Chapter 2:

Context-dependent learning in honeybees: a behavioral and neural analysis

Summary:

Context-dependent learning (CDL) has been shown in free flying bees and here we show similar learning in restrained bees. We took advantage of the classical learning proboscis extension response (PER) to combine behavior with extracellular recordings in alpha lobe of mushroom bodies. In one series of experiment, bees had to solve differential conditioning task under two different contexts. In one context, one odor was rewarded and in another context a second odor was rewarded. The contexts presented were two colors, two different temperatures or combination of both colors and temperatures. As expected, bees could solve the differential conditioning task easily under combination of two contexts such as colors and temperatures compared to either color or temperature alone. Neuronal response increased with increase in temperature and decreased with decrease in temperature. After context learning bees showed higher response towards rewarded context compared to unrewarded context, while there was no difference in neuronal response between rewarded and other odors. In another series of experiment, we made the context learning easier by removing the reversal learning rule and rewarded only one odor in either bright or dark context. Bees showed better learning in this context learning experiment. Neuronal response during such a context learning protocol was higher in the rewarded context compared to unrewarded context, while response towards rewarded odor was lower compared to unrewarded or neutral odor. This showed that neuronal responses of alpha lobe neurons of mushroom bodies have different mechanisms for cued responses and context responses.

Introduction:

Contexts are nothing but secondary cues for predicting a biologically relevant event. For example when an animal is trained to respond to a primary cue (eg. odor) by combining it with a biologically relevant reward such as food, the animal apart from the learning the primary cue remembers conditions such as time of day, temperature, visual cues etc. A cue occurs mostly in a deterministic way with a precisely defined temporal fashion along with reward, while context is always present and serves as a background making up the environment for the animal. For example, Tulving and Thomson in 1973 showed that pairs of words can be best recalled under conditions similar to when learning occurred. During learning, animals attend to cues that have the most biological relevance (like food), but the animal also encodes contextual cues to help facilitate learning (Gonzalez et al., 2003, Riccio et al., 1992). Absence of contextual cues can disrupt recall (Odling-Smee, 1975; Balsam, 1984). In mammals it has been shown that contextual learning depends on hippocampus (Kesner et al., 1983; Hirsh, 1974; Philips et al., 1992) while cued learning does not require hippocampus (Hirsh., 1974; Gaskin et al., 2005). Therefore, it is of general understanding that context learning is a complex task requiring cognitive abilities (Cohen et al., 1999; Umbricht et al., 2000).

Apart from honeybees (Collett et al., 1997; Cheng, 2005; Zhang et al., 2006) context learning has been shown in bumblebees (Dale et al., 2005), crickets (Matsumoto et al., 2004), cockroaches (Sato et al., 2006), ants (Chameron et al., 1998), spiders (Skow et al., 2005) etc. Studies have shown context learning in free flying bees (Collett et al., 1997; Cheng, 2005; Zhang et al., 2006) and also restrained bees (Gerber et al., 2000). Free flying bees can differentiate between patterns, places, landmark etc. Gerber et al. (2000) used different odors as contexts, while in our experiment we used non-olfactory cues as contexts. Insects have known to learn contexts in a short time (Chittka, 2003; Dyers et al., 2004) and we too attempted to train bees to quickly learn context, which was necessary for us to understand the neural mechanisms underlying context learning. Our aim was to understand how contextual cues contribute to learning and if they are different from elementary forms of learning such as differential conditioning. We investigated this by combining behavior with extracellular recording. We differentially conditioned the

bees to odors in presence of different contexts and recorded from alpha lobe neurons of mushroom bodies. We evaluated how these neurons respond to odors and contexts after learning.

Materials and methods:

Preparation of bees:

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

Setup for context learning:

The setup had three parts (Fig. 2.1):

1) Olfactometer- a custom-made computer-driven device, blowing continuous stream of air over the bee's antennae, was used to deliver the odors (Galizia et al. 1997, Komischke et al. 2002).
2) Light setup- Light source (KL 1500 LCD, Schott AG) was delivered with the help of light guides to the reflective paper of the experimental arena placed in front of the bee. Two color filters, Tokyo blue, #071 (460nm) and Medium yellow, #010 (>540nm) (Rosco) were placed in the filter slide of the light source.
3) Temperature setup- A custom-made device was built to deliver hot or cold air. Hot air was generated by passing compressed air through a hollow copper tube immersed in temperature-controlled water heater. Similarly, a copper tube containing air was immersed in a water coolant to generate cold air. Hot air was maintained at 32-34 °C and cold air at 18-20 °C.

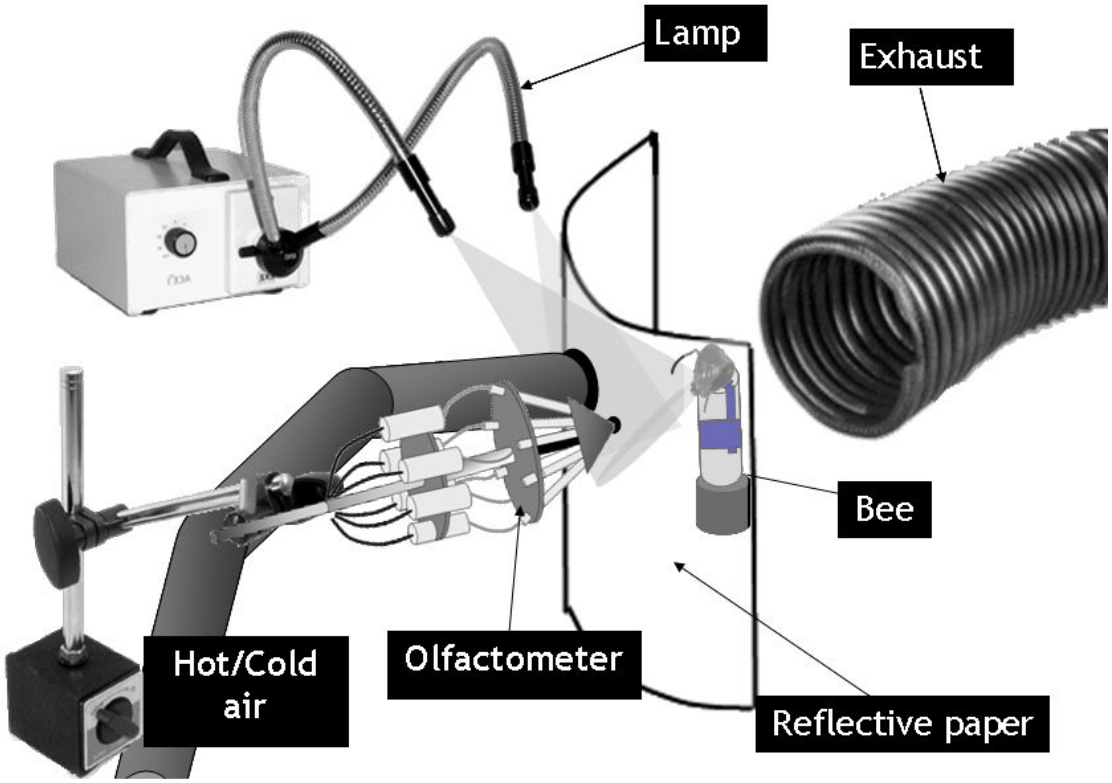

Fig. 2.1: Context learning setup: The setup consisted of an olfactometer which delivered odors to the bee. Air was passed through heated or cooled water (not shown) and hot or cold air was blown via a tube. The lamp with the help of light guides illuminated the reflective paper in front of the bee. The colors could be changed by changing the filters within the lamp. An exhaust pipe behind the bee removed the surrounding air including odors..

Training:

Bees were checked for PER (unconditioned response: UR) by lightly touching the antennae with 30% sucrose solution 10 minutes before training. Only bees which demonstrated the UR were trained (<5% were discarded). 2-octanol, limonene and peppermint were used as odor stimuli (CS). Odor will be referred to as A,B and C. 30% sucrose solution was used as an appetitive reinforcer (US). Contexts used were: a) Color context (Yellow and Blue), b) Temperature context (Hot and Cold) and c) Light context (Bright and Dark).

Bees were trained by presenting them with odors in the presence of one or combination of two contexts. Odors were prepared by pipetting 4 µl of odor onto half sq. inch filter papers, placing it into 1-ml syringes and fitting it into the holes of the olfactometer (Galizia et al. 1997). During the training each bee was placed in front of the

olfactometer with its antennae facing the air-stream and was conditioned to odors by pairing them with US in presence of different context conditions. After every trial, the exhaust system behind removed the odors.

Electrophysiology:

Setup: The entire electrophysiology setup was custom-made. A metal plate (100 x 100 x 3) mounted on an iron table formed the base of the setup. Rubber paddings between the stand and metal plate served as shock absorbers. A faraday cage, with its one side open, was mounted on the metal plate and was effective against reducing noise. A compound microscope, an electrode micromanipulator, an olfactometer, a camera and an infra-red lamp were placed on the metal plate and grounded. A differential 4-channel amplifier (A-M systems, USA), a cathode ray oscilloscope (CRO), a analog-digital converter (1401 micro MKII, CED, UK), an olfactometer-controller, a Humbug (Digitimer, UK) and a Windows PC was kept inside a metal rack and were all grounded to a common sink.

Electrodes: Custom-made electrodes were made as described before. (Mizunami et al., 1998; Okada et al., 1999 and 2007). A 14 micron thick copper wire (Electrisola, Switzerland), coated with polyurethane was used as an electrode. Two copper wires were glued together and attached to a 3cm long tungsten wire (100 micron thick) that was attached to a glass capillary. Therefore, an electrode comprised of, a glass capillary (Base portion), a tungsten wire (middle portion) and two copper wires (end portion). Glass capillary was fitted into an electrode holder and the loose ends of the copper wires were soldered to connectors of the electrode holder which was connected to a differential amplifier (A-M Systems, USA). If the resistance of the electrode was more than 5 M ohms, it was re-soldered to the connectos.

Dissection: A restrained bee was fixed into a magnetic stand and placed on the metal plate under the compound microscope. A silver wire (100 microns) was inserted into the eye of the bee, this served as the ground electrode. A second silver wire was inserted between ocelli and eyes on the back of the head and served as m17- muscle

electrode. Both wires were fixed using eicosane. A rectangular region of the head cuticle (between the two compound eyes and between ocelli and antenna) was cut open to expose the brain. Head glands and trachea sacks were removed until one of the alpha lobe was seen.

Extracellular Recording: The dissected bee was placed under the microscope inside the electrophysiology setup and the ground wire and muscle recording wire were connected. A differential copper wire electrode was lowered towards the right alpha lobe region of the MB and positioned just above the surface at 6 - 7 O'clock position. At this point a small cut was made on the alpha lobe surface using a sharp pin. Lowering of the electrode was resumed and when it touched the brain surface, electrode coordinates were noted down from the electrode micromanipulator.

The electrode was lowered carefully until 150 microns when small units started to appear. The position of the electrode was manipulated until the units were large enough for experiment. If necessary, electrode was removed from the brain and re-inserted at a different location (within 6-7 O'clock position of alpha lobe). This process was repeated until a reasonably large unit was found. A two-component silicon (WPI, Germany) was mixed together and added to the brain. In 5 minutes the silicon polymerized and hardened. This procedure kept the brain sufficiently wet which was important for stable recordings. After 20 minutes, protocol for conditioning the bee was started.

Signal processing: The recorded neuronal signals were amplified by the differential amplifier (A-M systems) which had a bandpass filter of 10 – 10 KHz. The signals where passed through a Humbug (Digitimer, UK) to remove 50Hz hum noise. Signals were digitized using analog-to-digital converter (1401 micro MKII, CED, UK) and stored in the PC using spike2 signal processing software (CED, UK). Spike2 was also used to control olfactometer via computer keyboard. The onset of odors and contexts were saved as markers in spike2. All recordings were passed through a digital filter of spike2 to reduce the fluctuations and improve signal-to-noise quality. Typical settings were, High pass frequency: 300Hz, Transition gap: 165 and Length: 311 (auto). These settings gave the best signal-to-noise ratios.

The resulting units or spikes were sorted with Spike2 template matching tool to separate different units. The important criteria for spike sorting were: spike amplitude, positive or negative spike and spike shape (crest peak, trough peak, crest-to-trough distance). After spike sorting, units were proof checked by overlapping all similar spikes and also manually going through all spikes. If necessary, spikes were re-sorted. All spikes, including spike-timing, odor markers, context markers were exported to a text file for analysis. A total of 44 bees were analyzed (Experiment 3: 7 bees, Experiment 4: 12 bees and Experiment 5: 25).

Analysis: For behavioral experiments, data of PER was analyzed and plotted in excel spreadsheet. For electrophysiology data, R (statistical and programming software) was used to import the text files and read the data. Specific scripts for analyzing the data were written in R. For calculating the response of the neurons following data was taken:

(a) Number of spikes 1 second **after** odor onset (odor response)
(b) Number of spikes 1 second **before** odor onset (spontaneous activity)
(c) Number of spikes 10 seconds **after** context onset (context response)
(d) Number of spikes 10 seconds **before** the context onset (spontaneous activity)

For normalizing odor responses, ratio of (a) and (b) was taken and for normalizing context responses, ratio of (c) and (d) was taken. Normalization was done for all tests, i.e. Pretests and Posttests. Change in spike frequency (Δ) from pretests to posttests was calculated. Therefore, the formula for Δ spike frequency was ([Posttest ÷ Spontaneous activity] ÷ [Pretest ÷ Spontaneous activity]). If, Δ spike frequency = 1, it meant no change in spike frequency. Results were plotted in excel spreadsheet and statistics was done in R. For testing statistical significance Paired t-tests and Wilcoxon tests were used.

Experiments:

Experiment 1: *Context learning with temperature and color*

The restrained bees were conditioned inside the context learning setup (Fig. 2.1).They were randomly allocated to 4 context groups and each group was conditioned to two odors using a differential conditioning paradigm in the presence of two contexts.

In context-1, odor-1 was rewarded and odor-2 not rewarded. In context-2, odor-2 was rewarded and odor-1 was not rewarded. The four context groups were:

Group-1: Yellow versus Blue colors.

Group-2: 26°C versus 32°C (Narrow-Temp)

Group-3: 19°C versus 32°C (Broad-Temp)

Group-4: 32°C+ Yellow versus 19°C+ Blue

The context learning protocol for all groups was as follows (Fig. 2.2):

One training trial consisted of about 10 minutes; Experiment started with onset of context-1 (CX1) for 1 minute, onset of odor-1 (A+) for 4 seconds followed by 1-second overlap with 3-second sucrose reward (US). After 2.5 minutes a second odor (B-) was presented without reward. After one minute CX1 was turned off and immediately a second context (CX2) was turned on, after 1 minute the odor B+ was presented with US and after 2.5 minutes odor A- was presented without US. Training trial ends with offset of CX2. Therefore, in every trial, consisting of 2 context and 4 odor presentation, 2 different odors in 2 different contexts were rewarded. After the 5 such training trials, a 6^{th} trial was presented after 5 minutes to the bees without any US. Each of four groups had one control where the protocol for context learning was exactly the same except that they were not rewarded. The orders of context and odor presentations were changed in every experiment.

Based on the behavior displayed by the bees, responses were grouped (Table: 2.1) as, Complete learning (CL), Partial learning (PL) and Generalization (GL). Complete learning (CL): when bees show correct responses to rewarded and unrewarded odors in both contexts. Partial learning (PL): when bees show correct responses only in one of the contexts. Generalization (GL): when bees show same response in both the contexts. All kinds of responses were noted down during training and test trials. The term “responses” refers to partial learning unless mentioned otherwise.

	Context -1	Context - 2
Complete learning (PL)	A+ B-	B+ A-
Partial learning (PL)	A+ B-	B- A-
	A+ B-	B+ A+
	A+ B+	B- A-
Generalization (GL)	A+ B-	B- A+
	A+ B+	A+ B+

Table 2.1: Types of PER responses during context learning. A+ means response to odor-A and B- means no response to odor-B. The expected response after context learning (Fig. 2.1) was A+B- in context-1 and B+A- in context-2. This is complete learning. Apart from this partial learning and generalization was also seen.

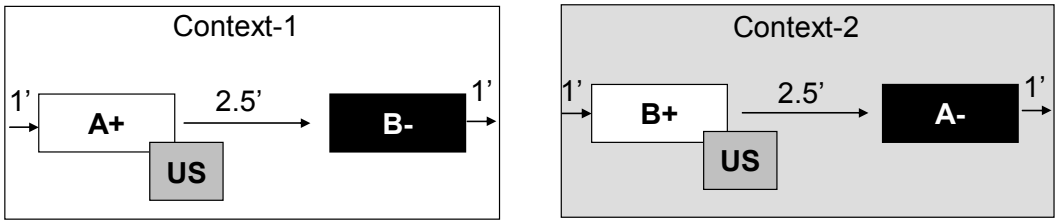

Fig. 2.2: Context learning protocol: Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds followed by sucrose reward (US) for 3 seconds overlapping for 1 second with A. After 2.5 minutes odor-B was presented but without any US. After 1 minute context-1 was turned off and immediately context-2 was presented. After 1 minute odor-B was presented and paired with US. After 2.5 minutes odor-A was presented but without any US. Context-2 was turned off after 1 minute. This comprised of one trial. Bees were subjected to 5 such trials followed by one test trial where context and odors were presented in same sequence but without any reward.

Experiment 2: *Context learning with Bright and Dark*

In this experiment, bees were presented with two odors in the presence of two contexts. But only one odor in one context was rewarded (Fig. 2.3) and the odors in the second context were not rewarded. The two contexts were; Bright white light (Bright) and absence of light (Dark). One training trial consisted of 10 minutes and started with onset of first context (CX1+) and after 1 minute first odor (A+) was presented for 4 seconds along with 3 second sucrose reward (US). After 2.5 minutes a second odor (B-) was presented without US. Immediately after the offset of CX1+, a second context (CX2-) was turned on. After 1 minute odor A- was presented without any reward and 2.5 minutes later B- was also not rewarded. Trial ended with the offset of CX2-. Therefore, in every trial, consisting of 2 context and 4 odor presentations, only one odor and one context were rewarded. After the 5 such training trials, a 6^{th} trial was presented after 5

minutes to the bees without any US. The orders of context and odor presentations were changed in every experiment. The PERs were noted down during training and test trials.

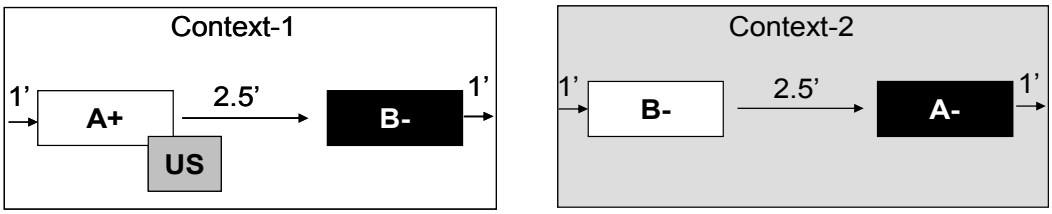

Fig. 2.3: Context learning protocol: Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds followed by sucrose reward (US) for 3 seconds overlapping for 1 second with A. After 2.5 minutes odor-B was presented but without any US. After 1 minute context-1 was turned off and immediately context-2 was presented. After 1 minute both odors B and A were presented without any US with inter-stimulus interval of 2.5 minutes. Context-2 was turned off after 1 minute. This comprised of one trial. Bees were subjected to 5 such trials followed by one test trial where context and odors were presented in same sequence but without any reward.

Experiment 3: *Extracellular recordings and differential conditioning:*

After the bee was dissected and stable recordings were obtained (see dissection and extracellular recording above) it was subjected to a differential conditioning protocol (Fig. 2.4). Experiment started with a pre-test phase in which the bee was presented with 3 different odors A, B and C in random sequence at 1 minute intervals. This was repeated twice. After 5 minutes break, conditioning phase was started. One odor (A+) was rewarded (US) and after 1 minute a second odor (B-) was not rewarded. After 5 such trials, posttest phase started. In this phase bees were presented with 3 odors; A, B and C at 15 minutes, 1 hour and 2 hours after the last conditioning trial. The sequence of presentation was same as in the pretest. The PERs were noted down for all the 3 phases. The normalized odor responses of posttests were compared with normalized odor responses of pretest (averaged) by taking a ratio between them. Since we compared change in responses from pretest to posttest, the unit for neuronal responses was Δ spike frequency.

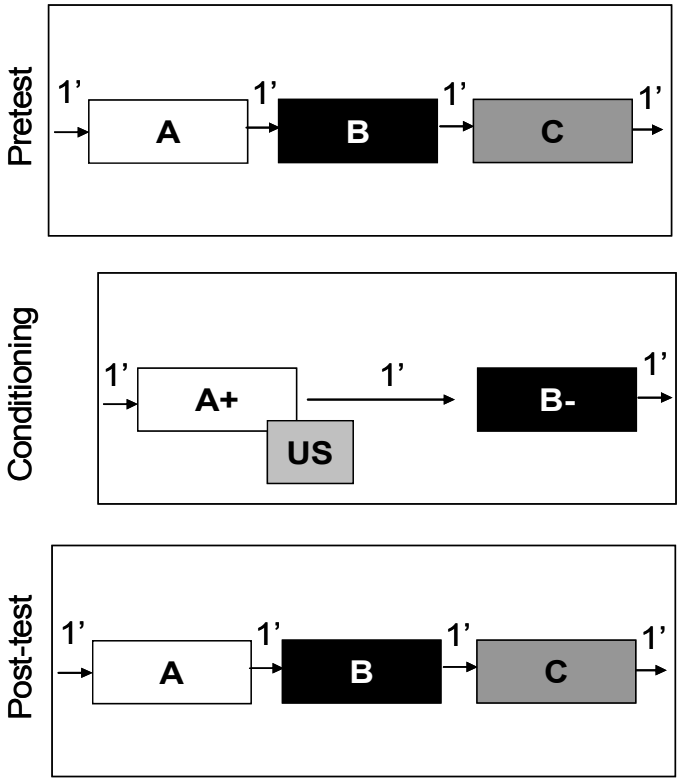

Fig. 2.4: Differential conditioning protocol for electrophysiology:
Pretest: Bees were presented with odors A, B and C with inter-stimulus interval of 1 minute between them.
Conditioning: Odor-A was presented for 4 seconds followed by sucrose reward (US) for 3 seconds overlapping for 1 second with A. After 1 minute odor-B was presented but without any US. This comprised of one trial. Bees were subjected to 5 such trials.
Posttest: Similar to pretest but one trial was presented at 15 minutes, 1 hour and 2 hours after conditioning.

Experiment 4: *Extracellular recording + Context learning with Temperature & Color*

Dissected bee was placed inside the context learning setup and was subjected to context learning protocol (Fig. 2.5). Experiment had 3 phases; the first phase was pretest phase in which the bee was subjected to 3 odors A, B and C without US in each of two contexts, 32°C + Yellow and 19°C + Blue. This procedure was repeated twice. The protocol was: 32°C + Yellow context was presented for one minute followed by A, B and C with 1 minute between them. After one minute, 32°C + Yellow was turned off and the second context, 19°C + Blue was turned on. Again, odors A, B and C were presented with inter-stimulus interval of 1 minute. Trial ended when 19°C + Blue context was turned off. Pretest phase consisted of 2 such trials.

The second phase was conditioning phase in which the bee was subjected to 2 odors in each of the two contexts, 32°C + Yellow and 19°C + Blue. The protocol was: 32°C + Yellow context was presented for one minute followed by A+ which was rewarded with US and after 2.5 minutes B- was presented without any US. After one minute, 32°C + Yellow was turned off and the second context, 19°C + Blue was turned on. This time odor B+ was rewarded and after 2.5 minutes A- was not rewarded. The trial

ended when 19°C + Blue context was turned off. Conditioning phase consisted of 5 such trials.

The last phase was the posttest phase which was exactly similar to the pretest. However, there was only one posttest trial at 15 minute, 1 hour and 2 hours after the conditioning phase. The sequence of context and odor presentation was similar to pretest for direct comparison of responses. Note that the order of context and odor presentations changed from experiment to experiment.

In the control animals, the procedure was exactly the same as above except that they were not rewarded during the entire experiment. PERs were noted down during the pretest, conditioning and posttests. The normalized odor responses of posttests were compared with normalized odor responses of pretest (averaged) by taking a ratio between them. Similarly, ratio of posttest context responses and pretest responses were taken. Since we compared change in responses from pretest to posttest, the unit for neuronal responses was Δ spike frequency.

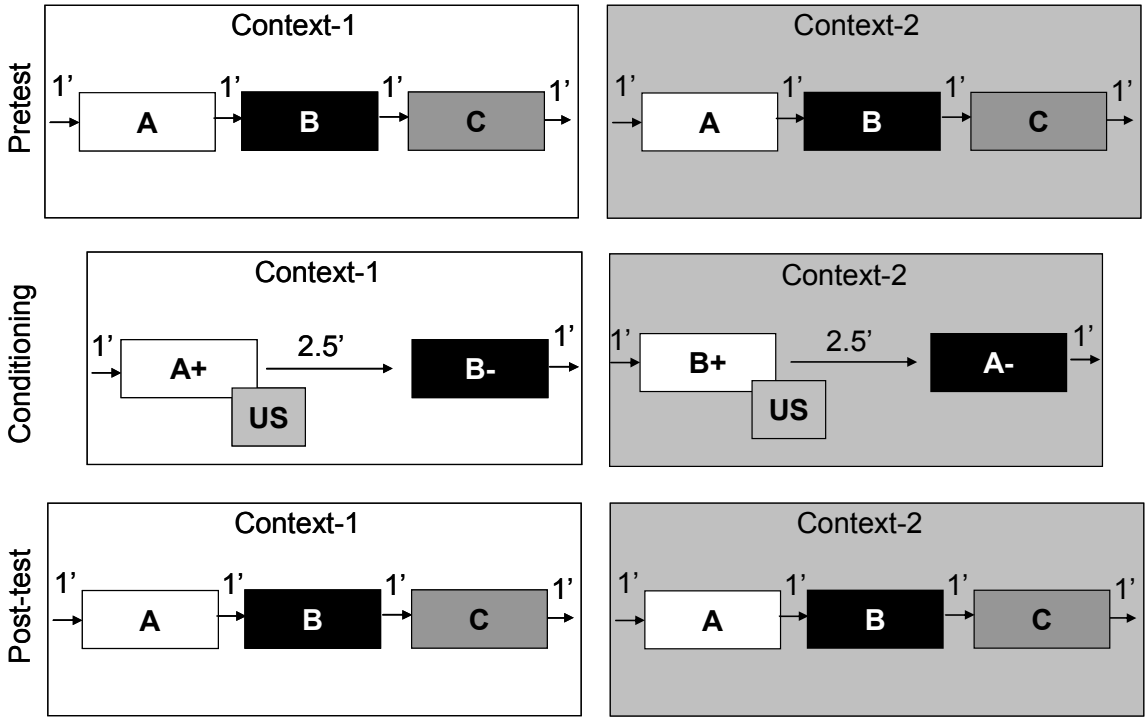

Fig. 2.5: Context learning protocol for temperature and color contexts:
Pretest: Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds. Similarly odor-B and odor-C were presented after 1 minute. After 1 minute context-1 was turned off and immediately context-2 was presented. Odors A, B and C were presented as in context-1. Context-2 was turned off after 1 minute. This comprised of one pretest trial. Bees were subjected to 2 such trials. *Conditioning:* Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds followed by sucrose reward (US) for 3 seconds overlapping for 1 second with A. After 2.5 minutes odor-B was presented but without any US. After 1 minute context-1 was turned off and immediately context-2 was presented. After 1 minute odor-B was presented and paired with US. After 2.5 minutes odor-A was presented but without any US. Context-2 was turned off after 1 minute. This comprised of one trial. Bees were subjected to 5 such trials.
Posttest: Similar to pretest but one trial was presented at 15 minutes, 1 hour and 2 hours after conditioning.

Experiment 5: *Extracellular recording + Context learning with Bright and Dark*

Dissected bee was placed inside the context learning setup and was subjected to context learning protocol (Fig. 2.6). Experiment had 3 phases; the first phase was pretest phase in which the bee was subjected to 3 odors A, B and C without US in each of two contexts, Bright and Dark. This procedure was repeated twice. The protocol was: Bright context was presented for one minute followed by A, B and C with 1 minute between them. After one minute, Bright context was turned off which is the second context Dark. In the Dark context, again odors A, B and C were presented with inter-stimulus interval of 1 minute. Trial ended when room lights were turned on, which was different from Bright context. Pretest phase consisted of 2 such trials.

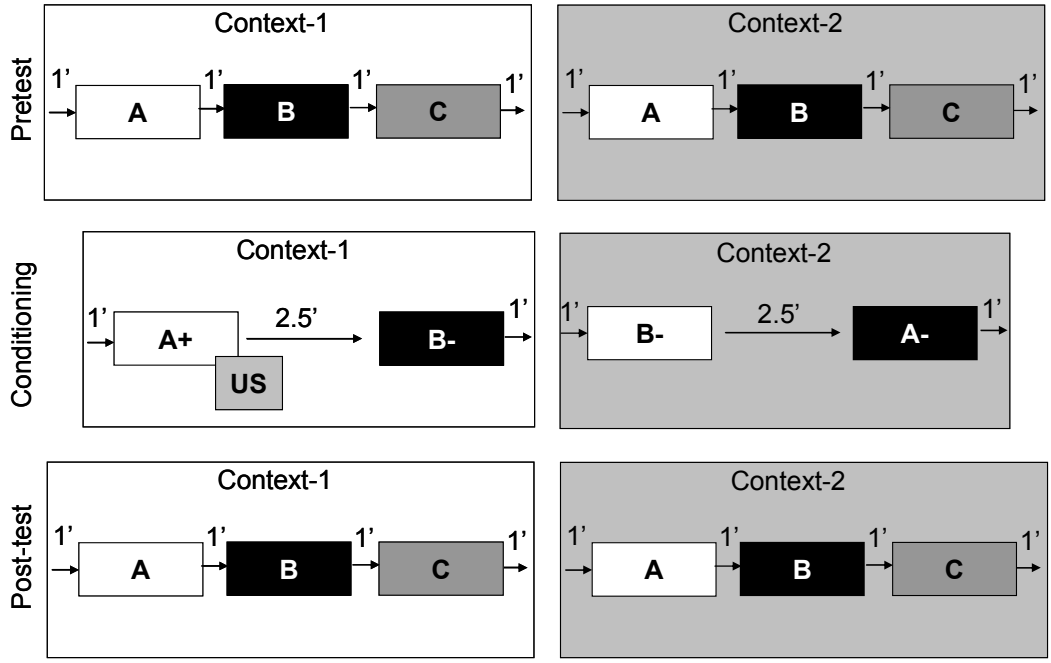

Fig. 2.6: Context learning protocol for bright and dark context:
Pretest: Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds. Similarly odor-B and odor-C were presented after 1 minute. After 1 minute context-1 was turned off and immediately context-2 was presented. Odors A, B and C were presented as in context-1. Context-2 was turned off after 1 minute. This comprised of one pretest trial. Bees were subjected to 2 such trials.
Conditioning: Bees were presented with context-1 and after 1 minute odor-A was presented for 4 seconds followed by sucrose reward (US) for 3 seconds overlapping for 1 second with A. After 2.5 minutes odor-B was presented but without any US. After 1 minute context-1 was turned off and immediately context-2 was presented. After 1 minute odor-B was presented without any US and 2.5 minutes later odor-A was also presented without any US. Context-2 was turned off after 1 minute. This comprised of one trial. Bees were subjected to 5 such trials.
Posttest: Similar to pretest but one trial was presented at 15 minutes, 1 hour and 2 hours after conditioning.

The second phase was conditioning phase in which the bee was subjected to 2 odors in each of the two contexts, Bright and Dark. The protocol started by presenting Bright light context for one minute followed by A+ which was rewarded with US and after 2.5 minutes B- was presented without any US. After one minute, Bright context was turned off which is the second context Dark. In this context, odors A- and B- were not rewarded. The inter-stimulus interval was 2.5. The trial ended when room lights were turned on. Conditioning phase consisted of 5 such trials.

The last phase was the posttest phase which was exactly similar to the pretest. However, there was only one posttest trial at 15 minute, 1 hour and 2 hours after the conditioning phase. The sequence of context and odor presentation was similar to pretest for direct comparison of responses. Note that the order of context and odor presentations changed from experiment to experiment.

In the control animals, the procedure was exactly the same as above except that they were not rewarded during the entire experiment. PERs were noted down during the pretest, conditioning and posttests. The normalized odor responses of posttests were compared with normalized odor responses of pretest (averaged) by taking a ratio between them. Similarly, ratio of posttest context responses and pretest responses were taken. Since we compared change in responses from pretest to posttest, the unit for neuronal responses was Δ spike frequency.

Results:

Experiment 1: *Context learning with temperature and color*

Group-1 bees responded gradually during the conditioning and at the end of conditioning about 35% of the bees showed partial learning and about 7% showed complete learning. Yellow color served as a better context than blue context. Generalization was 47%.

Group-2 bees showed the lowest response of all groups. During the 10 conditioning trials the response towards the rewarded context increased gradually. After 6 trials of 26°C and 6 trials of 32°C, 30% of bees showed partial learning and none of the bees showed complete learning. During the conditioning trials there was more than 52% generalization.

In Group-3, the response towards the rewarded context increased gradually to 45% until 4^{th} trial and dropped to 35%. Response was stronger during the 32°C context compared to 19°C context. After conditioning, nearly 39% of the bees showed partial reversal and 5% of the bees showed complete reversal. Generalization was 41%.

Groups-4 bees showed highest response. During the conditioning trials nearly 55% of the bees showed partial learning in the 3^{rd} trial itself, but dropped to 42% at 5^{th} trial. Like in the group-3 bees response was stronger towards 32°C context compared to 19°C context. After the conditioning the response increased slightly to 47%. Also about 15% of the bees showed complete learning. Generalization was 36%.

Each of the above groups had controls which were presented only contexts and odors but no reward. None of the groups showed complete learning and partial learning was below 15%.

Experiment 2: *Context learning with Bright and Dark*

In this experiment bees were rewarded in one of the two contexts. Bees showed higher response (Fig. 2.10) towards rewarded context compared to unrewarded context. Bright context had a slightly higher preference (58%) against dark context (42%) but was not significant. Responses during unrewarded contexts were less than 20%. Also, control bees which were placed in both contexts and presented with odors without rewards

showed less than 20% response. "Unrewarded" in Fig. 2.10 represents responses of control and unrewarded bees.

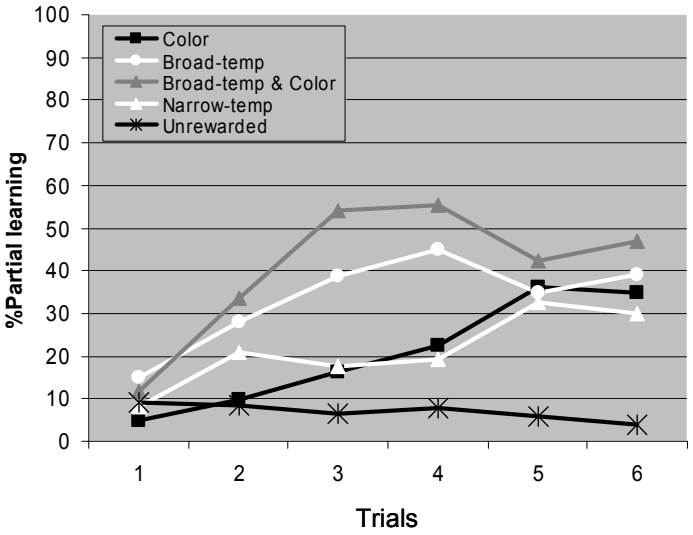

Fig. 2.7: Context learning with temperature and color: The combination of broad-temp (32 or 19°C) context and color (yellow or blue) context gave the best partial learning scores. In single contexts, broad-temp gave the best partial learning followed by color context and narrow-temp context. Learning scores towards unrewarded contexts (all contexts pooled) were near baseline.

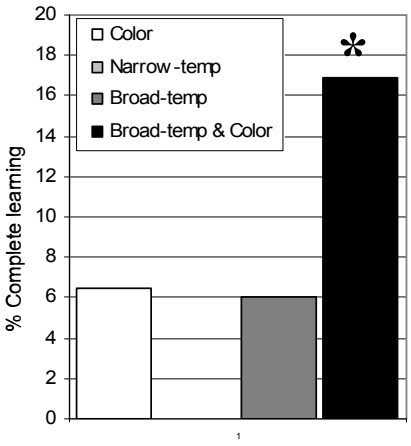

Fig. 2.8: Complete learning: Bees conditioned with a combination of Broad-temp & color contexts showed the highest complete learning ($P<0.01$) compared to other groups of bees.

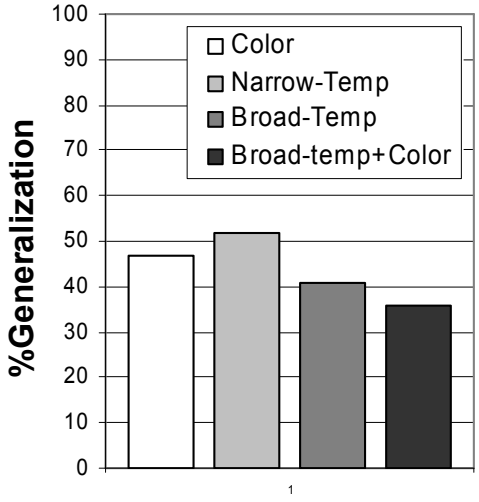

Fig. 2.9: Generalization: Bees generalized less in broad-temp & color context compared to other contexts. Highest generalization was seen in the group which showed poor partial and complete learning.

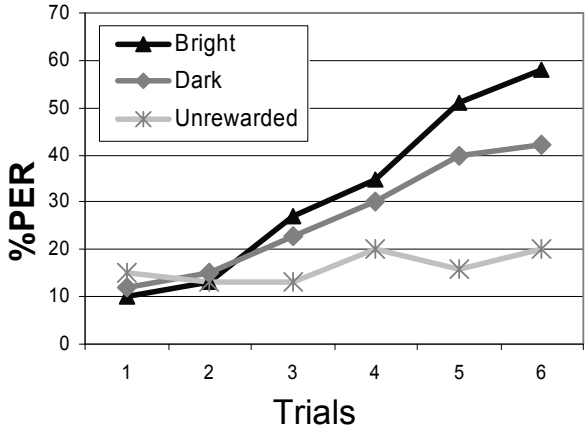

Fig. 2.10: Context learning with bright and dark contexts: Bees showed higher PER towards bright context compared to dark context (Not significant), but overall rewarded contexts (bright and dark) showed significantly higher PER compared to unrewarded context.

General properties of neurons:

The neurons chosen for analysis were consistent in the following ways: They were between 6 -7 O'clock position of the right alpha lobe neuron of the mushroom bodies, they were between the depths 170 – 210 microns from the surface of the brain, spikes had an initial positive potential reaching a crest followed by a negative potential reaching a trough (Fig. 2.12), the crest-to-trough times for most neurons analyzed were between 1200 – 2200 hz. All neurons responded to 3 odors; 2-octanol, Limonene and Peppermint. Based on the above criteria, neurons were pooled together for analysis.

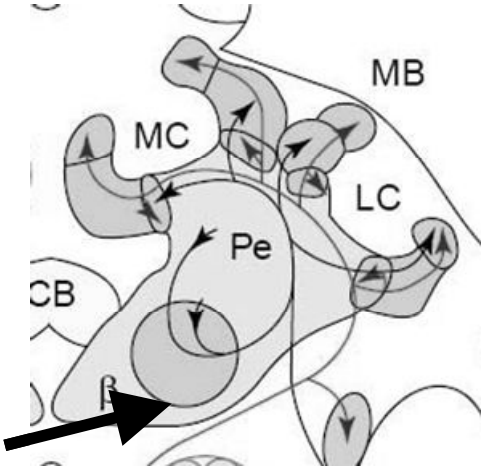

Fig. 2.11: Alpha lobe neurons: All recordings were obtained from 6-7 O'clock position (arrow) of right alpha lobes of mushroom bodies. Depth of electrode was between 170 - 210 microns.

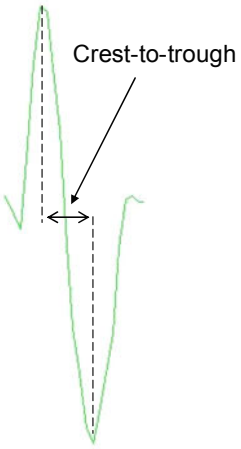

Fig. 2.12: Spike shape: Most of the recorded neurons had typical positive (crest) and negative (trough) peaks. The crest-to-trough time was mostly between 1200 – 2200 hz.

For all the neurons, inter-spike-intervals (ISI) were calculated (in milliseconds - ms) during the spontaneous activities. It was found that after conditioning the ISI reduced indicating an increase in spiking frequency (Fig. 2.13). The ISI decreased from 27.8 ms during pretest to 13.2 ms during 2h posttest. Additionally spiking activities became more uniform during the 1hr and 2hr posttests compared to pretest and 15m posttest. This was indicated by a decrease in standard deviation from 20.9 in pretest to 6.12 in 2h posttest.

Experiment 3: *Extracellular recording and differential conditioning:*

After differential conditioning, about 42% of the bees showed PER towards the rewarded odor. Each posttest neuronal response was normalized with pretest (average) response and is indicated by a black line at 1 Δ spike frequency (Fig. 2.14). After differential conditioning, the neuronal response towards the rewarded odor (A+) showed a slight decrease compared to unrewarded (B-) and neutral odors (C) (Fig. 2.14). This effect was statistically significant only at 2 hr posttest ($P < 0.05$).

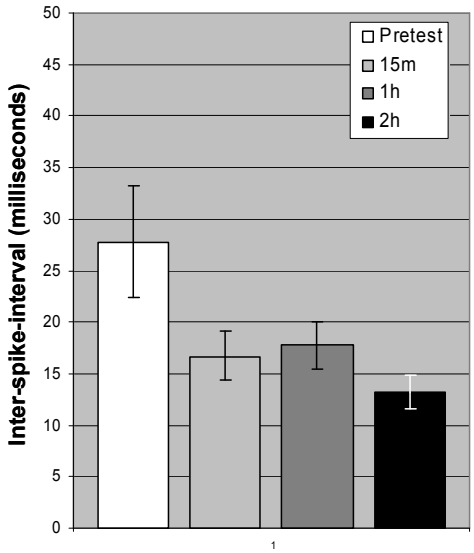

Fig. 2.13: Inter-spike-interval (ISI): ISI lowered from 27.8 ms in pretest to 13.2 ms in 2hr posttest indicating increase in spike frequency. Also the variance was reduced from pretest to posttest indicating stable firing of neurons.

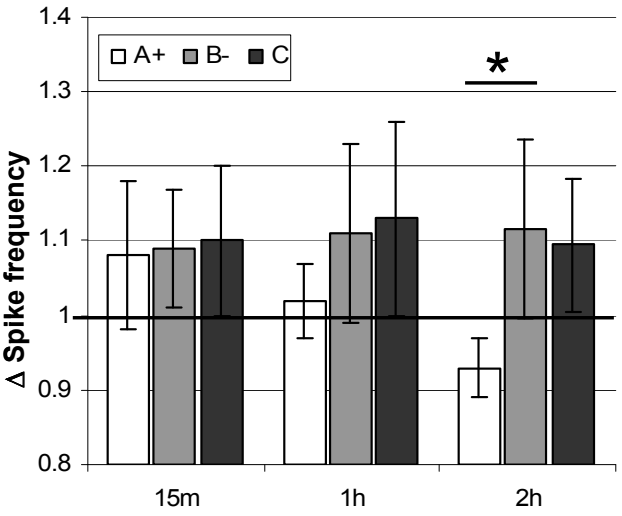

Fig. 2.14: Neuronal response after differential conditioning: After normalizing all posttests with pretest (black line at 1 Δ spike), the rewarded odor A showed a significantly ($P<0.05$) reduced response (Δ spike frequency) at 2h posttest compared to unrewarded odor B and neutral odor C.

Experiment 4: *Extracellular recording + Context learning with Temperature & Color*

The behavior showed that during the conditioning trials, about 37% of the bees showed partial learning and during the posttests 25% showed partial learning. Most bees learnt 32°C + Yellow context. The control bees which were placed in the context but not rewarded showed no preference to any particular context.

During the 32°C + Yellow context, frequency of spikes increased and during 19°C + Blue context frequency of spikes (spikes per second) decreased. The frequency of

spikes at 32°C + Yellow was more than ten-fold compared to that at 19°C + Blue. Figure 2.15 shows the spike frequency of a neuron during 19°C and 32°C contexts. At 19°C the frequency was <8 Hz but when temperature was increased frequency increased to about 70 Hz in 2 minutes and at 4 minutes the frequencies reached a saturation (~80 Hz). When temperature was decreased the frequency decreased to <25 Hz in 2 minutes and saturated after 4 minutes. Similarly the amplitude of the spikes increased during 32°C + Yellow context and decreased or disappeared during 19°C + Blue context (Data not shown).

Analyzing spikes for odor effects and context effects during rise and fall of amplitudes was very difficult since one of the criteria for spike sorting was amplitude. But the template sorting method in spike2 software was flexible enough for us to specify separate templates for increasing set of spikes and decreasing set of spikes. This made sure that all spikes were included in the analysis. Also, normalization procedure nullified the fluctuating effect of temperature changes and Δ spike frequency was true representative of neuronal changes (not temperature changes). In more than 65% of the bees the neuronal responses during the 19°C + Blue context were complete absent. Therefore, only a subset of the neurons was analyzed for context learning.

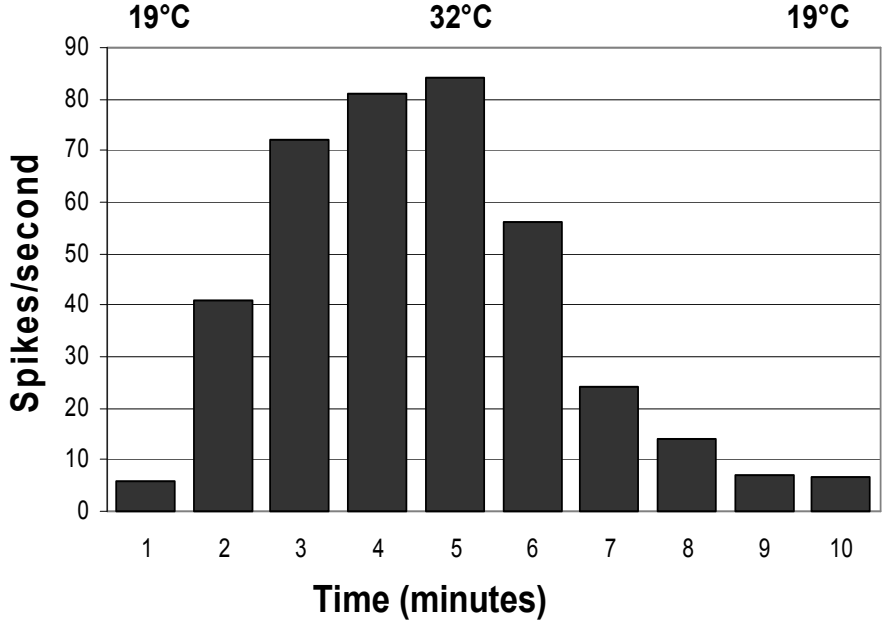

Fig. 2.15: Neuronal response to temperature: Spike frequencies increased with increase in temperature and decreased with decrease in temperature. The change in frequencies was highest (60 - 65 Hz) during the first 2 minutes after hot or cold context was started. Frequencies saturated after 4 minutes of hot or cold context.

During both contexts, there was no significant difference between any of the odor responses. However the context responses showed a significant increase (P<0.05) at 32°C + Yellow context compared to 19°C+ Blue context at 1hr and 2hr posttests.

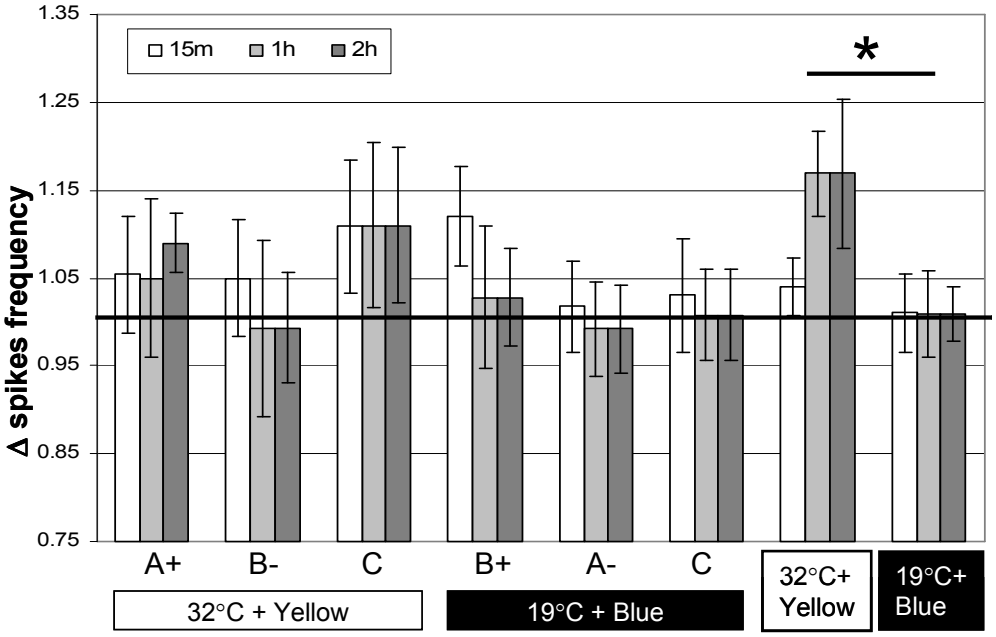

Fig. 2.16: Neuronal response after context learning with temperature and colors: There was no difference in odor responses in either of the contexts, but the context response was significantly higher (P<0.05) at 1h and 2h after conditioning in the 32°C+Yellow context compared to 19°C+Blue context.

Experiment 5: *Extracellular recording + Context learning with Bright and Dark*

After context learning, >40% bees showed PER towards rewarded odor. The control bees showed no particular preference to any odor in either context. The extracellular recordings from alpha lobe neurons showed that only the rewarded odor (A+) in the rewarded context (CX+) had a reduced response compared to other odors at 2h posttest (P < 0.01). But the rewarded context (CX+) itself had a higher response (P<0.05) compared to unrewarded context (CX-) at 2h posttest. However, the response towards odors in unrewarded contexts was not significantly different.

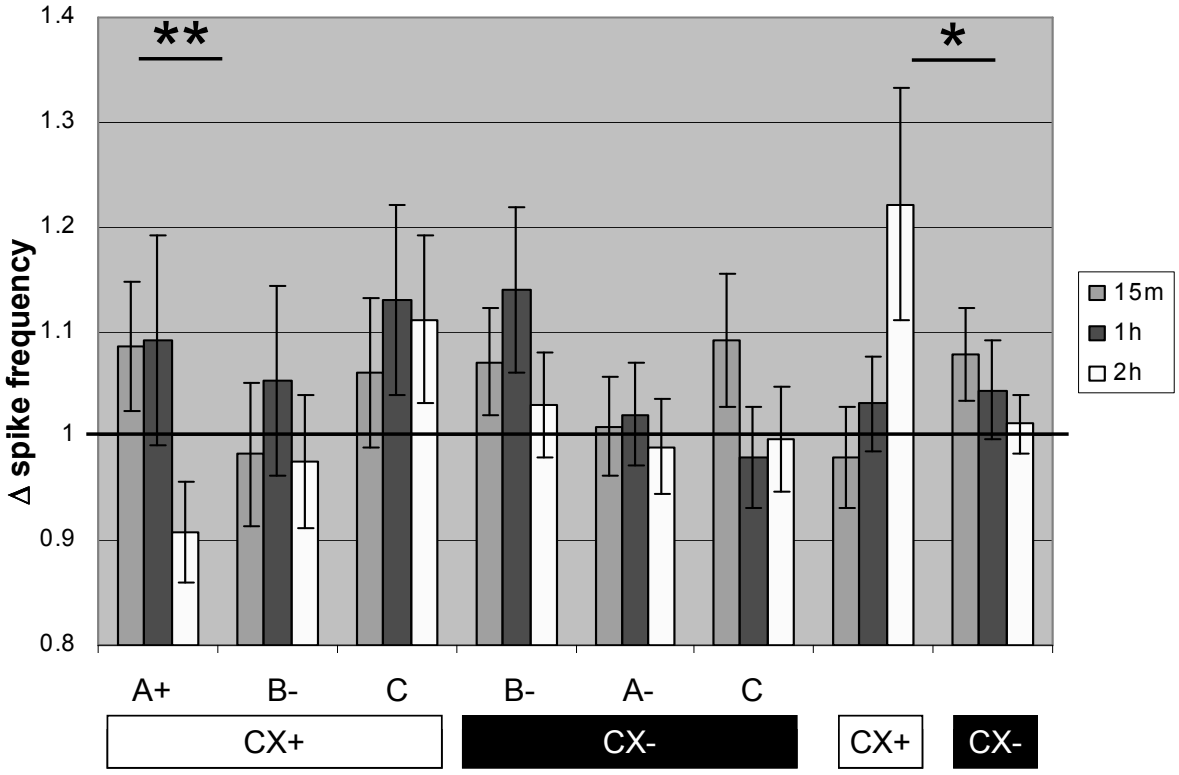

Fig. 2.17: Neuronal response after context learning with bright and dark: There was a significant difference ($P<0.01$) in odor response of rewarded odor A at 2h posttest compared to unrewarded odor B and neutral odor C in rewarded context CX+. The odor responses of A, B and C were not different in unrewarded context CX-. But context response in CX+ was higher ($P< 0.05$) compared to context response at CX- at 2h posttest.

Discussion:

Experiments on free flying bees have shown that they learn contexts quickly (Zhang et al., 2006). In this chapter we showed that bees can learn contexts in restrained conditions too.

The first experiment showed that bees learn context learning better and faster if two contexts were combined together. We had four groups of bees, one group was trained to yellow and blue context, second to 25°C and 32°C temperature, third to 32°C and 19°C temperature context and fourth to a combination of 32°C + yellow and 19°C and blue context. The fourth group showed the highest (55%) and fastest (3rd trial) learning compared to other groups. However, the learning after 4th trial dropped considerably (42%) but recovered slightly during test trial (47%). The bees respond quicker (3rd trial) towards two combined contexts and slower (4 or 5th trial) towards single contexts, this means that combined contexts are easier to learn than single context. This is also true in free flying bumblebees which forage slower during difficult discrimination tasks (Chittka, 2003; Dyers et al., 2004). In experiment 1, the context learning rule was difficult; i.e., bees had to respond to an odor in one context and had to ignore the same odor in another context. In other words, bees had to perform reversal learning in every conditioning trial. Also, the motivation of the bees dropped quickly because they were rewarded in both contexts equally and in a very short time (2 times in 10 minutes).

In experiment 2, we made the task easier by rewarding bees in only one out of the two contexts; Bright and Dark. Therefore, the task was to respond to the correct odor in the correct context. This task was less complicated than the reversal learning rule in experiment 1 and therefore bees showed better learning. After conditioning, bees learnt to respond to an odor in one context while showing no response to that same odor in another context. This showed that bees indeed learnt the context learning rule. However, bees showed slightly better learning in Bright context compared to Dark context (not significant), showing their innate preference. Bees are diurnal insects showing more activity during day than night and also showing strong positive phototaxis. Therefore, in our experiments it is not very surprising that they show better olfactory learning in bright context compared to dark context.

In Experiment 3, we combined behavior with extracellular recording of alpha lobe neurons of mushroom bodies. We first performed simple differential conditioning experiments to study general responses of neurons. After conditioning, the inter-spike-intervals reduced (indicating increased firing) and became more stable (smaller deviations). The neuronal response towards rewarded odors was lower compared to unrewarded and control odors at 2hr posttest. These results were similar to previous studies (Ryuichi et al., 2007) which showed reduced responses of PE1 (pedunculus-extrinsic neuron number 1) neurons towards rewarded odors. However, even non-PE1 neurons showed a trend (non significant) of reduced response towards rewarded odors compared to other odors. Since, we did not identify any PE1 neurons our neurons fall into non-PE1 category. Our Experiment 3 was out of scope of context learning experiment, but was done for subsequent comparison with experiments 4 and 5 which dealt with context learning.

In Experiment 4, we performed extracellular recordings while subjecting bees to context learning protocol (Fig.2.5). We observed that during heating (32°C) and cooling (19°C) of bees the responses of alpha lobe neurons also changed strongly. Honeybees sense temperature by a thermoreceptive sensillum on the antenna called “sensillum coelocapitulum” (Yokohari, 1983). It has been previously shown that the hippocampus neural activity changes when temperature is changed (Schiff et al., 1985; Moser et al., 1993; Shibasaki et al., 2007). In our experiment, increasing the temperature increased the neuronal firing and decreasing temperature decreased or abolished neuronal firing. This effect was reversible at will. We mimicked the daytime and nighttime conditions by providing bees with 32°C + yellow and 19°C + blue contexts respectively. During conditioning, we rewarded two different odors in two different contexts. For example, odor A was rewarded in 32°C + yellow context and odor B was rewarded in 19°C + blue context. Bees showed a bias towards 32°C + yellow context and hence quickly learnt odor A in 32°C + yellow context while odor B in 19°C + blue context was not learnt. The neuronal response was also higher towards 32°C + yellow context compared to 19°C + blue context at 1h and 2h posttests. But the neuronal response towards odors in 32°C + yellow context was not different from 19°C + blue context. This might be because of weak context learning (25%) effect. However, this experiment shows that cued (odors)

learning and context (temperature + color) learning are different because on one hand neurons respond differently to different contexts while on other hand response towards cues does not seem to change. Differences in cued learning and context learning are discussed later.

Experiment 4 had drawbacks such as strong increase and decrease of spikes during hot and cold contexts which made sorting of spikes difficult. Successive hot and cold air on exposed brain was invasive and undesirable making several extracellular recordings unusable. This prompted us to use bright and dark contexts which was shown to work in other insects like crickets (Matsumoto et al., 2004) and cockroaches (Sato et al., 2006) but untested in honeybees. We showed that honeybees show better context learning with bright and dark contexts (Experiment 2) compared to other single contexts (Experiment 1). This was partly because we removed reversal learning rule from our context learning experiment. This meant that bees had to learn to respond to one odor in one context and ignore the same odor in another context. This rule was sufficient to show context learning in bees. We therefore used bright and dark contexts for our subsequent extracellular recording experiments.

Experiment 5 was similar to experiment 2 except that we had 2 trials of pretests and 3 posttests after conditioning. As expected, bees showed better context learning (~40%) compared to experiment 4 (25%). Extracellular recordings showed that the odor response for the rewarded odor was significantly less from other odors at 2h posttest. This was similar to differential conditioning (Experiment 3) result. However, the context response during the rewarded context was higher than context response during unrewarded context. Again, this showed that cues and contexts are different.

Why do rewarded cues have different meaning than rewarded contexts? In mammals it has been shown that cued learning and context learning have different mechanisms (Philips et al., 1992). It appears like context learning requires an intact hippocampus (Chen et al. 1996; Logue et al. 1997; Holland et al., 1999; Anagnostaras et al. 2001; Corcoran et al., 2001; Wallenstein et al., 2001) and cued learning does not require hippocampus (Gould et al., 2002). Similarly in honeybees, previous studies have shown that mushroom bodies are not required for simple olfactory learning tasks (Scheiner et al., 2001; Malun et al., 2002). Previous studies on honeybees showed that

after differential conditioning a mushroom body neurons (PE1 neurons and some non-PE1 neurons) show inhibitory effect towards rewarded odor (Okada et al., 2007). This is consistent with our experiments 2 and 5 which involved differential conditioning too. But neuronal response towards rewarded context showed an increase in both context learning experiments (experiment 4 and 5). Therefore, in our experiments neuronal response towards odors is decreased and towards context is increased.

The neuronal response towards rewarded odors in experiment 4 was inconsistent with experiment 3 and 5 probably because it was reversal learning and not a true differential learning. In experiment 4 bees had to reverse their responses in every trial which was not an elementary task, but a complex task. Therefore, no reduction in response towards rewarded odor was observed. A more parsimonious explanation is that, since the rewards are equally balanced on both sides the neuronal response does not show a decrease which would otherwise be evident with differential condition situation.

Considering that complex form of learning such as context learning requires mushroom bodies, we propose that mechanisms involved in elementary form of learning and complex form of learning in honeybees are different.