, brown colors short sleep periods and light color long sleep periods. Again a high variability in the sleep can be seen. Still, most sleep phases, especially long sleep phases occurred during the night.

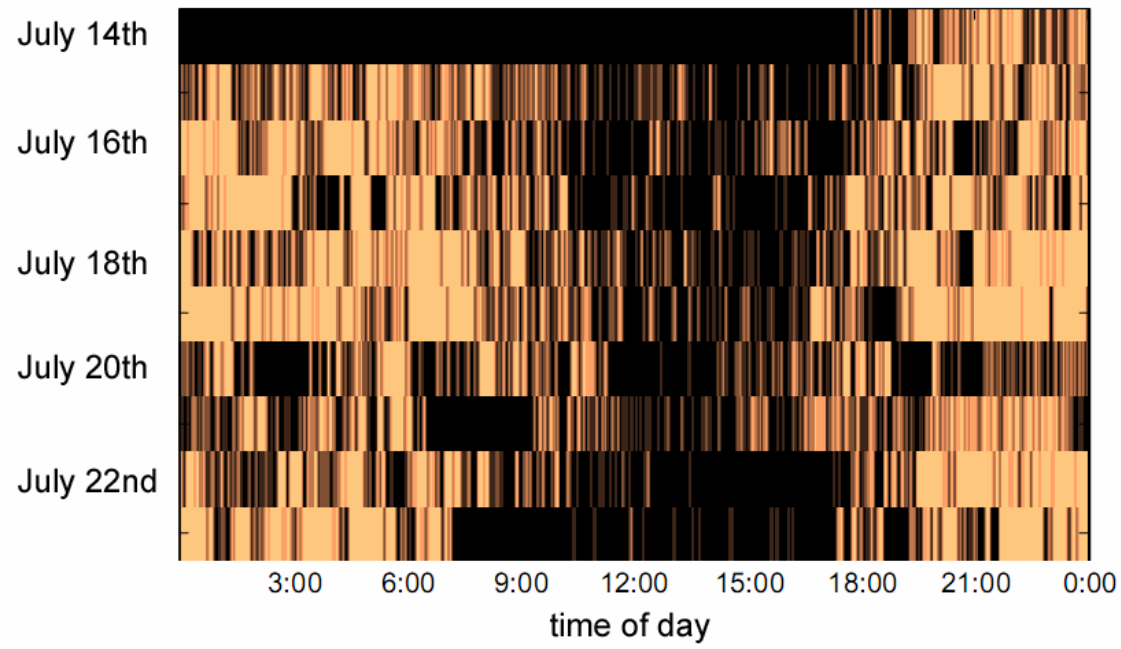

Figure 3.10 Rest of a single forager bee during the entire observation period. Black color indicates no sleep, long sleep periods are light, shorter periods are darker. Long phases of sleep occur mainly during the night. The amount and the profile of sleep seem to be different in different days, but most sleep occurs during the night.

3.2.2 Bees have special sleep sites

By observing the bees on the hive surface with the RFID scanning device, I could not only determine the time and duration of the sleep behavior of marked bees, but also their approximate location in the hive. The observed bees spend their active time mainly close to the brood area of the hive. During sleep, however, they often retreated to less crowded locations outside of the brood area. Figure 3.11A shows an example of the position frequency of a single bee during activity (figure 3.11A) and sleep (figure 3.11B) at the hive surface. The brood area is marked with a red circle, a location with high frequency of sleep behavior outside the brood area with a green circle.

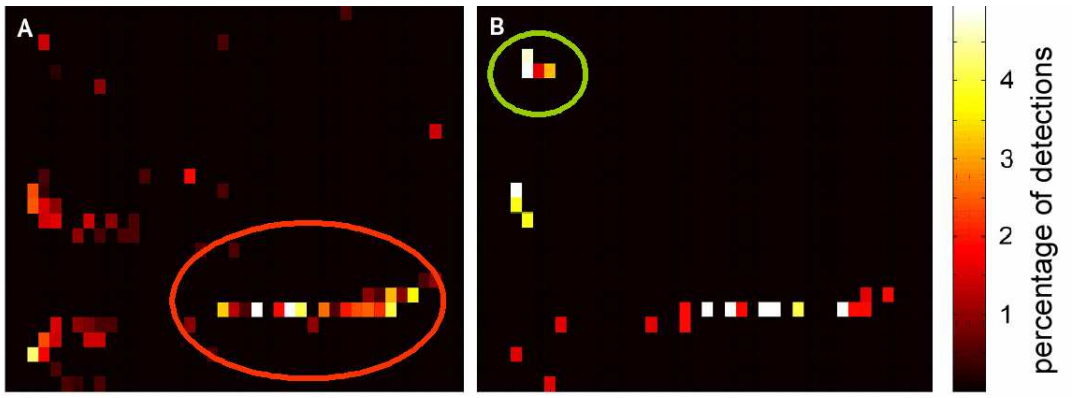

Figure 3.11 Hive surface (25x40cm). Hive detections of a single bee over 24h in percent. (A) active bee, (B) sleeping bee. The colors show the percentage of detections at the individual points from 0% (black) to 5% (white). Awake bees were found at more positions than sleeping bees but both awake and sleeping bees were most of the time detected in the main brood and food area (red circle). Sleeping bees also spend a lot of time in a less crowded area (green circle). Bee 8065, Sept. 4th 2008

3.2.3 The RFID tag does not impair flight performance

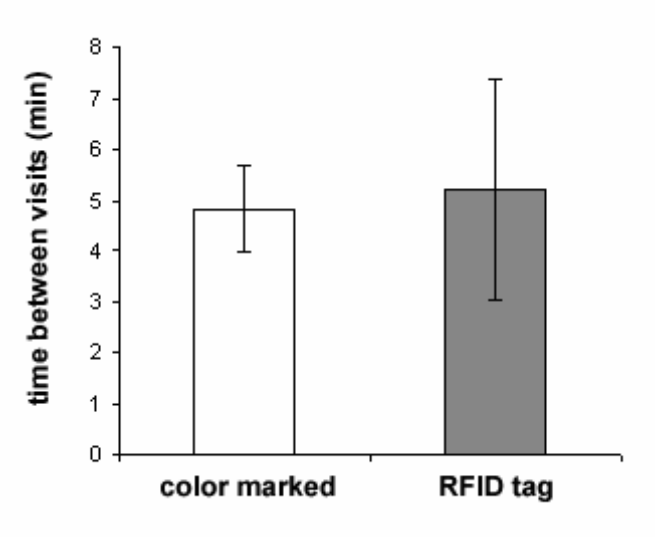

Figure 3.12 Mean time between visits (n = 12) at feeding site. Control bee (n = 1) with only color marking on the thorax (white bar) compared with bees marked with a microchip (n = 4). The time the bees need to fly back to the hive, give the collected sugar to hive workers and return to the feeder. T-test, ns.

The first flight experiment performed with the RFID marked bees should reveal if the RFID tags impaired the foraging performance. The foraging of RFID and color control marked bees was compared at the feeder they had been trained to. The time between visits at the feeder did not differ between the color marked and the RFID marked bees (figure 3.12).

3.2.4 The flight time does not influence the sleep time

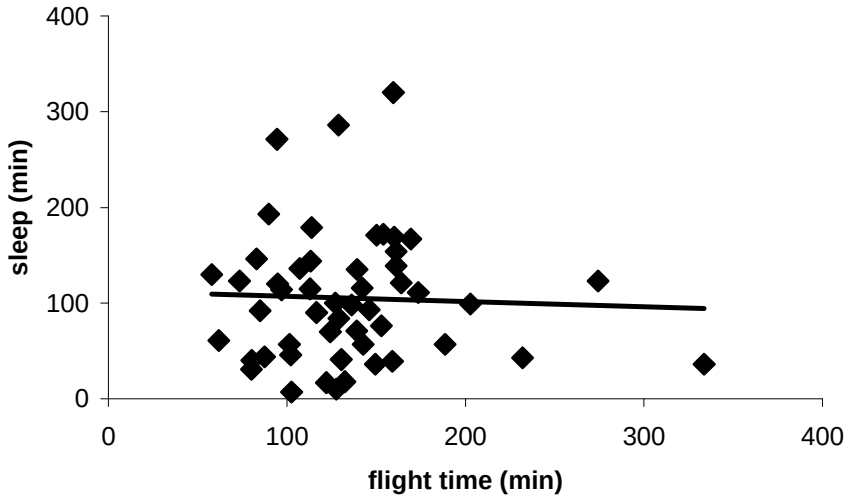

Figure 3.13 flight time correlated to sleep time during the first part of the night. The amount of sleep is independent from the time spend foraging. X^2-Independence test. N = 52, regression line R^2= 0.0016

In figure 3.13 the overall sleep time during the night is shown against the overall flight during the previous day for individual bees. The sleep time seemed to be independent of flight time. There was also no correlation between flight time and sleep time for single parts of the night. Bees that had been exposed to different tasks did also not show a correlation between flight times and sleep time (data not shown).

Similar results are shown in figure 3.14. Here, in contrast to the results shown in figure 3.13, I looked at resting times instead of sleeping times in comparison to the foraging time. Resting is in this case defined as no movement for longer than one minute. The resting phases directly after each flight were analyzed. In figure 3.14A the individual resting periods are shown against the duration of the previous foraging flight. Duration of resting and duration of foraging flights were not correlated. Only about half of the foraging flights were followed by rest. The percentage of flights followed by rest was not different for long flights (≥ 10 min) and short flights (< 10 min) (figure 3. 14B). In figure 3.14C the length of the individual flights and the following rest period are shown in detail.

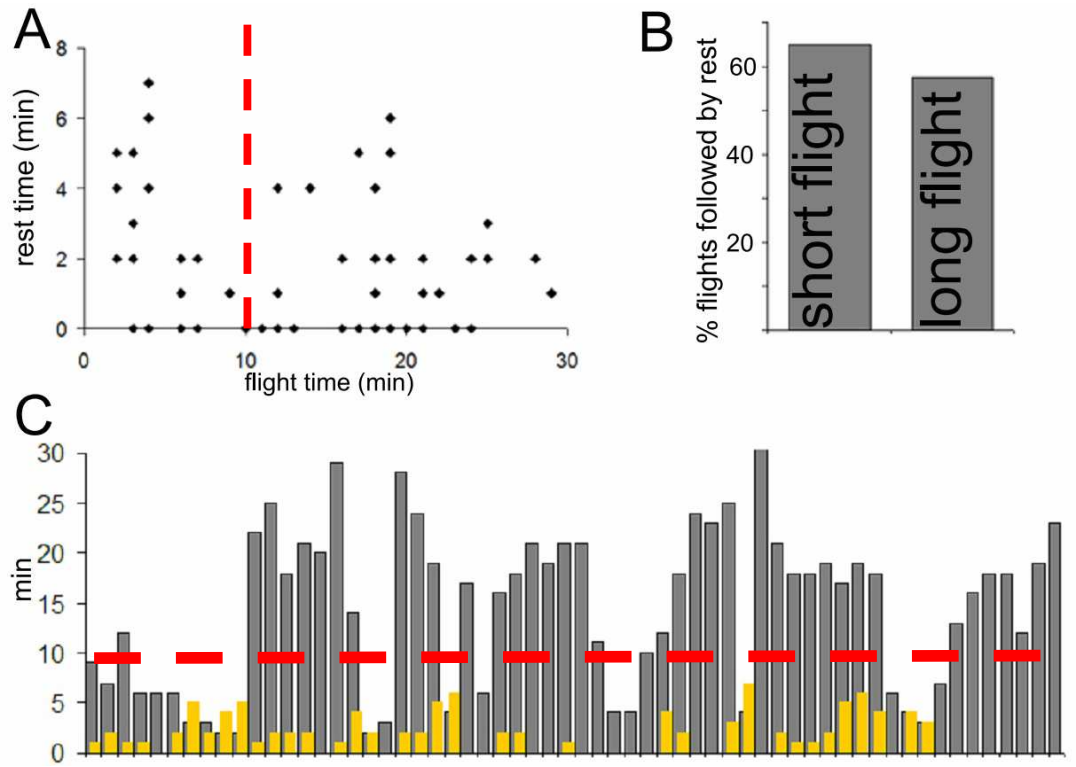

Figure 3.14 Resting behavior after foraging trips is independent of flight time. The data shown are from one foraging day of a single bee. The flights were separated into short and long flights with a flight time threshold of ten minutes. (A) shows the individual flight times compared to the resting times. The red line marks the threshold for long flights. (B) shows the percentage of short and long flights that were followed by rest. (C) shows all flights of one foraging day of one bee (grey bars) and the rest phases following the flights (yellow bars). Threshold for long flights is 10 min (red line). Bee 8002

Median number of flights and flight duration of bees which had to perform a navigation task are shown in figure 3.15. The same data are also shown for bees foraging at the feeder but not displaced (feeder foragers) and bees not foraging at the feeder but at natural sources (free foragers). Free foragers performed significantly less foraging flights compared to feeder foragers and navigation task bees (figure 3.15A). However the time the free foragers spend outside the hive was not significantly different from the time the other two groups spend outside the hive (figure 3.15B). For feeder foragers forced navigation tasks had no significant effect on the number of flights performed per day (figure 3.15A). Nevertheless the overall time spend outside the hive is significantly higher in bees after forced navigation tasks (figure 3.15B).

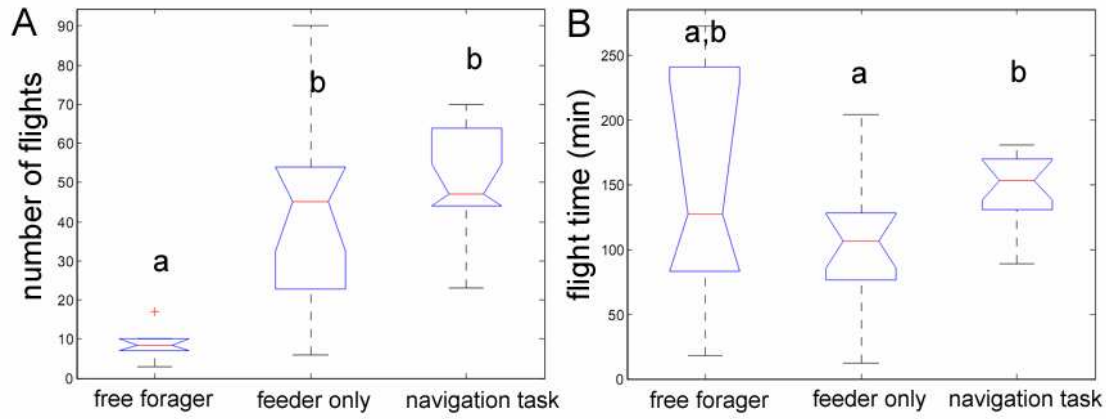

Figure 3.15 (A) Average number of flights. Normal forager bees performed around 10 foraging trips per day. Bees foraging on a nearby feeder did significantly more foraging trips. Displaced bees did not have less foraging trips than those simply foraging on the feeder (One way ANOVA $p < 0.001$). $N_{free} = 6$; $N_{feeder} = 15$; $N_{navigation} = 16$; (B) Overall flight times/day. The mean foraging time/day is highly variable in freely foraging bees. Neither simple foraging on a feeder nor three displacements changed the flight time compared to natural behavior, One Way ANOVA p=0.10. However, bees which were displaced spend significantly more time flying than bees simply foraging on the feeder Shown are medians and interquartile ranges. Mann Whitney u-test $p < 0.05$. $N_{free} = 6$; $N_{feeder} = 15$; $N_{navigation} = 16$;

3.2.5 Forced navigation leads to increased sleep

Figure 3.16 shows the influence of three forced navigation tasks on the sleep behavior of the foragers. Shown is the second part of the night where the differences were strongest. In the night before the displacement the observed bees slept in the median less than 20 min. In the night after the forced navigation tasks the sleep time was significantly increased to about the fourfold. In the second and third night after the navigation tasks the median sleep time was still higher than on the first night. During the first part of the night (4PM to 12AM) no significant differences were seen. Also bees exposed to one forced navigation task on two consecutive days slept significantly longer during the night after the first forced navigation task (data not shown).

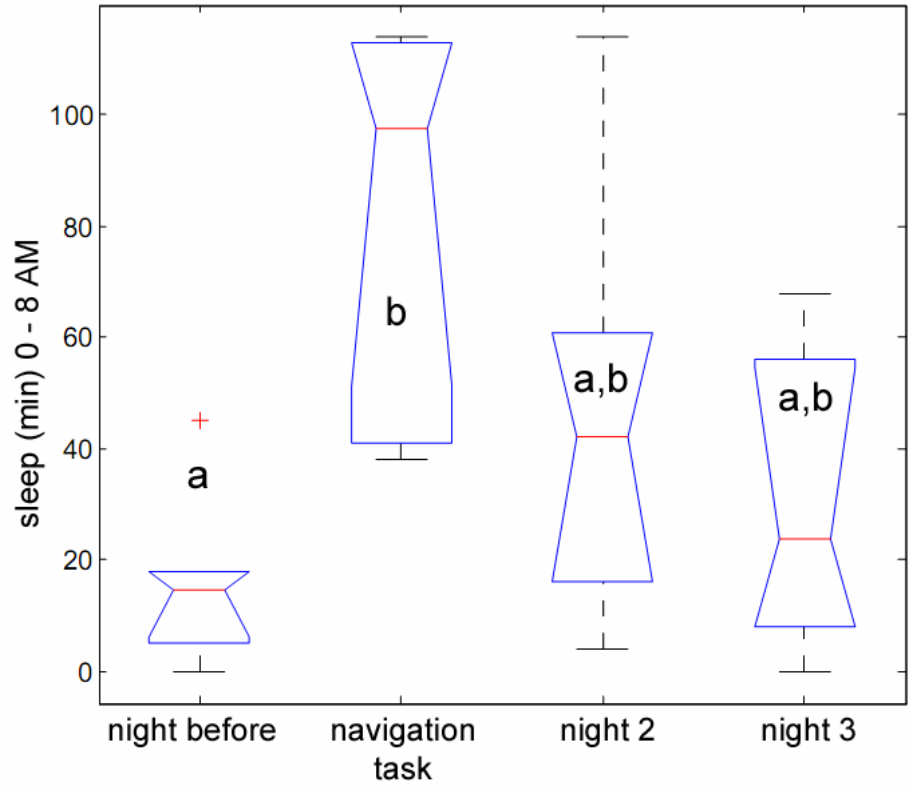

Figure 3.16 median sleep time over several days. Shown are medians and interquartile ranges. N = 4, One Way ANOVA $p < 0.01$

Figure 3.17 and 3.18 show the sleep time of differently displaced bees. The sleep times in the different displacement groups did not differ from each other when sleeping times of the whole night were considered. However, sleep time during the first part of the night (5-12PM) was higher for bees after navigation tasks than for free foragers (figure 3.18A), but not significantly higher than the sleep time of feeder foragers. No significant differences in the sleep time of the differently treated bees were found for the second part of the night (figure 3.18B). The difficulty of a navigation task was not reflected in the sleep time (data not shown).

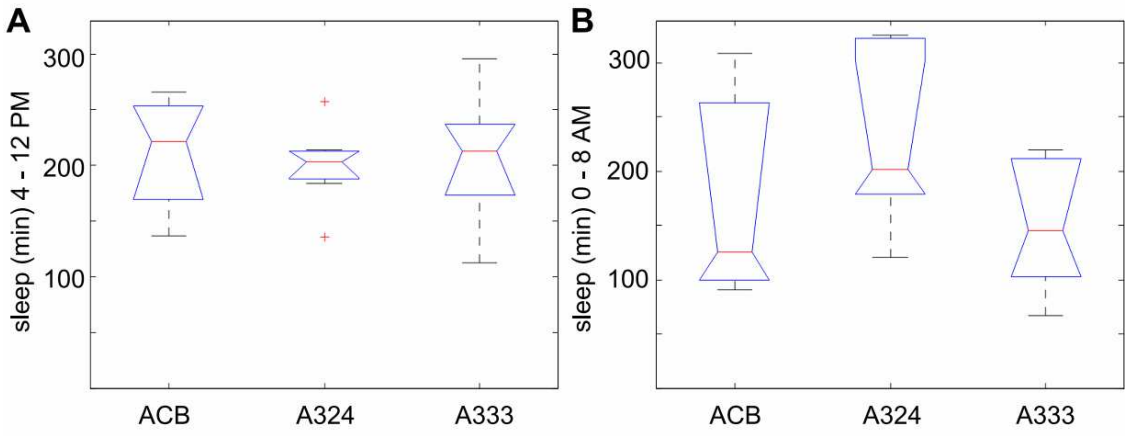

Figure 3.17 Sleep in the night after displacement. (A) First part of the night, (B) Second part of the night. The difficulty of a navigation task was not reflected in the sleeping time. Shown are medians and interquartile ranges. First part of the night One Way ANOVA, ns; N_{ACB}= 3; N_{A324} = 6; N_{A333}=5. Second part of night One Way ANOVA, ns; N_{ACB}= 3; N_{A324} = 6; N_{A333}=5.

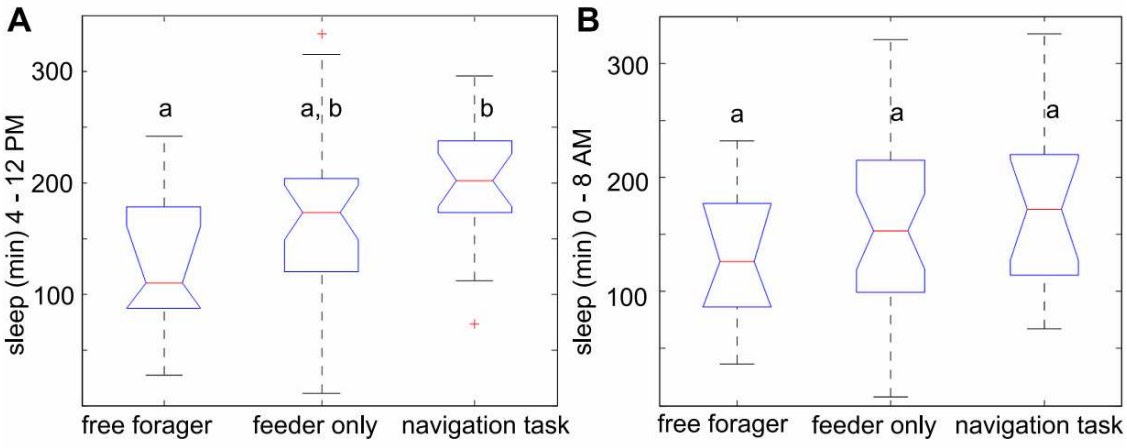

Figure 3.18 Sleep in the night after displacement (A) sleep during the first part of the night. Freely foraging bees slept less during the first part of the night (4-12PM) than displaced bees which were taken from feeder foraging bees One Way ANOVA $p < 0.05$. There was no difference between undisturbed feeder foraging bees and displaced bees. (B) sleep during the second part of the night. No differences between the groups. Shown are medians and interquartileranges. N_{free} = 8; N_{feeder} = 33; $N_{displaced}$ = 14.

Bees displaced once per day on two consecutive days slept significantly less in the night after the second displacement compared to the night after the first displacement (figure 3.19). The differences were strongest during the first part of the night (4PM to 12AM)

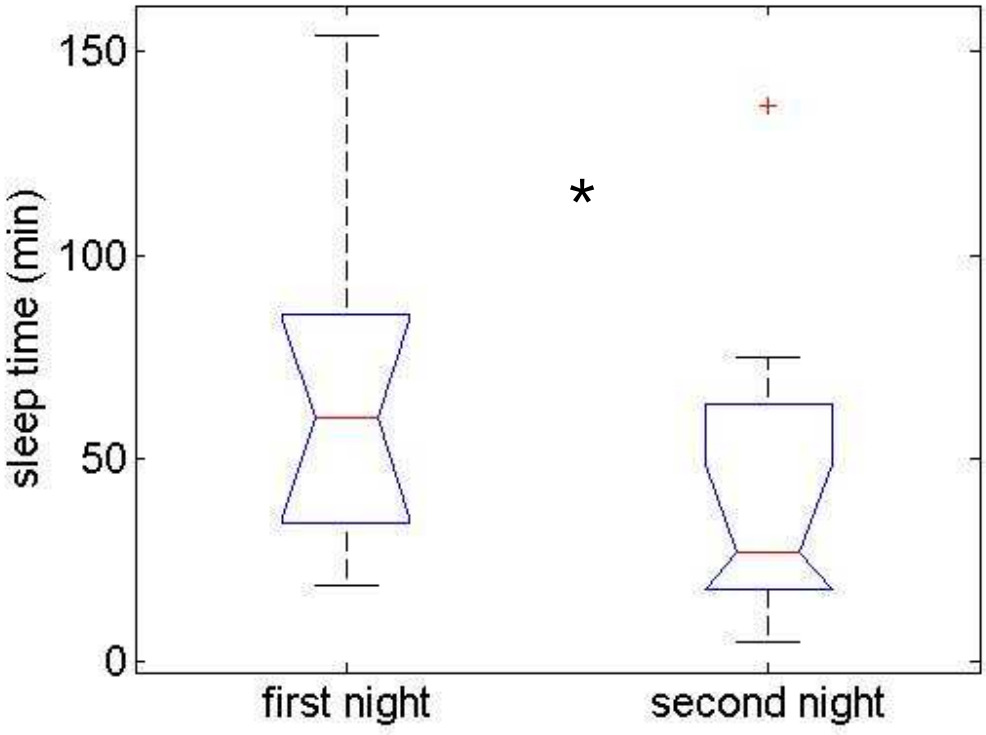

Figure 3.19 Differences in sleep time before midnight for single bees between first day of displacement and second day of displacement. During the first part of the night most bees slept less after the second day of displacement N = 9; Wilcoxon-signed-rank test $z > 1.96$, $p < 0.05$.

3.2.6 Sleep Deprivation changes sleep during the following night

Bees removed from the colony and shaken for sleep deprivation as described in 2.2.8 behave normally after being placed back into the hive. They are accepted by their colony and usually start foraging after a short delay in the hive. Shaking had no effect on foraging performance at the well known feeder.

Figure 3.20 shows the sleep time in the night after the second forced navigation task. In the first part of the night the sleep time was significantly shorter for bees which had been sleep deprived the night between the navigation tasks. In the second part of the night no difference was seen.

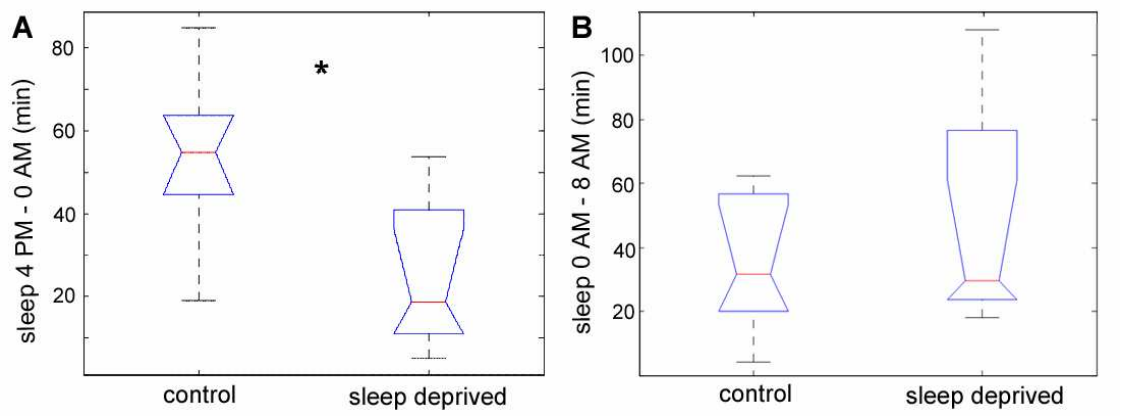

Figure 3.20 Sleep time for control and sleep deprived bees. (A) sleep time during the first part of the night. Bees in the hive showed significantly longer sleep time during the first part of the night (4PM to) compared to bees that had been sleep deprived the previous night. Mann-Whitney U-test $p < 0.05$; $N_{hive} = 7$; $N_{shaken} = 7$ (B) Sleep time during the second part of the night. No significant differences between the sleep times of control and sleep deprived bees. Shown are medians and interquartile ranges. Mann-Whitney U-test ns; $N_{hive} = 7$; $N_{shaken} = 7$

Directly after being placed back into the hive after the sleep deprivation but before foraging onset the bees rested some time in the hive. This resting time was not different from the resting time before foraging onset in control bees which had spend the night in the hive (figure 3.21). Also the rest time performed during the day following sleep deprivation was not significantly different for sleep deprived bees and the not sleep deprived control bees.

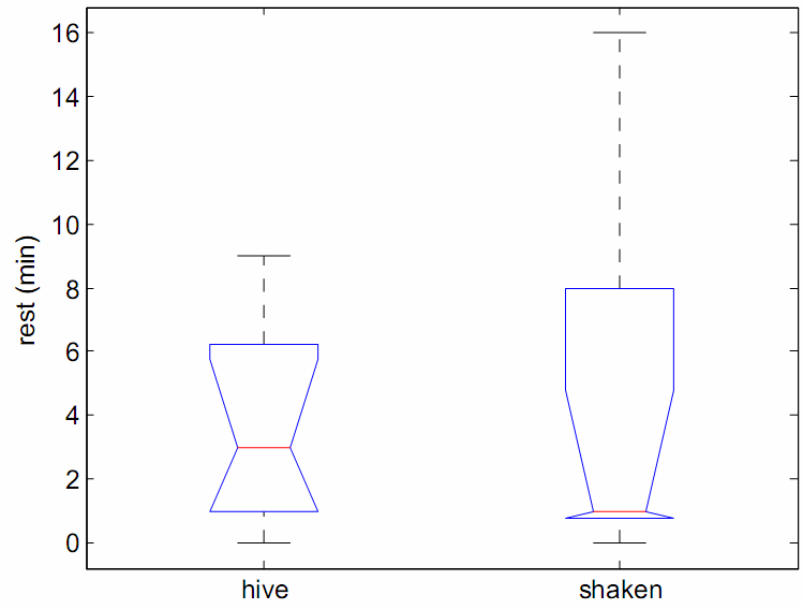

Figure 3.21 mean rest time before 1st flight of a day. Shown are medians and interquartile ranges. Shaken bees did not rest significantly more or less between their return to the hive and their first foraging flight than control bees during the same time. Mann Whitney U-test ns; N = 9

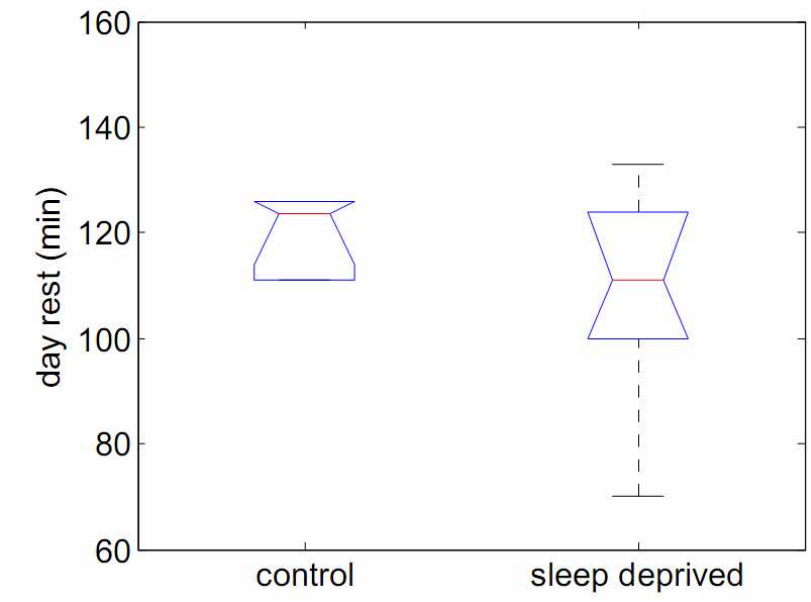

Figure 3.22 day rest. The day rest time did not differ significantly between control and sleep deprived bees. Shown are medians and interquartile ranges. Mann-Whitney U-test ns; N = 9

Sleep deprivation had no influence on the time the displaced bees needed to find back to the hive after the second displacement. Neither sleep deprived bees nor control bees showed a significant improve in return time (figure 3.23).

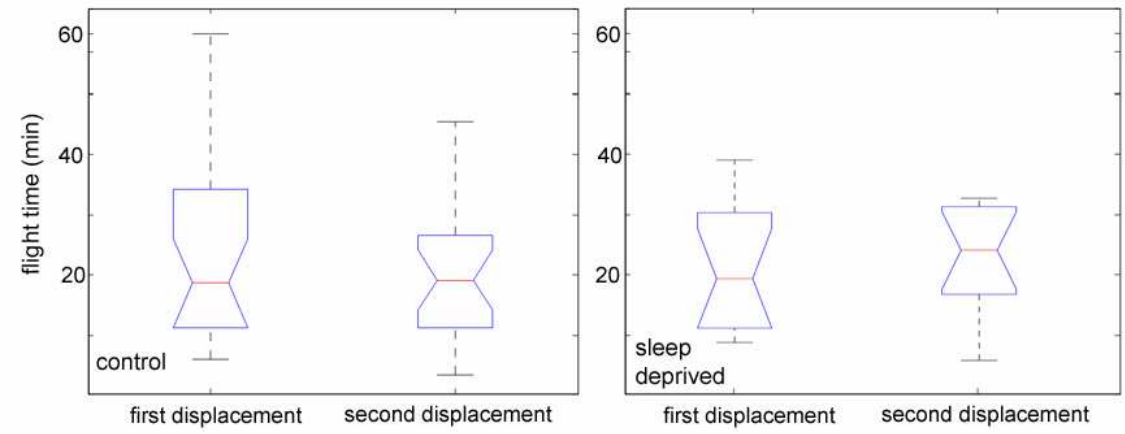

Figure 3.23 Flight times (min) after displacement of forager bees. Shown are medians and interquartile ranges. Mann-Whitney u-test ns; $N_{hive} = 7$; $N_{shaken} = 7$

3.2.7 Sleep deprivation impairs the consolidation of newly acquired navigation memory

After the first forced navigation trial less than 60 % of the bees returned to the hive while more than 40 % were lost. After the second forced navigation trial less than 20 % of the control bees were lost. The bees which had been sleep deprived, however, showed no significant decrease in the loss rate compared to the first forced navigation trial. Compared to the control bees the loss rate after the second trial was significantly increased (figure 3.24). The loss rate of the deprived bees was significantly higher than that of control bees.

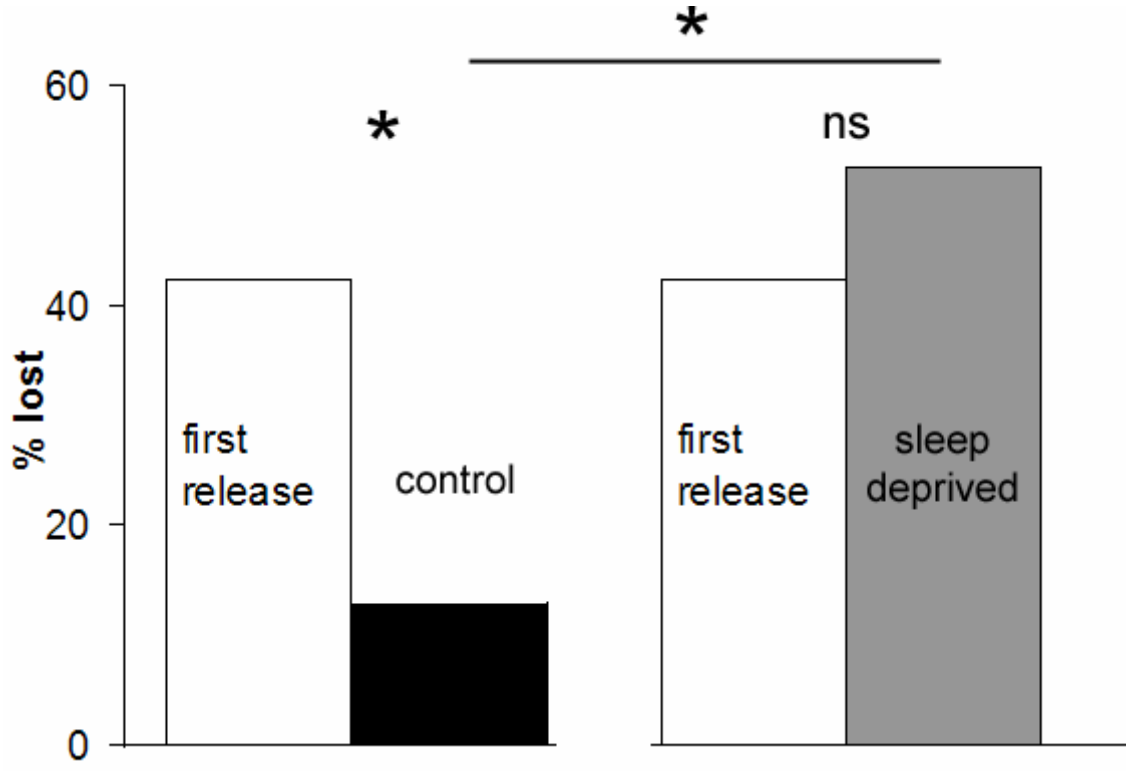

Figure 3.24 Loss rates after displacement. A high percentage of bees did not find their way back after the first displacement (white bar). After a night in the hive (black bar) the loss rate after the second displacement was significantly reduced. The loss rate in sleep deprived bees (grey bar) stayed at the level of the first displacement. Fisher exact test: first displacement vs. control $p < 0.05$. First displacement vs. sleep deprived ns. $N_{hive} = 23$; $N_{shaken} = 13$; $N_{first\ displacement} = 55$

3.2.8 Color learning tasks did not alter sleep behavior

I also tried to find an effect on sleep after color learning tasks. The bees were exposed to color tasks of varying difficulty. But even though the learning performance reflected the difficulty of the tasks, no effect on sleep behavior could be found.

3.3 Sleep alters brain and muscle activity in honeybees

3.3.1 Antennal activity can be used as a criterion for sleep

I used antennal activity to record sleep in individual harnessed bees. The antennal activity was high during the day (figure 3.25 A) and reduced during the night (figure 3.25 B+C). Still, even during the night, the antennae were sporadically active. After light offset the activity phases became shorter and were followed by antennal inactivity (figure 3.25 C). Later short antennal activity interrupted prolonged phases of inactivity (figure 3.25 C). These activity bursts lasted for at least one minute (figure 3. 25 D).

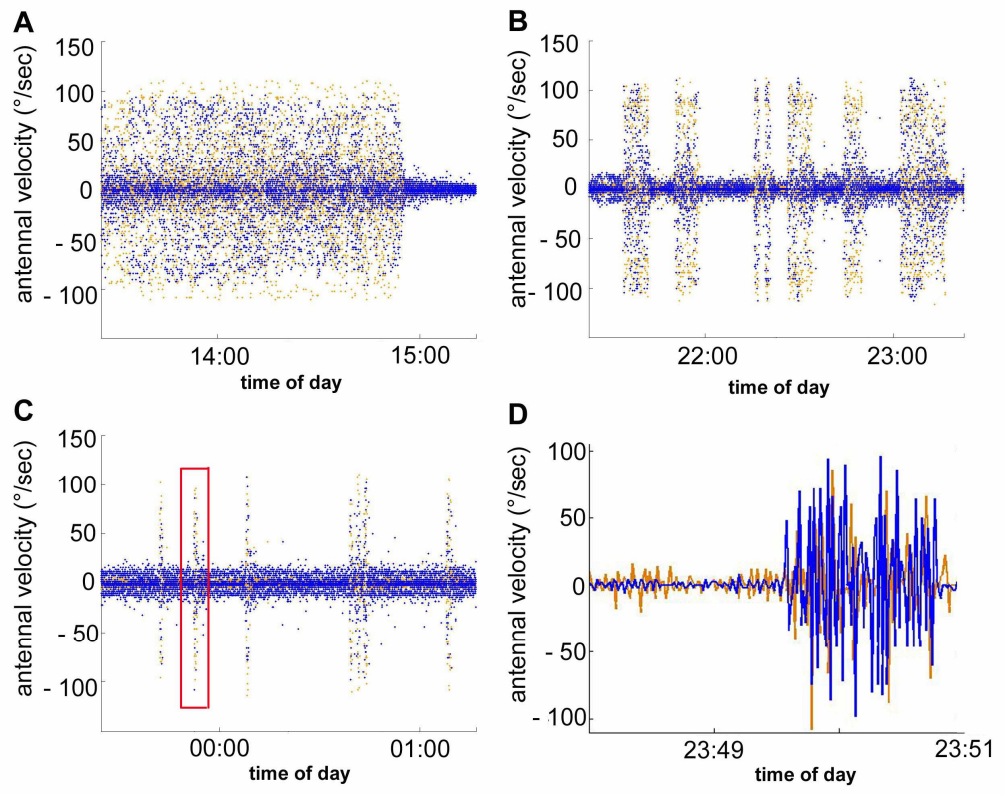

Figure 3.25 Antennal velocity. Blue shows the movements of the right antenna, yellow the movements of the left antenna. Movements towards the head capsule are positive values. Movements away from the head capsule are negative values. (A) During the day, the bee was mostly active. (B) After light offset the activity phases were interrupted by inactive phases. (C)

Later in the night the antennae were mostly inactive but short activity bursts persisted. These bursts lasted for about one minute. (D) shows an activity burst highlighted by the red box in (C) in high resolution.

I further looked at the distribution of sleep throughout the day. Bees showed significantly more phases of antennal immobility during the night than during the day (figure 3.26).

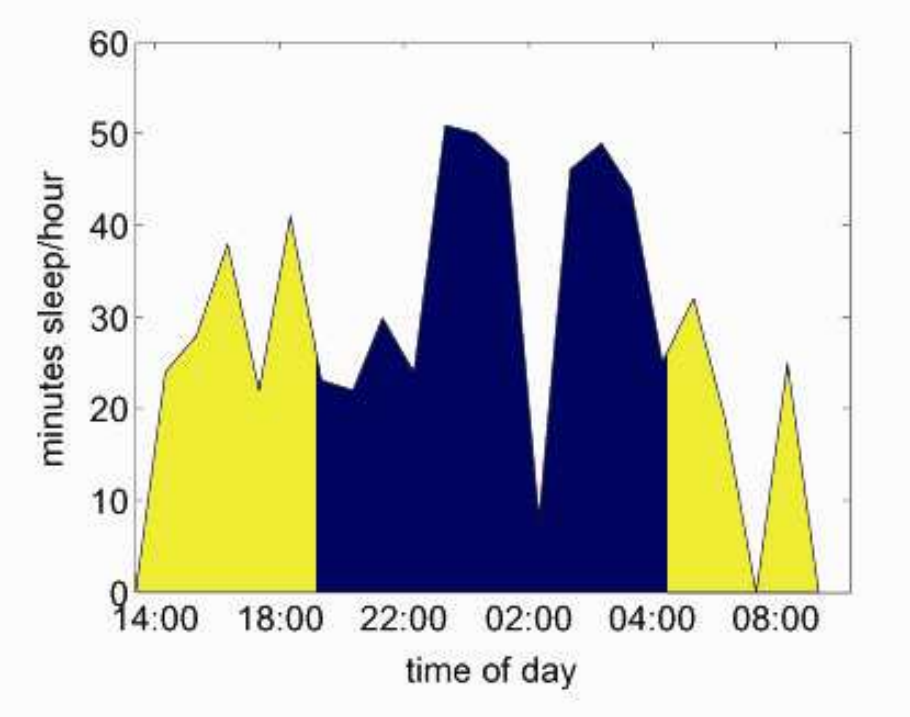

Figure 3.26 sleep distribution of one bee. The bees slept less during the day (yellow) than during the night (blue).

3.3.2 Muscle activity is decreased during sleep

In this experiment I recorded the electrical activity of the flight muscle of a harnessed bee and compared its activity with the antennal activity. The bee showed phases of low antennal activity, especially during the night. Low antennal activity seemed to be correlated to lower muscle activity (figure 3.27).

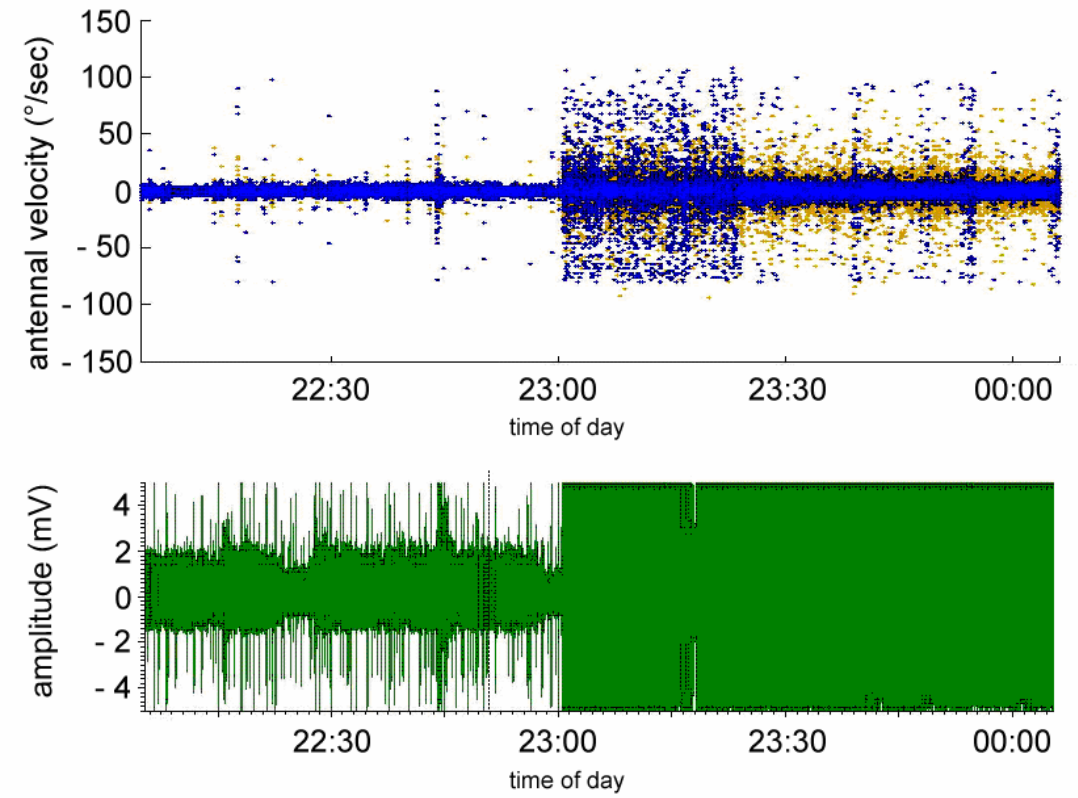

Figure 3.27 antennal movements and flight muscle activity over 120 min during the night from 10:05PM to 1:05 AM. Lower activity in the flight muscle is correlated with lower antennal activity.

3.3.3 The activity of some α-lobe extrinsic neurons is decreased during sleep

In this experiment an electrode was placed into the α-lobe of one mushroom body of a harnessed bee to record extracellularly from mushroom body extrinsic neurons. To measure sleep the antennal movements of the bee were recorded with a camera. The antennal activity was lower during the night but even then long phases of inactivity were interrupted by shorter phases of activity. After sorting the neurons I compared the antennal movement patterns and the electrical activity of mushroom body extrinsic neurons. In one case I found a clear correlation between antennal activity and neuronal activity. The recorded

neuron was much more active while the bee was awake and showed very little activity when the bee was asleep (figure 3.28). Nevertheless most mushroom body extrinsic neurons did not show a correlation with sleep and activity.

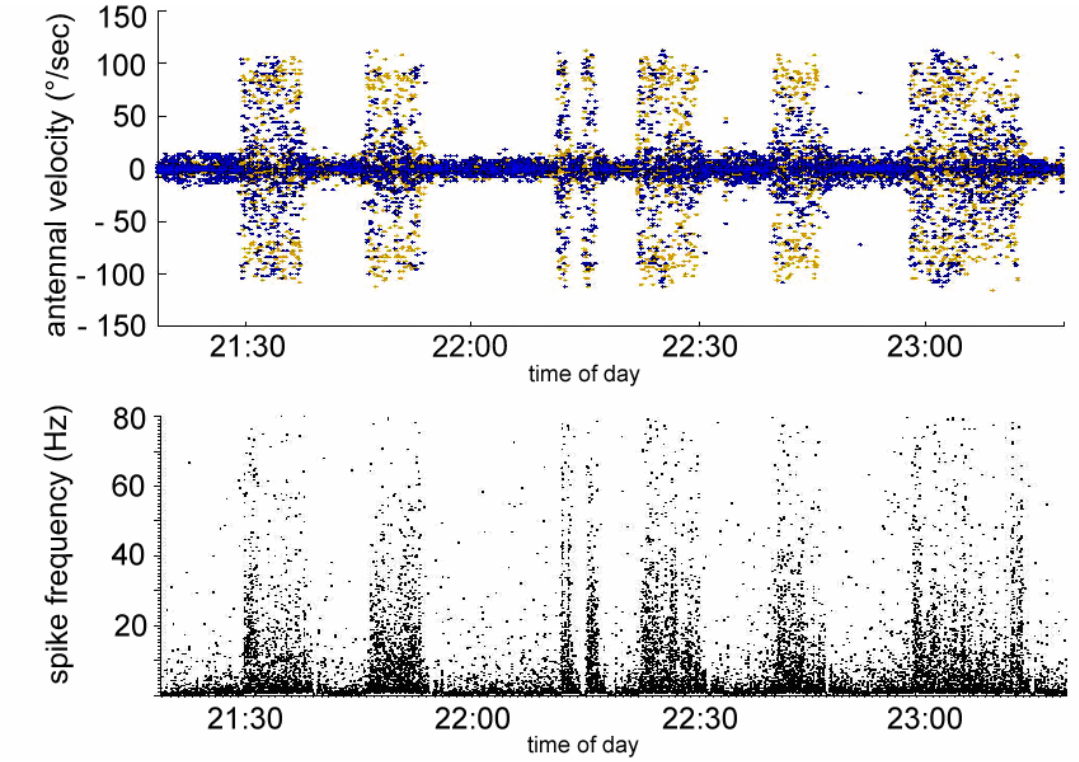

Figure 3.28 antennal and neuronal activity over 120 min during the night from 9:20PM to 11:20PM. (A) Antennal velocity, blue right antenna, orange left antenna. Periods of low antennal activity are interrupted by phases of high activity. (B) Spiking pattern of mushroom body extrinsic neuron. Phases of high antennal activity and high spiking frequency in the mushroom body extrinsic neuron are correlated.

4. Discussion

4.1 The effects of sleep and sleep deprivation on olfactory memory

In the first part of my PhD thesis I performed PER experiments to analyze the role of sleep in olfactory memory consolidation. For this purpose different groups of bees were sleep deprived and tested for memory persistence. The bees were trained in the morning before foraging onset and in the evening after the end of the foraging period. They were tested for extinction and retention.

I could show that morning and evening trained bees had different learning scores. Evening trained bees learned significantly better than morning trained bees (figure 3.1). Not only was the acquisition curve higher in evening trained bees, the bees also learned faster compared to morning trained bees (figure 3.2 A+B). This shows that learning and memory consolidation in honeybees is dependent on the time of the day. Before foraging onset the ability to learn seemed to be impaired. At the end of the light period of the day, when the bees in their normal environment would already have stopped foraging, the learning ability was much higher.

I sleep deprived some bees either during the day or during the night. It has been shown by Abid Hussaini in his PhD thesis that this treatment resulted in sleep loss when the bees were shaken during the night, while shaking during the day had no effect on sleep. In this thesis I could show that in morning trained bees neither shaking during the day nor during the night led to a changed extinction learning after three CS-only trials compared to the control group (figure 3.2C). Evening trained bees, sleep deprived by shaking during the night, showed stronger extinction learning compared to evening trained bees that were shaken during the day but not during the night or not shaken at all (control) (figure 3.2D). The difference in the response rate was gone when the different groups of bees were tested for retention 24h after the extinction training.

When evening trained bees were sleep deprived during the day directly before the extinction training, they had, although they did not show changed extinction learning (figure 3.3B), a reduced retention score compared to the not shaken control (figure 3.3C). The same effect was found for bees sleep deprived during the night directly after extinction training but not before. Only the not sleep deprived control bees showed spontaneous recovery from extinction (figure 3.3C).

These data suggest a time dependent role of sleep for olfactory memory consolidation. The time of the day plays an important role in the stringency of memory acquisition. Sleep deprivation during the night shortly after acquisition training leads to a faster extinction. This might be due to a changed consolidation of the acquisition memory. A simple negative influence of the mechanical disturbance on memory stimulation is unlikely since the same treatment during the day did not have the same effect. Though I, like Abid Hussaini, did not see an effect when the memory was tested after 24h, the faster extinction of the memory suggests that the balance between consolidation and extinction learning had been altered by sleep deprivation.

I found a reduced retention score compared to the control, when the bees were sleep deprived after three trial extinction learning, but also when the bees were shaken before the extinction training. Abid Hussaini has shown in his PhD thesis that sleep deprivation after two extinction trials resulted in a higher retention score compared to a not sleep deprived control. There are many possible explanations for this difference though it is unlikely that minor differences in the experimental setups like the shaking frequency or the feeding were the only reasons for these opposite results. It is possible that the timing of the sleep deprivation in context to the extinction trials is very important and leads to different results. This could explain the difference between the control group and the group that has been shaken before the extinction trials, but both Abid Hussaini and I trained the bees in the afternoon and sleep deprived a group of them over night. Thus the differences between those groups can not be easily

explained as a timing effect. One major difference between Abid Hussaini's and my experiments was the number of the extinction trials. In his experiments Abid Hussaini used two extinction trials, while I used three. It has been shown previously that the number of acquisition and extinction trials has a strong influence on the retention score (Sandoz et al. 2004). It has been shown that two extinction trials are sufficient to induce extinction but that the extinction can be blocked by protein synthesis inhibition. However, after five extinction trials bees show spontaneous recovery from extinction. This can be blocked by protein synthesis inhibition (Stollhoff et al 2005). This indicates two different events occurring during extinction learning. One event seems to be the acquisition of a new memory, which can be blocked after weak learning, i.e. only two new learning trials. The other event is more likely a reactivation of the older memory, which is first seen after prolonged extinction learning. In this case the reactivation of the old memory can be blocked. The effects of a protein synthesis inhibitor could be repeated for two extinction trials by sleep deprivation (Hussaini et al. 2009). Three extinction trials led to a result that can be interpreted as spontaneous recovery from extinction in the control group and a lack of spontaneous recovery in the sleep deprived groups. This is comparable to result Stollhoff et al. showed for five extinction trials. Thus three trials seem to be sufficient for the reactivation of the conditioned memory. In my studies it did not seem to matter if the sleep deprivation took place before or after the extinction training. There seems to be a persistent effect of the shaking, which might be an ongoing disruption of the normal protein synthesis. From rat studies it is known that sleep deprivation alters gene expression (Terao et al. 2006). This might also be an explanation for the disrupted reconsolidation.

It is known that satiation decreases acquisition of a trained odor in the PER paradigm (Ben-Shahar & Robinson 2001) and that glycogen levels in *Drosophila* brains vary throughout the day and are reduced after sleep deprivation (Zimmermann et al. 2004). Therefore the bees were tested if they were differently motivated to get a sugar reward. In contrast to satiated bees that only responded in a low percentage to higher sugar concentrations and not at all to water, a low

percentage of starved bees responded even to water. At a 10% sugar solution, a concentration clearly under the conditioning concentration of 30%, almost all starved bees responded. I found no difference in sucrose responsiveness between bees tested in the morning and in the evening. There was also no difference in the sucrose responsiveness between sleep deprived and not sleep deprived bees. The bees were equally motivated to get a sugar reward. Thus it is unlikely that the differences in the response rates during the acquisition and extinction trials are caused by differences in satiation.

4.2 The effects of sleep and sleep deprivation on navigation memory

In this part of my thesis I used RFID technology to track individual bees. They were tracked inside a one frame observation hive. Prolonged immobility was recorded as sleep (figure 3.6). Sleep occurred mainly during the night with sleep peaks around sunset and sunrise (figure 3.7). Consisting with earlier studies (Bloch 2001; Klein et al. 2008) I found that the analyzed forager bees show a strong diurnal rhythmicity in their sleep behavior with increased sleep during the night, while young bees showed a much more evenly distributed sleep pattern (figure 3.8). The day-night pattern in foragers was consistent for several bees over the same day (figure 3.9) as well as for individual bees over up to 14 consecutive days (figure 3.10). During sleep bees spent a high amount of the time at special sleep sites away from the main brood and food areas (figure 3.11). Both diurnal rhythmicity and specific sleep sites are characteristics for sleep that have been described previously in honeybees (Kaiser 1988). Together with video recordings showing the tracked bees in characteristic sleep positions this is a strong indication for the validity of RFID recordings to measure sleep.

The RFID-chip did not alter the foraging performance of the bees (figure 3.12), which meant that the bees were not strongly impaired by the additional weight and could be seen as normal foragers. I found that sleep times were independent of foraging times (figure 3.13). I also looked at the behavior of forager bees directly after the single foraging trips. Since daytime sleep was almost absent, I could not restrict the analysis to long sleep phases. Instead I analyzed all resting times which lasted for more than one minute. The resting time was also independent of the flight time and longer flights were not significantly more often followed by rest than short flights (figure 3.14). These data suggest that sleep and rest are not necessary to compensate for the consumed energy.

To investigate navigation learning I captured bees at a feeder and released them at sites unknown to the bees. This displacement initiated orientation learning (Menzel et al. 2000). Displacement of feeder trained bees to an unknown

position did not change the number of foraging flights compared to bees which foraged at the feeder without being displaced. The overall flight time of bees exposed to the navigation tasks increased compared to the feeder bees, but stayed well within the time window freely flying bees spend foraging (figure 3.15). A forced navigation task during foraging flights significantly increased the sleep time during the second part of the following night (figure 3.16). The difficulty of the task did not have an effect on the sleep time (figure 3.17), though bees slept significantly more after navigation tasks than freely flying foragers (figure 3.18). When the bees were exposed to a difficult task on two consecutive days, the sleep time during the night after the second displacement was significantly reduced compared to the preceding night (figure 3.19). Together these data indicate that newly acquired memory is followed by an increased sleep time. The additional time of sleep might be needed for memory consolidation.

A subset of bees was exposed to navigational tasks and sleep deprived. After sleep deprivation the bees, after being placed back into the hive, behaved like their nest mates which had spend the night undisturbed in the hive. The sleep deprived bees did not show any sleep rebound, neither during the day (figure 3.21 + figure 3.22) nor during the following night when they even showed reduced sleep time (figure 3.20). This is surprising since sleep rebound has been described in *Drosophila* (Huber et al 2004) as well as in honeybees after sleep deprivation in the lab (Sauer et al. 2004; Hussaini et al. 2009). It is still likely that sleep rebound happens inside the hive after sleep deprivation, but it might be necessary to monitor the bees much more closely to find it.

Sleep deprivation did not have a significant effect on the time it took the bees to return from the release site they had learned the previous day. But it did have an effect on the return level. While almost all bees that had spend the night undisturbed in the hive returned after the second navigation task, the loss rate of sleep deprived bees was still on the same high level as bees after the first navigation task. This indicates that the formation of the new navigation memory had been disturbed by the sleep deprivation.

In contrast to navigation learning I could not find an effect of color learning on sleep. There was no difference in the sleep time after color learning tasks of differing complexity.

Since learning of navigational tasks is rather complicated, I expected visible differences in task performance and sleep time between sleep deprived bees and the control group. In fact sleep deprived bees did not perform as well as the control group that spend the night in the hive, even though they behaved normally in the hive and remembered the already well trained feeder, suggesting that the difference is not simply a result of a general impairment.

Sleep in honeybees has been studied previously (Kaiser 1988; Bloch et al. 2001; Hussaini et al. 2009). But most studies have been done in the lab or under restricted conditions in the hive. Though some studies found differences between young in hive bees and foragers (Meshi & Bloch 2007), there are to my knowledge no studies about the influence of foraging related learning tasks on sleep behavior of bees in the hive. I therefore looked for a correlation between foraging and sleep. Since the foraging time and the amount of sleep are not correlated and bees do not rest more after returning from longer foraging trips, it is likely that there are others reasons for sleep in honey bees than just energy conservation.

For *Drosophila melanogaster* a role of sleep in courtship memory has been found (Ganguly-Fitzgerald 2006). Also the short term memory in *Drosophila* is specifically impaired by sleep deprivation, but not by other stressing factors (Li et al. 2010). This, and the differences I found between shaking during the day and during the night (see results in 3.1), makes it likely that the effect of shaking is not simply due to stress.

4.3 Electrophysiological sleep signs

I measured antennal and flight muscle activity to define sleep states in harnessed honeybees. Furthermore I recorded extracellularly from the α-lobe region of the honeybee mushroom body. I used the data from the antennal activity measurements to correlate sleep with neuronal activity in this region.

I could confirm that harnessed forager bees have a diurnal sleep rhythm with increased sleep during the night and less sleep during the day.

Previously it has been shown that the lack of antennal activity is a sign of sleep in harnessed bees (Hussaini et al. 2009). Since reduced muscular activity is another sign of sleep (Kaiser 1988) and I could show a decreased activity in the flight muscle during phases of low antennal activity, I could further confirm the reliability of antennal tracking to define sleep states in honeybees. In some cases measurements of flight muscle activity could be a useful alternative to measurements of the antennal activity.

Extracellular recordings from mushroom body extrinsic neurons of the α-lobe revealed that some neurons are more active during wake phase than during sleep. This region is known to be involved in learning processes (Okada et al. 2007). Therefore the finding that some neurons are less active during sleep might have some relevance for sleep related memory formation.

4.4 Conclusions and outlook

Both under laboratory conditions and in the field I could show effects of sleep deprivation on memory formation. In both cases the bees seemed to be rather resistant to sleep deprivation. Given the high plasticity of behavior in honeybees and the possibility that bees, which usually sleep in the crowded environment of a bee hive, are able to compensate external disturbances, this is not too surprising. Nevertheless this study provides data that strongly suggest a role of sleep in memory consolidation in the honeybee. This is consistent with studies done in *Drosophila melanogaster* (Ganguly-Fitzgerald et al. 2006) as well as in humans (Diekelmann & Born 2010).

This study improves our understanding of bee sleep especially in the context of natural behavior like foraging and thus adds to various studies over the last thirty years that showed how bees sleep. It further gives more insight into the reason for sleep in honeybees.

The picture we have of sleep in honeybees remains far from complete. Though this study, along with observations made by Abid Hussaini, suggests that specific forms of memory are sensible to sleep deprivation and thus to a more or less large extent sleep dependent, the mechanisms of memory formation during sleep are still unclear. Additional recordings of different neuron populations in the mushroom body during sleep might help to better understand the underlying neuronal principles of memory consolidation during sleep. Since the timing of sleep and sleep deprivation seems to be important for memory consolidation, additional experiments to reveal the exact temporal relations could be helpful. In the field, radar experiments with sleep deprived bees could clarify how lack of sleep intervenes with consolidation of navigational memory.

5. Abstract

Despite many years of research the exact purpose of sleep is still not known. However, sleep seems to be ubiquitous within the animal kingdom, indicating its important role. One possible function of sleep might be a sleep dependent processing of experiences. This is supported by many studies performed both with humans and different model organisms, including insects, which suggest that sleep is in fact important for the consolidation of memory. The fruit fly *Drosophila melanogaster* and the honeybee *Apis mellifera* have been used previously as model organisms to study sleep in insects. While the honeybee lacks the advantages of the genetic tools widely used in the fruit fly, the honeybee has much more complex learning capabilities and is therefore a good choice for studying the effect of sleep on learning and memory in insects. In this thesis, I used the honeybee to study the impact of sleep on memory consolidation in three different sets of experiments. In the first set I analyzed the effect of sleep deprivation on the olfactory memory. I could show a time dependent effect of sleep deprivation on extinction learning and an effect of sleep deprivation on spontaneous recovery from extinction. In the second set I studied the link between sleep and navigation memory. Bees exposed to navigational tasks showed increased sleep in the following night. Sleep deprivation impaired the consolidation of newly acquired navigation memory. Third, in electrophysiological studies, I could show reduced activity of mushroom body neurons during sleep. In summary I conclude that memory of bees is sensitive to sleep deprivation, and demands on the navigational skills at day are reflected in a need for sleep at night.

6. Zusammenfassung

Trotz jahrelanger Forschung ist die genaue Bedeutung von Schlaf immer noch unbekannt. Trotzdem scheint Schlaf im Tierreich allgemein verbreitet zu sein, was eine wichtige Rolle des Schlafs nahe legt. Eine mögliche Funktion von Schlaf könnte eine Schlaf abhängige Prozessierung von Erfahrungen sein. Dies wird unterstützt von vielen Studien, die sowohl beim Menschen als auch diversen Modellorganismen, einschließlich Insekten, gefunden haben, dass Schlaf tatsächlich wichtig für die Konsolidierung von Gedächtnis ist. Bei der Taufliege *Drosophila melanogaster* und der Honigbiene *Apis mellifera* wurden bereits verwendet, um Schlaf in Insekten zu untersuchen. Obwohl die Honigbiene genetisch schlecht manipuliert werden kann, hat sie ein wesentlich komplexeres Lernverhalten als die Taufliege und ist deshalb eine gute Wahl, um den Effekt von Schlaf auf Lernen und Gedächtnis in Insekten zu untersuchen. In dieser Arbeit habe ich drei verschiedene experimentelle Ansätze verwendet, um die Bedeutung des Schlafs auf die Gedächtniskonsolidierung bei der Honigbiene zu untersuchen. Erstens habe ich den Effekt von Schlafentzug auf das olfaktorische Gedächtnis untersucht. Ich konnte einen zeitabhängigen Effekt von Schlafentzug auf das Extinktionslernen und auf die spontane Erholung von der Extinktion zeigen. Zweitens habe ich die Verbindung zwischen Schlaf und Navigationsgedächtnis untersucht. Bienen schliefen mehr nach einer Navigationsaufgabe. Schlafentzug verschlechterte die Konsolidierung von neu erworbenem Navigationsgedächtnis. In einem dritten Ansatz konnte ich mittels elektrophysiologischer Ableitungen eine verringerte Neuronenaktivität in Pilzkörper-Neuronen finden. Zusammenfassend schließe ich, dass das Gedächtnis von Honigbienen empfänglich für Schlafentzug ist und Navigationslernen zu einem erhöhten Schlafbedarf führt.

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