Olfactory learning and coding in honeybee: behavioral and physiological evidences

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…… To Atanu, Southik, Tudor and Amlan

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Authors and Contributions: Neloy Kumar Chakroborty and Randolf Menzel

Designed the experiment: NKC and RM. Gathered the data: NKC. Analyzed the data: NKC. Wrote the paper: NKC and RM.

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Olfactory adaptation changes the glomerular response strengths and representations of odors in honeybee antennal lobe
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Zusammenfassung

Honigbienen besitzen die Fähigkeit eine Vielzahl von Düften anhand von unterschiedlich langen Kohlenstoffketten und unterschiedlichen funktionalen Gruppen zu unterscheiden. Düfte sind für diese Tiere während der Futtersuche und im Stock zur Kommunikation von höchster Wichtigkeit. Bienen lernen Düfte im Labor in unterschiedlich komplexen Lernexperimenten. Hierfür bedient man sich der olfaktorischen Konditionierung des Proboscisstreckungsreflexes (PER). Mit Hilfe solcher Konditionierungsexperimente untersuchte ich die Einflüsse von verschiedenen Lern- und Gedächtniseigenschaften auf die Performance von Bienen in komplexen Formen olfaktorischen Lernens. Zusätzlich habe ich (opto-)physiologische Messungen an olfaktorischen Neuronen im Bienengehirn vorgenommen, um den olfaktorischen Code in Gegenwart von komplexen Hintergrunddüften zu untersuchen. Im ersten Kapitel dieser Dissertation untersuchte ich die Rolle von Olfaktion bei der Aufspürung der pathogenen Milbe *Varroa* in den Brutzellen von Honigbienen. Die Ergebnisse zeigen, dass Bienen mit einer höheren Resistenz gegen *Varroa*-Milben besser zwischen den Düften von gesunder und infizierter Brut unterscheiden konnten. Dies deutet stark darauf hin, dass resistente Bienen von *Varroa* parasitierte Brut durch olfaktorische Merkmale erkennen können. Im zweiten Kapitel wurden die Bienen mit Hilfe einer kumulativen (komplexen) Form der olfaktorischen Konditionierung trainiert um die Ergebnisse der verschiedenen Typen von lernbezogenen Performerklassen interpretieren zu können. Ich fand heraus, dass die Geschwindigkeit des Duftlernens und Duftunterscheidbarkeit die beiden Eigenschaften sind, die die Performance von allen Typen von Performerklassen am meisten beeinflussen. Im letzten Kapitel untersuchte ich unter Anwendung der opto-physiologischen Technik *in vivo* Calcium Imaging die Effekte von olfaktorischer Adaption auf den Duftcode im Antennallobus. Meine Ergebnisse zeigen, dass Adaption an einen komplexen Hintergrundduft die Antwortstärke erhöht und das glomerulare Aktivitätsmuster von zusätzlich präsentierten Einzeldüften verändert. Diese Ergebnisse tragen dazu bei, die Rolle von Hintergrunddüften in der Umgebung beim neuronalen Coding und das Lernverhalten von Duftinformation in der Honigbiene besser zu verstehen.

Summary

Honeybees have superior abilities to learn and discriminate between enormous number of odors with different carbon chain length and functional group. They learn odors outside the colony during foraging as well as inside the colony while communicating with the hive comrades. Bees can be trained to learn odors in the laboratory in simple and complex forms of learning assays using the popular conditioning paradigm namely, the olfactory conditioning of proboscis extension reflex (PER). I used the same olfactory PER conditioning assay and investigated the influences of different learning and memory related features on the overall performance of bees in complex form of olfactory learning. In addition, I recorded physiological responses from the olfactory neurons in honeybee brain to understand the olfactory coding in presence of the complex background odor used for adaptation. In the first chapter of this dissertation I investigated the role of olfaction in honeybees to detect the presence of pathogenic *Varroa* mite inside the brood cells. Results showed that bees with higher resistance against the *Varroa* mite

were able to distinguish between the odors of the healthy and infected brood better than the less resistant bees. This strongly indicated that resistant bees possibly detect the *Varroa* parasitized brood through recognizing their abnormal odors in the colony. This also indicated that honeybees can possibly learn the odors associated with the *Varroa* infection in presence of the adapting background odor of the honeybee colony. In the second chapter a cumulative form (complex form) of olfactory conditioning assay was used to train bees to identify and understand the behavioral characteristics of the different types of learning related performer classes present in the population of honeybee. I found that speed of odor learning and odor discriminability were the two most important features that strongly influenced the overall performances of all types of performer classes in honeybee. Furthermore, in the third chapter I used the popular neurophysiological technique of *in vivo* calcium imaging and investigated the effects of olfactory adaptation on the odor coding of the antennal lobe glomeruli. My results showed that adaptation with the background of complex odor stimuli changed the response strengths and representation patterns of odor in the glomeruli which together confirmed the change in odor coding scheme of the glomerular coding space. These results altogether contributed further to the understanding of neural coding and behavioral learning of odor information in honeybee.

Key words: Hygienic behavior, *Varroa* mite, Cumulative conditioning assay, Performer classes, Adaptation, Glomeruli

Abbreviations

ABPV	Acute bee paralysis virus
AL	Antennal lobe
AS	Adaptation stimulus
CCD	Colony collapse disorder
CR	Conditioned response
CS	Conditioned stimulus
DC	Differential conditioning
DWV	Deformed wing virus
EAG	Electroantennogram
ED	Euclidean distance
Ger	Geraniol
IAA	Isoamyl acetate
IAPV	Israeli acute bee paralysis virus
KBV	Kashmir bee virus
l-ACT	lateral- antennocerebral tract
LA	Linolenic acid
LIB	Länderinstitut für Bienenkunde
Lina	Linalool
LN	Local neuron
MB	Mushroom body
m-ACT	median- antennocerebral tract
OA	Oleic acid
OB	Olfactory bulb
ORN	Olfactory receptor neuron
PEA	Phenethyl acetate
PER	Proboscis extension reflex
PN	Projection neuron
PRC	Pyramidal cell
RM-ANOVA	Repeated measurement analysis of variance
US	Unconditioned stimulus
WMP (test)	Wilcoxon matched pairs (test)
SAP	Sting alarm pheromone
GRP	Glomerular representation pattern

Contents

Chapter -1 Introduction 11
Role of olfaction to elicit the hygienic behavior against the Varroa mite 13
Typical characteristics of learning of the individual bees 15
Behavioral characterization of the different learning and memory performer classes of the honeybee population 17
More information are available on the olfactory learning than the adaptation related neural plasticities in honeybee antennal lobe 21
Effects of olfactory adaptation on the glomerular odor coding of the honeybee antennal lobe 21
Bibliography 24

Chapter -2 Investigating the potential differences in olfactory learning between the hygienic and non-hygienic honeybees 26
Introduction 27
Goals of the study 34
Materials and Methods 35
Preparing honeybees for the olfactory PER conditioning 35
Olfactory PER conditioning protocols 36
Results 39
Differential conditioning using the wax caps isolated from the healthy and the Varroa infected brood cells 39
Differential olfactory conditioning using the healthy and the Varroa infected pupae 43
Absolute conditioning with the sting alarm pheromone odor, isoamyl acetate 47
Differential conditioning with the floral odors 59
Discussion 68
Differential conditioning of the hygienic and non-hygienic bees with the wax caps 68
Differential conditioning of the hygienic and non-hygienic bees with the brood specific volatiles 69
Absolute conditioning of the two types of bees with the sting alarm pheromone odor, isoamyl acetate 71
Differential conditioning of the hygienic and non-hygienic bees with the floral odors 72
Bibliography 74

Chapter -3 Characterizing the learning and memory performances of the individual honeybees using the cumulative olfactory conditioning paradigm 78
Introduction 79
Goals of the study 83
Honeybee colonies used in the assay 84
Backcrossing Scheme 85
Materials and Methods 87
Preparing honeybees for the olfactory PER conditioning 87
General information about the differential olfactory PER conditioning; appetitive paradigm 87
Protocol and purpose of the cumulative olfactory PER conditioning assay 88
Odors used 91
Scoring scheme 94
Data analysis 96
Results 97

Cumulative learning and memory performances of the backcrossed colonies 97
Variability in the learning and memory performances of the individuals 103
Selection of the best and the poor cumulative scorers 107
Selection of the best and poor cumulative scorers using the scores of the learning and memory related features 117
Analysis of performance histories of the different selected groups of performers 122
Bivariate histogram analysis revealed the pair-wise relationships of the features 136
Discussion 150
Heterogeneity in learning and memory performances inside the population of honeybee 150
Individual's analyses of learning and memory picked up the different performer classes from the performance heterogeneity 153
The next step 157
Outlook 159
Bibliography 159
Appendix-1 163

Chapter-4 Olfactory adaptation changes the glomerular response strengths and representations of odors in the honeybee antennal lobe 170
Introduction 171
Aim of the investigation 176
Materials and methods 177
Preparation of honeybees for the backfilling of projection neurons 177
Experimental protocol 179
Confocal Microscopy to check for the staining of the PNs and glomerular anatomy 183
Data processing 184
Statistical analysis 187
Results 189
Olfactory adaptation of the AL glomeruli with the background odor stimuli 189
Olfactory adaptation changed the odor response strength of the glomerular ensemble 190
Individual glomeruli showed the similar or dissimilar types of change in odor responses due to adaptation 201
Adaptation induced changes in glomerular responses were found to vary with the odor identity 207
Categories of test odors 220
Adaptation changed the odor representation pattern in glomeruli 226
Discussion 238
Methodological considerations 238
Colony odor and synthetic odor mixture differentially influenced the odor response strength of the glomeruli 239
Odor responses of the Glomeruli did not show the adaptation recovery 240
Glomeruli showed similar or dissimilar types of change in odor response strength 241
Odor categories 243
Dissimilarities in the odor representation patterns due to adaptation 244
Comments and outlook 245
Bibliography 246
Appendix-2 250

Chapter -5 Discussion 259

Discussion of results; chapter-2 259
Future perspective 262
Common aspect; chapter-2 and chapter-3 263
Discussion of results; chapter-3 263
Future perspective 268
Contradictory aspect; chapter-2 and chapter-4 268
Discussion of results; chapter-4 269
Future perspective 273
Bibliography 273

Chapter -1

Introduction

Honeybee, member of the insect order Hymenoptera is a eusocial insect with the characteristics of cooperative brood care by the members of the colony (society), overlapping generations of members and the reproductive division of labor amongst the comrades of the colony. Worker bees, the labor force of the colony start their working life as caretakers of the in-hive requirements such as cleaning the hive, preserving the honey in the tightly sealed combs, taking care of the developing brood, attending the queen, feeding the drones, building the new comb and coating the hive walls with the propolis etc. As the workers grow older they start to switch from performing the set of intranidal tasks into the more outside-task such as foraging (Seeley and Kolmes 1991). Foraging for food (nectar, pollen) is a common feature in this regard which builds up the food reserve of the honeybee colony during the spring and summer when the outside is enriched with the food sources. However, summer life span of the workers is limited to few weeks compare to the few months of life time during the long winters (Ribbands 1964; Sakagami and Fukuda 1968). Hence, worker bees learn the necessary skills for successful food foraging within shorter period of time during the summer. Learning the color and olfactory information of flowers are of high importance for efficient food foraging on the floral patches. Honeybees are well equipped to learn both types of sensory stimuli as showed in previous studies when bees were trained on different colors and odors to associate the sucrose reward in the free flying conditions (von Frisch K 1914; Menzel and Erber 1978; von Frisch K 1919; Laska *et al*., 1999). Among the different sensory modalities olfactory pathway is well studied in honeybee from the behavioral manifestation until the cellular correlates of learning and memory. Conditioning the proboscis extension reflex in honeybee with the olfactory stimulus or the olfactory PER conditioning paradigm in this regard has contributed substantially to our understanding of olfactory learning behavior of honeybee (Kuwabara 1957). Bees in this Pavlovian or associative conditioning paradigm can be trained in the laboratory to

associate an odor stimulus with the food (sucrose) reward to develop the long term memory of the odor as predictor of the food reward (Bitterman *et al*., 1983). This popular appetitive paradigm has been used to train bees in many different ways such as with one odor paired with the sucrose reward (absolute conditioning: Bitterman *et al*., 1983), with two odors where one odor follows the reward and the other is unrewarded (differential conditioning: Bitterman *et al*., 1983), with reversal conditioning where the rewarded odor of the first differential conditioning (DC) become unrewarded during the second round of DC and *vice versa* (Hadar and Menzel 2010) and with the extinction learning paradigm where the rewarded odor during the first phase of conditioning follows no reward in the second phase of conditioning (Eisenhardt and Menzel 2007). The robust olfactory learning ability of honeybees in this paradigm has been utilized not only to understand the behavioral and neural mechanisms of olfactory learning and memory in general (Menzel *et al*., 1996; Menzel *et al*., 2001; Sandoz *et al*., 2001; Rath *et al*., 2011) but also to untwist the role of olfaction in the specific behavioral aspect such as the hygienic behavior or resistance against the pathogens (Nazzi *et al*., 2004; Swanson *et al*., 2009). The term hygienic behavior was originally coined by Rothenbuhler to define the genetic ability of the worker bees to detect, uncap and remove the abnormal or diseased larvae from the colony to stop the spread of infestation early (Rothenbuhler 1964). This defensive behavior is vital for survival of the colony in the face of pathogenic burden since honeybee probably do not have more number of immune effector genes in their genome (Evans et al. 2006). Amongst the different bee diseases, western honeybee *Apis mellifera* has the effective resistance against the American foulbrood (Spivak *et al*., 2001) but no effective resistance is found against the ectoparasitic mite *Varroa destructor*. However, some of the honeybee colonies in Europe and America were reported to develop higher level of resistance than others against the *Varroa* pathogen (Ibrahim *et al*., 2006; Ehrhardt *et al*., 2006). These colonies breed as genetic lines for the trait of higher behavioral resisatnce against the *Varroa* mite are popularly known as the hygienic or tolerant lines and colonies with lower levels of resistance are called as the non-hygienic or sensitive lines. Bees of the hygienic lines can detect and remove the *Varroa* parasitized brood early and more frequently than bees of the non-hygienic lines which lead to the better resistance against this ectoparasitic mite; however the mechanism of higher

behavioral resistance of hygienic bees is not clearly known. Results of the study conducted by Nazzi and colleagues although showed the strong connection between the olfactory cues emanated from the body of the *Varroa* parasitized brood to initiate the hygienic behavior inside the hygienic bee colonies however; usage of their hygienic bees without the non-hygienic bees in parallel did not answer the important question as whether the hygienic bees compare to the non-hygienic bees possess better olfactory perception or learning ability to the odors associated with the *Varroa* infection which contributes to the higher brood-removal hence, better hygienic behavior of these bees against the *Varroa* mite.

Role of olfaction to elicit the hygienic behavior against the Varroa mite

In chapter-2 of this dissertation we tried to answer this question by comparing the olfactory learning abilities of bees from colonies with higher (hygienic colonies) and lower (non-hygienic colonies) levels of resistance against the *Varroa* parasite. Olfactory learning in the PER paradigm was used to answer this question. Honeybees can detect the *Varroa* infection present inside the brood cells while inspecting the brood health from the other side of the sealed brood cells as part of their in-hive activity. Hence, bees definitely detect some form of chemical signals which affirm the presence of *Varroa* in the brood cells. At first we checked whether bees can distinguish between the odor profiles of the wax caps sealing the healthy and the *Varroa* parasitized brood cells in the differential olfactory paradigm. Bees from both genetic lines were failed to learn the discrimination between the volatile odor profiles of the two types of wax pieces. The following experiment investigated the same issue more closely when bees from the two lines were conditioned to learn the discrimination between the volatile odor profiles of the parasitized and un-parasitized (healthy) pupae. Whole pupae were taken into the syringes for delivering odor stimuli. Pupal volatiles although elicited lower overall conditioned responses in bees of the two lines compared to the wax-volatiles; however, unlike the last experiment *hygienic bees but not the non-hygienic bees were found to discriminate between the two volatile odor-bouquets* while the parasitized pupae were used as the rewarded or CS+ stimulus and the healthy pupae as the unrewarded or CS- stimulus but not in the opposite combination of the two CSs. The successful discrimination between

the odors of the healthy and the parasitized pupae by the hygienic bees indicated that these *two honeybee lines differed in their olfactory learning abilities for the brood odors*. This also indicated the possible general scenario that hygienic bees use the olfactory cues emanating from the *Varroa* parasitized brood to detect the infection (or abnormality) which leads to the better hygienic behavior of these bees against this parasitic mite.

However, these results did not confirm whether these two lines had general differences in their olfactory learning abilities. To test that, hygienic and non-hygienic bees were conditioned with the sting alarm pheromone odor, isoamyl acetate (IAA) but were tested during the memory retention tests with the novel or untrained odor (new CS), 1-hexanal along with the CS+ odor IAA. This protocol tested the effect of olfactory generalization between the trained and the novel odors (CS new). Bees from both genetic lines were found to learn the CS+ (IAA) odor similarly during the absolute conditioning but non-hygienic bees compared to the hygienics showed significantly higher odor generalization between the CS+ and CS new both during the short-term and long-term memory retention tests. In addition, the non-hygienic bees showed consistent strong responses to the stimulus, like filter paper (may be a neutral stimulus) during the two memory retention tests. When bees from these two lines were conditioned differentially with the high concentrations of the floral odors such as geraniol and 1-hexanol, non-hygienic bees were again failed to learn the discrimination both during the conditioning and the retention tests. Hygienic bees on the other hand learned the discrimination task between these two odors, like bees in our institute's garden as reported previously (Malun *et al*., 2002).

Hygienic bees although showed significantly better discrimination between the pupal odors however, poor olfactory learning and memory performances of the non-hygienic bees throughout the experimental season of 2009 (summer) did not confirm the general differences in olfactory learning between these two lines. Possibly, the non-hygienic honeybee line had some general deficit to learn the olfactory information in PER paradigm. Hence, the question of 'general differences in olfactory learning between the hygienic and non-hygienic lines' was not clearly answered in this chapter but the findings indicated the possible role of olfactory chemoreception processes to elicit the behavior of brood-removal in hygienic bees against the *Varroa* mite.

Chapter-2 of the thesis dealt with the olfactory learning of honeybees in the simple (absolute and differential) conditioning paradigms but using both the simple (pure floral or pheromonal odors) and complex forms of odor stimuli (odor mixtures emanated from the wax pieces or pupae). In chapter-3, a complex form of odor training protocol namely the cumulative olfactory conditioning (multiple phases of differential conditioning and memory retention test) was applied to train and test the bees with single or pure odors (in place of complex odor stimuli). Results of chapter-2 although did not confirm the possible differences in olfactory learnability between the hygienic and non-hygienic bees but using the backcrossed hygienic bee colonies in chapter-3 (why backcross? see the section 'Honeybee colonies used in the assay' in chapter-3) *we investigated the learning and memory performances of individual bees of the population to characterize the different performer classes behaviorally*. Bees from the hygienic colonies were used for this purpose as it was particlularly interesting to test the different single brood odors (odors released from the healthy and parasitized brood) on these bees in the differential conditioning during the cumulative assay. Differential conditioning (DC) of the hygienic bees in the laboratory background using the brood odors was somehow represented the simpler version of the natural behavior of removal of the diseased brood when bees detect them through the abnormal olfactory cues in presence of the constant background of colony odor (e.g. hygienic behavior against the *Varroa* mite as studied in chapter-2). Although the disease-associated single odor (phenethyl acetate) used in the cumualtive assay was isolated from the chalkbrood infected larvae (Swanson *et al*., 2009) in place of the *Varroa* parasitized brood; however, testing the hygienic bees with these type of odors made the connection between the motivations of investigations reported in chapter-2 and 3 as depicted in Fig. 1. Additioanlly, these two chapters generally made the investiagtions on the 'olfactory learning and memory processes of honeybee'.

Typical characteristics of learning of the individual bees

Olfactory learning and memory mechanisms in honeybee were studied for long time for the population, rather than for the individual bees. However, recent study by Pamir and colleagues (Pamir *et al*., 2011) revealed some interesting aspects of the learning behavior of individual bees which were never detected in the population based performance

analyses. This study analysed the results of the different PER conditioning paradigms and found that individual bees in the population were variable in their rate of olfactory learning which created the population heterogeneity. Individuals were found to learn the odor information in a switch-like or abrupt manner unlike the gradually increasing conditioned or learned responses found commonly in the population learning curve. Additionally, it was also found that once the individual bees learned the association or no

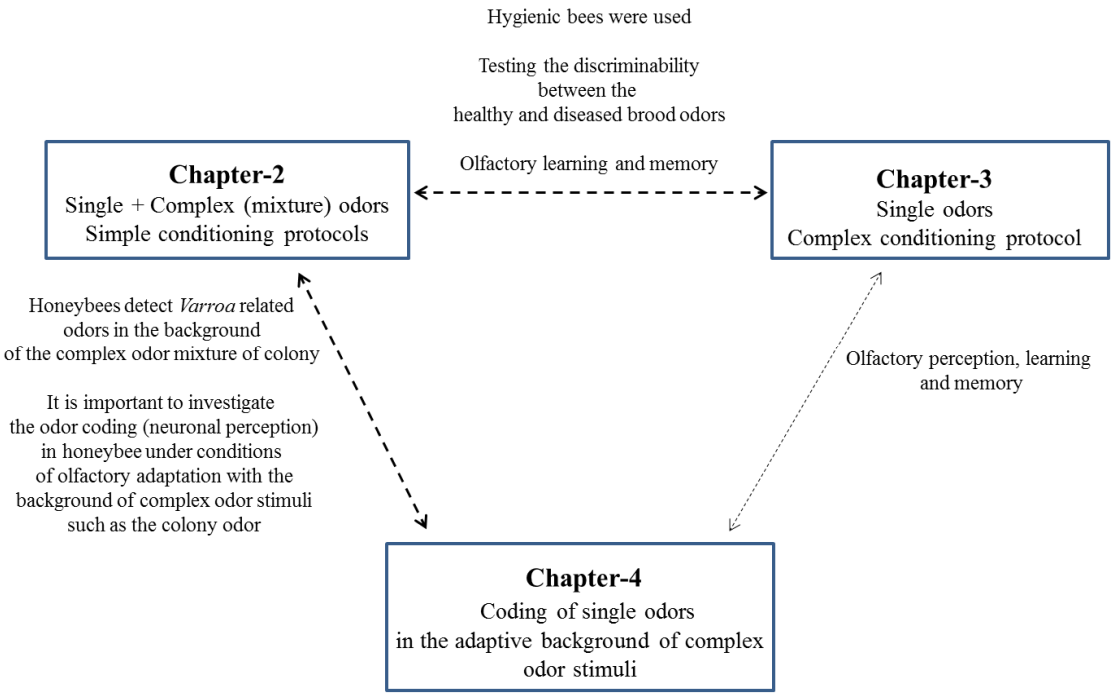

Fig. 1: Schematic representation of the connections between the individual chapters of this dissertation in terms of their respective goals: As part of the common introduction it was important to provide the possible connections between the different chapters of the dissertation which was represented in this schematic diagram. In chapter-2 (top left rectangular box) the possible roles of olfaction in recognition of the *Varroa* infected brood inside the colony was investigated using the simple olfactory PER learning paradigms and both single and multi-component odor stimuli. Although, all experiments in chapter-2 using the hygienic and non-hygienic honeybees were performed in the laboratory not inside the honeybee colony; however, bees were trained to discriminate between the volatile odor profiles of the healthy and the *Varroa* parasitized brood. This was important to understand the possible natural scenario of whether bees can recognize the presence of the parasitic mite through the olfactory cues. The same hygienic bees were used in chapter-3 (top right rectangular box) and conditioned in the complex form of olfactory conditioning assay (cumulative olfactory conditioning assay) with the goal to behaviourally characterize the different learning and memory classes of the honeybee population. The goals apparently were different between the chapter-2 and 3 but in the cumulative conditioning assay bees were conditioned to discriminate between the pure odors represented the

healthy and the diseased brood. Although, the disease-associated odor was isolated from the chalkbrood infected larvae rather from the *Varroa* infected brood, but the basic ideas of these two chapters were related. Differential conditioning of hygienic bees in the laboratory background using these pure odors in chapter-3 was to some extent mimicked the natural behavior of brood-removal from the colony through the recognition of the disease-associated olfactory cues in presenece of the adapting background of the colony odor e.g. the hygienic behavior against the *Varroa* mite as studied in chapter-2. Additionally, these two chapters commonly investiagted the olfactory learning and memory processes in honeybee (connection showed with the bold dotted double arrow). In chapter-4 (lower rectagular box) calcium imaging of the antennal lobe (AL) neuropil was performed unlike the behavioral learning experiments performed in the other two chapters. However, in chapter-4 we investigated the possible effects of olfactory adaptation on the neural perception of odors or odor coding in the AL neuropil. Adaptation in one of the two sets of experiments was achieved with the background odor stimulus extracted from the honeybee colony. Hence, chapter-4 although did not investigated the olfactory learning in the AL neuropil; however, chapter-2 and 4 shared the common interest to understand the phenomena of odor perception and / or learning in honeybees in general (in chapter-2 bees were trained with the isoamyl acetate under the condition of olfactory adaptation with the colony odor) or with respect to the specific behavior (e.g. hygienic behavior) under the condition of olfactory adaptation with the background odor of the colony (connection showed with the bold dotted double arrow). Chapter-3 and 4 rather were only connected with respect to of the general interest to understand the processes of olfactory perception, learning and memory (connection showed with the faint dotted double arrow).

association (CS- trials of the differential conditioning or the extinction learning trials) between the CS and the US they remained stable in the learned state with high probability for rest of the odor conditioning. Conversely, bees which did not show the PER during the particular training trial also showed no-PER with high probability during the following trial and if they continued showing no-PER until the last conditioning trial then they also showed no conditioned responses during the memory retention tests again with high probability. These results confirmed the existence of the good and bad learning performers in the heterogeneous behaving population and opened up the possibility to characterize these extreme performer classes behaviorally as well as to investigate the possible other interesting aspects of the individual's learning behavior.

Behavioral characterization of the different learning and memory performer classes of the honeybee population

We analyzed the learning and memory performances of the individual bees in the cumulative conditioning assay to characterize the different performer classes of the population. The experimental protocol of the cumulative conditioning assay was

consisted of two phases using two different pairs of odors, with each phase had one round of differential conditioning (DC) followed by the two rounds of the memory retention tests. This paradigm offered the advantage to test the individual honeybees repeatedly with 56 odor trials for 6.5 hours to perform the specific set of learning and memory tasks which made the screening for performer classes (such as the cumulatively good or bad performers) stringent. In other words, performance evaluation of the individual bees based on the total number of correct and incorrect responses to the different CS stimuli decreased the chances of selection of performers based on random responses. The combination of the multiple phases of DCs and the retention tests also made it possible to score the different learning and memory related behavioral features such as the speed and consistency of the CS+ (rewarded odor) learning during the DCs, odor discriminability during the DCs and the retention tests, odor sensitivity to the dilutions of the conditioned odors, responses to the stimuli like filter paper and paraffin oil of the individual bees separately during the two phases of the assay. Performance scores of individual bees in these features were first evaluated using a simple scoring scheme (details given in chapter-3). This was followed by the selection of different scorer or performer classes (e.g. cumulatively high and low scorers) of these individual features as well as of the overall or cumulative performance (summation of performance scores of all features) to ask question such as

- how the performance scores of the different individual features such as the learning speed, odor discriminability, sensitivity or the overall responsiveness to the conditioned stimuli influenced the scores of other features generally (for whole population) and in the specific scorer categories?
- whether the performance scores (higher or lower scores) of any one or more of these features were able to select the two extreme classes of cumulative scorers (best and poor cumulative performers) with higher probabilities than the other features. The idea was to check whether any single or more of these quantified features was able to predict strongly or influenced the final performance levels of bees in the cumulative olfactory conditioning assay.

High variability in the performance scores of individuals for all of the learning related features such as the speed of learning, odor discriminability and sensitivity was the most

salient feature found in the data. This prohibited the formation of any visible scorer or performer cluster while the cumulative performance scores of the entire experimental population (152 honeybees) were plotted; hence, the cumulatively best and the poor performers were selected with the arbitrary criteria of higher and lower ranges of the cumulative score. Nearly 14% bees of the population were selected as the best (consistently good performance throughout the assay) and ~13% as the poor cumulative performers (consistently bad performance throughout the assay) with these criteria. However, independent of performance dissimilarities common high correlation (Pearson's linear correlation) between the performance scores of the learning speed and odor discriminability during the 1st differential conditioning was found for these two classes of cumulative performers. The high correlation between these two features was also found in other performer classes irrespective of the differences between their performances. Amongst the different learning related features, the higher and lower scores in odor discriminability were found to select respectively for the best and the poor cumulative scorers with the highest probabilities. In other words, ability to learn the CS+ and CS- stimuli concomitantly was found to influence strongly the final performance levels of these two types of cumulative scorer classes in this assay. Performance scores of Acq1 (speed and consistency of CS+ learning) and Disc1 (odor discriminability during 1st differential conditioning) were found to predict better the scores of the feature represented 'odor sensitivity' but not *vice versa*. Additionally, the cumulative performance scores and the speed of learning of the CS+ stimuli were found as the two key features strongly influenced the learning speed (dynamics) of the unrewarded or CS- stimuli in the different performer classes. Higher cumulative or Acq1 scorers showed the concomitant learning of the CS+ and CS- stimuli during the 1st differential conditioning. On the other hand, the moderate and lower cumulative or Acq1 scorers showed the faster learning of CS+ than the CS- stimuli.

Individual's analyses of performances also provided us with the chance to examine the possible correlations between the individual's learning related performances with the expression patterns of genes in their brain neuropiles involve with the processes of learning and memory. The cumulatively best and the poor performers of this assay were

studied for their expression patterns of the learning related genes in the mushroom body neuropil; but the results were not analyzed hence, not given in chapter-3.

In chapter-4 of this dissertation we reported the experimental results of the *in vivo* calcium imaging study performed on the projection neurons of the honeybee antennal lobe (AL) neuropil. Unlike the other two chapters, reported the findings about the olfactory learning behavior in honeybees, chapter-4 varied substantially with respect to the technique used. In chapter-4 we investigated the effects of olfactory adaptation on the glomerular odor coding (neuronal perception of odors) of the honeybee antennal lobe. Honeybees were exposed constantly for 20 min to the background of complex odor stimuli such as the odor mixture of honeybee colony to achieve the adaptation of olfactory glomeruli. The meaning of colony odor (represented the internal environment of the honeybee colony) was already learned by bees used in our experiments and in general this is one of the most frequent odor stimuli that bees encounter throughout their life. Although it was not understood as how bees perceived the colony odor in the context of the laboratory; however, measurements of odor responses under the adapted condition with the colony odor was an important step which contributed to the understanding of neuronal perception of single odors in the background of complex-adapting odor stimuli. This also somehow represented the scenario of perception of odor cues associated with the *Varroa* infection in presence of the constant adapting background odor of the colony. In chapter-2 although, no experiment was designed to find out the effects of olfactory adaptation (using the colony odor) on the learning of odors associated with the *Varroa*, infection, but bees were at least conditioned in the laboratory background to discriminate between the volatile odor mixtures of the healthy *vs*. *Varroa* parasitized pupae. Importantly, in chapter-2 bees in one of the experiments were also conditioned with the sting alarm pheromone (SAP) odor isoamyl acetate (IAA) in presence of the constant adaptation background of colony odor. The purpose of this experiment performed in chapter-2 *although was different from measuring the calcium signals in the AL to the same odor IAA in chapter-4*, but these two chapters shared the *common goal* to understand the phenomena of odor perception and / or learning in honeybees generally or with respect to the specific behavior (hygienic behavior) under the condition of olfactory adaptation with the constant background of complex odor stimuli (such as the odor of

honeybee colony). The possible connections between these two chapters with respect to the overall goals were described in Fig. 1, although in chapter-4 unlike the other two chapters we did not use bees from the hygienic or non-hygienic colonies, rather bees of our institute's garden were used. Investigations of chapter-3 and 4 were mostly related in terms of the general interest to understand the processes of olfactory perception, learning and memory (Fig. 1).

More information are available on the olfactory learning than the adaptation related neural plasticities in honeybee antennal lobe

Olfactory learning paradigms in honeybee were not only used to understand the dynamics and mechanisms of learning and memory processes behaviorally but also merged with the signal recordings from the different neuronal populations of brain to understand the cellular correlates of learning and memory. Early works of Till Faber (Faber *et al*., 1999) and recent report by Lisa Rath and colleagues (Rath *et al*., 2011) showed that associative olfactory learning transformed the odor representation patterns in the honeybee antennal lobe (AL) while they recorded the calcium signals both 'before' and after the differential olfactory PER conditioning. Olfactory learning was found to increase the response intensity of the glomeruli to the rewarded odor after the condition compared to 'before'. However, the population's activity to the unrewarded odor was found to remain same between the same two conditions. In comparison to our knowledge of the neural correlates of olfactory learning and memory in the honeybee antennal lobe, effects of olfactory adaptation on the neural representations of odors in the AL were much less known.

Effects of olfactory adaptation on the glomerular odor coding of the honeybee antennal lobe

Adaptation is an important plastic mechanism that reduces the responses of the neural system to the unchanging and often meaningless background stimuli due to the prolong exposure. Olfactory adaptation is a frequently encountered phenomenon in insect's life when performing tasks driven by the odor stimuli such as the search for food or mates or

the predator avoidance in presence of the complex odorous background. Successful performances in these tasks in the adapted state of the olfactory system indicate that either the neural representations of the crucial olfactory stimuli remain unchanged or get changed without changing the meaning of the relevant stimuli. These possibilities can be checked out through the recording of odor responses from the olfactory neurons while keeping them in the state of adaptation. In chapter-4, we recorded odor responses in the AL glomeruli to understand the adaptive odor coding of glomeruli using the already established scheme of spatio-temporal glomerular odor coding (Galizia *et al*. 1999; Sachse *et al.*,1999; Sachse *et al.*,2002). Two different odor mixtures were used for the adaptation of the antennal lobe glomeruli; the odor mixture drawn from the honeybee colony with the quantitative estimation of its individual components unknown, but mimicking the background environment of the hive and the mixture of four different pure odorants (1-hexanol, 1-nonanol, 2-octanone and limonene) with known composition (equal volume synthetic odor mixture). The second stimulus was used to test the *general effects of adaptation on the glomerular odor coding*, independent of the complexity of adaptation stimulus. This synthetic mixture of odors also weakly mimicking the background olfactory environment of floral patch (since numbers of odor components in the floral patch are generally much higher than four) as these four odors were isolated from the floral scents (Knudsen *et al.*,1993).

Possible changes in the glomerular response strength and representation pattern to the set of eight-test odors were considered as the result of change in the process of odor coding due to the olfactory adaptation. Glomerular responses to the different test odors were measured and compared between the conditions of before, during and after the adaptation for investigation of the potential effects of adaptation and its removal on the odor responses with respect to the initial state of no-adaptation (before adaptation). Adaptation was defined as the process that *declined the strength of calcium signals of the AL glomeruli to the adaptation stimulus with time from the onset until the point of no detectable responses*; considered as the point of physiological adaptation. Changes in calcium concentration were recorded 20 sec before the onset of the adaptation stimulus to capture the adaptation related events. Although, the individual bees were neither found to show the onset responses nor did they show the subsequent decline in the strength of

calcium responses over time to the adaptation stimuli (for both the colony odor and the synthetic odor mixture); however, the constant exposure for 20 min most likely adapted the glomerular responses to the background adaptation stimuli. Both adaptation stimuli increased the average response strength of the glomerular ensemble (pooled data of all glomeruli from all bees separately in the two adaptation experiments) to the set of 8-test odors during the time of adaptation compared to the initial un-adapted condition. For the analysis of individual glomeruli, 14 candidates were chosen which also showed the adaptation induced increase in the odor response strengths in most of the cases with both adaptation stimuli (e.g. Glomerulus 28 and 35 in the colony odor adaptation experiment; glomerulus 38, 42, 47 and 48 in the adaptation experiment with the synthetic odor mixture). However, decrease in the odor response strength of the AL glomeruli during the adaptation was also found with both types of background adaptation stimuli (Glomerulus 42 and 17 respectively in the adaptation experiment with colony odor and with the synthetic odor mixture). Same glomerulus showed different patterns of changes in odor responses with the two adaptation stimuli as well as little more number of glomeruli was found to increase their odor response strength when adapted with the synthetic odor mixture than with the colony odor. The differential effects of two background adaptation stimuli on the odor response strength of the glomeruli probably indicated that these two background odor activated the different forms or pathways of olfactory adaptation in the glomerular network of the honeybee antennal lobe. These possibilities although were not investigated further in this study. Amongst the different test odors only three were found to show the common pattern of change in glomerular responses with both adaptation stimuli; floral odor 1-hexanol and the sting alarm pheromone odor isoamyl acetate showed the adaptation induced increase and 1-octanal showed the decrease in glomerular responses. However, for all of the test odors common increase in the linear distances between their glomerular representation patterns (quantified through the measurement of Euclidean distances) was found due to the introduction of background adaptation stimuli compared to their removal. Increase in Euclidean distances brought about by the olfactory adaptation and its persistence even after the removal of the adaptation stimuli clearly showed that prolong exposure of the AL glomeruli to the habitat odor of honeybee (colony odor) or the mixture of the pure odorants enhanced the specific and stable forms

(although unknown) of odor discrimination in the glomerular odor coding space. At the end, it was concluded that olfactory adaptation of the honeybee antennal lobe with the odor mixture changed the glomerular response strengths and the representation patterns of odors which together indicated the change in the odor coding scheme of the antennal lobe neuropil.

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Chapter -2

Investigating the potential differences in olfactory learning between the hygienic and non-hygienic honeybees

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2.1 Abstract

Honeybees breed for the higher resistance against the *Varroa* mite or the hygienic bee lines can detect and remove the *Varroa* parasitized brood early and more frequently than the less *Varroa* resistant or the non-hygienic bee lines. The presence of *Varroa* must be detected by bees through the perception and recognition of the chemical signals emanating from the infected brood cells. Olfactory signals can be one of the possible candidates. In this study it was tested whether hygienic bees can recognize the odors associated with the *Varroa* infection better than the non-hygienic bees which possibly contributes to the better hygienic behavior of these bees against the *Varroa* mite. When conditioned differentially in the olfactory PER paradigm, bees of the hygienic line were found to discriminate better or generalized less between the odor bouquets of the healthy and the *Varroa* infected pupae than bees of the non-hygienic line. However, bees from both genetic lines were failed to discriminate between the volatile odor profiles of the wax caps isolated from the healthy and the *Varroa* parasitized brood cells. Apart from the differences in learning of the *Varroa* associated odors, these two lines showed differences when the general olfactory learning was tested with the floral and pheromonal odors. Bees from the hygienic and non-hygienic lines learned the alarm pheromone component, isoamyl acetate similarly both under the normal and adapted condition. However, hygienic bees showed significantly less generalization in both conditions than the non-hygienics when the effect of odor generalization was tested with an untrained odor during the multiple retention tests. Non-hygienic bees were even failed to learn the discrimination between the floral volatiles, geraniol and 1-hexanol in the differential conditioning paradigm. Honeybees in general were known to discriminate between these two odors in the PER paradigm. Hence, the poor learning performance of the non-hygienic bees precluded any conclusion about the possible differences in olfactory learning between the two genetic lines; however, better discrimination of the pupal odors by the hygienic bees provided the important clue about the possible use of olfactory signals for the detection of *Varroa* mite.

Author's contribution: This is a manuscript which will be submitted for publication in an international peer reviewed journal. Please refer to page number iii of this dissertation for details about the author's contribution.

2.2 Introduction

Honeybee colonies are populated mainly with the workers, performing bulk of the behavioral tasks which are required both inside and outside of the colony (Seeley 1985; Winston 1991). Worker bees start to switch from performing the set of intranidal tasks of taking care of the queen, brood, food reserve and cell cleaning into the more outside-task of foraging as they grow older (Seeley and Kolmes 1991). Inside the honeybee colony the whole set of tasks are allocated with exquisite precision amongst the hive comrades depending on their age. However, bees of the same age can display behavioral plasticity in their temporal task performances, which is governed by their genetic make-ups, social interaction with the hive mates and local demand of the colony (Page and Robinson 1991; Robinson 1992; Calderone and Page Jr 1991; Calderone and Page 1988, 1992). The phenomenon of task partitioning of the honeybee colony has been explained with the response threshold model, according to which individual bees start to perform certain task if the threshold of the task-driving stimulus exceeds the detection threshold of the individuals (Robinson 1992; Arathi and Spivak 2001; Page 1997; Arathi and Spivak 2001). Like the behavior of task allocation, it was reported previously that hygienic behavior of honeybee colony can also be explained with the response threshold model (Masterman *et al.*, 2001). This is an important behavior which determines the degree of disease resistance and hence the survival probability of the colony while facing the pathogenic challenges. The term hygienic behavior was originally coined by Rothenbuhler to define the genetic ability of the worker bees to detect, uncap and remove the abnormal or diseased larvae (Fig. 1; pictorial representation) from the nest (before the pathogen becomes virulent) to stop the dissemination of infection early (Woodrow and Holst 1942; Rothenbuhler 1964). Hygienic behavior was reported to consist of two task components- first 'uncapping'; the act that begins during the time of inspection of the brood health by the nurses or foragers, with a small whole made through their mandibles on top of the wax cap of the brood cell with the suspicion of abnormality, following the second task of 'removal' of the brood if it shows any disease symptoms (Rothenbuhler 1964). Due to the limited number of immune effector genes found in the honeybee genome the hygienic behavior is considered to be one of the most important social or group immune responses to cope up with the brood diseases (Evans *et al.*, 2006). The

importance of this behavior was further understood form the report that 15 – 20 days old bees (younger than the typical foragers) are also able to show this behavior inside the colony (Goode *et al.,* 2006). Hygienic behavior was previously studied at the colony level to understand the temporal dynamics of the uncapping and brood removal behaviors as well as for the purpose of selective breeding of these traits against the various brood diseases (Palacio *et al.,* 2000; Palacio *et al.,* 2005; Spivak and Gilliam 1993; Spivak and Reuter 1998). Investigations of this kind evaluated the efficiency of this behavior against the different brood diseases, however, did not provide any information about the behavioral resistance mechanisms of the individual bees which collectively contribute to the hygienic behavior of the whole colony. Research initiatives, a decade back started to investigate the behavioral and physiological mechanism(s) of the hygienic behavior of the individual bees as opposed to the whole population. In this context, Marla Spivak's group of the department of Entomology, University of Minnesota, USA contributed substantially as they showed that honeybees utilize the olfactory cues to detect the parasitized brood to elicit the brood-removal or hygienic behavior against the chalkbrood infestation (Masterman *et al.,* 2000; Masterman *et al.,* 2001; Gramacho and Spivak 2003; Swanson *et al.,* 2009). Masterman and colleagues found that honeybees from the different lines which were breed for the higher and faster removal of the freeze-killed brood (the hygienic lines) were also able to learn and discriminate (in the olfactory PER conditioning paradigm) between the volatile odor bouquets of the healthy and the chalkbrood infested pupae significantly better than the bees of the non-hygienic (slow removers of the freeze-killed brood) lines (Masterman *et al.,* 2000). Hygienic bees were also found to show lower response threshold and higher sensitivity to the diseased brood odors compared to bees of the non-hygienic lines (Masterman *et al.,* 2001). Furthermore, it was found that even bees from the same hygienic colony showed variability in their olfactory sensitivity to the brood odors (Gramacho and Spivak 2003). Hygienic bees collected during the time of uncapping (the 'uncappers') were found to show significantly higher sensitivity to the lower concentrations of the diseased brood odors (chalkbrood infection) than the hygienic bees collected while performing the task of removal (the 'removers'). These highly sensitive 'uncappers' were also able to discriminate significantly better between the volatile odor profiles of the healthy and the chalkbrood

infested pupae in the differential olfactory PER conditioning paradigm compared to the 'removers'. However, the superior olfactory sensitivity and discriminability of the hygienic bees compared to the non-hygienic bees were not found to encompass the naturally occurring floral odors. Masterman and colleagues found the similar olfactory learning and discriminability between their hygienic and non-hygienic bee lines while the bees were trained with the high concentrations of the floral odors, 1-hexanol and geraniol (Masterman *et al.,* 2000). Hence, the superior sensitivity and discriminability of the hygienic bees was thought to be limited to the odors related to the health status of the brood which can facilitate the early detection and removal of the abnormal brood to confer the disease resistance. However, a direct link between the olfactory recognition of the abnormal brood and the colony-level manifestation of the hygienic behavior was missing until the report by Swanson and colleagues (Swanson *et al.,* 2009). The authors in this study found the expression of multiple volatile compounds (phenethyl acetate, 2-phenylethanol and benzyl alcohol) in the chalkbrood infected larvae which were not expressed (qualitative) by the healthy larvae. Subsequently, phenethyl acetate amongst the other compounds was also found to elicit the larval removal (hygienic) behavior inside the hygienic colonies while the pure compounds were tested on the healthy larvae in the field bioassay. This evidence confirmed the involvement of olfactory cues as one of the chemical signals to evoke the hygienic behavior in honeybee against the chalkbrood pathogen *Ascophera apis*. Similar to the chalkbrood disease, honeybees also

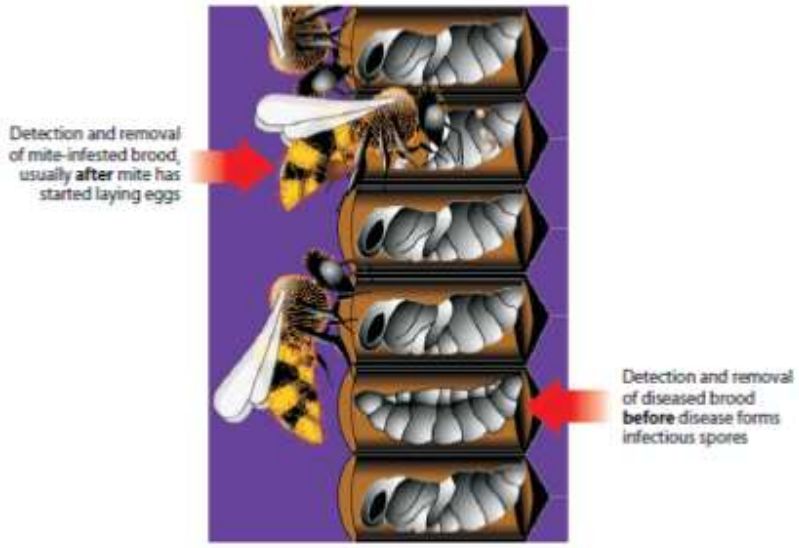

Fig. 1: Pictorial representation of the hygienic behavior in honeybee colony: Worker or nurse honeybees are detecting and removing the fungal, bacterial or *Varroa* mite infected brood from the nest before the dissemination of the disease (picture taken from the Annu. Rev. Entomol. 2009. 54, 405-423; Rich NW, Spivak M, Fefferman NH and Starks PT).

employ the same hygienic behavior to resist the common infestation of the American foulbrood bacterium *Paenibacillus larvae* (Spivak and Reuter 2001a; Spivak and Gilliam 1989a, 1998b). However, a direct link between the olfactory recognition of the foulbrood infected larvae and the hygienic behavior was never reported, although the conspicuous foul smells of the foulbrood infected combs suggest the possible connection between them. Hence, olfactory recognition of the diseased brood seemed to be a common behavioral mechanism eliciting the hygienic behavior against the different types of brood diseases. The same hygienic behavior as well as the grooming behavior was also reported to act as the resistance mechanisms against the threating ectoparasitic mite *Varro destructor* Anderson & Trueman (Peng *et al.*, 1987; Boecking and Spivak 1999).

Varroa destructor was found as the natural parasite of the Asian honeybee *Apis cerana* (Anderson and Trueman 2000) but the mite switched the host to the Western honeybee *Apis mellifera* probably in early 1960s during the incidence when the imported *A. mellifera* colonies in Philippines came into contact with the infected colonies of *A. cerana*. The life cycle of the adult female mite consists of two phases, the phoretic and the reproductive phase. During the phoretic phase the mite attaches itself in between the abdominal segments and feed on the haemolymph (blood) of the adult bees. This phase ends with the beginning of the reproductive phase which lasts for 10 days as the adult female mite leaves the mature bee and enters into the larval cells before the cells are capped by the attending bees. Within the next days the female foundress mite produces many offspring which finally mature and leaves the brood cell along with the emerging adult bee. During the reproduction cycle the foundress mite feeds on the haemolymph of the developing bee which compromises the fitness of the newly emerging adult. These parasitized bees were reported to show the abnormal development of their wings and body and died soon after the maturation (Yang and Cox-Foster 2007) or even cannibalized by the adult workers at the pupal stage (Shimanuki *et al.*, 1994). In addition, certain fraction of the infected brood which developed normally into the mature foragers

were reported to show the significantly compromised olfactory learning and foraging abilities compared to the uninfected hive comrades (Kralj *et al.,* 2007; Kralj and Fuchs 2004). Hence, unlike the *A. cerana* (original host of the *Varroa* mite), *A. mellifera* do not possess any effective resistance against the *Varroa* parasitization. Infestation of this parasitic mite can cause the alarming outbreak in the *A. mellifera* colonies namely varroosis, which probably is one of the important reasons of the recent time losses of the European and American colonies in high number. The over winter massive losses of the honeybee colonies in Europe and in other parts of the world are not only affecting the economy of the bee keeping industry but also threatening the pollination of the important crops and flowers (Potts *et al.,* 2010; Ratnieks and Carreck 2010). Viral pathogens such as the deformed wing virus (DWV), acute bee paralysis virus (ABPV), Israeli acute bee paralysis virus (IAPV) and the Kashmir bee virus (KBV) were reported to play an important role for this large scale loss of the honeybee colonies (Genersch and Aubert 2010; Potts *et al.,* 2010; Ratnieks and Carreck 2010). Additionally, *Varroa destructor* was found to transmit these viral pathogens vectorially (Ball 1989; Bowen-Walker *et al.,* 1999; De Miranda and Genersch 2010; Chen *et al.,* 2004; Di Prisco *et al.,* 2011; Yue *et al.,* 2007; Gisder *et al.,* 2009) and at least the DWV and ABPV were reported to become virulent and caused the overt infections only when they were transmitted by the *V. destructor* (De Miranda *et al.,* 2010; Genersch and Aubert 2010). Throughout the last decade the bee keeping industry in the USA suffered the devastating colony losses due to the phenomena of colony collapse disorder (CCD). *Varroa* parasitism is suspected to be one of the potent reasons of the CCD although no concrete connection is established. No strict clinical cases of the CCD was found in the Europe until now, although the drastic rise in the number of colony losses in Europe and worldwide indicate the possible connection with the *Varroa* infestation apart from the contributions of the other pests, pathogens (Ratnieks and Carreck 2010). Selective breeding programs in the USA (Spivak 1996; Spivak and Reuter 2001b) as well as in Europe (Büchler *et al.,* 2010) in recent time produced multiple genetic lines of the *Apis mellifera* which were reported to have higher resistance against the *Varroa* pathogenesis compared to the non-selected lines. These resistant honeybee lines are commonly known as the hygienic lines with the genetic background to perform the uncapping and removal behavior more efficiently than the

non-selected and less-resistant or non-hygienic lines (Rothenbuhler 1964; Moritz 1988). Development of the hygienic honeybee lines with the genetic (natural) defense mechanisms against the ectoparasitic *Varroa* mite is important for both the long-term goals of the bee keeping industry as well as to avoid the health hazards of the toxic chemical treatments in bees and in the human consumers of the colony products. The resistance mechanisms of the American hygienic colonies although were not clearly known, however, it was found that colonies breed for the hygienic behavior against the commonly encountered brood diseases such as the chalkbrood and the American foulbrood also showed higher resistance against the *Varroa destructor* (Ibrahim and Spivak 2006). Hence, it was hypothesized that like other brood diseases honeybees can perceive the *Varro*a infestation using the olfactory cues of the parasitized brood or the foundress mites. Reports from the different research groups, however, were controversial about the source of the key olfactory stimuli eliciting the hygienic behavior against the *Varroa* mite. Le Conte's group reported that volatile chemical mixtures present on the cuticle of the foundress mite were mostly distinct from the volatiles of the healthy and the parasitized pupae (Martin *et al.,* 2002). Polar compounds (some acids and esters) of the cuticular fractions of the foundress mites were reported as the possible interesting candidates since the *Varroa*-resistant honeybees showed higher olfactory sensitivity to these chemical moieties. The authors suggested that honeybees preferably use these polar volatile compounds of the *Varroa* mite to detect the parasitized brood present inside the capped cells. However, the authors neither tested the potential of these volatile chemicals to elicit the brood removal behavior in the colony (field bioassay), nor they showed that resistant bees were more sensitive or able to discriminate better between these odor cues than bees of the non-resistant lines. However, other field bioassays excluded the possibilities of mite-volatiles or the movements of foundress mites inside the capped cells as the potential stimuli to elicit the hygienic behavior in the Africanized and Carniolan honeybee colonies (Aumeier 2001). At this point the obvious candidate left to be tested was the parasitized brood itself, which Nazzi and colleagues showed to emanate the crucial volatile chemicals which were able to elicit the brood removal behavior inside the hygienic colony (Nazzi *et al.,* 2004). The authors of this study found the expression of multiple unsaturated hydrocarbons such as the pentadecenes (both Z-(6) and Z-(7)

isomers) and heptadecenes in higher amount (quantitative) in the volatile profiles of the intact *Varroa* infected brood cells compared to the uninfected cells. In the subsequent bioassay they also found that at least the isomer, Z-(6) pentadecene was able to elicit the removal of the chemically treated brood from the hygienic colonies significantly higher than the solvent (hexane) controls. Although, they never compared the brood removal behavior between the hygienic and non-hygienic colonies, however, this evidence strongly suggested that like other brood diseases, hygienic bees also utilize the olfactory recognition mechanisms to detect the *Varroa* infestation in the colony. In recent time Schöning and colleagues effused more lights regarding the possible vital role of the olfactory cues to recognize the *Varroa* infected brood (Schöning *et al.*, 2012). The authors of this study found that brood removal from the hygienic colonies was dependent on the extent of damage inflicted on the brood during the process of *Varroa* infestation. Honeybee broods infected artificially with the *Varroa* mites harboring the virulent forms of the deformed wing virus (DWV) with the potential to cause overt infection in brood, were removed in significantly higher number compared to the broods which were either uninfected or parasitized with the mites carrying the non-virulent DWV strains with the potential to cause the covert infection. They also found that volatile odor profiles of the brood infected with the mites with high viral load (virulent DWV) had quantitative differences compared to the other two brood types such that the former contained the compounds like acetoin, isovaleric acid and 2, 3-butanediol in significantly higher amount (some of these compounds were known to be associated with the bacterial spoilage). These results for the first time showed the direct involvement of the viral infection to dictate the fate of the *Varroa* pathogenesis and again indicated the important roles of the volatile odor cues emanated from the parasitized brood to elicit the brood removal behavior in the hygienic honeybee colonies. Other mechanism such as the contact chemoreception elicited by the surface texture of the wax caps of the infected brood cells might also contributed to the results of these field assays, which were not tested; however, the roles of the olfactory chemoreception were more apparent. Additionally, the *Varroa* tolerant or hygienic line bees compared to the sensitive or non-hygienic bees were reported to show the up-regulation in expression levels of the genes involve with the processes of olfaction, neuronal excitability and the neuroblast

proliferation of the mushroom body; associated with olfactory learning (Navajas *et al.*, 2008). The early evidences were able to make the connection between the recognition of *Varroa* by the hygienic bees using the olfactory signals released from the infected brood cells. The result of the gene expression study on top of this strongly indicated the possible superior olfactory perceptibility or learning ability of the *Varroa* tolerant bees compared to the bees of sensitive line which contributes to the better hygienic behavior of the tolerant line against the *Varroa* mite. One interesting point found in the data of Caspar Schöning was, that majority of the broods infected with the high viral-load mites developed normally apart from the few which showed the apparent signs of spoilage (discolored, disfigured with foul smell). This probably indicates the adaptation strategy of the *Varroa destructor* for the successful pathogenesis over the host *Apis mellifera*, where the workers are only able to perceive the presence of the mite inside the sealed brood cells if the brood develops the clear symptoms of spoilage due to an overt or virulent DWV infection.

2.3 Goals of the study

Several studies in honeybee although indicated the involvement of the olfactory signals to elicit the hygienic behavior against the *Varroa* mite (Nazzi *et al.*, 2004; Schöning *et al.*, 2012); however, these studies only used their more-resistant or hygienic bee lines but never compared the brood-removal behavior between the hygienic and the non-hygienic honeybee lines. Hence, the piece of evidence was missing which tested these two types of honeybees for their abilities to recognize the odors associated with the *Varroa* infection such as the volatile odors emanate from the infected hive materials (infected brood or wax pieces from the infected brood cells). Possible superior performance of the hygienic bees than the non-hygienic bees can indicate the important contribution of the olfactory chemoreception processes in the higher behavioral resistance of the hygienic bees against the pathogenic mite. This issue was investigated in this study through the conditioning of honeybees from the behaviorally more (hygienic) and less resistant (non-hygienic) genetic lines in the olfactory PER paradigm. Bees from the two genetic lines were trained to discriminate between the volatile odor profiles of the healthy and the *Varroa* infected

pupae or the wax caps isolated from these two types of the pupal cells and their performances were compared. These two genetic lines used were delivered from the honeybee institute of Hohen Neurndorf (Länderinstitut für Bienenkunde, Hohen Neuendorf), Berlin. One of the two genetic lines were breed for the higher (hygienic) and the other for the lower (non-hygienic) level of behavioral resistance (uncapping and removal of parasitized brood) against the ectoparasitic mite *Varroa destructor* since 1997 (Boecking *et al.,* 2000; Ehrhardt *et al.,* 2006; Garrido *et al.,* 2005). Additionally, the possible differences in the general olfactory learning and memory performances of the two lines were also checked with the absolute and differential olfactory PER conditioning paradigms using the high concentrations of the floral and pheromonal odors. Masterman and colleagues although previously found no difference in the floral odor learning between their hygienic and non-hygienic honeybee lines (Masterman *et al.,* 2000), however, it was imperative to clarify this issue with the two genetic lines that were used here in the experiments.

2.4 Materials and Methods

2.4.1 Preparing honeybees for the olfactory PER conditioning

The general procedure of preparing the honeybees for the olfactory PER conditioning was explicitly written in the previous articles (Bitterman *et al.,* 1983; Menzel 2001; Stollhoff *et al.,* 2005). Here, I used the same protocol with the minor changes. Honeybee colonies of the two genetic lines (hygienic and non-hygienic) were placed in the institute's bee-garden between the month of July and October 2009 for the behavioral experiments. Forager bees were caught at the entrance of the hives during the afternoon, around 16.00 hours; the day before the experiment, taken to the laboratory and were anesthetized on ice to fix them in the small plastic tubes. Only, the antennae and mouthparts such as mandibles, proboscis and antennae were allowed to move freely with rest of the animal's body fixed within the tube with a sticky tape. Equal number of bees from both types of colonies was used during each round of the experiment, to compare their performances afterwards. In the evening, around 18.00 hours all bees were fed with the 30% (W/V) sucrose solution (0.87 M) and were satiated. Bees were kept inside the

small, dark and humid (~ 24^{o}C) Styrofoam box for overnight. The very next morning bees were taken out form the box and placed in the experimental arena at least 30-45 min before (to adapt the bees with new surrounding) the beginning of the olfactory PER conditioning.

2.4.2 Olfactory PER conditioning protocols

General information about the differential olfactory PER conditioning is given in chapter-3. During the differential conditioning bees were conditioned with the rewarded (CS+) and the unrewarded (CS-) odors for a total of 12 trials (6 CS+ and 6 CS- conditioning trials) with the alternate presentations of the two stimuli (not pseudoradomized). A constant amount of 10 µl. of pure odors soaked on a piece of filter paper (1 cm^2) was used as the stimulus both during the conditioning trials and the memory retention tests. Olfactory conditioning in all experiments was performed with an already established odor delivery protocol (Stollhoff *et al.*, 2005) where the odor stimulus (CSs) was manually delivered from the 20 ml. volume syringe for 5 sec. During the reinforced CS presentation (CS+) the sucrose reward (30% sucrose solution) was offered to the animal by first touching the antenna with the sucrose solution to elicit the PER, followed by feeding though the proboscis. The US was presented 3 sec after the onset of the CS (total time of the CS+ trial was 7 sec) for the total time of 4 sec with an overlap of 2 sec between the CS and the US. The unreinforced CS or CS- trials lasted for 5 sec where only the odor was presented without any US. Bees were placed in front of an exhaust for 20 sec before and 20 sec after the odor trials (otherwise mentioned). Honeybees, after the conditioning trials and the mid-term memory retrieval test were feed with the same 30% (W/V) sucrose until satiation and kept overnight for the memory retention test performed on the next day (day-2). At the end of the retention test on day-2, PER responses of the bees were checked by touching the antennae with the sucrose (without feeding) and only the performance data of the bees were incorporated into the analysis which showed the PER until this point of the experiment.

Differential conditioning using the wax caps as the stimuli was performed with the inter-stimulus interval (ISI: the time interval between the dissimilar CS trials; between the CS+

and CS- trials) of 15 min and inter-trial interval (ITI: the time gap between the similar CS trials) of 30 min. Fifteen freshly isolated wax caps from the brood cells kept at a constant temperature of 37^{o}C were used as the odor stimulus. Two types of wax caps were used in this experiment, one type was isolated from the healthy brood cells and the other type was collected from the brood cells artificially infected with the *Varroa* mites for 7 days. However, reproduction status (presence or absence of multiple offspring) of the foundress mites was checked during the isolation of wax caps and wax caps only from the parasitized brood cells with the reproducing mites were used in the experiment. Memory retention tests were performed twice after 2 hours and 1 day of the differential conditioning using the identical doses of the two types of wax caps.

Differential conditioning with the pupae was performed with the ISI and ITI of 14 min and 21 min respectively. For the pupae experiment bee larvae were artificially infected (brood cells were opened with the fine needle and mites were introduced with the help of a soft brush) with the three foundress mites. The idea of using three in place of one mite was to raise the level of the brood-damage inflicted by the *Varroa* mites for the purpose of producing in sufficient amount, the disease-associated volatile chemicals for the PER conditioning experiments. The infected and control or un-parasitized brood cells were kept inside the incubator at 37^{o}C for 7 days before the pupae were removed and used for the experiment. During the experiment 10 infected and un-parasitized (and healthy) pupae were taken out of the brood cells (cells were previously marked on top of a transparent sheet), carefully placed without damaging their skin inside the 12 ml. volume syringes and used as the stimuli. Used batches of the stimuli were periodically replaced with the fresh stock of pupae during every round of the differential conditioning. Importantly, the parasitized pupae which supported the mite reproduction were only used for the conditioning. However, during the isolation only a few of the infected pupae were found to show the obvious symptoms of spoilage (black colored, disfigured and malodorous pupae), but majority did not show any apparent abnormalities, even though they were infected with the three foundress mites. Memory retrieval tests were performed twice after 1 hour and 1 day of the differential conditioning using the fresh stocks of the healthy and the *Varroa* infected pupae (same developmental stage as used for conditioning).

Differential conditionings with the pure odors were performed using the constant amount (10 µl.) of fresh odors with all stimuli delivered through a custom built olfactometer. Differential conditioning with the geraniol (Aldrich, purity: 98%) and 1-hexanol (Sigma Aldrich, 98%) was performed using the same ISI of 14 min and ITI of 21 min. However, conditioning with 1-octanol (Roth, purity: 99.6%) and 1-hexanal (Sigma, Germany) was conducted with the ISI of 10 min and ITI of 20 min.

Absolute conditioning experiments were performed using the alarm pheromone odor, isoamyl acetate (IAA; purchased from Sigma, Germany) with the inter-trial interval of 30 min. Memory retention tests were conducted after 1 hour and 1 day of the odor training. During the memory retention bees were presented with the novel or untrained odor (1-hexanal) along with the conditioned odor, IAA to test for the effect of olfactory generalization. Responses to the stimulus like filter paper were also recorded during tests.

Absolute conditioning with the isoamyl acetate was performed again but this time prior to the odor conditioning; bees were adapted behaviorally with the background odor of the honeybee colony. The previous experiment was conducted with the background condition of the laboratory, but this time additional background odor stimulus was used on top of the laboratory background. Honeybees often were found to extend their proboscis while exposed to the colony odor, probably due to the dominant odor cues of the honey and wax. With an exhaust fan, the air inside the colony was sucked and delivered to the bees continuously until they stopped showing the PER and / or stoppage the directed movement of their antenna towards the adapting stimulus. This particular point was considered as the point of behavioral adaptation when the bees were conditioned with the IAA. Behavioral adaptation was achieved within 3-4 min after the onset of the adaptation stimulus and bees were conditioned under adapted condition with isoamyl acetate using the inter-trial interval of 60 min. Honeybees were conditioned with 4 training trials and were tested twice (the same set of bees) after 1 hour and 1 day of the conditioning. During the memory retention tests bees were first adapted with the colony odor and then were tested sequentially with the presentation of the three CS stimuli: CS+, CS new (1-hexanal), and the filter paper. Like the previous experiment effect of olfactory generalization was tested with the new odor 1-hexanal along with the conditioned odor, IAA and the filter paper.

The overall learning and memory performances of bees were represented with the group-averaged conditioned responses to the rewarded (CS+) and unrewarded (CS-) odors during the conditioning and memory retention tests. Repeated measurement ANOVA and chi-square tests were performed to analyze the PER data respectively for the differential and absolute conditioning experiments.

2.5 Results

2.5.1 Differential conditioning using the wax caps isolated from the healthy and the Varroa infected brood cells

Nazzi and colleagues reported the important voltiles (pentadecene isomers) from the intact *Varroa* parasitized brood cells which were able to elicit the brood removal behavior inside the hygienic colonies (field bioassay: Nazzi *et al.*, 2004). This meant that volatile compounds emanated from the body of the parasitized brood were able to impregnate and released through the wax matrix of the brood cells which eventually were detected in the chemical analysis. Hence, in the first experiment it was investigated whether bee's form the two genetic lines were able to discriminate between the volatile

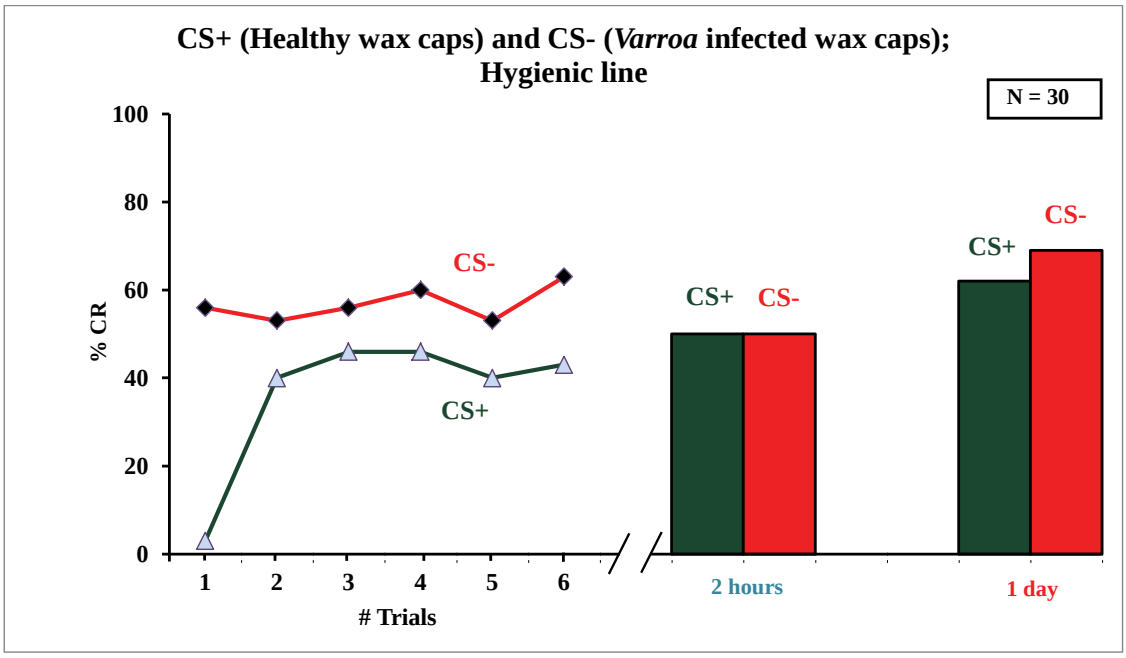

Fig. 2a: Learning and memory performances of bees of the hygienic line in the differential conditioning using the wax caps of the healthy brood cells as the CS+ and from the *Varroa* infected brood cells used as the CS-: The first set of line graphs in the figure showed the conditioned responses (y-axis represented the percent conditioned response or % CR) of the hygienic bees to the CS+ (dark green line graph: wax caps collected from the healthy brood cells) and CS- (red color line graph: wax caps isolated from the *Varro*a infected brood cells) stimuli during the conditioning trials (x-axis represented the number of conditioning trials; from 1-6). Hygienic bees generalized between the CS stimuli throughout the conditioning as well as during the memory retention tests (CS+: dark green bar and CS-: red bar) performed after 2 hours and 1 day of the conditioning (the bars after the line graphs). 'N' represented the number of hygienic bees used in the experiment.

odor profiles of the wax pieces sealing the healthy and the *Varroa* parasitized brood cells. The idea behind was that if bees can detect the disease-associated chemicals while attending the brood, then the pieces of wax caps isolated from the infected brood cells probably contain these compounds in sufficient amount that they can be discriminated in the olfactory PER conditioning paradigm. Fresh wax caps covering the healthy and infected brood cells were collected and heated at 37^{o}C for the constant release of the volatile chemical cues during the experiment. Results of the differential conditionings with all four combinations of the CSs viz. wax caps from the healthy brood cells as the CS+ and caps isolated from the *Varroa* parasitized brood cells as the CS- and *vice versa*

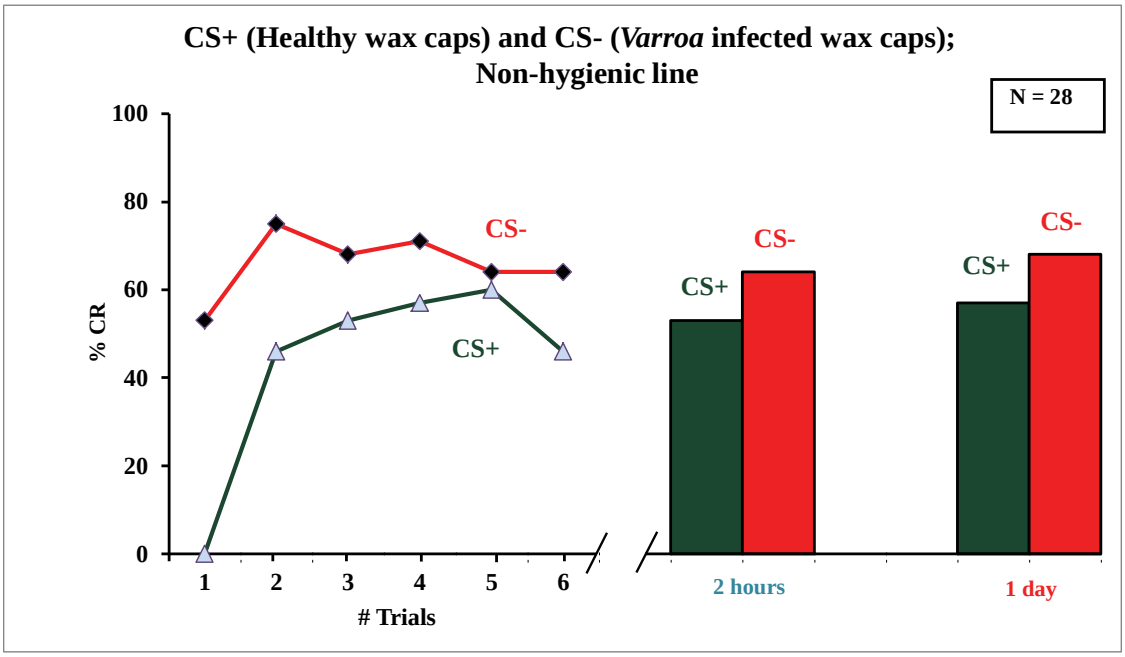

Fig. 2b: Learning and memory performances of bees of the non-hygienic line in the differential conditioning using the wax caps of the healthy brood cells as the CS+ and from the *Varroa* infected brood cells used as the CS-: The line graphs in the figure showed the conditioned responses of the non-hygienic bees to the CS+ (dark green line graph: wax caps collected from the uninfected and healthy brood cells) and CS- (red color line graph: wax caps isolated from the *Varro*a infected brood cells) stimuli during the conditioning trials. X and y axes in the figure represented the same variables as described in Fig. 2a. Non-hygienic bees like the hygienics generalized between the two stimuli during the conditioning as well as the memory retention tests (CS+: dark green bar and CS-: red bar) performed after 2 hours and 1 day of the conditioning (the bars after the line graphs). 'N' represented the number of non-hygienic bees used in the experiment.

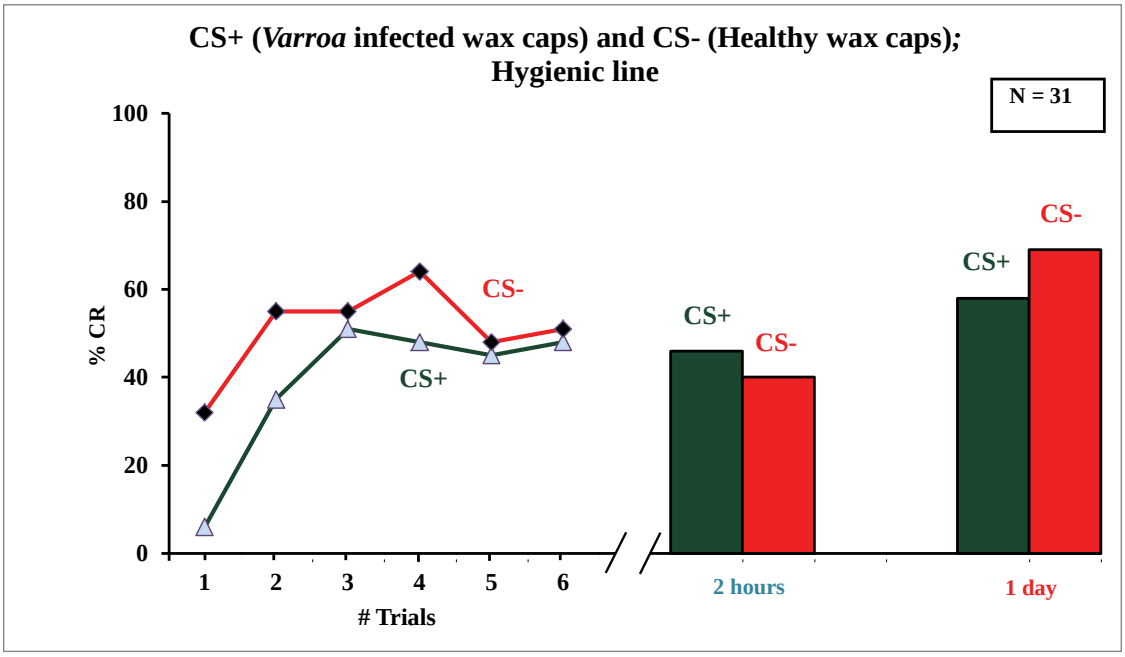

Fig. 3a: Learning and memory performances of the hygienic line in the differential conditioning using the wax caps of the infected brood cells as the CS+ and from the healthy brood cells used as the CS-: The line graphs in the figure showed the conditioned responses of the hygienic bees to the CS+ (dark green line graph: wax caps from the *Varro*a infected brood cells) and CS- stimuli (red color line graph: wax caps from the uninfected and healthy brood cells) during the conditioning. X and y axes represented the same variables as described in Fig. 2a. Hygienic bees like before generalized between the two stimuli during the conditioning as well as the memory retention tests (CS+: dark green bar and CS-: red bar) performed after 2 hours and 1 day of the conditioning (the bars after the line graphs).

showed the high generalization in conditioned responses (CRs) between the CSs with no discrimination showed by bees of the hygienic and non-hygienic lines (Fig. 2a, 2b, 3a and 3b). No statistical test was performed to find out whether the responses to the CS- stimuli were higher compared to the CS+; since the generalization in CRs to the volatile odor

profiles of the two types of wax pieces were clearly visible in all four experimental groups. The continuous release of the wax volatiles during the conditioning and the CRs of the experimental populations to the CS stimuli excluded the possibility that bee's only perceived the constant air flow of the olfactometer (although there was no air-only group tested in parallel).

Honeybees showed higher conditioned responses to the CS- compared to the CS+ stimuli throughout the differential conditioning in all experimental groups. These higher CS- responses probably appeared due of two reasons; bees generalized completely between the two CS stimuli during conditioning and conditioned with the alternate CS+ and CS- stimulus trials. Hence, complete generalization between the two CS stimuli resulted in little more number of responses during the CS- trials with the anticipation of receiving the sucrose US again like the preceding CS+ trial. Alternately, no US of the CS- trials reduced the conditioned responses during the following CS+ trial by little.

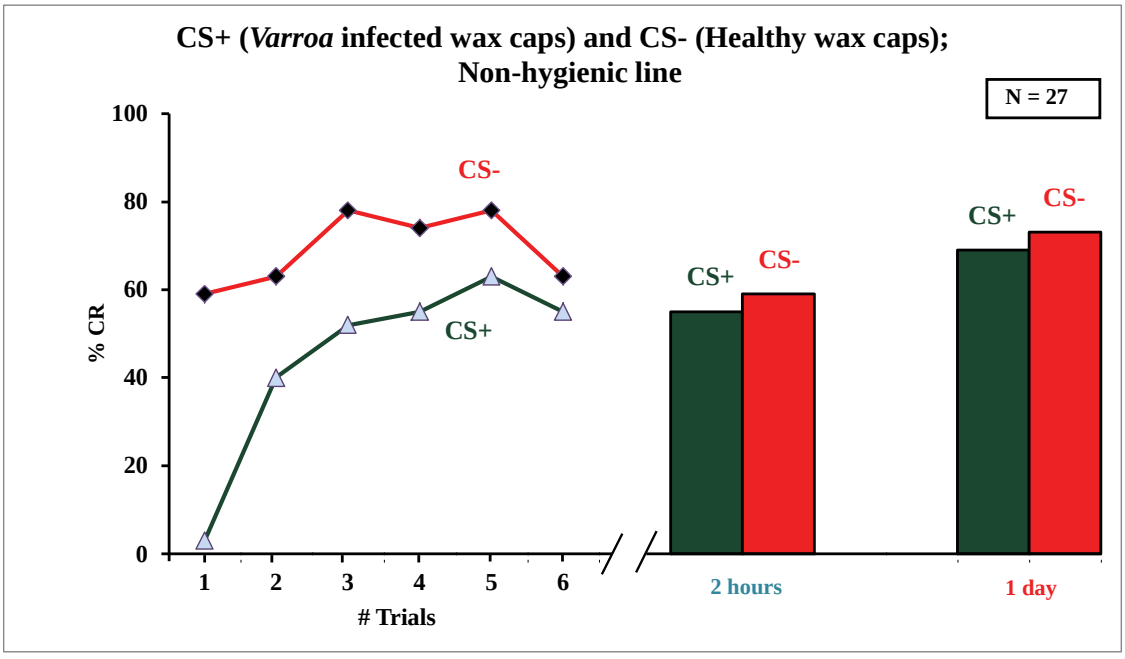

Fig. 3b: Learning and memory performances of the non-hygienic line in the differential conditioning using the wax caps isolated from the infected brood cells as the CS+ and from the healthy brood cells used as the CS-: The line graphs showed the conditioned responses of the non-hygienic bees to the CS+ (dark green line graph: wax caps isolated from the *Varroa* infected brood cells) and CS- stimuli (red color line graph: wax caps collected from the uninfected and healthy brood cells) during the conditioning trials. X and y axes in the figure

represented the same variables as described in Fig. 2a. Non-hygienic bees like before generalized between the two CS stimuli during the conditioning as well as the memory retention tests (CS+: dark green bar and CS-: red bar) performed after 2 hours and 1 day of the conditioning.

The reason for complete generalization in CRs to the two types of wax stimuli was not known. However, one possible explanation might be that presence of the common wax compounds in higher concentrations than the disease-associated chemicals in the volatile odor profiles of the infected wax caps probably overshadowed the perception of the disease-associated volatiles during the PER conditioning. This eventually led to the complete generalization in conditioned responses to these wax stimuli. Furthermore, unlike the study conducted by Schöning and colleagues, the levels of the replicating virulent forms of the deformed wing virus (DWV) in the infecting foundress mites were not checked in these experiments. The presence of the virulent DWV was the prime reason in Schöning's study that elicited the higher number of brood removal as well as elevated the production of the specific volatile chemical cues associated with the parasitization. Hence, conducting the same set of differential conditioning experiments as reported here with the wax caps isolated from the brood cells containing the overtly infected and damaged pupae might change the results. Interestingly, one previous study conducted by Feller & Bienefeld (unpublished results) found differences in the volatile chemical profiles (using the SPME-GC-MS analysis) of the cell caps of the *Varroa* infected and the uninfected brood cells. Together these, it was concluded that the lack of discrimination showed by the bees did not fully confirm the fact that odor profiles of the wax pieces sealing the healthy and the *Varroa* infected brood can't be discriminated in the PER assay.

2.5.2 Differential olfactory conditioning using the healthy and the Varroa infected pupae

The last experiment found that honeybees from the hygienic and non-hygienic lines were unable to discriminate between the volatile odor profiles of the wax caps isolated from the healthy and the *Varroa* infected brood cells. As the next step, honeybees from the two genetic lines were conditioned to learn the discrimination between the volatile odor bouquets emanated from the body of the healthy and the *Varroa* parasitized pupae.

Hygienic bees were able to discriminate between the two odor profiles during the differential conditioning when the *Varroa* parasitized pupae were used as the CS+ and the (un-parasitized) healthy pupae as the CS- (Fig. 4a). Repeated measurement-analysis of variance (RM-ANOVA) showed significant stimulus (CS+/CS-) × conditioning trial ($F(5, 530) = 2.67$, $p = 0.02$) effect as well as the significant trial effect ($F(5, 530) = 5.28$, $p = 0.00096$), although the stimulus effect was found non-significant ($F(1, 106) = 2.25$, $p = 0.13$). This showed that hygienic bees were able to learn the difference in contingencies between the two CS stimuli during the conditioning trials. During the memory retention tests performed after 1 hour and 1 day of the conditioning, hygienic bees showed the significantly higher responses (Fig. 4a) to the CS+ compared to the CS- stimuli (1 hour: Fisher LSD post hoc test: $p = 0.0005$, 1 day: Fisher LSD post hoc test $p = 0.01$). In comparison, the non-hygienic bees were failed to learn the discrimination (Fig. 4b) between the two stimuli both during the conditioning (RM-ANOVA: non-significant stimulus × trial effect $F(5, 410) = 0.75$, $p = 0.5$) and non-significant stimulus effect $F(1, 82) = 0.41$, $p = 0.52$) and the retention tests conducted after 1 hour (Fisher LSD post hoc test: $p = 0.17$) and 1 day (Fisher LSD post hoc test: $p = 1.0$) of the conditioning. When

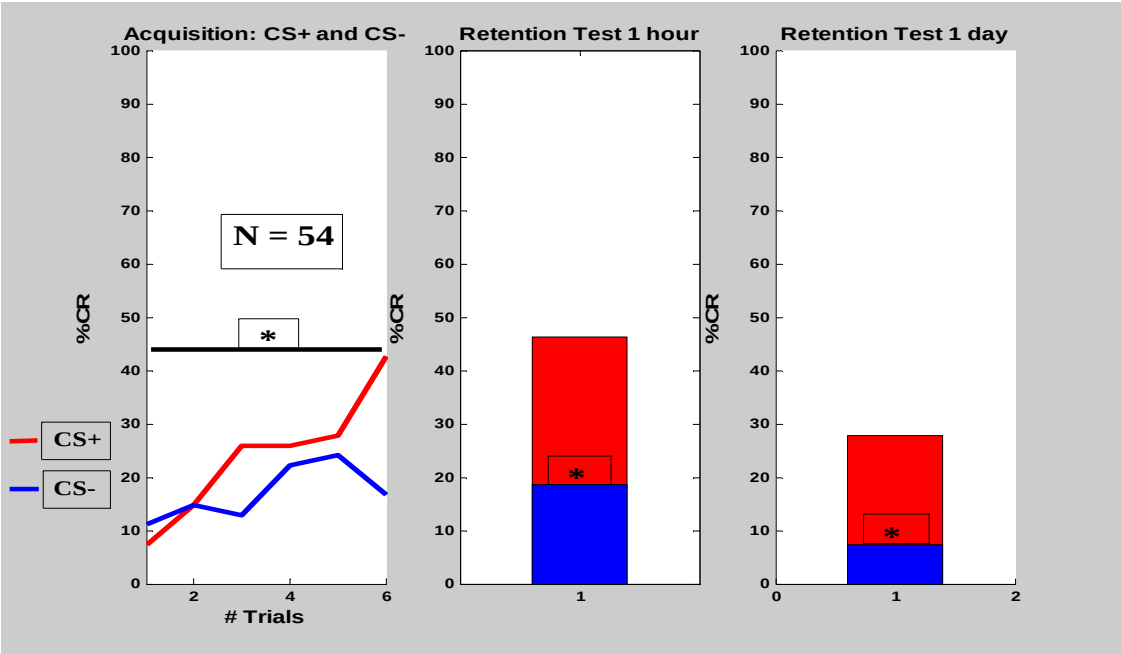

Fig. 4a: Learning and memory performances of the hygienic line in the differential conditioning using the *Varroa* parasitized pupae as the CS+ and un-parasitized pupae as the CS-: Hygienic bees were trained differentially with the volatile odors of the *Varroa* infected and the uninfected pupae in this experiment. The 1st sub-plot (line graphs) showed the percent conditioned responses (y-axis represented the percent conditioned response or CR) to the CS+ (red color line graph: *Varroa* infected pupae) and the CS- (blue line graph: healthy and un-parasitized pupae) stimuli during the conditioning trials (x-axis showed the number of conditioning trials; 6 trials). Hygienic bees were able to discriminate between the two CS stimuli during conditioning (significant differences between the CRs to the CSs together were denoted by the asterics inside the rectangular box on top of the black line). The percent CRs to the CS+ and CS- stimuli during the memory retention tests were represented together in the same bar graphs with the same two colors as used in line the graphs (red: CS+ and blue: CS-). Hygienic bees showed significantly higher responses (denoted by the asterix inside the rectangular box at the demarcation between the red and blue colors) to the CS+ (red part of the bars) compared to the CS- (blue part of the bars) during the retention tests performed after 1 hour (the 2nd sub-plot) and 1 day (the 3rd sub-plot) of the conditioning. 'N' represented the number of hygienic bees used in the experiment.

these two lines were trained and tested with the opposite combination of the CSs, hygienic bees were found to generalize highly between the CS+ and CS- stimuli during the conditioning (Fig. 5a). The RM-ANOVA showed the significant stimulus × trial effect ($F(5, 470) = 7.25$, $p = 0.000001$) due to the higher number of responses shown to the CS- stimulus; albeit the stimulus effect was found non-significant ($F(1, 94) = 1.99$, $p = 0.16$). The strong generalization effect was found to continue during the memory retention test performed after 1 hour of the conditioning (Fisher LSD post hoc test $p = 0.22$); however, during the 1 day retention test hygienic bees showed significantly higher responses to the CS+ compared to the CS- stimuli (Fisher LSD post hoc test: $p = 0.015$). On the other hand bees of the non-hygienic line were again found to generalize completely or failed to learn the discrimination between the volatile odor bouquets of the healthy (CS+) and the parasitized pupae (CS-) both during the conditioning and the memory retention tests (Fig. 5b). Significant stimulus × trial ($F(5, 410) = 2.75$, $p = 0.018$), stimulus ($F(1, 82) = 7.57$, $p = 0.0072$) and trial effects ($F(5, 410) = 2.83$, $p = 0.015$) found in the RM-ANOVA confirmed that non-hygienic bees responded significantly higher to the CS- compared to the CS+ stimuli throughout the conditioning. The strong effects of odor generalization were found continuously during the retention tests performed after 1 hour (Fisher LSD post hoc test: $p = 0.49$) and 1 day ($p = 0.36$) of the conditioning. In addition, non-hygienic bees also showed higher conditioned

responses to the CS- compared to the CS+ during the conditioning with odors of the pupae like before with the wax odors.

Hygienic bees showed high generalization or no discrimination between the volatile odors of the healthy and the diseased pupae when the contingencies of these two stimuli were reversed. Similar result was reported by Gramacho and colleagues, as they found that bees of their hygienic line were able to discriminate significantly better and generalized less between the pupal (healthy *vs.* diseased) odors when the volatile odors emanated from the chalkbrood infected pupae were used as the CS+ but not when the odors of the healthy pupae were used as the CS+ (Gramacho and Spivak 2003). *The asymmetric salience of the volatile odor bouquets of the healthy and the infected pupae can be a general scenario irrespective of the identity of the infection although the reasons are unclear.* However, in contrast to the chalkbrood or foulbrood diseases, *Varroa* parasitized broods often were found to develop normally without any signs of abnormality (Schöning *et al.,* 2012). In our experiments, majority of the parasitized brood developed normally except the few which showed the apparent symptoms of spoilage (discoloration, disfiguration and foul odors). Furthermore, the volatile odor profiles of the healthy and the *Varroa* parasitized pupae were reported to differ largely in the quantitative rather in the qualitative manner (Nazzi *et al.,* 2004; Schöning *et al.,* 2012).

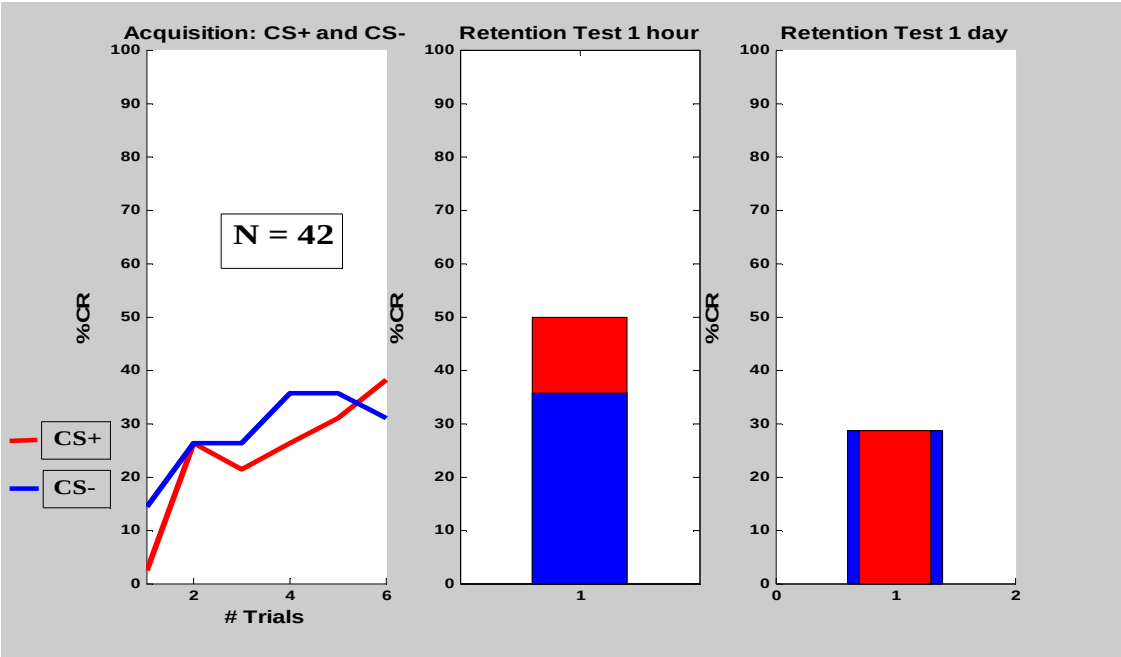

Fig. 4b: Learning and memory performances of the non-hygienic line in the differential conditioning using the *Varroa* parasitized pupae as the CS+ and un-parasitized pupae as the CS-: The 1st sub-plot showed the percent conditioned responses of the non-hygienic bees to the CS+ (red color line graph: *Varroa* infected pupae) and the CS- (blue line graph: healthy and uninfected pupae) stimuli during the conditioning trials. X and y axes in the figure represented the same parameters as described in Fig. 4a. Non-hygienic bees were unable to discriminate between the volatile odor profiles of the two types of wax caps during the conditioning as well as during the retention tests conducted after 1 hour (2nd sub-plot) and 1 day (3rd sub-plot) of the conditioning. The percent CRs to the CS+ and CS- stimuli during the memory retention tests were represented together in the same bar graphs with the same two colors used in the 1st sub-plot (red: CS+ and blue: CS-).'N' represented the number of non-hygienic bees used in the experiment.

Together with these factors, the possible (unchecked) low viral load in the parasitizing foundress mites used in our experiments probably contributed to the overall low levels of CRs of the experimental groups to the CS stimuli. The reasons for the higher odor generalization showed by the non-hygienic bees compared to the bees of the hygienic line although were not understood however; successful discrimination between the odors of the healthy and the parasitized pupae by the hygienic bees indicated that these *two honeybee lines differed in their olfactory learning abilities for the brood odors*. This also indicated the possible general scenario that hygienic bees can recognize the odors of the *Varroa* parasitized brood as abnormal or disease-associated signals which leads to the better hygienic behavior of these bees against the *Varroa* mite.

2.5.3 Absolute conditioning with the sting alarm pheromone odor, isoamyl acetate

PER conditioning experiments until now showed that bees form the hygienic line discriminated better or generalized less than the non-hygienic bees between the volatile odor profiles of the *Varroa* infected and uninfected brood, although both lines failed to discriminate between the odor bouquets of the wax caps isolated from the healthy and the parasitized brood cells.

Whether the better performance of bees of the hygienic line was specific for the brood odors used in the second set of experiments or these two genetic lines had general differences in olfactory learning were unclear. To test that, bees from both genetic lines were trained in the absolute conditioning paradigm with the sting alarm pheromone odor,

isoamyl acetate (also known as isopentyl acetate). The choice of this odor was based on the report that honeybees conditioned with the isoamyl acetate (IAA) showed higher odor generalization to the floral and other pheromonal odors than if they were conditioned with the floral odors (Sandoz *et al.*, 2001). This satisfied our idea to check for the potential differences both in olfactory learning and odor generalization processes between

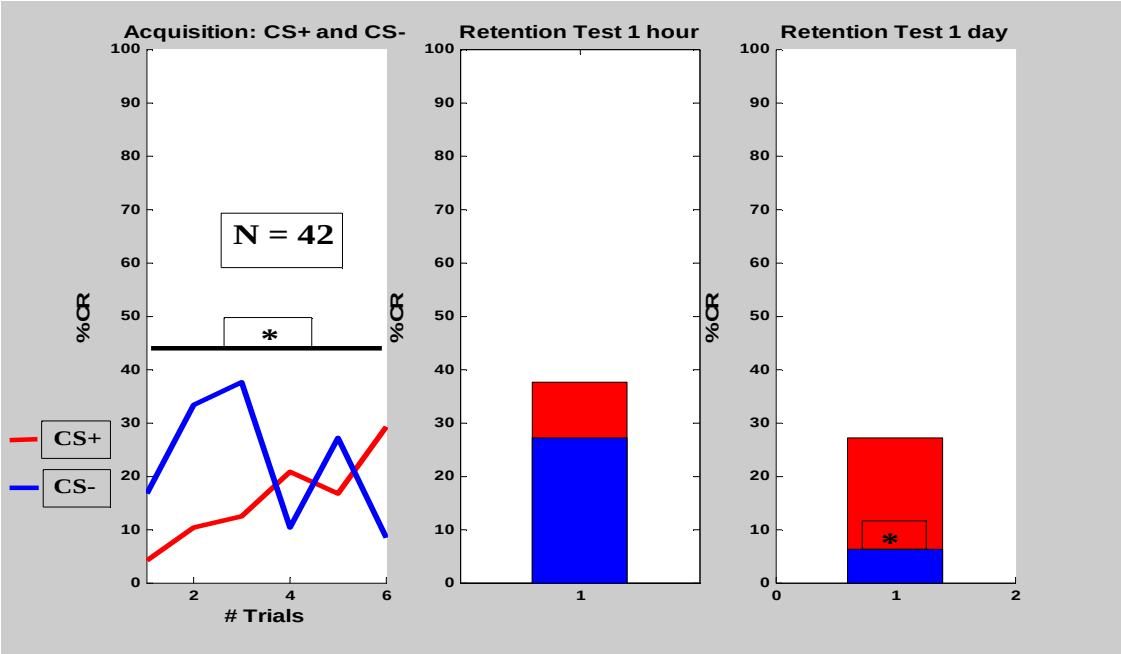

Fig. 5a: Learning and memory performances of the hygienic line in the differential conditioning using the un-parasitized pupae as the CS+ and *Varroa* parasitized as the CS-: Bees were trained differentially with the volatile odor profiles of the *Varroa* infected and uninfected pupae and the 1st sub-plot (line graphs) represented the percent conditioned responses (y-axis represented the percent conditioned response or % CR) to the CS+ (red color line graph: un-parasitized pupae) and CS- (blue line graph: *Varroa* parasitized pupae) stimuli during the conditioning trials (x-axis showed the number of conditioning trials; 6 trials). Unlike the last occasion (Fig. 4a), this time hygienic bees strongly generalized between the two CS stimuli during the conditioning. The overall significantly higher number of responses to the CS- compared to the CS+ was denoted by the asterix on top of the black line (results of RM-ANOVA). The generalization effect was found to continue during the 1 hour retention test (2nd sub-plot); however, bees showed significantly higher responses to the CS+ during the 1 day retention test (3rd sub-plot). Significant difference in the CRs (the 3rd sub-plot) was denoted by the asterix inside the rectangular box at the demarcation between the red and blue colors (red bar: CS+ and blue bar: CS-).

these two lines. Olfactory generalization was tested during the memory retention tests (1 hour and 1 day after conditioning) when the PER responses to the conditioned odor

(IAA) were compared with the responses to the untrained or new odor, 1-hexanal (CS new) and with the filter paper. Hygienic (Fig 6a) and non-hygienic bees (Fig 6b) conditioned in parallel with the IAA showed no significant difference in the conditioned responses during the four conditioning trials (χ^2 test: $p > 0.05$ for all 4-trials). However, during the retention tests performed after 1 hour and 1 day of the conditioning, hygienic bees (Fig. 6a) showed significantly lower responses both to the untrained CS, 1-hexanal (1 hour: $\chi^2 = 13.08$, df =1, $p = 0.02$; 1 day: $\chi^2 = 29.47$, $p = 5.6\times10^{-8}$) and to the filter paper (1 hour: $\chi^2 = 22.53$, $p = 2.06\times10^{-6}$; 1 day: $\chi^2 = 51.69$, $p = 6.4 \times 10^{-13}$) compared to the conditioned (CS+) odor, IAA. Hygienic bees also showed significantly higher responses to the natural odor 1-hexanal compared to the filter paper during both tests (1 hour: $\chi^2 = 7.5$, $p = 0.006$; 1 day: $\chi^2 = 6.54$, $p = 0.01$). Responses to 1-hexanal did not reduce significantly (between 1 hour and 1 day: $\chi^2 = 4.03$, $p = 0.06$) between the two retention tests, however, for the filter paper significant decrease in responses was found during the 2nd compared to the 1st retention test (between 1 hour and 1 day: $\chi^2 = 3.5$, $p = 0.06$). Additionally, the initial response level of the hygienic bees to the filter paper was also

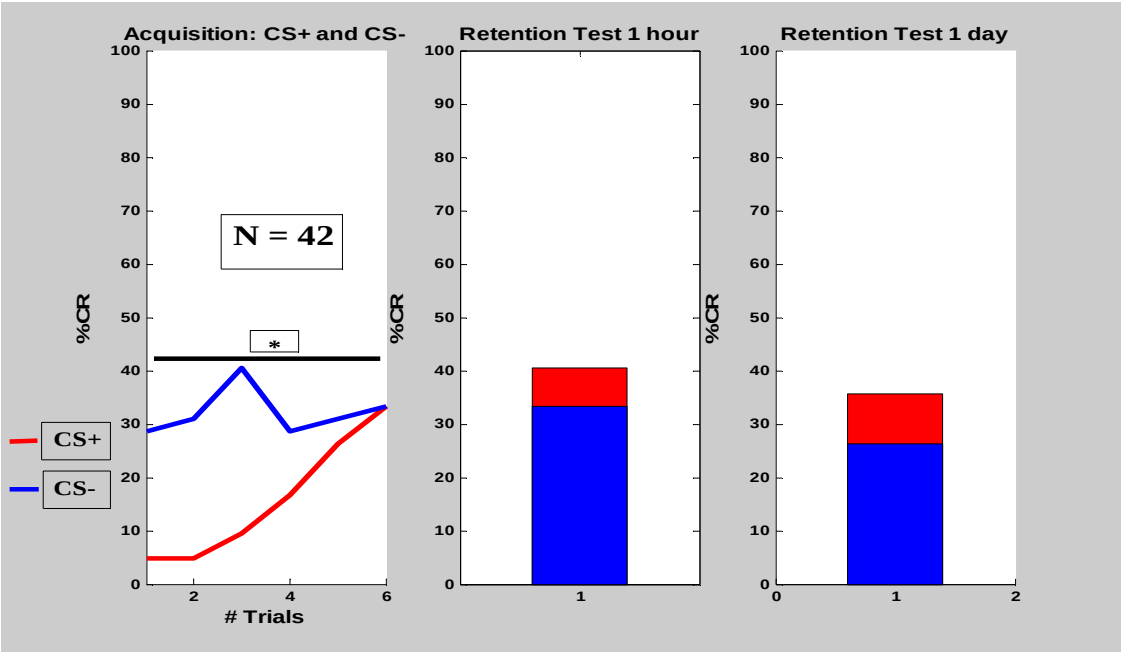

Fig. 5b: Learning and memory performances of bees of the non-hygienic line in the differential conditioning using the un-parasitized pupae as the CS+ and *Varroa* parasitized pupae as the CS-: The 1st sub-plot showed the percent conditioned responses of the non-hygienic

bees to the CS+ (red-colored line graph: healthy and un-parasitized pupae) and the CS- (blue line graph: *Varroa* parasitized pupae) stimuli during the conditioning trials. X and y axes represented the same parameters as described in Fig. 5a. Non-hygienic bees showed high generalization between the two CSs during the conditioning as denoted by the asterics (in the rectangular box on top of the black line) due to the significantly higher number of responses to the CS- compared to the CS+. Memory retention tests performed after 1 hour (2^{nd} sub-plot) and 1 day (3^{rd} sub-plot) of the conditioning also showed the high odor generalization between the two CS stimuli (red bar: CS+ and blue bar: CS-).

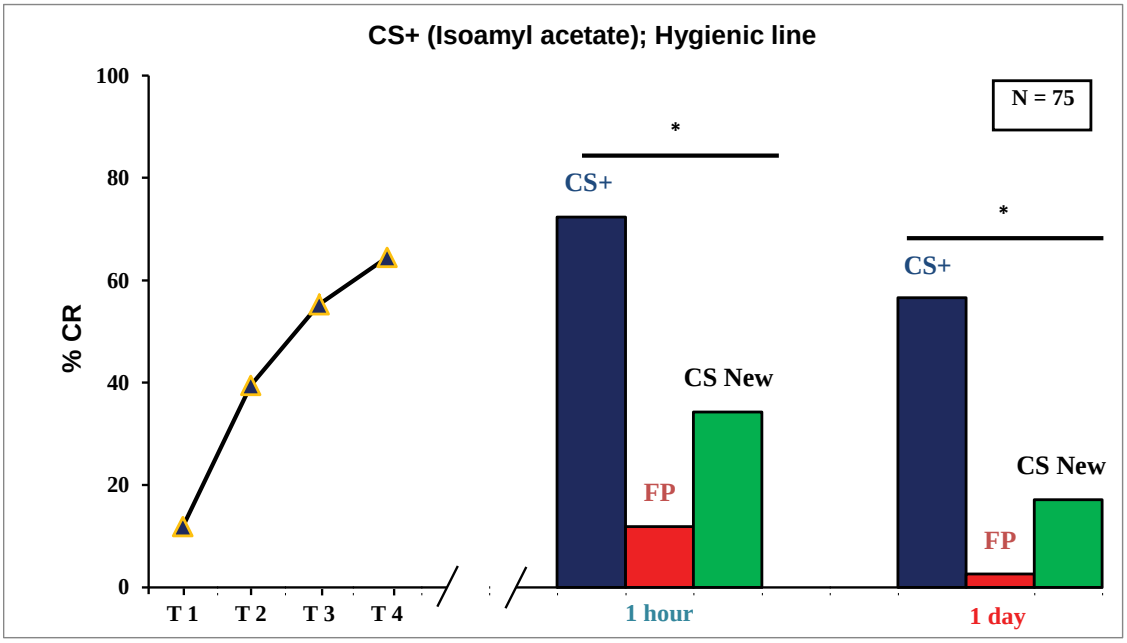

Fig. 6a: Learning and memory performances of the hygienic line in the absolute conditioning with isoamyl acetate: Bees of the hygienic line were trained in the absolute conditioning paradigm with 4 training trials using the alarm pheromone odor, isoamyl acetate (IAA) as the CS+. The 1^{st} line graph (dark green color) showed the learning performance of the bees (y-axis represented the percent conditioned response or CR) along the conditioning trials (shown on the x-axis: the number of training trials denoted with T1 until T4). The next two sets of bar graphs represented respectively the % CRs to the CS+ (dark blue bar), CS new (1-hexanal; green bar) and to the filter paper (red bar) during the memory retention tests performed after 1 hour and 1 day of the conditioning. Hygienic bees showed significantly higher (χ^2 test: $p < 0.05$) responses to the CS+ compared to the CS new and the filter paper during both tests (denoted by the black lines and asterics). Percent CRs to 1-hexanal were also found significantly higher ($p < 0.05$) than the filter paper during the two tests. 'N' represented the total number of hygienic bees trained and tested.

found low (~ 10% CR). Olfactory conditioning with isoamyl acetate elevated the effects of odor generalization in honeybees as reported previously (Sandoz *et al.*, 2001); however, hygienic bees always responded significantly lower to the novel CS compared

to the conditioned odor. Hence, we concluded that hygienic bees showed less odor generalization or recognized the novel CS as different from the conditioned odor. The overall low response levels to the filter paper during both retention tests also supported this conclusion. The lowering in CRs to the CS+ during the 1 day compared to the 1 hour test was not found significant (χ^2 = 2.16, p = 0.14) but probably involved the component of extinction learning since the same group of bees were tested twice. Bees of the non-hygienic line in contrary showed strong generalization (Fig. 6b) between the sting alarm

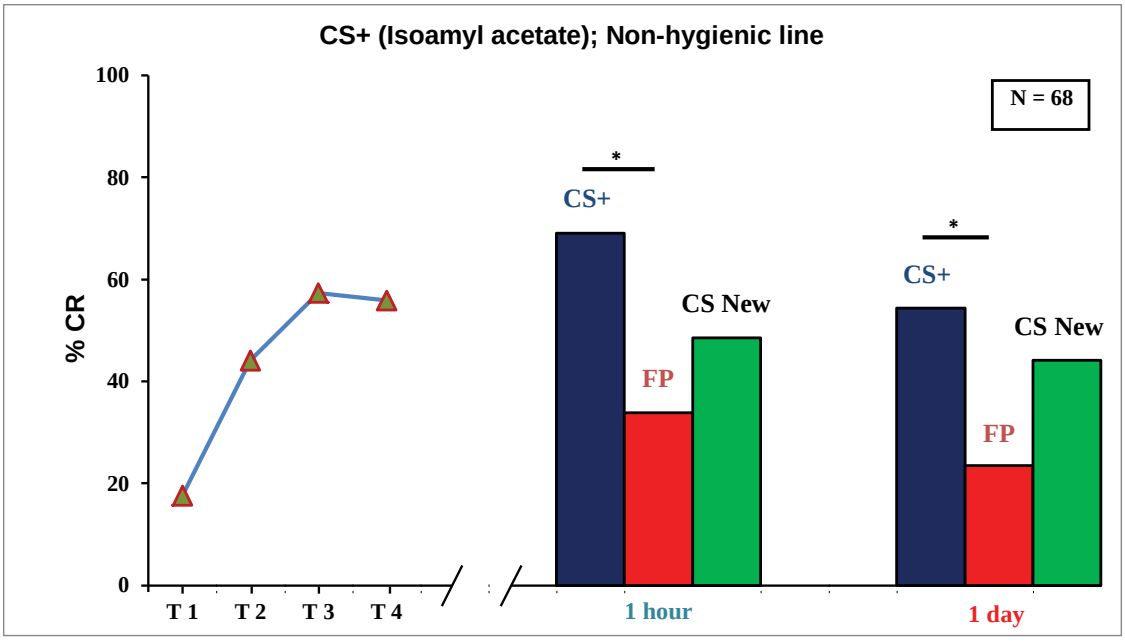

Fig. 6b: Learning and memory performances of the non-hygienic line in the absolute conditioning with isoamyl acetate (IAA): The line graph (dark green color) showed the learning performance of bees of the non-hygienic line during the conditioning with the IAA. The x and y axes represented the same variables as described in Fig. 6a.The next two sets of bar graphs were respectively represented the % CRs to the CS+ (dark blue bar), CS new (1-hexanal; green bar) and to the filter paper (red bar) during the memory retention tests performed after 1 hour and 1 day of the conditioning. Chi-square test compared the responses to these stimuli during the retention tests showed that conditioned responses to the CS+ were only significantly higher than the responses to the filter paper (χ^2 test: $p < 0.05$; denoted by the black lines and asterics). Non-hygienic bees generalized strongly between the CS+ and the CS new as well as between the filter paper and 1-hexanal during the two retention tests. Responses to the filter paper and the CS new did not change significantly between the two tests. N' represented the total number of non-hygienic bees used in the experiment.

pheromone odor, isoamyl acetate (CS+) and the natural odor (CS new), 1-hexanal. Responses to these odors did not show any significant difference during the retention tests (1 hour: $\chi^2 = 2.77$, $p = 0.09$; 1 day: $\chi^2 = 0.74$, $p = 0.38$) performed after 1 hour and 1 day of the conditioning. Unlike the hygienic bees, these bees showed no significant difference in responses between the CS new and the filter paper during the two tests (1 hour: $\chi^2 = 1.68$, $p = 0.19$; 1 day: $\chi^2 = 3.76$, $p = 0.05$). However, responses to the filter paper were significantly lower than isoamyl acetate (1 hour: $\chi^2 = 8.6$, $p = 0.003$; 1 day: $\chi^2 = 7.6$, $p = 0.005$) during the retention tests. The non-hygienic bees like the hygienics showed no significant change in responses to the CS new between the retention tests ($\chi^2 = 0.14$, $p = 0.7$), but unlike the hygienic bees the responses to the filter paper did not decrease significantly between the retention tests ($\chi^2 = 1.06$, $p = 0.3$).

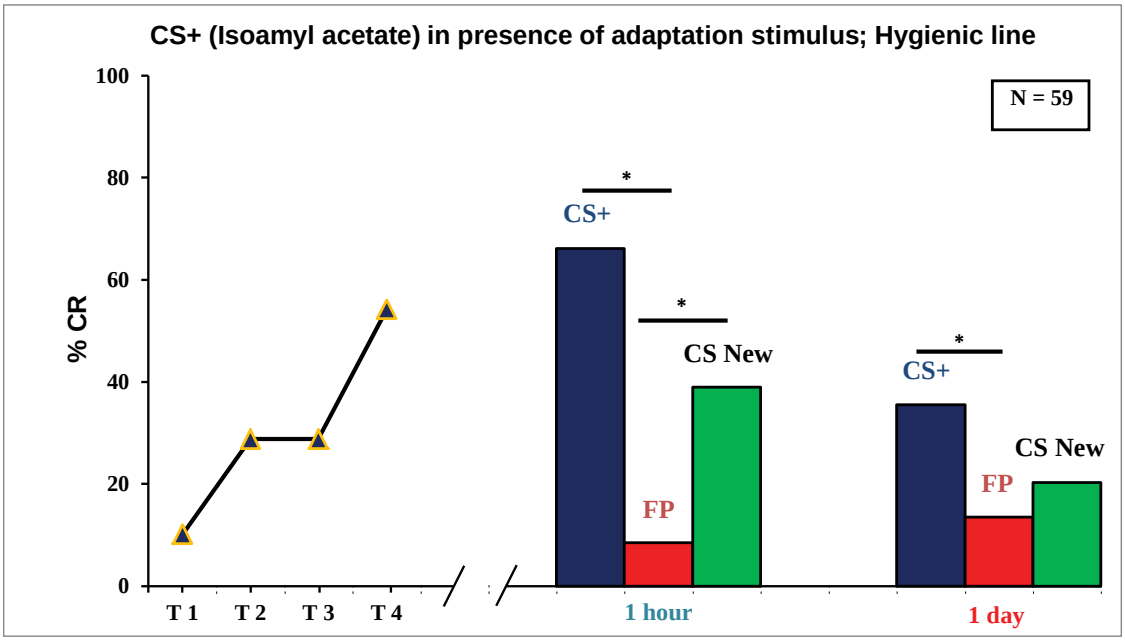

Fig. 7a: Learning and memory performances of the hygienic line in the absolute conditioning with isoamyl acetate under condition of olfactory adaptation: Bees of the hygienic line were adapted behaviorally with the constant background of colony odor and then conditioned with the isoamyl acetate (IAA) using the 4-conditioning trials. The 1st line graph (dark green color) showed the learning performance of bees (% CR represented on the y-axis) along the training trials (x-axis represented the 4-training trials; denoted with T1 until T4). The next two sets of bar graphs represented respectively the % CRs to the CS+ (dark blue bar), CS new (1-hexanal; the green bar) and to the filter paper (the red bar) during the memory retention tests performed after 1 hour and 1 day of the conditioning. Hygienic bees were found to show significantly higher (χ^2 test: $p < 0.05$) responses to the CS+ compared only to the filter paper

during the two tests (significant difference: denoted by the black lines and asterics). Responses between IAA and 1-hexanal did show any significant difference in the two tests. However, hygienic bees showed significantly higher responses to 1-hexanal compared to the filter paper only during the 1 hour test (denoted by the black lines and asterics). Percent CRs to 1-hexanal and the filter paper did not change significantly between the two tests. 'N' represented the total number of bees trained and tested.

Comparison between these two lines revealed that non-hygienic honeybees showed significantly higher responses to the filter paper during both retention tests compared to the hygienic bees (No figure shown: Mann-Whitney U test: 1 hour $U = 2065.00$, $Z = -2.71$, $p = 0.04$, 1 day $U = 2018.00$, $Z = -2.15$, $p = 0.03$) as well as to the CS new, 1-hexanal during the 1 day (Mann-Whitney U test: 1 hour $U = 2200.00$, $Z = -1.66$, $p = 0.09$, 1 day $U = 1829.50$, $Z = -3.64$, $p = 0.00026$). The differences in odor generalization found between these two lines were not due to the differences in olfactory learning since they learned similarly. The differences also did not arise due to some unknown effect of the sequence of odor presentation during the memory retention since both lines received the same sequence of odor presentation during the two tests (CS+, CS new and lastly filter paper). However, keeping in mind the effects of isoamyl acetate on odor generalization in honeybees and the absence of any other honeybee lines conditioned and tested in parallel, no conclusion was drawn between the two possibilities; whether the non-hygienic bees performed poorly in the odor generalization task due to some general deficit of this genetic line to learn in the olfactory PER paradigm or the hygienic bees were generally better performers than both the non-hygienic and other honeybee lines. Previous reports of PER conditioning in honeybees (not selectively breed for any specific genetic trait) with isoamyl acetate indicated the second possibility (Sandoz *et al.,* 2001).

The next experiment although did not test between these possibilities; however the olfactory learning and effects of odor generalization between the two lines were again compared using the same conditioned odor, isoamyl acetate but under the condition of olfactory adaptation. Adaptation was achieved with the constant background of colony odor. In addition to the comparison of performances between the two lines, performances of the individual lines in the adaptation experiment were compared with the performances in the previous experiment to find out the possible effects of adaptation on the olfactory learning and generalization processes.

Hygienic line

Hygienic bees (Fig. 7a) learned the conditioned odor during the conditioning trials and showed significantly higher responses to the CS+ compared to the filter paper (1 hour: χ^2 = 19.8, p = 8.27×10^{-6}; 1 day: χ^2 = 15.76, p = 7.15×10^{-5}) during the two retention tests. However, unlike the last experiment (Fig. 6a), responses between the CS+ and CS new (1-hexanal) in this occasion were found no longer significantly different during the two retention tests (1 hour: χ^2 = 3.53, p = 0.06, 1 day: χ^2 = 1.73, p = 0.18). The responses between the filter paper and 1-hexanal were found significantly different only during the 1 hour (χ^2 = 7.9, p = 0.0004) but not during the 1 day test (χ^2 = 0.52, p = 0.4). The higher odor generalization in this experiment compared to the previous experiment (Fig. 6a) was also associated with the significantly lower conditioned responses to the CS+ during the 1 day compared to the 1 hour retention test (χ^2 = 4.55, p = 0.03). Responses to the filter paper were found lowest amongst the test stimuli but unlike the previous experiment (Fig. 6a) did not show any decrease during the 1 day compared to the 1 hour test (χ^2 = 0.43, p = 0.5). The responses to the CS new, 1-hexanal also did not vary (χ^2 = 2.47, p = 0.11) between the two tests.

During the 1 day unlike the 1 hour retention test hygienic bees were unable to distinguish between the filter paper and the novel CS, 1-hexanal. The differences in responses between the conditioned and novel odors although were never statistically significant, but were higher during the 1 hour than the 1 day test. Responses to the filter paper unlike the last experiment (Fig. 6a) did not reduce between the two tests, rather were found to increase during the 1 day test. These results together showed that olfactory adaptation with the colony odor elevated the effects of odor generalization in the hygienic bees. This effect was found stronger during the 1 day retention test (early-long term memory) compared to the 1 hour (mid-term memory) test. In addition, significant reduction in CRs to the CS+ odor during the 1 day compared to the 1 hour test was found when the bees were adapted with the colony odor but not when bees were conditioned in the background of the laboratory. Adaptation with the colony odor decreased the consolidation of CS+ memory (IAA) between the 1 hour and 1 day test. Two retention tests conducted on the same group of bees although incorporated an extinction component in the conditioned responses, but this component was weaker in the hygienic bees when they were not

adapted with the colony odor. Whether the extinction learning became stronger due to the olfactory adaptation with colony odor in hygienic bees was unclear. However, hygienic bees were found to show lower conditioned responses during the conditioning trials when adapted with the background stimulus of colony odor compared to the condition of no-adaptation (Fig. 7b). The CRs only during the 3rd conditioning trial showed the significant difference between the two experiments but the overall CRs were lower during the adaptation (Mann-Whitney U test: trial-3 adaptation *vs.* no-adaptation U = 1640.50, Z = 2.98, p = 0.0028). Hence, adaptation with the background stimulus of the colony odor seemed to inhibit (or interfere) the olfactory learning of hygienic bees while isoamyl acetate was used as the conditioned odor. This reduction in CS+ learning probably led to the deficit in consolidation of the CS+ memory or in other words the possible effect of extinction learning was stronger over the reduced effect of CS+ learning which subsequently reduced the CRs to the CS+.

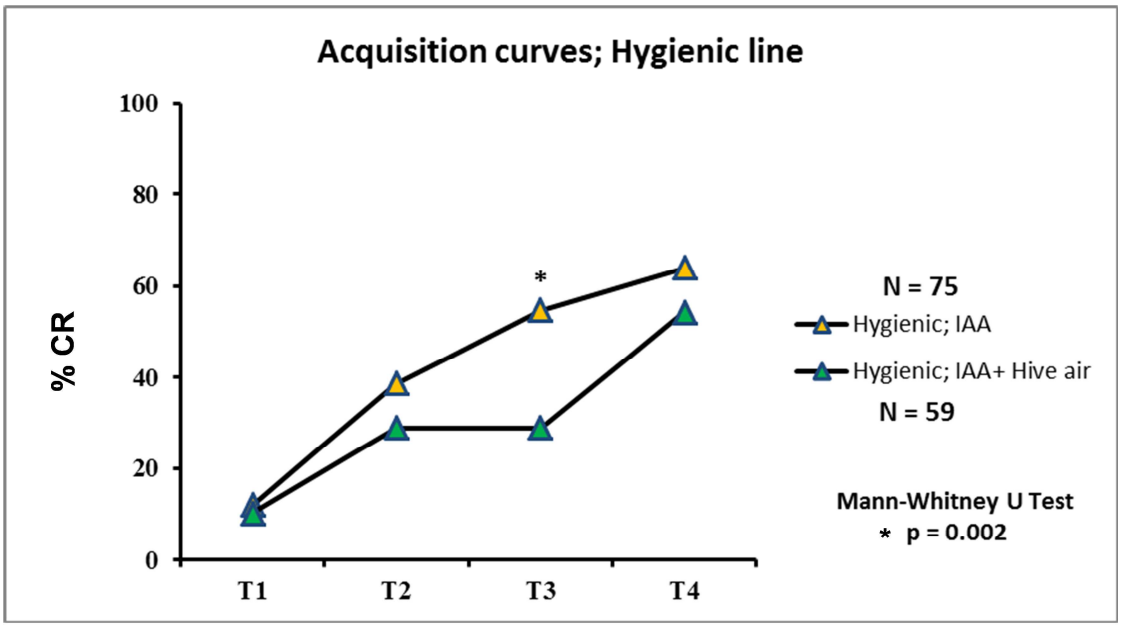

Fig. 7b: Comparison between the population-averaged learning functions of bees of the hygienic line found during the conditions of olfactory adaptation and no-adaptation: The average learning or acquisition functions (for isoamyl acetate) found during the condition of no-adaptation (line graph with green triangular data points) and background olfactory adaptation with the colony odor (line graphs with yellow triangular data points) were compared for the hygienic bees using the Mann-Whitney U test. Significant reduction in CR was found during the 3rd conditioning trial (p value = 0.002) under the condition of adaptation. The x and y-axes in the

figure were respectively represented the number of conditioning trials (denoted with T1 until T4) and the percent CR (conditioned response). Overall, olfactory adaptation inhibited the learning of isoamyl acetate in the hygienic bees. 'N' represented the total number of hygienic bees in each experimental group.

Non-hygienic line

Non-hygienic bees showed high generalization (Fig. 8a) between the odor stimuli during the two retention tests. No significant difference in the PER responses was found between the CS+ and 1-hexanal (1 hour: $\chi^2 = 0.48$, $p = 0.48$ 1 day: $\chi^2 = 0.71$, $p = 0.39$), between the CS+ and filter paper (1 hour: $\chi^2 = 2.84$, $p = 0.09$ 1 day: $\chi^2 = 0.3$, $p = 0.58$) as well as between the CS new and filter paper (1 hour: $\chi^2 = 0.99$, $p = 0.3$ 1 day: $\chi^2 = 0.08$, $p = 0.76$) during the two retention tests. The responses to the CS+ ($\chi^2 = 3.29$, $p = 0.06$), CS new ($\chi^2 = 3.81$, $p = 0.05$) and filter paper ($\chi^2 = 0.46$, $p = 0.49$) also did not change significantly between the two tests. Hence, like the previous experiment (Fig. 6b), the non-hygienic bees showed the strong generalization between isoamyl acetate and 1-hexanal, along with

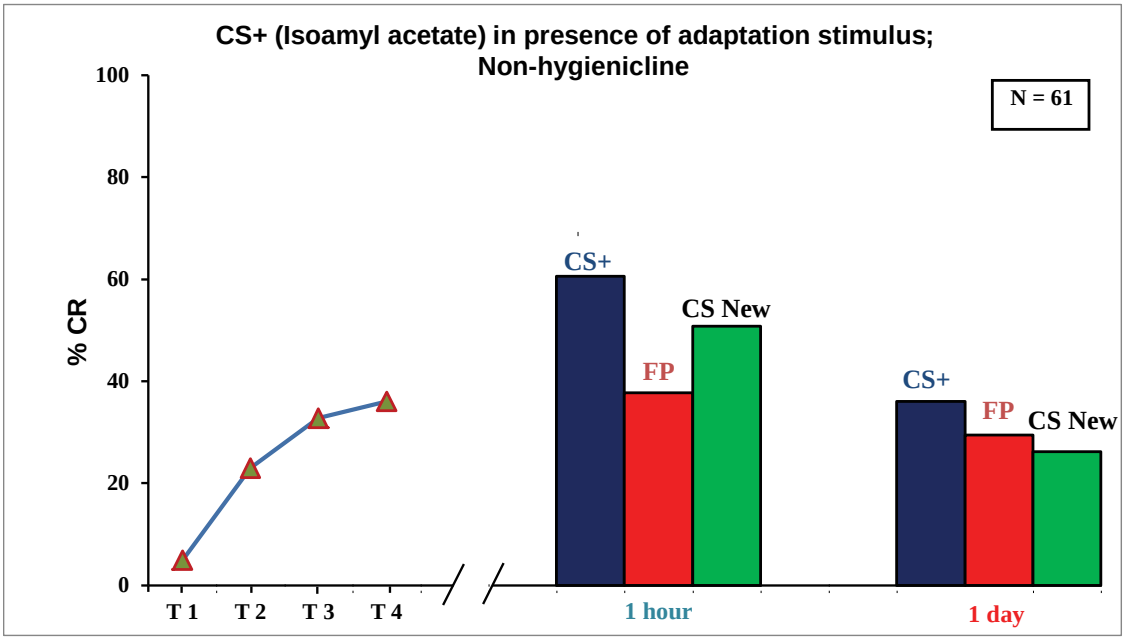

Fig. 8a: Learning and memory performances of the non-hygienic line in the absolute conditioning with isoamyl acetate (IAA) under condition of olfactory adaptation: Non-hygienic bees were adapted with the colony odor and conditioned with 4 training trials using IAA as the CS+. The 1st line graph (blue color) showed the learning performance of the bees along the conditioning trials. The x and y axes in the figure represented the same variables as describes in Fig. 6a. The next two sets of bar graphs represented respectively the % CRs to the CS+ (dark blue

bar), new CS (1-hexanal; green bar) and to the filter paper (red bar) during the retention tests performed after 1 hour and 1 day of the conditioning. Non-hygienic bees generalized completely between the CS+ and CS new, between the CS+ and filter paper as well as showed similar responses to the filter paper and the CS new during the two tests. Responses to the filter paper and CS new did not change significantly between the two tests. 'N' represented the total number of non-hygienic bees used in the experiment.

the constant high responses shown to the filter paper. Adaptation with the background of colony odor led to more reduction in learning in these bees compared to the hygienic bees. Non-hygienic bees showed significant reduction in conditioned responses throughout the four conditioning trials during the adaptation (Fig. 8b) compared to the previous condition of no olfactory adaptation (Mann-Whitney U test: trial-1 between adaptation and no-adaptation $U = 1810.00$, $Z = 2.24$, $p = 0.024$, trial-2 between the two conditions $U = 1635.00$, $Z = 2.52$, $p = 0.011$, trial-3 between the two conditions $U = 1530.00$, $Z = 2.97$, $p = 0.0029$, trial-4 between the two conditions $U = 1697.00$, $Z = 2.05$, $p = 0.03$).

The inhibitory effect of adaptation on olfactory learning was found in both honeybee lines, although the effects were more severe in the non-hygienic bees. However, it was unclear whether this effect was *specific for the isoamyl acetate or specific of the colony odor*, which was not tested further in this study. Hygienic bees showed higher odor generalization in the adapted compared to the unadapted state but overall they showed significantly less generalization than the non-hygienic bees. Non-hygienic bees on the other hand showed complete generalization in odor responses during both adapted and unadapted conditions. In addition to the strong odor generalization, these bees also showed strong responses to the filter paper in both experiments. Honeybees probably can perceive the smell of the filter paper but in comparison to the odor stimuli such as IAA or 1-hexanal with much higher salience, filter paper possibly acted like a neutral stimulus in these experiments. Hence, strong responses to such a stimulus strengthened the doubt that non-hygienic bees either had some general deficit to learn the olfactory information or had the specific problem to learn odors in the PER paradigm.

Important point

A separate honeybee colony was used for the purpose of delivering the adaptation stimulus to the hygienic and the non-hygienic bees. The air flow delivered from this colony probably had some stress related odors (since bees were restrained from flying out during the experiments) apart from the pheromones, kin-related compounds and the dominant odors of the wax and honey. Since, bees can recognize and discriminate between the different kin-related and the various pheromonal odors; it was possible that these volatile chemicals had the influences on olfactory learning and generalization of the two genetic lines. This issue remained unresolved in this study as the hygienic and non-hygienic bees were not adapted with the odors of their own colonies.

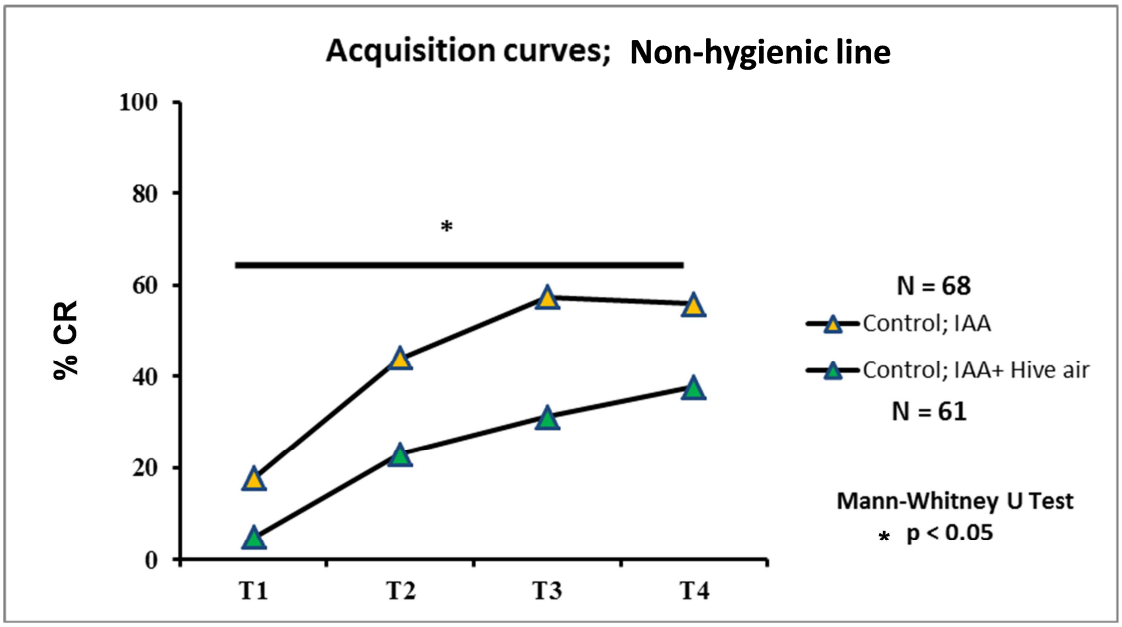

Fig. 8b: Comparison between the average learning functions of bees of the non-hygienic line found during the conditions of olfactory adaptation and no-adaptation: The average learning functions (for isoamyl acetate) of the non-hygienic bees found during the conditions of no-adaptation (line graph with green triangular data points) and background olfactory adaptation with the colony odor (line graphs with yellow triangular data points) were compared using the Mann-Whitney U test. The x and y-axes were respectively represented the number of conditioning trials (represented with T1 until T4) and the percent CR (conditioned response). Significant reduction in CRs throughout the 4-conditioning trials was found during the adaptation compared to the condition of no olfactory adaptation. This inhibitory effect of adaptation was found stronger in the non-hygienic than in the hygienic bees. 'N' represented the total number of bees in each experimental group.

2.5.4 Differential conditioning with the floral odors

The last experiment was designed to resolve the previous dilemma about the poor learning ability of the non-hygienic line. Honeybees from the two genetic lines were conditioned again in the differential conditioning paradigm using the common floral odors, 1-hexanol and geraniol. Masterman and colleagues in 2000 already reported that bees from their hygienic colonies although were able to discriminate significantly better between the volatile odors of the healthy and the chalkbrood infected pupae compared to the non-hygienic bees, however; both lines were able to discriminate similarly between the high concentrations of the floral odors, geraniol and 1-hexanol. In our experiment hygienic bees were found to discriminate (Fig. 9a and 9b) well between the two CS stimuli during the differential conditionings with both combinations of the CS+ and CS-. Repeated measurement ANOVA showed significant stimulus (CS+/CS-) × conditioning trial effect $F(5, 460) = 9.84$, $p = 0.00000$ and significant trial effect ($F(5, 460) = 3.31$, $p = 0.0059$) although, the stimulus effect was not found significant $F(1, 92) = 2.18$, $p = 0.14$ when geraniol was used as the CS+ and 1-hexanol as the CS- (Fig. 9a). When 1-hexanol was used as the CS+ and geraniol as the CS- (Fig. 9b) RM-ANOVA tests showed the significant stimulus × trial $F(5, 460) = 3.6130$, $p = 0.0032$ as well as the trial effects ($F(5, 460) = 5.05$, $p = 0.00000$) although, the stimulus effect was again found non-significant $F(1, 92) = 3.36$, $p = 0.00016$). The stimulus effects in hygienic bees although were not found significant however, the significant trial effects as well as the significant interaction between the stimulus and trial confirmed that hygienic bees were able to learn the discrimination between these two odors or in other words these bees learned the different contingencies of the two odor stimuli along the conditioning trials during both differential conditionings. During the memory retention tests performed after 1 hour and 1 day of the conditioning (both experiments) hygienic bees showed the significantly higher number of responses to the CS+ compared to the CS- stimuli (Fisher LSD post hoc test: 1hour $p = 0.000000$, 1 day $p = 0.000000$). Responses to the filter paper were also found significantly lower in these bees compared to the CS+ odors during the two experiments (Fisher LSD post hoc test: geraniol as CS+ 1 hour $p = 0.000000$ and 1 day $p = 0.000001$, 1-hexanol as CS+ 1 hour $p = 0.000001$ and 1 day $p = 0.000092$). However, when 1-hexanol was used as the CS+, these bees showed significantly higher responses

(Fisher LSD post hoc test: p = 0.04) to the filter paper during the 1 hour compared to the 1 day retention test (Fig. 9b). The responses to the filter paper also were not found significantly different from the CS- stimuli in the two differential conditionings (data not shown: Fig. 9a and 9b).

On the contrary, the non-hygienic bees were failed to discriminate or showed high generalization like before between the CS stimuli (Fig. 10a and 10b) during the differential conditionings with alternate combinations of the CS+ and CS-. RM-ANOVA showed the non-significant stimulus × trial effect F (5, 440) = 2.17, p = 0.05634 and significant stimulus effect F (1, 88) = 14.21, p = 0.00029 when geraniol was used as the CS+ and 1-hexanol as the CS-. In the other combination of the two CSs (1-hexanol as CS+ and geraniol as CS-) the non-significant stimulus × trial effect F (5, 460) = 0.72, p = 0.60273 was associated with the significant stimulus effect F (1, 92) = 8.89, p = 0.0036). Non-hygienic bees showed strong responses to the CS- stimulus which resulted in the significant stimulus effect but non-significant interaction between the stimulus and trial in both differential conditionings. These results confirmed that non-hygienic honeybees were failed to learn the different contingencies of the two CS stimuli along the training trials during both differential conditionings. During the memory retention tests, non-hygienic bees responded significantly higher to the CS+ odors (Fisher LSD post hoc test:

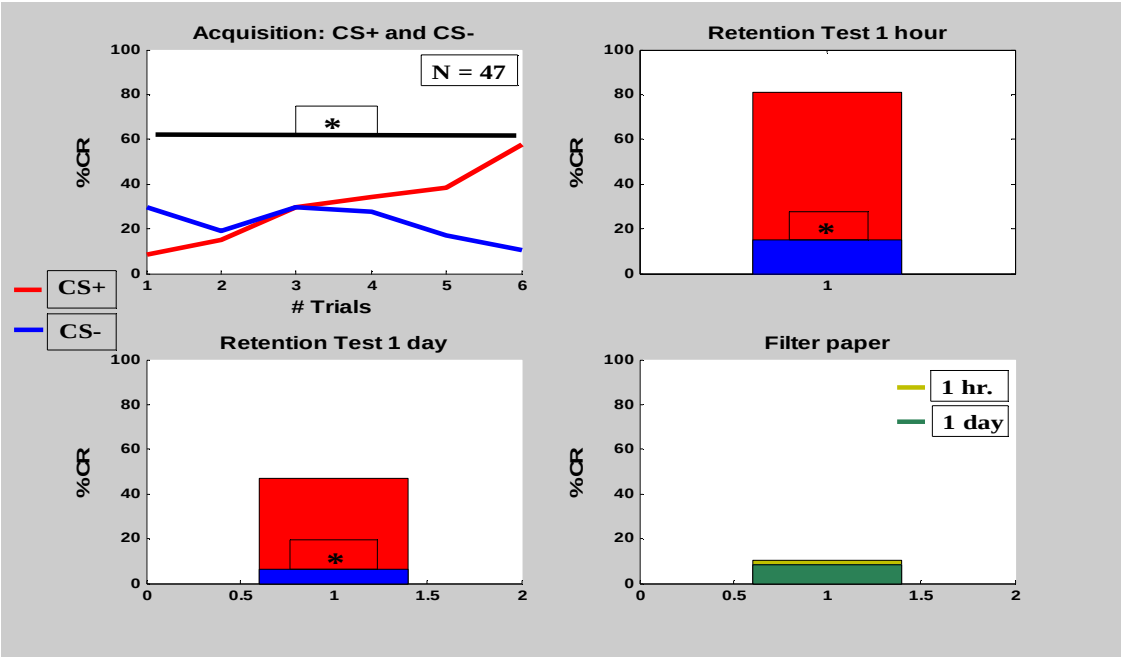

Fig. 9a: Learning and memory performances of bees of the hygienic line in the differential conditioning with geraniol as the CS+ and 1-hexanol as the CS-: Hygienic bees were trained differentially with geraniol as the CS+ and 1-hexanol as CS-. The 1st sub-plot (line graphs) represented the percent conditioned responses (percent CR plotted on the y-axis) to the CS+ (red line) and CS- (blue line) during the conditioning trials (the number of trials were given on the x-axis; 6 trials). During the conditioning hygienic bees discriminated well between the two stimuli (significant differences between CRs of the conditioning trials were together denoted by the asterics on top of the black line). The 2nd and 3rd sub-plots showed the percent CRs to the two CS stimuli respectively during the retention tests performed after 1 hour and 1 day of the conditioning. The percent CRs to the CS+ and CS- stimuli during the memory retention tests were represented together in the same bar with the same color codes as used in the line graphs (red: CS+ and blue: CS-). Hygienic bees showed significantly higher responses to the CS+ compared to the CS- at both time points (denoted by the asterics inside the rectangular box at the demarcation between the red and blue colors). The 4th sub-plot showed the responses to the filter paper during the same two time points of test (1 hour: light green bar and 1 day: dark green bar). N' (in the 1st plot) represented the total number of hygienic bees used in the experiment.

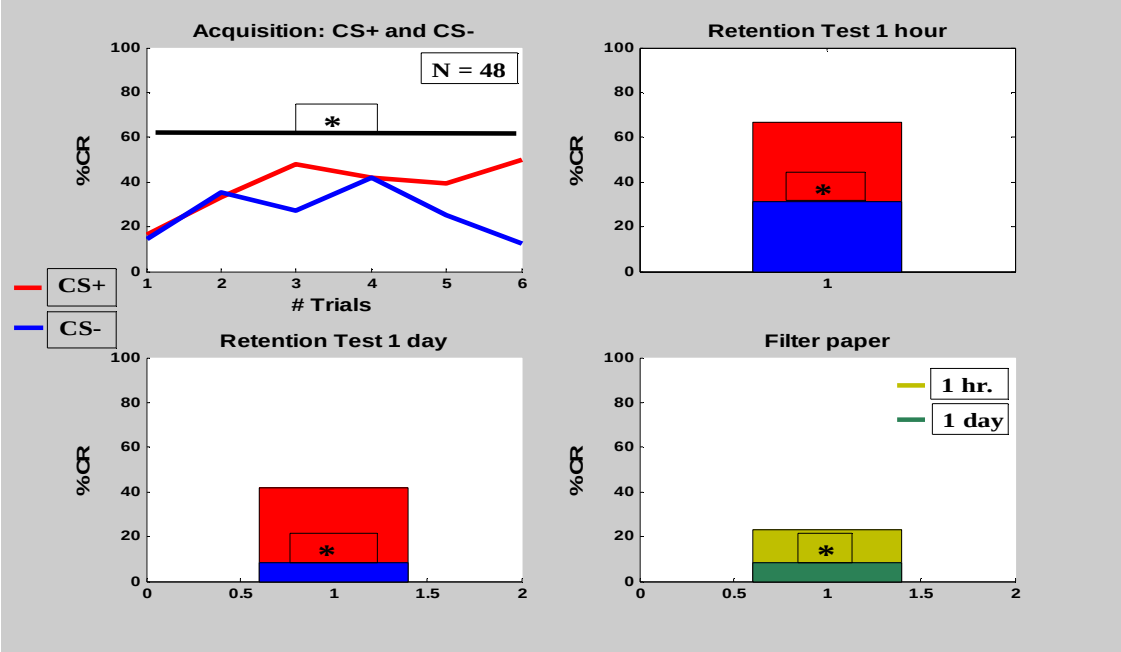

Fig. 9b: Learning and memory performances of the hygienic line in the differential conditioning with 1-hexanol as the CS+ and geraniol as the CS-: Hygienic bees were trained differentially with 1-hexanol as the CS+ and geraniol as the CS-. The 1st sub-plot (line graphs) showed the conditioned responses to the CS+ and CS- stimuli during the conditioning trials. The x and y axes represented the same parameters as described in Fig. 9a with the same color codes used to represent the CS stimuli. Hygienic bees were able to discriminate between the two stimuli during conditioning (significant differences in CRs along the training trials: denoted by the black line with the asterics on top). The 2nd and 3rd sub-plots represented the percent CRs to the CS stimuli during the retention tests performed after 1 hour and 1 day of conditioning. Significant differences were denoted by the asterics inside the rectangular box at the demarcation between the red and blue colors. The 4th and last sub-plot showed the responses to the filter paper during

the same two time points (1 hour: light green bar and 1 day: dark green bar) of the test. Responses to the filter paper declined significantly during the 1 day compared to the 1 hour test (denoted by asterics in the box); however, no significant differences were found between the CRs to the CS- and responses to the filter paper. N' represented the total number of hygienic bees.

1 hour p = 0.04) during the 1 hour time point (Fig. 10a) when geraniol was used as the CS+ and 1 day after (Fisher LSD post hoc test 1 hour: p = 0.003) the conditioning when 1-hexanol was used as the CS+ (Fig. 10b). The other two conditioned responses during the retention tests reflected the strong effects of odor generalization as showed by these bees during the conditioning. Hence, the significantly higher responses to the CS+ stimuli in two cases did not confirm the response specificity of the non-hygienic bees. When geraniol was used as the CS+ stimulus, the increased responses to the CS+ were lost between the mid-term (1 hour) and the early long-term memory (1 day) retention. Opposite type of conditioned responses were found when 1-hexanol was used as the CS+. These two responses were not easy to be explained form the viewpoint of the consolidation of CS+ memory after the conditioning trials since in one case the CRs to the CS+ compared to the CS- were higher during the mid-term and in the other case during the early long- term retention test. However, when we looked at the responses to the filter paper, we found (Fig. 6a and 8b) that the non-hygienic bees like before again

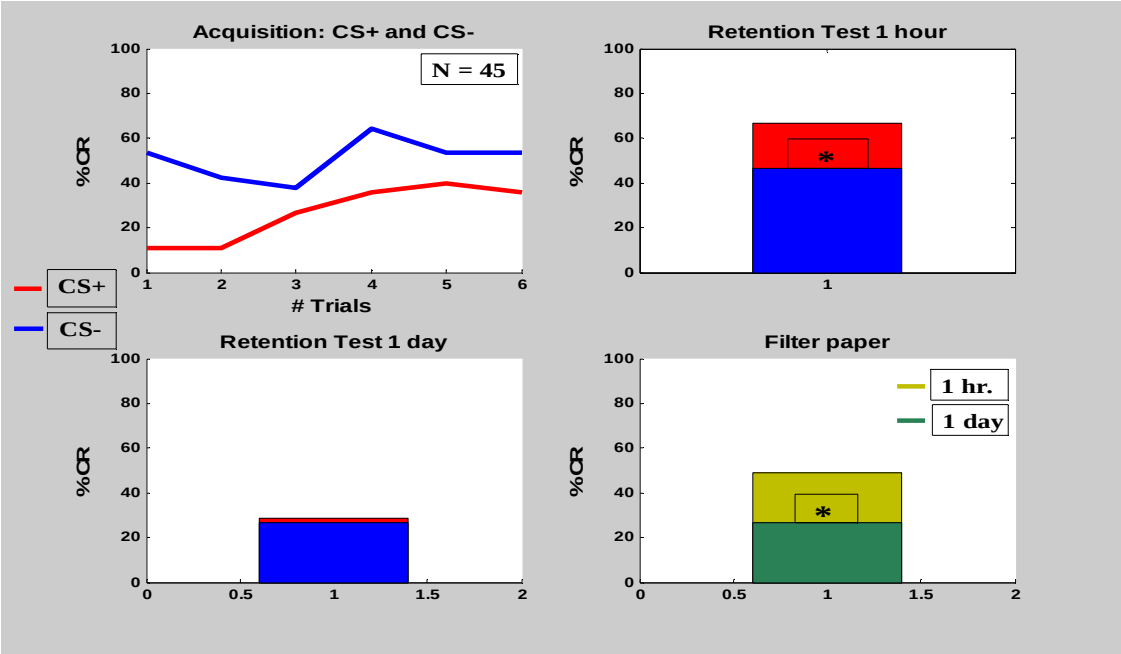

Fig. 10a: Learning and memory performances of the non-hygienic line in the differential conditioning with geraniol as the CS+ and 1-hexanol as the CS-: Non-hygienic bees were trained differentially with geraniol as the CS+ and 1-hexanol as CS- in this experiment. The 1st sub-plot (line graphs) showed the conditioned responses to the CS+ and CS- stimuli during the conditioning trials. The x and y axes represented the same parameters as described in Fig. 9a with the same color codes were used to represent the CS stimuli. During conditioning non-hygienic bees generalized strongly between the two CS stimuli. The 2nd and 3rd sub-plots respectively represented the percent CRs to the CS stimuli (same color codes as in Fig. 9a) during the retention tests performed after 1 hour and 1 day of conditioning. Significantly higher responses to the CS+ compared to the CS- were shown by these bees during the 1 hour test (as denoted by the asterics inside the rectangular box at the demarcation between the red and blue colors) but not during the 1 day test. The 4th sub-plot was showed the responses to the filter paper during the 1 hour and 1 day tests with the same color codes as used in Fig. 9a. Responses to the filter paper were found significantly higher during the 1 hour compared to the 1 day test, but no significant differences were found between the responses to the filter paper with both the CS+ and CS- odors during the two tests.

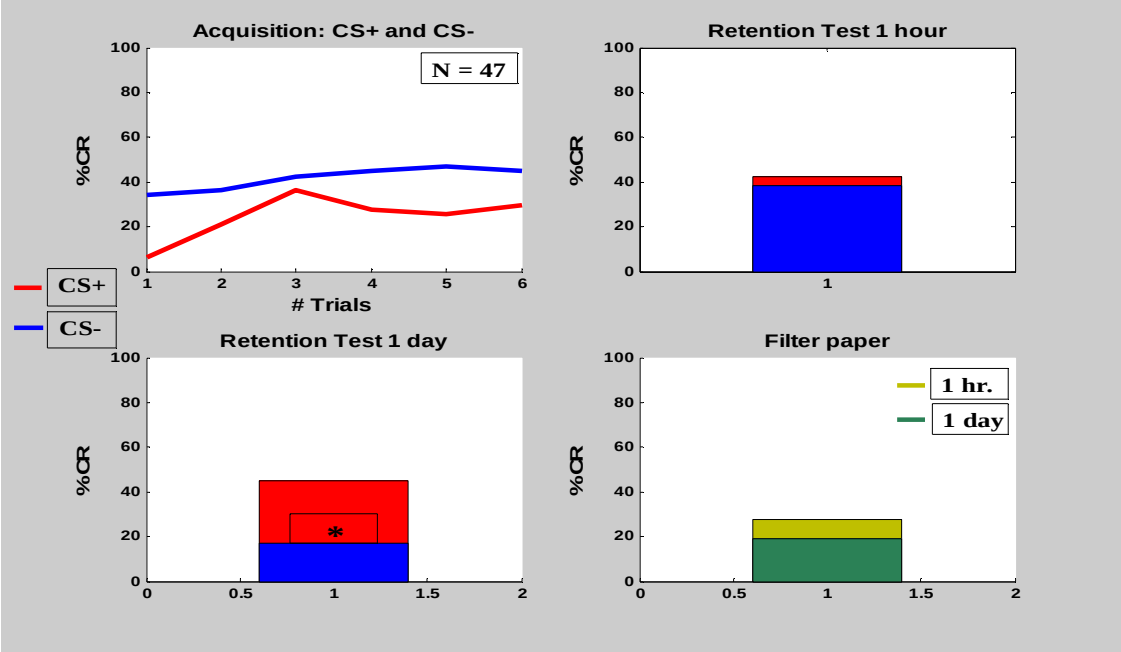

Fig. 10b: Learning and memory performances of the non-hygienic line during the differential conditioning with 1-hexanol as the CS+ and geraniol as the CS-: Non-hygienic bees were trained differentially with 1-hexanol as the CS+ and geraniol as CS-. The 1st sub-plot (line graphs) showed that bees were unable to discriminate or generalized highly between the two CS stimuli during conditioning. The x and y axes test the same parameters as described in Fig. 9a with the same color codes were used to represent the CS stimuli. Percent conditioned responses to the two CS stimuli (with the same color codes as in Fig. 9a) during the retention tests were represented in the 2nd and 3rd sub-plots. Significantly higher responses to the CS+ than the CS- were shown by these bees during the 1 day test (denoted by the asterics inside the rectangular) but not during the 1 hour retention test. The 4th sub-plot represented the responses to the filter paper

during the 1 hour and 1 day tests with the same color codes as used in Fig. 9a. Responses to the filter paper did not change between the two tests.

showed the strong generalization in responses between the filter paper and CS+ and between the filter paper and CS- (no significant differences were found in both comparisons: data not shown) in both experiments (Fig. 10a and 10b). Honeybees in general (bees from our institute's garden) can discriminate between the high concentrations of these two floral odors, geraniol and 1-hexanol in the olfactory PER paradigm (Malun *et al.*, 2002). Non-hygienic bees however, were failed to learn the discrimination between the highest concentrations of these two odors. Hence, the strong responses to the stimulus like filter paper in all experiments along with the high generalization in odor responses during the conditioning and retention tests together strengthened the possibility that non-hygienic bees had some general problem to learn the

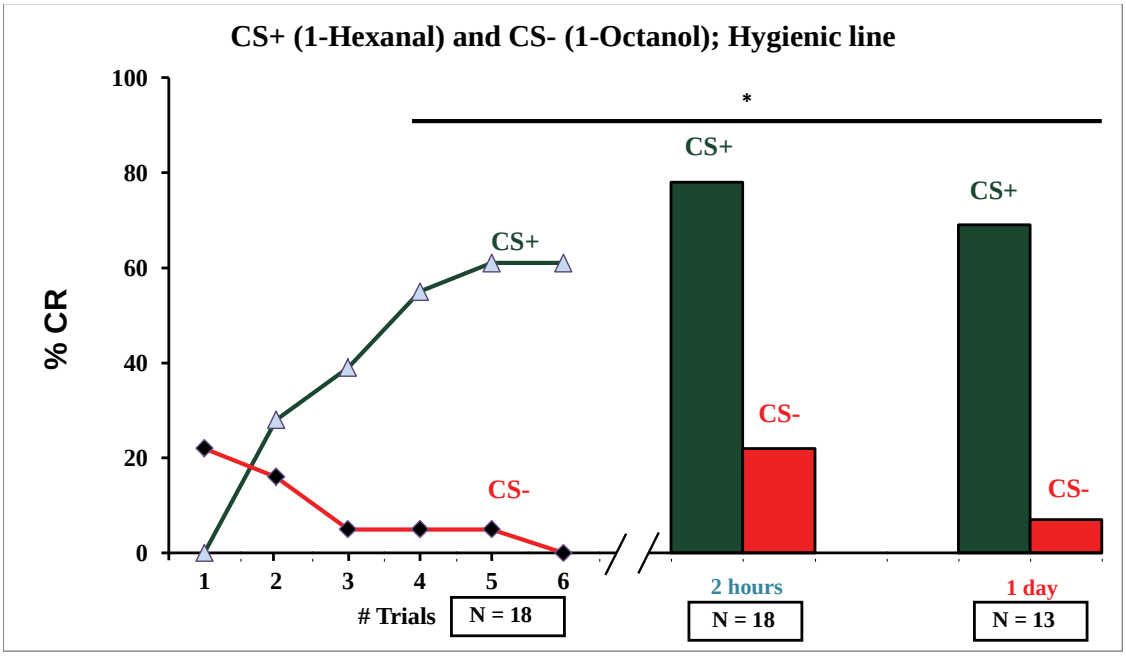

Fig. 11a: Learning and memory performances of the hygienic line during the differential conditioning with 1-hexanal as the CS+ and 1-octanol as the CS-: Hygienic bees were conditioned differentially using 1-hexanal as the CS+ and 1-octanol as CS- with the 6 alternate presentations of each of the CS+ and CS- stimuli. The x and y axes in the figure respectively represented the number of conditioning trials and the percent conditioned responses (CR). During the conditioning as well as the retention tests conducted after 2 hours and 1 day of training, bees showed significantly higher responses to the CS+ compare to the CS- (χ^2 test: $p < 0.05$; denoted

by the black line to show all significant differences with the asterics on top). The x and y-axes respectively represented the number of conditioning trials and the percent CR to the CSs. 'N' represented the total number of hygienic bees used in the experiment which was in between the 1 day and 1 hour test (due to death).

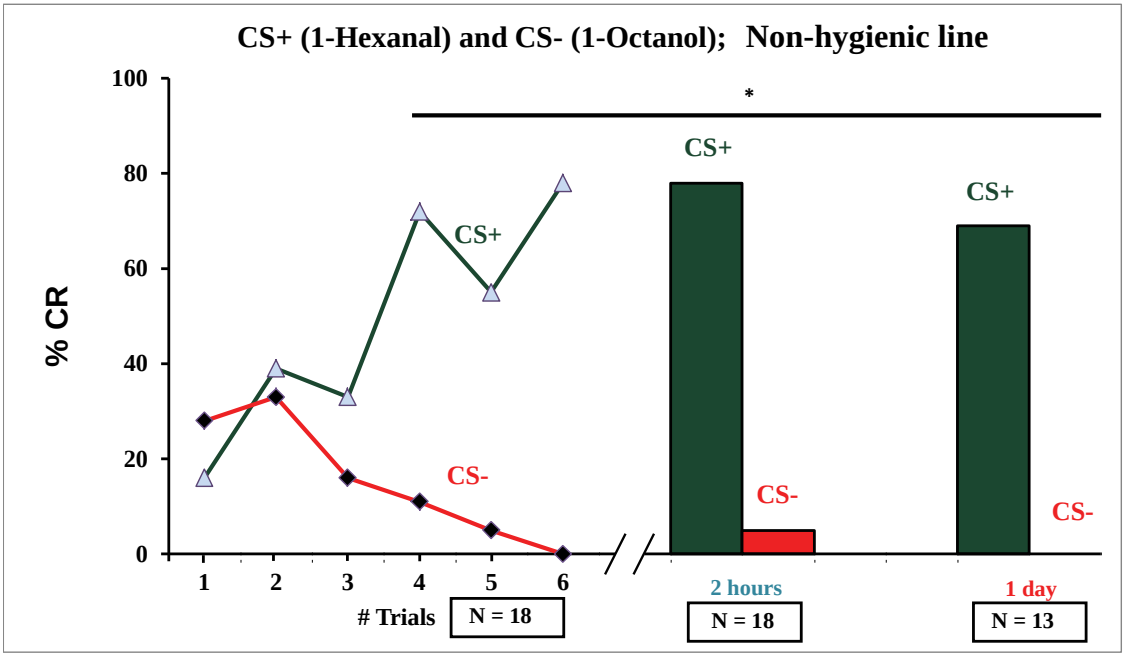

Fig. 11b: Learning and memory performances of the non-hygienic line during the differential conditioning with 1-hexanal as the CS+ and 1-octanol as the CS-: Non-hygienic bees were conditioned differentially using 1-hexanal as the CS+ and 1-octanol as CS- with the 6 (each) alternate CS+ and CS- trials. The x and y axes in the figure respectively represented the number of conditioning trials and the percent conditioned responses (CR). During the conditioning as well as the retention tests non-hygienic bees showed significantly higher CRs to the CS+ compared to the CS- (χ^2 test confirmed the significant differences, $p < 0.05$; denoted by the black line to show all significant differences with the asterics on top). The x and y-axes respectively represented the number of conditioning trials and the percent CR to the CSs. N' represented the total number of hygienic bees used in the experiment which was in between the 1 day and 1 hour test (due to death).

olfactory information in the PER paradigm. Alternately, these bees might have suffered with some kind of sensitization effect which lasted long during and after the experiments and contributed to the increased responses to all kinds of CSs (strong generalization effect). We concluded that *the differences in the olfactory learning behavior found between the two genetic lines were most likely reflected the poor overall performance of the non-hygienic honeybees rather the true superior performance of the hygienic honeybees.* Surprisingly, during the latter part of the season (beginning of autumn; 2009)

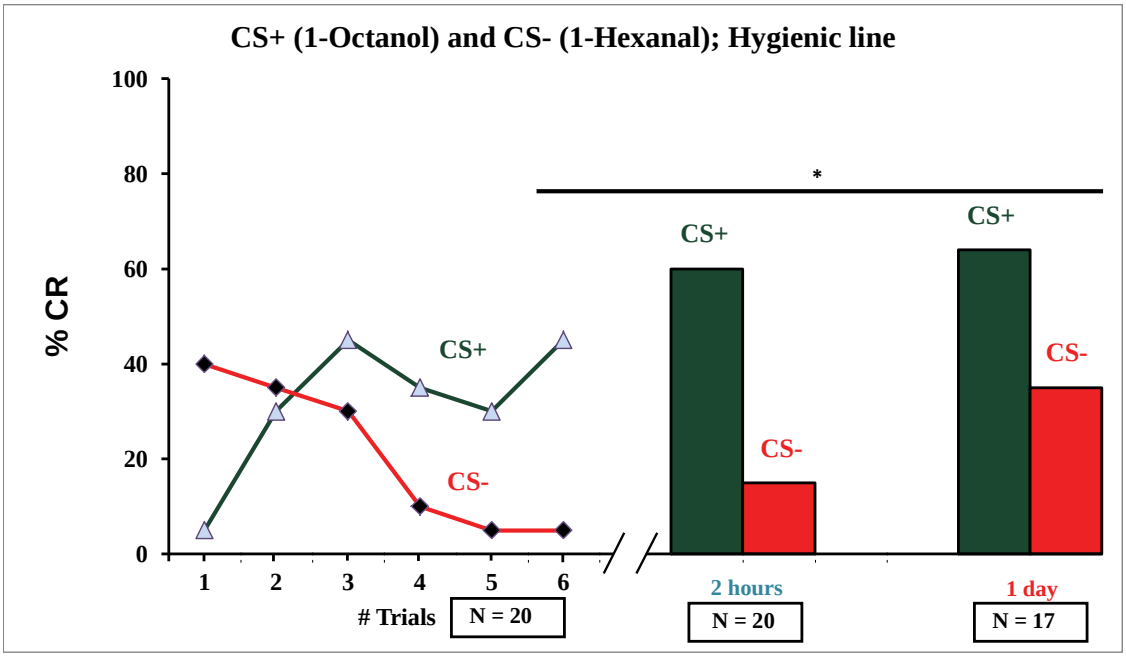

Fig. 12a: Learning and memory performances of the hygienic line during the differential conditioning with 1-octanol as the CS+ and 1-hexanal as the CS-: Hygienic bees were trained differentially using 1-octanol as the CS+ and 1-hexanal as CS- with the 6 each alternate CS+ and CS- trials. The x and y axes in the figure respectively represented the number of conditioning trials and the percent conditioned responses (CR). During the conditioning and the retention tests these bees showed significantly conditioned responses to the CS+ compare to the CS- stimuli (χ^2 test: $p < 0.05$; denoted by the black line to show all significant differences with the asterics on top). N' represented the total number of hygienic bees used in the experiment which was in between the 1 day and 1 hour test (due to death).

the non-hygienic bees started to perform similarly with the hygienic bees in the olfactory discrimination tasks. This was found when bees from both genetic lines were conditioned differentially with the two other naturally occurring odors namely 1-hexanal and 1–octanol. Both hygienic and non-hygienic bees learned the discrimination between the CS+ and CS- stimuli during conditioning (Fig. 11a, 11b, 12a and 12b) with significantly higher responses shown to the CS+ odors during the memory retention tests performed after 2 hours and 1 day of the conditioning. The last group (Fig. 12b) of the non-hygienic bees although showed the differences in CRs to the two CS stimuli but they were not found significant probably due to the low sample size. These experiments were repeated during this time with the younger bees (1-2 weeks of age) in place of the foragers from both genetic lines. We found that young bees were also able to learn the discrimination

between the same two odors (data not shown). This was the first time during the entire season when the similar learning performances were found between the two types of bees. The reasons for the overall poor performance of the non-hygienic bees during the summer and the sudden improvement in performance during autumn were not understood, especially when the non-hygienic colonies were well maintained throughout the summer and autumn (sufficient food, healthy queen and good population size). However, these results disproved our previous conclusion that non-hygienic bees had general deficit to learn in the PER conditioning paradigm. Under this circumstance, no specific conclusion was made about the possible differences in olfactory learning between the two genetic lines.

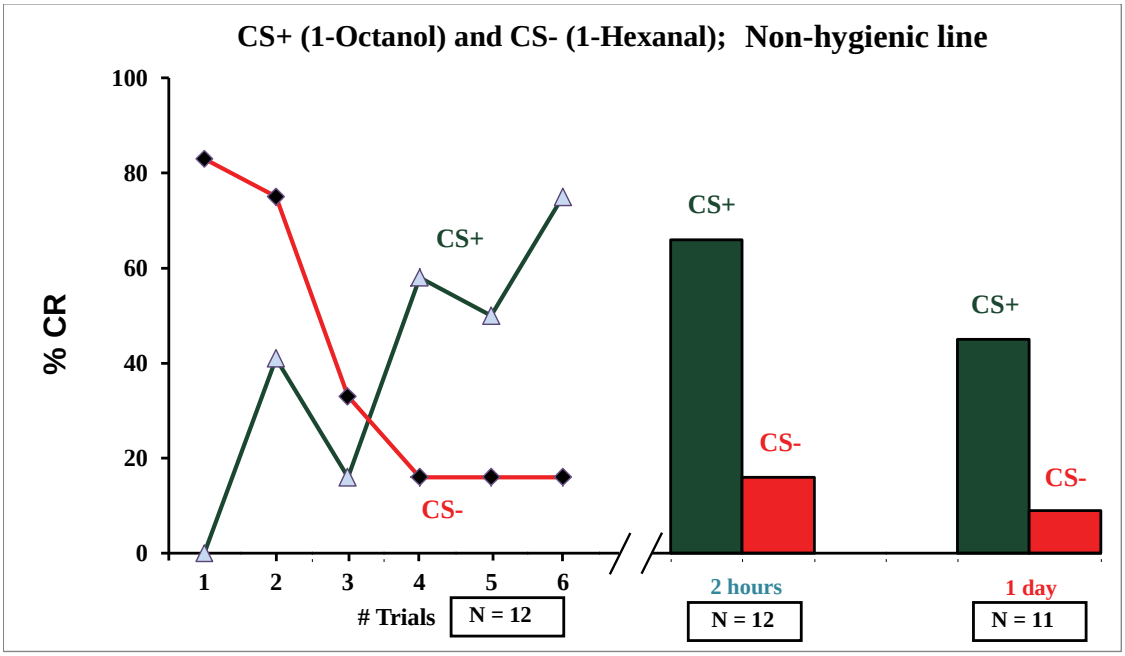

Fig. 12b: Learning and memory performances of the non-hygienic line during the differential conditioning with 1-octanol as the CS+ and 1-hexanal as the CS-: Non-hygienic bees were conditioned differentially using 1-octanol as the CS+ and 1-hexanal as CS-. The x and y axes in the figure respectively represented the number of conditioning trials and the percent conditioned responses (CR). During the conditioning and the retention tests, bees were able to discriminate between the CS stimuli, however, the differences were not found significant (χ^2 test: $p > 0.05$) due to the small sample size in this experimental group. N' represented the total number of hygienic bees used in the experiment which was in between the 1 day and 1 hour test (due to death).

2.6 Discussion

2.6.1 Differential conditioning of the hygienic and non-hygienic bees with the wax caps

Both the olfactory and contact chemosensory mechanisms can operate together to elicit the uncapping component of the hygienic behavior inside the colony. To test the olfactory component of the hygienic behavior honeybees from the hygienic and non-hygienic lines were trained to discriminate between the volatile odor profiles of the wax pieces sealing the un-parasitized (and healthy) and the *Varroa* parasitized brood cells. The wax pieces were heated at 37^{o}C for the constant release of the volatile chemical cues during the conditioning. However, bees from both genetic lines were failed to learn the discrimination task between the volatile odor profiles of the two types of wax caps. Beeswax mainly contains the esters of fatty acids and long chain alcohols; compounds such as the esters of palmitate, palmitoleate, hydroxypalmitate and oleate with the long chain (30 – 32 carbons) aliphatic alcohol predominates the composition. However, volatile chemical compounds such as butanediol, isovaleric acid (Schöning *et al*., 2011) or Z- (6) pentadecene (Nazzi *et al*., 2004) which were reported as the possible crucial compounds to elicit the hygienic behavior against the *Varroa* mite, have more hydrophilicity than the lipid components of the wax caps. The polar nature of these compounds probably inhibits their incorporation into the lipid matrix of the wax caps in higher concentrations. Hence, the higher concentrations of the common lipid components over the lower concentrations of the disease-associated chemicals in the infected wax caps probably overshadowed their detection in the PER conditioning assay. This might be one of the reasons why bees from both genetic lines failed to discriminate or generalized highly between the volatile chemical profiles of the two types of wax caps.

Francesco Nazzi's experiment used the whole brood cells, in comparison we only used the small pieces of wax caps; hence, it was possible that the infection associated chemical cues were lost in these preparations due to the use of small caps in place of the entire brood cells. Additionally, the isolation procedure might also have affected the chemical composition of the wax caps. Caspar Schöning's study showed that removal of the *Varroa* parasitized brood predominantly involved with the incidence of overt infection of

the brood by the replicating, virulent forms of the deformed wing virus (Schöning *et al*., 2011). The virulent form of the deformed wing virus (DWV), transmitted by the foundress mite during the infection was able to inflict more damage on the parasitized brood. The extent of brood damage finally governed the brood removal behavior in the hygienic colonies. Otherwise, *Varroa* parasitized brood carried the covert from of infection and the non-virulent forms of the mite-transmitted DWV were often not detected as abnormal by the bees and hence, were not removed. In our experiments the viral loads of the foundress mites were never tested. It was possible that foundress mites used in these experiments only carried the non-replicating or non-virulent forms of the DWV; hence, caused the covert infections in the infected brood which eventually led to the formation of the spoilage-associated volatile cues in amounts which were not recognized by the hygienic and non-hygienic bees in the olfactory PER assay.

2.6.2 Differential conditioning of the hygienic and non-hygienic bees with the brood specific volatiles

Schöning's study found that the volatile chemical profiles of the healthy and the *Varro*a infected pupae with the replicating DWV differed quantitatively, with many of the infected pupal-odor profiles contained some of the rare compounds such as isovaleric acid and 2, 3-butanediol in high proportions. These short chain volatiles especially the isovaleric acid was known to be involved with the process of microbial spoilage or decay (Allison *et al*., 1978). Nazzi and colleagues also reported the quantitative difference between the chemical compositions of the volatile profiles of *Varroa* parasitized and un-parasitized brood (Nazzi *et al.*, 2004). Honeybees probably use these odor cues to detect the *Varroa* parasitized brood present inside the capped cells. Hence, in the next experiment it was checked whether bees from the hygienic and non-hygienic lines were able to discriminate between the volatile odor profiles of the healthy and the *Varroa* parasitized pupae in the olfactory PER paradigm. Unlike the severe brood-damage inflicted by the chalkbrood or foulbrood pathogens, *Varroa* parasitization often produces no abnormality in the developing brood (Schöning *et al.*, 2012). In our experiments ~ 15 - 20 % of the pupae used in the single rounds of differential conditioning were found to

show the prominent signs of spoilage included the black coloration, disfiguration and malodors. The viral load and the reproduction status of the individual foundress mite although were unchecked in our experiment however; sustenance of the three mother mites (originally used to infect individual brood) on the haemolymph of one brood led to the occasional spoilage amongst the pupae. Hygienic bees in this set of experiments were able to recognize and discriminated the infection-associated volatiles (probably expressed quantitatively) released by the spoiled pupae from the healthy pupal-volatiles when the parasitized pupae were used as the CS+ stimulus. Non-hygienic bees, in comparison were failed to learn the discrimination between the volatile chemical profiles of the healthy and parasitized pupae. They showed high generalization in responses to the CS stimuli throughout the conditioning. In the reciprocal training, with the healthy pupae used as the CS+, both genetic lines were failed to learn the contingencies of the two CS stimuli during the conditioning. Similar asymmetry in the salience of the diseased and the healthy brood odors was reported before by Masterman and colleagues as they found that hygienic bees were able to discriminate better and generalized less when the chalkbrood infected pupae were used as the CS+ but generalized more when the healthy pupae were used as the CS+ (Masterman *et al.*, 2001). The reasons for the asymmetric salience of these two volatile profiles were unknown however; the general weaker nature of the pupal-odors (low amount of odors released from the pupal body) compared to the pure odor stimuli probably contributed to the low overall levels of PER and low odor discrimination of the two honeybee lines. Additionally, unlike the previous experiment with the wax caps, the temperature of the syringes in this experiment was not be raised to increase the release of the volatile odors from the pupae since; it could have caused some thermal damage on the pupae. Hence, it was unclear from these results whether the poor performance of the non-hygienic bees indicated the general problem of this line to learn the olfactory information in the PER paradigm or the hygienic bees were better performers in the olfactory learning tasks. However, irrespective of these possibilities the successful discrimination of pupal-volatiles by the hygienic bees at least in one of the two experiments was a *key finding* as it strongly indicated the *possible use of olfactory signals by the hygienic bees for the recognition of Varroa mite in the colony, which eventually contributes to the better hygienic behavior of these bees against the parasitic mite*.

2.6.3 Absolute conditioning of the two types of bees with the sting alarm pheromone odor, isoamyl acetate

Effects of olfactory generalization in the two genetic lines was tested during the mid-term and early long-term memory retention tests using the plant odor 1-hexanal when bees were conditioned with the sting pheromone compound isoamyl acetate. Both hygienic and non-hygienic bees learned and subsequently formed the memory of the CS+ odor, IAA; however, non-hygienic bees showed higher odor generalization than the hygienic bees. No significant difference in conditioned responses (CRs) between the conditioned and the novel odor (1-hexanal) was found for the non-hygienic bees. In comparison hygienic bees always showed the significantly higher responses to the CS+ compared to the novel CS. Stronger effect of odor generalization found in the non-hygienic bees supported the results of Sandoz's study (Sandoz *et al*., 2001) however, unusually high and constant responses to the stimulus, like filter paper questioned the general olfactory learning ability of these bees. In absence of any other group of honeybees trained and tested in parallel with these two lines we did not make any conclusion about the superior performance of the hygienic bees or the general difference in olfactory learning between these two lines. In the next experiment we tested the possible differences in olfactory learning between these two lines however; bees were adapted before the conditioning with the background odor of honeybee colony. Honeybees used in our conditioning experiments already learned the meaning of colony odor. It was interesting to test whether such a learned stimuli had any influence on the learning of the sting pheromone odor IAA in both genetic lines. Like the previous experiment we found that non-hygienic bees generalized between the IAA and novel odor 1-hexanal more than the hygienic bees. However, unlike the last experiment both type of bees were found to show odor generalization in their responses. When the possible effects of olfactory adaptation on the odor learning and generalization in honeybees were investigated, we found the common decrease in olfactory learning in both types of bees. The reduction in olfactory learning was found stronger in the non-hygienic compared to the hygienic bees. These results did not confirm the possible general deficit in olfactory learning of the non-hygienic line, since this type of experiment was never reported on any other honeybee line. However, behavioral (olfactory) adaptation with the habitat odor was found to inhibit the olfactory

learning and elevate the effect of odor generalization in honeybees of the hygienic and non-hygienic lines. The deficit in odor learning might be specific effect of the colony odor background or for the training odor isoamyl acetate, rather not general. To test between these possibilities, further experiments are required where one has to test the adaptation effects of the single and mixture odor backgrounds on the PER learning of odors with different carbon chain lengths and functional groups.

2.6.4 Differential conditioning of the hygienic and non-hygienic bees with the floral odors

Masterman and colleagues (Masterman *et al.,* 2000) showed that although their hygienic bees had higher discriminability between the volatile odors of the healthy and the chalkbrood infested pupae than the non-hygienic bees; however, both lines were able to discriminate similarly between the high concentrations of the floral odors geraniol and 1-hexanol. Previous reports also showed that honeybees were able to discriminate between these two odors in the olfactory PER paradigm (Malun *et al.,* 2002) as well as in the free flying condition (Laska *et al.,* 1999). In our experiments, hygienic bees were able discriminate between the floral odors geraniol and 1-hexanol which supported the previous results. On the contrary, the non-hygienic bees were failed to learn the discrimination tasks during the two conditionings. In addition, the non-hygienic bees showed strong effect of generalization between their responses to the conditioned odors and filter paper during the memory retention tests. The strong effect of generalization was associated with the consistently higher conditioned responses (CRs) to the CS- compared to the CS+ stimuli during the differential conditioning with wax odors, pupal odors and floral odors. This common effect found in the CRs probably did not arise only due to the combined effects of stimulus generalization and alternate CS+ and CS- trials, but indicated some form of prolong arousal or sensitization effect in odor responses of the non-hygienic bees. However, non-hygienic foragers definitely contributed substantially to the sufficient food reserve (monitored by us) of these colonies during both summer and autumn. Hence, keeping in mind the superior olfactory learning ability of the free flying forager bees (von Frisch K 1919; Laska et al. 1999), we discarded the possibility that

non-hygienic foragers used in our experiments had general deficit in olfactory learning. We concluded that the overall poor learning and memory performances of the non-hygienic bees during the summer were most likely due to some general deficit of this genetic line to learn the odors in the PER paradigm. To our surprise, the non-hygienic bees showed the similar olfactory learning and memory performances with the hygienic bees when conditioned with the other naturally occurring odors (1-hexanal and 1-octanol) during the autumn. This sudden improvement in learning performances precluded our previous conclusion that these bees had general problem to learn in the PER paradigm. Additionally, in our study no other group of honeybees (from other colonies or genetic lines) was trained in parallel to compare the performances of the two genetic lines with another group or honeybee line. Hence, in absence of the third group of honeybees and the lack of our understanding about the switch in learning behavior of the non-hygienic bees we did not conclude on the specific or general differences in olfactory learning between the hygienic and non-hygienic lines.

2.7 Comment and Outlook

Results showed that hygienic bees were able to discriminate better or generalized less between the volatile odor bouquets of the healthy and the *Varroa* parasitized pupae compared to the non-hygienic bees. However, amongst the batches of the infected pupae both the healthy looking and the discoloured and odorous pupae were used in the differential conditioning experiments. These deformed pupae probably contributed the abnormal odors to increase the distinctness between the volatile odor profiles of the two types of stimuli which were better perceived and discriminated by the hygienic bees. However, for some unknown reasons the hygienic bees only were able to discriminate more or generalized less when the diseased odor bouquet was used as the CS+ but not when it was used as the CS-. Odors specifically expressed and emanate from the *Varroa* parasitized brood were not clearly known. However, use of such odors in pure form along with the healthy brood odor such as β-ocimen might confirm the potential superior olfactory learning ability of the hygienic bees than the non-hygienic bees for the brood odors.

For comprehensive testing of possible superior abilities of the hygienic bees to learn and discriminate between the brood volatiles one can design a merged or double-test assay. In this assay bees from the hygienic and non-hygienic honeybee lines at first can be observed for their performances to remove the *Varroa* parasitized brood when exposed to the healthy and the artificially-infected brood cells inside an observation hive. These bees then can be followed for their performances in the olfactory PER conditioning to discriminate between the healthy and the diseased brood odors (single odors). Correlation in performances of the observation hive and of the PER conditioning assay can provide important confirmation about the abilities of bees to detect the health status of the brood using the olfactory signals. This type of double-test assay not only can reveal the important aspects of the individual's hygienic behavior but also can be used to study the expression patterns of genes related to the olfactory learning and / or hygienic behavior in the different brain neuropiles of the individual bees.

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Chapter -3

Characterizing the learning and memory performances of the individual honeybees using the cumulative olfactory conditioning paradigm

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3.1 Abstract

Olfactory learning and memory performances of the individual honeybees were analyzed with the aim to characterize the different types of learning related performers present in the population. Honeybees were first trained and tested in the cumulative olfactory conditioning paradigm with multiple phases of differential conditionings and memory retention tests. The overall performance of an individual bee was evaluated based on the scores of the different learning and memory related features such as the speed of odor learning, odor discriminability and odor sensitivity during the assay. Performance scores of the individuals showed high variability in each of these features. Under this circumstance, the overall or cumulatively best and the poor performers were selected with the arbitrary criteria of higher and lower range of cumulative scores (summation of scores of all features) and their performances were compared. Common high correlation between the learning speed and odor discriminability was found in these two types of cumulative scorers and in other types of performers selected with different criteria. The higher and lower scores of 'odor discriminability' among the other features were found to select respectively the best and the poor cumulative scorers with highest probabilities. In other words, the cumulative performances of the two types of extreme scorers were strongly influenced by the ability to discriminate odors during the differential conditioning. The analysis also showed that speed of learning of the rewarded (CS+) odor and the cumulative performance levels were the two important features that determined the learning speed of the unrewarded (CS-) odor during the differential conditioning. Apart from these, other interesting aspects such as the differences in odor generalization in several types of performers, consolidation of the short-term memory, and details about the relationship between the different learning related features were also discussed in this chapter.

Author's contribution: This is a manuscript which will be submitted for publication in an international peer reviewed journal. Please refer to page number iii of this dissertation for details about the author's contribution.

3.2 Introduction

Classical and operant conditioning paradigms have been extensively used for decades to understand the behavioral and physiological mechanisms of learning and memory both in the vertebrate and invertebrate models. In the classical or Pavlovian conditioning paradigm bees receive the training trials to learn the association of a neutral stimulus called the conditioned stimulus (CS) with a biologically meaningful or reinforcing stimulus (commonly a food reward or an electric shock) called as the unconditional stimulus (US). After multiple of such training trials bees learn the association between these two stimuli and start to respond to the presentation of the CS while anticipating the presence of the US following the CS. This is known as the conditioned response where a neutral stimulus (CS) acquires a different meaning through the association of a motivationally important stimulus. In the other version of the conditioning paradigm (operant) certain behavioral response of the bees decides the occurrence of the events of reinforcement. During the training trials bees learn in an operant manner the contingency between the appropriate responses with the appearance of the reinforcing stimuli.

The study reported here was based on the popular Pavlovian or classical conditioning paradigm in the honeybee, namely the olfactory conditioning of the proboscis extension reflex (PER). This is an appetitive learning paradigm (Kuwabara 1957; Takeda 1961; Bitterman *et al.,* 1983) where the proboscis extension reflex of the honeybee is conditioned with the olfactory stimulus (conditioned stimulus) through the presentation of odor stimulus overlapping with the delivery of the sucrose reward (unconditioned stimulus) first to the antenna to elicit the PER and then to the proboscis which allows the bees to feed. This particular conditioning procedure is able to create the robust and long-lasting memory in the honeybees as they start to show the conditioned response (the PER) to the presentation of the odor after the conditioning with different time intervals between the conditioning-trials (Menzel *et al.,* 2001). However, it was always found in the results of the olfactory PER conditioning, that certain proportion of the honeybees in the training population was able to learn the association faster than the others, along with the proportion which failed to learn. Similar results were found in the different vertebrate learning paradigms such as the autoshaped key pecking in pigeons (Gamzu and Williams 1973), plus maze learning in rats (Pellow *et al.,* 1985), water maze learning in mouse

(D'Hooge and De Deyn 2001) and the eye blinking conditioning in rabbit (Hinson 1982) where the learning performance of the individuals was found to differ (Gallistel *et al.,* 2004) from each other. Before, the work of Gallistel and colleagues, the individualistic variability in learning was not investigated carefully since; popularly learning performances (conditioned responses) in the different paradigms were always explained for the group or population of bees through the measures of the population-averaged learning parameters. A popular measure of this kind is the population learning curve, which represents the average performance of all the bees, trained identically over the training trials. The gradually rising population probability of the conditioned response (CR) commonly found in the population learning curves described 'learning' as the process where the associative strength of the CR rises gradually as a function of the number of the training trials. However, Gallistel and his colleagues clearly showed for the different vertebrate conditioning paradigms that the learning curves of the individual bees looked different from each other as well as from the population-averaged measurements. Individuals were found to vary with respect to their latency in responses and the asymptotic levels of their CRs as well as they neither were found to show the gradual increment nor the prolonged acceleration of the CR probability which were found in the population learning curve. Instead, individual bees showed the step-like increase in the probability of the CR with the combination of both faster and slower slopes found in their learning curves before arriving to their respective asymptotes. This confirmed the fact that individual animals in the eye-blinking, water maze or in other paradigms did not learn gradually, rather abruptly or in the switch-like manner from the unlearned to the learned states. Additionally, it was found that once the individuals achieved the learned state they remained stable during rest of the conditioning trials. Gallistel's analysis strongly indicated that individual's learn the conditioned stimuli with an 'all-or-none' dynamics which also was previously reported for the other learning experiments (Restle 1965; Bower 1961; Estes 1960). However, these interesting features of the individual's learning were not captured in the population estimates of the learning parameters since, it only represented the average of the learned and unlearned responses of all bees; hence, bees which showed the CR dictated the group-averaged probabilities of the CR during the conditioning trials. Similar inadequacy of the population-averaged measurement to

represent the individual's behavior was also reported previously with the other conditioning paradigms (Krechevsky 1932; Estes 2002).

In contrast to the vertebrate literature limited number of reports was available in the invertebrate models regarding the learning dynamics and the performance heterogeneity of the individuals in the popularly used conditioning paradigms. In the fruit fly, *Drosophila melanogaster* it was reported time back by Quinn (Quinn *et al.,* 1974) and colleagues that population measures were able to adequately represent the individual's probability of learning behavior in the aversive olfactory learning paradigm. In the appetitive olfactory learning paradigm of the *Drosophila* Chabaud and colleagues (Chabaud *et al.,* 2006) also showed that individualistic probabilities of showing the proboscis extension response or PER (conditioned response) were represented by the population PER probabilities during the conditioning trials. These results disclosed the fact that all members of the experimental populations of the adult fruit fly were homogeneous with respect to their rates and the final levels of olfactory learning; hence, excluded the possibilities of existence of the intelligent or poorly performing sub-groups. Furthermore, unlike the vertebrate conditioning paradigms, flies did not show the stable learned responses (PERs) over time and developed only the short-term memory in the appetitive paradigm which was successfully retrieved after 15 min of the conditioning but disappeared within an hour time. The weaker effects of conditioning trials as seen in the *Drosophila* model indicated the possibilities of low levels of learning or even a faster extinction however; did not match with the results of the olfactory PER conditioning performed with the honeybee. Apart from these two reports, two other studies investigated the olfactory and visual learning of the individual *Drosophila* larvae were relevant to mention in this context (Scherer *et al.,* 2003; Gerber *et al.,* 2004). Although, the authors of these studies looked at the behavior of the individual larvae however; they only monitored the memory retention of the individual's without analyzing the individual's learning (acquisition functions). In their experimental paradigms, they trained the larvae either to learn the odor cues or the visual conditions in groups and tested their memories individually. Hence, these results also did not contribute much information about the dynamics of the individual's learning.

To this end we started to investigate the issue of the individualistic heterogeneity in learning by systematically analyzing the learning data generated with the different olfactory PER conditioning paradigms (absolute, differential and extinction learning) in the honeybee. We found that individual honeybees inside the different experimental populations were variable in their rate of olfactory learning which created the population heterogeneity (Pamir *et al.,* 2011). During any conditioning trial of the different training protocols, two types of bees were found, one with the associative strength higher than the threshold and high probability to show the CR (PER) and the other type with the associative strength lower than the threshold and showed no CR with high probability. This analysis confirmed the existence of the good and the bad learning performers in the different experimental populations which received the different types of odor training. We also found that learning curves of the individual honeybees did not show the gradual and prolonged accelerated increase in the CR, rather like the vertebrate models they were found to show the abrupt or step like increase in responses from an unlearned or naïve level to the level of complete mastery. Additionally, it was found that once they learned the association or no-association (CS- trials of the differential conditioning or during the extinction learning) between the CS and the US in a switch-like manner they showed high probability to remain stable in the learned-state for rest of the experimental procedure until the memory retention test. Conversely, bees which did not show the PER in a certain training trial also showed no-PER with high probability during the following trial and if they continued showing no-PER until the last conditioning trial then they also showed no conditioned responses during the memory retention tests again with high probability. Although in the fruit fly model individualistic heterogeneity in the learning probability was never found both in the aversive and in the appetitive paradigms however; in honeybees the serial correlation analysis clearly revealed the individualistic differences in the learning behavior in the different appetitive paradigms. But it is not know whether the other invertebrates also learn in the switch-like fashion similar to the honeybees or the individual's learning in the other model system and in the other paradigms takes place classically. The finding of (Pamir *et al.,* 2011) heterogeneity in the learning performance of the individual honeybees although opened up the next set of questions as whether there are behavioral signatures that can be used to characterize the

different classes of the learning and memory performer in the middle of the heterogeneously behaving population.

3.3 Goals of the study

The purpose of this study was to analyze the olfactory learning and memory performances of the individual honeybees to characterize the different performer classes present in the population. To fulfill the goal I designed a cumulative form of olfactory conditioning assay to train the honeybees in two different phases with each phase consisted of one round of differential conditioning followed by the two rounds of memory retention tests using the multiple dilutions of the conditioned odors. The assay offered the advantage of testing the bees repeatedly for long period of time (6.5 hours) with the total number of 56 conditioning and retention test trials to screen for the different types of learning and memory performers in comparison to the simpler odor training protocols that bees received in the data sets analyzed recently by Evren Pamir (Pamir *et al.,* 2011). The cumulative manner of odor conditionings with the pure odorants and retention tests using the different dilutions of these odors made the protocol particularly suitable *to score for the different learning and memory related features* such as the speed and reliability of the rewarded (CS+) odor learning, discriminability between the CS+ and the CS- (unrewarded) odors both during the conditioning trials and the memory retention tests, sensitivity to the odor dilutions and responses to the stimuli like paraffin oil and filter paper *using a simple scoring scheme for multiple times during the assay*. Individual's analysis of performances based on the scores in these quantified behavioral features and the cumulative scores (summation of scores of all features) allowed me to answer the questions such as:

1. How the different learning related features were correlated or in other words how the performance scores of the single features influenced the scores of others in the different group of performers such as the best and the poor cumulative performers (behavioral characterization of the two extreme groups of performers), bees with the higher odor sensitivity or higher odor discriminability or for the entire experimental population?

The performance histories for the individual learning related features were rigorously analyzed to understand their influences over each other and on the overall performances

of the bees e.g. whether bees with higher scores in 'odor discrimination task' during the 1st differential conditioning maintained their specific responses during the immediate conditioning trials of during the retention tests as well as during the 2nd differential conditioning and the following tests, whether the cumulative performance levels of the bees affected their responses to the filter paper and paraffin oil and so on.

2. How the learning dynamics of the rewarded (CS+) and the unrewarded odor (CS-) stimuli varied between the different performer classes?

3. The cumulative assay provided the chance to analyze whether the performance scores (higher or lower scores) of any one or more of the different learning related features were able to select the two extreme classes of cumulative scorers (best and poor performers) with higher probabilities compared to the others. The idea was to check whether any single or more of these quantified features was able to predict or influenced the overall or cumulative performance levels of bees in the cumulative conditioning assay.

The other major purpose of this assay was to study the expression patterns of the learning related genes among the different performer classes with the motivation to find out the possible genetic signatures of the olfactory learning and memory performances. The performed gene expression study was limited to the mushroom body neuropil of the best and the poor cumulative performers. However, the data analysis of this study is currently ongoing with our colleague Dr. Gérard Leboulle (Freie University, Berlin Germany); hence, the findings of the behavioral data were only discussed in this chapter.

3.4 Honeybee colonies used in the assay

Honeybee colonies belong to the specific genetic lines from the Länderinstitut für Bienenkunde or LIB, Hohen Neuendorf, Berlin were used to perform the cumulative conditioning assay. These genetic lines were selected for (breed for generations; since 1997) their higher resistance against the ectoparasitic mite *Varroa destructor* (details were given in chapter-2) and were called as the 'Hygienic line' in this dissertation. Hygienic behavior (details given in chapter-2) is defined as the ability of the worker bee's to detect and remove the diseased or abnormal brood from the colony before the dissemination of the disease. Olfactory learning in honeybee was previously reported to show strong heritable components as bees from different colonies with the different

genetic backgrounds were found to perform differentially in the different olfactory PER conditioning paradigms (Latent inhibition and reversal learning: Chandra *et al.,* 2000; Ferguson *et al.,* 2001, Absolute conditioning: Brandes 1988). Hence, in the cumulative conditioning assay we wanted to use honeybees with less genetic variability to reduce the possible variability in olfactory learning. For this purpose we used bees from the LIB-hygienic lines since they were raised from the drones with very similar genetic background (few colonies, not all) hence, probably had low genetic variability. Additionally, we (with my collaborator Dr. Gérard Leboulle; Freie Universität, Berlin) investigated the possible correlations between the behavioral performances of the individual bees with the expression patterns of the learning and memory related genes in their brain neuropiles. For the gene expression study it was necessary to reduce the genetic variability in the experimental population of bees without compromising with the idea to test bees from the different colonies for their learning and memory performances. Hence, multiple backcrossed hygienic colonies (lines) were used in the cumulative assay which eventually reduced the experimental population of the worker bees into two types; one type contained both copies of the hygienic alleles and the other one contained the genetic background with one copy each of the hygienic and the non-hygienic alleles. This procedure reduced the overall variability in the genetic backgrounds of the worker bees and given us the chance to test the effects of the gene or allelic dosage (homozygous *vs.* heterozygous) on the olfactory learning which eventually raised our chances to find out the potential genetic signatures of the learning behavior in the gene expression study, although both the homozygous and heterozygous workers actually still represented many different allelic combinations in their genome.

3.4.1 Backcrossing Scheme

In the backcrossing scheme (Fig. 1) queen bees from the non-hygienic (+/+) colonies were artificially inseminated with the sperms taken form the hygienic drones (H). Heterozygous queens (H/+) were selectively raised from the F1 progeny of the inseminated (+/+) parental queens. During the last step of the backcross, the (H/+)-F1 queens were again artificially inseminated with the sperms of their paternal hygienic

drones (H). These inseminated, heterozygous queens were used to generate the colonies which were populated by the workers with either of the two genetic backgrounds; homozygous workers (H/H) with two copies of the hygienic alleles in their genome and the heterozygous (H/+) progeny with one each copy of the alleles inherited from the hygienic and the non-hygienic parents. These two types of worker progenies were solely used in the cumulative olfactory condition assay.

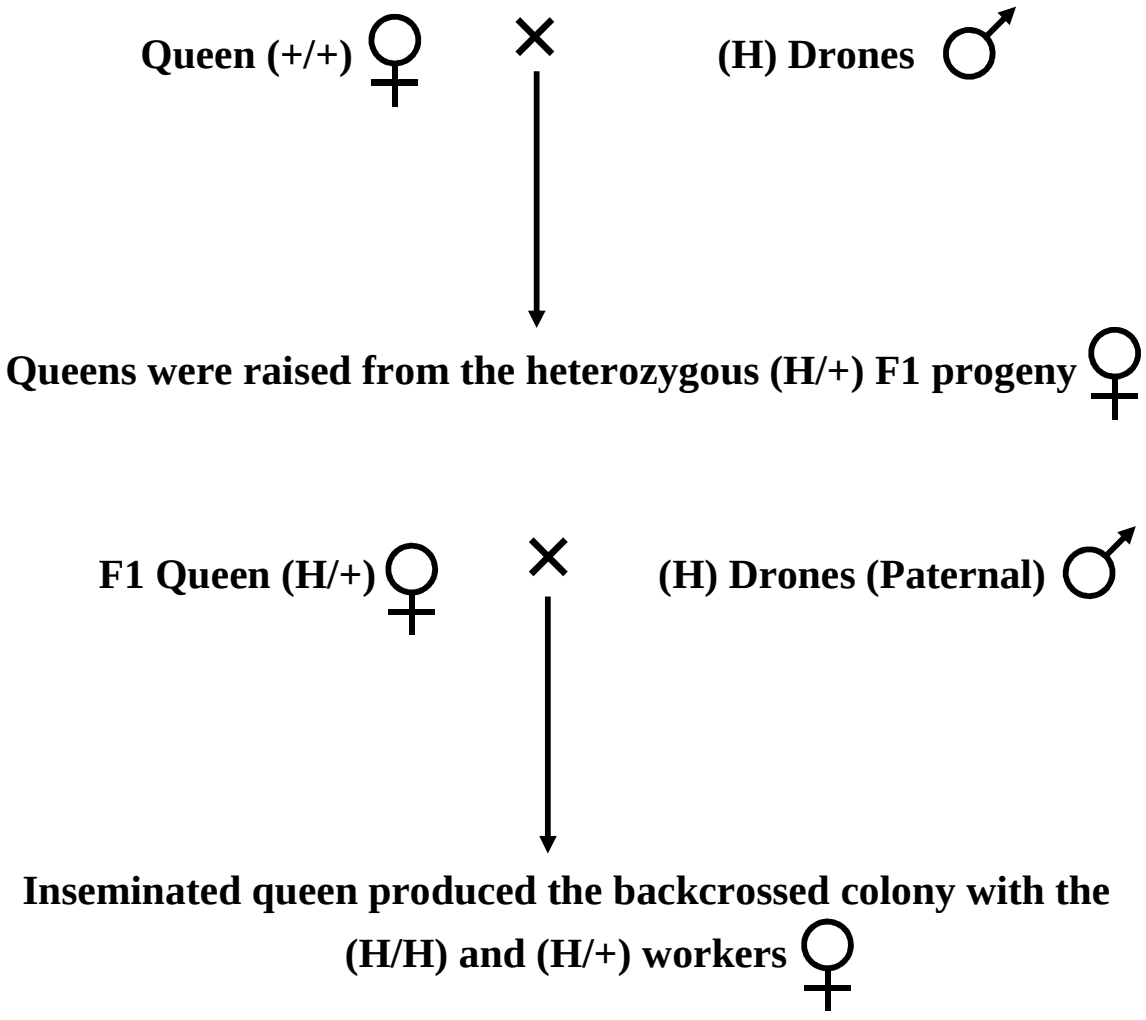


Fig. 1: Schematic representation of the successive genetic crosses of the backcrossing scheme: At first the non-hygienic queen (+/+) bees were inseminated with the sperms of the hygienic drones (H), which produced the heterozygous F1 progenies (H/+). Queen bees were raised from the heterozygous progenies which again were inseminated with their paternal hygienic sperms to produce the backcrossed population of the workers with either two copies of the hygienic alleles (homozygous: H/H) or one copy each of the hygienic and non-hygienic (heterozygous: H/+) alleles in their genomes.

3.5 Materials and Methods

3.5.1 Preparing honeybees for the olfactory PER conditioning

The general procedure of preparing bees for the olfactory PER conditioning was explicitly mentioned in the previous articles (Bitterman *et al.,* 1983; Menzel *et al.,* 2001; Stollhoff *et al.,* 2005) which was also followed in our experiments with minor changes. Honeybee foragers were caught at the entrance of the hives (a total of 3 different backcrossed colonies were used) during the afternoon, around 16.00 hrs; the day before the experiment. All colonies were placed in the institute's bee garden and remained there for the summer of 2010. The whole set of the conditioning experiments were conducted between the month of July and the October' 2010. Honeybees were caught with the help of an UV translucent catching box, taken to the laboratory, immobilized on the ice and harnessed into the small plastic tubes. Only, the antennae and mouthparts such as the mandibles, proboscis and antennae were allowed to move freely with rest of the animal's body fixed within the tube with a sticky tape. For every day's experiment, equal number of bees were caught and trained in parallel from the three colonies to be able to compare their performances and to avoid the effects of the seasonal and the day-to-day variations on the olfactory learning. In the evening, around 18.00 hrs all bees were fed with the 30% (W/V) sucrose solution (0.87 M) until they were satiated. After the feeding procedure they were kept for overnight inside a small, humid (~ 24^{o}C and ~ 70% humidity) Styrofoam box for the next day's experiment. Bees were taken out of the box on the next morning (10:00 am) and were placed in front of the experimental arena for at least 30-45 min before (to adapt bees with the new environment) the experiment.

3.5.2 General information about the differential olfactory PER conditioning; appetitive paradigm

In the appetitive paradigm of the differential olfactory PER conditioning bees receive the training with two odors; the two conditioned stimuli (CSs). Presentation of one of these odors namely the reinforced CS or CS+ is associated with a food reward (sucrose solution) known as the unconditioned stimulus or US and the other odor, the non-

reinforced CS or CS- is presented with no US following. Honeybees trained in this paradigm learn the contingencies of the two CSs during the conditioning trials and start extending the proboscis to the rewarded odor and showing no PER to the unrewarded one (pictorial description: Fig. 2A). This protocol forms the long-term memory of the odor identities in the bees which can be seen during the time of memory retrieval tests (Fig. 2B). Differential PER conditioning in the cumulative assay was performed with an already established odor delivery protocol where the odors (CSs) were manually delivered with a syringe of 20 ml. volume for 5 sec (Stollhoff *et al.,* 2005). During the reinforced CS presentation (CS+) the sucrose reward (30% sucrose solution) was offered to the bees 3 sec after the onset of the CS (total time of CS+ trial was 7 sec) for a total time of 4 sec with an overlap of 2 sec between the CS and the US. The unreinforced CS or CS- trials lasted for a total of 5 sec when the odor was only presented without any US. Honeybees were placed in front of an exhaust (conditioning arena) for 20 sec before and 20 sec after the CS+ or CS- conditioning trials. The time interval between the two similar CS trials (inter-stimulus interval or ISI) was 16 min and between the two successive and dissimilar CS trials (between CS+ and CS- trials: inter-trial interval or ITI) was 8 min. During the retention test only the two CSs were presented (Fig. 2B) for 5 sec like the conditioning trials to check for the formation of memories.

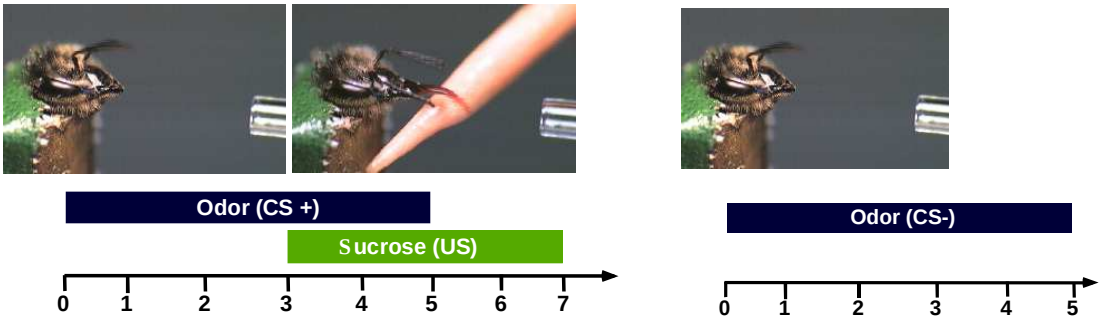
3.5.3 Protocol and purpose of the cumulative olfactory PER conditioning assay

The experimental protocol of the cumulative olfactory PER conditioning assay was consisted of two different phases with each one consisted of one round of the differential conditioning (DC) followed by the two rounds of memory retention tests. The completion of the first round of DC during the first phase was followed by a pause of 20 min, which immediately followed by the two consecutive rounds of retention tests to test for the short-term memory in the bees. No time gap was allocated between the two rounds of retention tests in each of the two phases however; a pause for 30 min was applied between the two phases of the cumulative conditioning assay. Retention tests were performed using the inter-stimulus interval of 8 min and the inter-trial interval of 4 min (half of the time intervals used during the conditioning). The completion of the whole

protocol required 6.5 hours where the single bees received the total number of 56 training and test trials. Differential conditionings (DCs) of the two phases were conducted with two different pairs of odors and the memory retention tests were performed using the trained and the untrained dilutions of the CS+ and CS- odors along with the presentation of the filter paper and paraffin oil. A total number of 152 honeybees were used in this assay from the 3 backcrossed colonies and their learning and memory performances were evaluated. During the DCs bees were trained with the pure concentrations of the CS+ and the CS- odors for a total number of 12 trials (6 CS+ and 6 CS- trials) with an alternate presentations of the CS+ and CS- stimuli (never pseudoradomized). However, during the retention tests they were exposed sequentially to the increasing concentrations of the CS+ and CS- odors (CS+ and CS- were again presented in alternation) along with the two other stimuli; filter paper and paraffin oil. Each round of retention test was comprised off 6 trials with two dilutions of the CS odors viz. $10^{(-3)}$ and $10^{(-2)}$ were used apart from their training concentrations (pure). A constant amount of 10 µl. of pure odors and their dilutions soaked on a piece of filter paper (1 cm^2) was used for the conditioning and for the retention tests. All dilutions were prepared with the paraffin oil (Sigma Aldrich, Germany) using a serial dilution procedure with the first dilution of the respective odors was made in the oil with 1:10 (v/v)-ratio from the highest (pure) concentration. The 30 min time gap after the completion of the first phase of the assay was followed by the beginning of the second phase which also was comprised of the same number of conditioning and the retention tests but conducted with a different odor pair. After the completion of the second phase all bees were checked for their overall fitness through the proboscis extension response to the sucrose (only touching the antenna with 30% sucrose solution), before they were sacrificed inside the refrigerator at -20^oC and then preserved immediately in another refrigerator at -80^oC and kept for the gene expression study. This paradigm offered the advantage to ask the individual honeybees repeatedly with the 56 odor trials for 6.5 hours to perform the specific set of learning and memory tasks which made the screening procedure more stringent for the cumulatively good or bad performers. In other words, the performance evaluation of the individual bees based on the total number of correct and incorrect responses to the different CS stimuli increased

the chances to isolate the truly and consistently superior and inferior classes of performers. The combination of the multiple

(A)



(B)

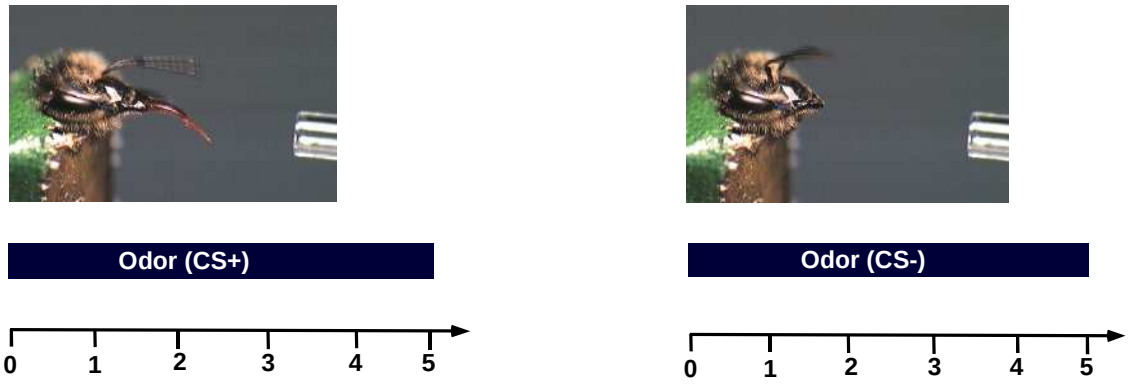


Fig. 2A and 2B: Pictorial representation of the differential conditioning (A) and memory retention (B) trials: During the CS+ conditioning trial (A-left picture) honeybees were trained to associate the odor (CS) with the sucrose reward (US). Bees were exposed to the CS+ for 3 sec followed by the sucrose reward delivered for the next 4 sec with the 2 sec overlap in between the CS and US (the time protocols were given below the pictures). Sucrose reward was delivered first to the antennae and then to the proboscis with the help of a toothpick. During the CS- (A-right picture) trial, bees only received the odor CS (CS-) for 5 sec without any US. Figure 2B (both pictures) represented the memory retention trials where bees were tested with the CS+ and CS- odors for 5 sec without any US. During the conditioning if bees learned the association between

the CS and US, then they showed PER to the CS+ and no-PER to the CS- odors during the retention tests (Adapted from Prof. Dr. Dorothea Eisenhardt, Freie Universität, Berlin Germany).

differential conditionings and retention tests also made it possible to score for the different learning and memory related behavioral features such as the speed and consistency of odor learning, odor discriminability and sensitivity of the individual bees separately during the two phases of the assay (details given in the 'scoring scheme') to answer the set of questions that I mentioned before (mentioned in the 'Goals of the study').

Other complex learning paradigms such as the single or multiple-reversal learning could be used to understand the heterogeneity in the individual's learning behavior and for the characterization of the different classes of learning performers. However, the design of the cumulative conditioning assay focusing on the successive rounds of the learning and memory trials discarded the incorporation of the extinction learning component (an important component of the reversal learning paradigm) on top of the already quantified set of learning related features (as mentioned previously). In addition, it was previously showed that cumulative training and test procedures were able to select for the different classes of learning performers in the honeybee (Brandes 1988; Brandes and Menzel 1990). Hence, the cumulative protocol was chosen with multiple rounds of the conditioning and retention test. *Multiple phases of memory retention tests might incorporate an extinction component in this assay* which was reflected in the reduction of the conditioned responses during the 2^{nd} compared to the 1^{st} retention test (among the pairs of retention tests) however; this component *probably never had the chance to consolidate due to the quick-fire procedure of the whole assay*, hence, probably did not influence the performance of the bees.

3.5.4 Odors used

Floral or pheromonal odors were commonly used in the olfactory PER conditionings, however, in this assay I used some special odors which were found to emanate from the body of the brood or present on the cuticle of the adult bees.

Why so?

Honeybee workers remove the unhealthy or diseased brood from the colony as part of their house keeping activity to stop the dissemination of the pathogen. This particular behavior is popularly known as the 'hygienic behavior'; a term that was originally coined by Rothenbuhler (Rothenbuhler 1964). Honeybees in general manifest this behavior inside their colonies but only a few of these bees (some are genetic lines) have acquired more resistance to the different bee diseases through the rigorous and efficient hygienic behavior. The more-resistant honeybee lines popularly called as the 'hygienic line' were reported many times in the literature (details given in chapter-2: Arathi *et al.,* 2000; Arathi *et al.,* 2006; Arathi and Spivak 2001; Ibrahim *et al.,* 2007; Spivak and Reuter 2001), and for the cumulative conditioning assay I used the hygienic bee lines from the LIB, Berlin (LIB, Hohen Neuendorf, Berlin) which were breed for the higher resistance against the parasitic mite *Varroa destructor*. Research for the past decade investigating the underlying neuronal mechanism(s) of this behavior showed multiple evidence of involvement of the olfactory chemoreception processes for the detection and removal of the diseased brood from the colony. In the year 2000 Masterman and colleagues reported that their honeybee lines breed for the higher and faster removal of the freeze-killed brood and higher level of overall hygienic behavior were able to learn and discriminate (in the olfactory PER conditioning paradigm) between the volatile odor profiles of the healthy and the chalkbrood infested pupae significantly better than the bee lines (non-hygienic lines) which were only capable of performing the slow removal of the freeze-killed brood (Masterman *et al.,* 2000). Another report of the same research group showed that bees from the hygienic line possess lower threshold and higher sensitivity to the diseased brood odors compare to the bees of the non-hygienic line (Masterman *et al.,* 2001). However, bees from the hygienic and the non-hygienic lines were also found to learn and discriminate similarly between the higher concentrations of the floral odors (Masterman *et al.,* 2001). Hence, the superior olfactory sensitivity and discriminability of the hygienic bees were probably directed towards the brood-specific odors as bees from both lines were able to learn the floral odors similarly. Apart from the possible behavioral mechanisms, report about the genetic control of this behavior revealed that hygienic behavior is a *quantitative trait* (potentially controlled by many genes: Lapidge *et al.,*

2002). This aspect was confirmed in the variability in *behavioral and physiological (olfactory sensitivity) responses* of the individual bees of the hygienic colony *to the healthy and the diseased brood odors* (Gramacho and Spivak 2003).

The cumulative conditioning assay reported here, although did not incorporate any non-hygienic bees however; the backcrossed hygienic colonies had heterozygous workers with one copy of the genome containing the non-hygienic alleles (H/+) along with the homozygous hygienic progenies (H/H). Hence, these colonies were probably comprised of members with differences in hygienic behavior due to the differences in their allelic composition. I decided to use the brood-specific odors for this reason (represented the healthy and diseased brood) to select for bees with differential performance levels in the olfactory learning and memory tasks influenced by the gene dosages from their hygienic and non-hygienic alleles. However, experiments of this kind could also be conducted with honeybees from any other colony (not breed as a genetic line) and using the floral or pheromonal odors.

Two brood-specific volatiles namely ocimen (beta-ocimen) and phenethyl acetate (PEA) were used in the cumulative assay. Ocimen was found as the constitutive component of the brood volatiles, on the contrary PEA was reported to produce specifically from the body of the larvae infected with the chalkbrood pathogen *Ascosphaera apis* (Swanson *et al.,* 2009). Hence, these two odors were chosen for one of the two differential conditionings. This particular conditioning procedure somehow mimicked the natural scenario inside the colony, when bees recognize and discriminate between the healthy and (chalkbrood) the parasitized brood based on their volatile odor profiles. No other pair of brood specific volatile was found which differed in their expression pattern qualitatively between the healthy and the parasitized brood such as these two. In this situation, I chose two long-chain cuticular lipids of the adult honeybee namely the oleic acid (OA) and the linolenic acid (LA) for the second round of differential conditioning. These two compounds were reported to be involved in the phenomena of kin-discrimination in honeybee (Breed *et al.,* 2004). These cuticular hydrocarbons were high molecular weight (MW) and less volatility compounds compared to the low MW and volatile brood odors however; the whole idea behind this selection was to direct the conditioning procedure more towards the phenomena of kin-recognition (nest mate

recognition) in absence of a suitable second pair of brood-specific volatiles. Ocimen (purity > 90%) and phenethyl acetate (99%) were purchased from the Sigma-Aldrich, Germany and both OA (> 99%) and LA (> 70%) were purchased from a Belgian company name TCI-Europe nv. In the assay only two combinations of the CS+ and CS- pairs were used; in one combination ocimen was used as the CS+ and PEA as CS- for the first round of the differential conditioning (DC) and linolenic acid was used as the CS+ and oleic acid as CS- for the second round of the DC, and in the other combination PEA was used as the CS+ and ocimen as CS- for the first DC and oleic acid was used as the CS+ and linolenic acid as the CS- during the second round of DC.

3.5.5 Scoring scheme

Eight different features related with the olfactory learning and memory processes were quantified to evaluate the performance of the individual honeybees throughout the cumulative conditioning assay. Three of these features viz. speed and reliability of the CS+ odor learning, odor discriminability during the conditioning and the memory retention test were quantified twice for each of the two phases of the assay. The features of 'odor sensitivity' and 'responses to the filter paper and paraffin oil' were quantified once, using the response data of the two phases of this assay. Only five of the six CS+ and CS- trials (after the first) of the DC were used to score the features associated with the conditioning. Scoring was performed on the binary data set of '1' and '0' which were respectively represented the extension and no extension of the proboscis to the CSs during the 56 conditioning and retention test trials; details of the scoring scheme were given below.

Feature-1 (F1) Speed and reliability of learning during the 1st differential conditioning (Acq1): Total number of PER responses showed by the bees only to the CS+ odors within the 3rd until the 11th trial of the differential conditioning during the 1st phase of the assay; divided by 5. This feature represented the speed and consistency of learning of the association between the CS and the US during the 1st DC.

Feature-2 (F2) Speed and reliability of learning during the 2nd differential conditioning (Acq2): Total number of PER responses shown by the bees only to the CS+

odors within the 31st until the 39th trial of the differential conditioning during the 2nd phase of the assay; divided by 5. This feature represented the speed and consistency of learning of the association between the CS and the US during the 2nd DC.

Feature-3 (F3) Odor discriminability during the 1st differential conditioning (Disc1): The number of CS+ responses during the 1st DC (trial number 3 to 12) that were respectively followed by no response to the CS- stimuli; divided by 5. This feature represented the behavioral discrimination between the CS+ and CS- odors during the 1st DC.

Feature-4 (F4) Odor Discriminability during the 2nd differential conditioning (Disc2): The number of CS+ responses during the 2nd DC (trial number 31 to 40) that were respectively followed by no response to the CS-; divided by 5. This feature represented the same behavioral aspect as the F3 but for the 2nd DC.

Feature-5 (F5) Discrimination during the memory retention tests 1 and 2 (T 1, 2): Total number of CS+ responses both during the test 1 (trial number 13, 15 and 17) and test 2 (trial number 21, 23 and 25) that were respectively followed by no response to the CS- during the test 1 (trial number 14, 16 and 18) and test 2 (trial number 22, 24 and 26); divided by 6. Feature-5 represented the learned discrimination between the CS+ and CS- odors (including the dilutions) during the short-term memory retention test (1st phase of the assay).

Feature-6 (F6) Discrimination during the memory retention tests 3 and 4 (T 3, 4): Total number of CS+ responses both during the test 3 (trial number 41, 43 and 45) and test 4 (trial number 49, 52 and 53) that were respectively followed by no response to the CS- during the test 3 (trial number 42, 44 and 46) and test 4 (trial number 50, 52 and 54); divided by 6. Feature-6 like feature-5 represented the learned discrimination between the CS+ and CS- odors during the short-term memory retention test (2nd phase of the assay).

***Feature-7 (F7) Odor sensitivity*:** Total number of responses to the lowest concentration of the CS+ ($10^{(-3)}$) odors during the 1st and 3rd retention tests (trial number 13 and 41); divided by 2. The feature 'sensitivity' represented the ability of the bees to detect the learned CSs at untrained dilutions during the retention tests.

***Feature-8 (F8) Response to the Filter paper and Paraffin Oil (FP + Oil)*:** Total number of responses to the filter paper and paraffin oil during the 4 retention tests (trial number

19, 20, 27, 28, 47, 48, 55, 56); divided by 8. Responses to the filter paper and paraffin oil represented both the overall responsiveness and odor-response specificity (odor generalization effect) of the honeybees.

A score range with the maximum of +1 and minimum of 0 was possible for all of the quantified features described above. Apart from these eight individual features, an overall score or the 'cumulative score' was calculated which represented the summation of scores of the individual features to evaluate the gross performance of the individual bees; ranging from 0 (minimum) until +8 (maximum).

These features apparently looked redundant as some of them were quantified twice, but to evaluate the total performance of an animal in this assay with two phases of identical sequence of conditioning and retention tests, it was necessary to score these features separately for the individual phases.

3.5.6 Data analysis

Overall learning and memory performance graphs: Overall performance graphs of the bees selected with specific criteria (specific performance scores in the learning and memory related features) showed the group-averaged conditioned responses to the CS+ and CS- stimuli during the conditioning and retention test trials of the cumulative assay. This also included the responses to the filter paper and paraffin oil.

Repeated measurement ANOVA was performed on the response data of the individual colonies to compare between the conditioned responses (CRs) to the CS+ and CS- stimuli during the conditioning. Wilcoxon matched pairs test was applied to compare between the CRs to the different dilutions of the CS+ and CS- stimuli during the memory retention tests.

Performance scores of the different quantified features (Feature-1 to 8) in the different selected group of bees were used to calculate the Pearson's correlation coefficients between them. These values were represented with false colors in the color coded correlation plots.

Bivariate histogram analysis was performed with the original binary response (PER) data of the pooled population of honeybees of the three backcrossed colonies. This analysis showed the relationships between the response histories to the different CS stimuli during the phases of conditioning and retention tests.

3.6 Results

3.6.1 Cumulative learning and memory performances of the backcrossed colonies

Cumulative or overall performances of bees (N = 152) in the pooled population of the three backcrossed colonies were represented at first in Fig. 3. The individual line-graphs (sub-plots) in Fig. 3 were showing the conditioned responses of the bees to the CS+ and CS- odors with all combinations, during the two phases of the assay. The 1st sub-plot (1st row) in Fig. 3 was showing the percent conditioned responses (CRs) of the honeybees to the reinforced (CS+: red line) and non-reinforced (CS-: blue line) odors during the 1st differential conditioning. The 2nd and 4th sub-plots were respectively represented the CRs during the 1st and 2nd memory retention tests to the different concentrations of the CS+ and CS-, started with the lowest dilution until the training concentrations. The 3rd and 5th sub-plots (black lines) represented the responses to the filter paper and paraffin oil. Similarly all line graphs in the lower panel (2nd row) of Fig. 3 represented the conditioned responses of the bees during the second phase of the differential conditioning and the two retention tests along with the responses to the filter paper and paraffin oil. Although, no statistical test was performed on the pooled data to check for the learning effects, however, it was apparent in Fig. 3 that bees learned the discrimination between the CS+ and the CS- odors both during the conditioning and the retention tests in the two phases of the assay. The overall learning and memory performances of the individual backcrossed colonies were shown below from Fig. 4 until Fig. 6. Honeybees from all three colonies were conditioned with two combinations of the CS+ and CS- odors (as mentioned before: ‘odors used’). In one of the two, bees were trained with ocimen as the CS+ and phenethyl acetate (PEA) as the CS- during the 1st differential conditioning (DC)

and with linolenic acid (LA) as the CS+ and oleic acid (OA) as the CS- during the 2nd DC. Honeybees conditioned with this combination of odor stimuli were called as the group-1. The other group of bees called the group-2 was trained with PEA as the CS+ and ocimen as the CS- followed by the OA as CS+ and LA as the CS-. Each colony had honeybees trained in both ways (both group-1 and 2).

Statistical analyses were performed on the response data of the individual colonies to check for the 'learning effect' however; during the analyses the PER data of these two groups were pooled for the individual colonies. This approach of data-pooling was possible, since no significant difference in the conditioned responses was found between the group-1 and 2 for the individual colonies along the conditioning trials with the repeated measurement ANOVA test (RM-ANOVA). Although, parametric ANOVA is not recommended for the binary data (e.g. the PER data), however, statistical techniques confirmed the (Lunney 1970) permissibility of the ANOVA for the dichotomous data under certain conditions. The data generated in the cumulative conditioning assay fulfilled these conditions of the equal cell or group frequencies and at least 40 degrees of freedom of the error term.

RM-ANOVA conducted for colony 98 showed the non-significant interaction effect between the group (group 1 and 2), stimulus (CS+/CS-) and the conditioning trial (group × stimulus × trial: $F_{5,1000} = 0.13$, $p = 0.98$) which validated the pooling of the data for the two groups. In the pooled data although no significant trial effect was found ($F_{5, 1000} = 2.18$, $p = 0.053$), but, the significant stimulus ($F_{1, 200} = 27.73$, $p = 0.000000$) effect and the significant stimulus × trial ($F_{5, 1000} = 37.54$, $p = 0.000000$) effect confirmed the learning of contingencies of the CS+ and CS- stimuli during both rounds of differential conditionings.

Similar non-significant interaction between the group × stimulus × conditioning trial ($F_{5, 980} = 1.08$, $p = 0.36$) was found for the colony or genotype 299 which confirmed the permissibility of the data-pooling. For colony 299, significant stimulus ($F_{1, 96} = 48.30$, $p = 0.000000$), trial ($F_{5, 980} = 3.96$, $p = 0.0014$) and stimulus × trial effects ($F_{5, 980} = 63.53$, $p = 0.000000$) confirmed the learning of the CS+ and CS- stimuli along the conditioning trials during the two phases of the differential conditioning.

In contrary, significant interaction between the group, stimulus and conditioning trials (RM-ANOVA: $F_{5, 490} = 2.35$, $p = 0.039$) was found for the colony 73 along with the significant stimulus ($F_{1, 98} = 56.14$, $p = 0.000000$), trial ($F_{5, 490} = 11.88$, $p = 0.000000$) and stimulus × trial ($F_{5, 490} = 29.87$, $p = 0.000000$) effects. The later three results confirmed that bees learned the discrimination between the rewarded and the unrewarded stimuli during the conditioning trials however; the significant group × stimulus × trial interaction prohibited the pooling of the conditioning data between the two training groups (1 and 2). Further analysis of the PER data of the colony 73 revealed the non-significant difference between the CRs for the alternate combinations of (alternate CS+ and CS- stimuli) the ocimen and phenethyl acetate used during the 1st differential conditioning (non-significant group × CS+ stimulus interaction: $F_{4, 196} = 1.68$, $p = 0.15$ as well as the non-significant group × CS- stimulus interaction: $F_{2, 98} = 0.78$, $p = 0.45$). Separate analyses

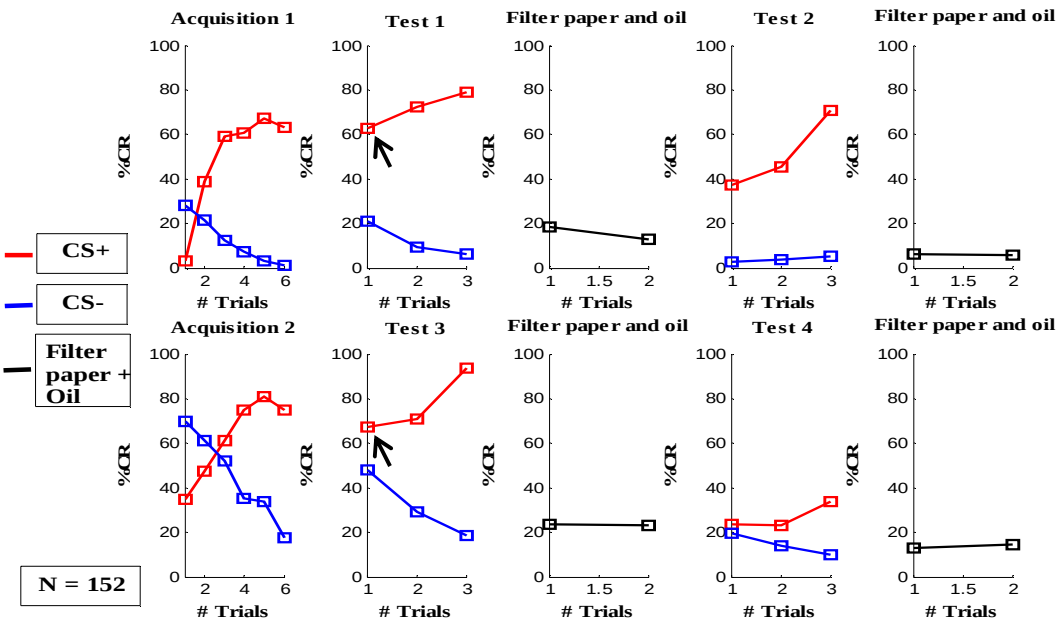


Fig. 3: Overall learning and memory performance graphs of the pooled population of honeybees in the cumulative olfactory conditioning assay. The overall learning and memory performances of the pooled honeybee population ('N' represented the total number of honeybees) from the 3 backcrossed colonies were represented here with the 8 sub-plots. Each sub-plot showed either the performance during conditioning or retention tests (written on top of the sub-plots) in course of the 56-trial assay. The whole assay was divided into two phases with the individual phases (each of the two rows) consisted of one round of differential conditioning

followed by the two retention tests and two additional tests with the two other stimuli (filter paper and paraffin oil). The x-axis and y-axes respectively represented the number of trials and the percent conditioned responses (CRs) during the conditioning or retention tests. Conditioned responses to the CS+ and CS- stimuli were always represented respectively with the red and blue lines and percent responses to the filter paper and oil were represented with the black lines. The 5 sub-plots (1st phase) of the 1st row of the figure showed the percent conditioned responses to the CS stimuli during the first differential conditioning (acquisition 1) and the two memory retention tests (test 1 and 2) along with the responses to the filter paper and filter paper + paraffin oil; tested twice during each phase. The 2nd row (2nd phase) similarly represented the CRs to the CSs of the second differential conditioning (acquisition 2) and the following retention tests performed with a different pair of CS+ and CS- stimuli. The black arrows in both rows indicated the retention test trial number 13 and 41 respectively of the 1st and 2nd phases of the assay. CS+ responses of bees of these two test trials were used to score for the feature 'sensitivity'.

between the alternate CS+ and CS- combinations of the oleic acid and linolenic acids used in the 2nd DC showed the similar non-significant difference between the CRs (non-significant group × CS+ stimulus interaction: $F_{5, 245} = 1.07$, $p = 0.37$ as well as non-significant group × CS- stimulus interaction: $F_{5, 245} = 0.52$, $p = 0.75$). These effects were

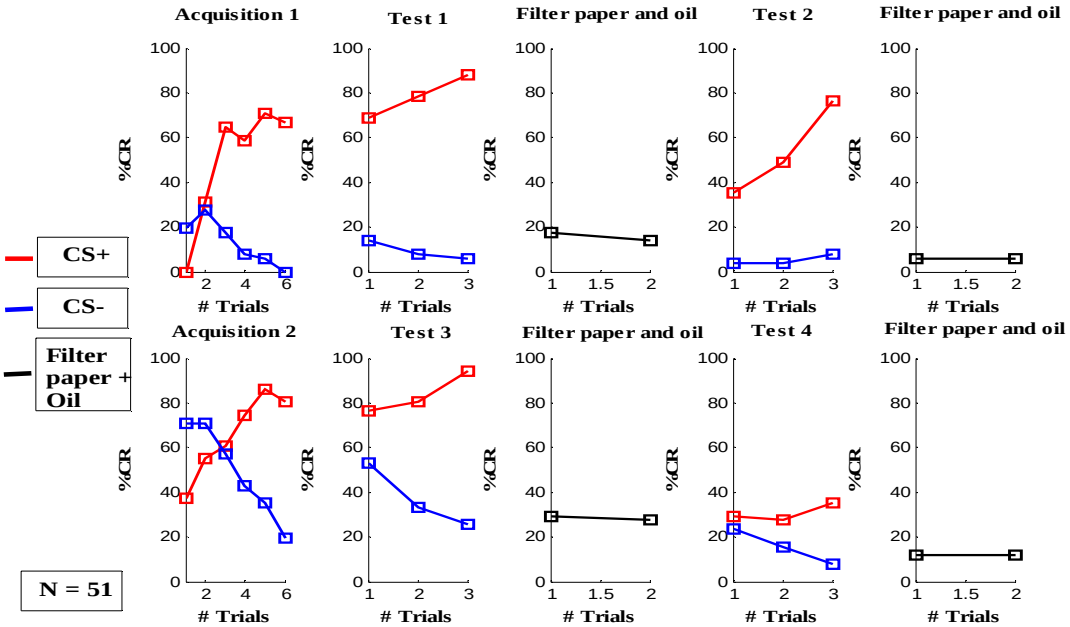


Fig. 4: Olfactory learning and memory performances of honeybees from colony (genotype) 73: The overall performances of bees from colony 73 were shown here with the x and y axes represented the same parameters as mentioned in Fig. 3. The 8 sub-plots in the figure also

represented the performances during the same phases of conditionings and the memory retention tests as described in Fig. 3. 'N' represented the total number of bees.

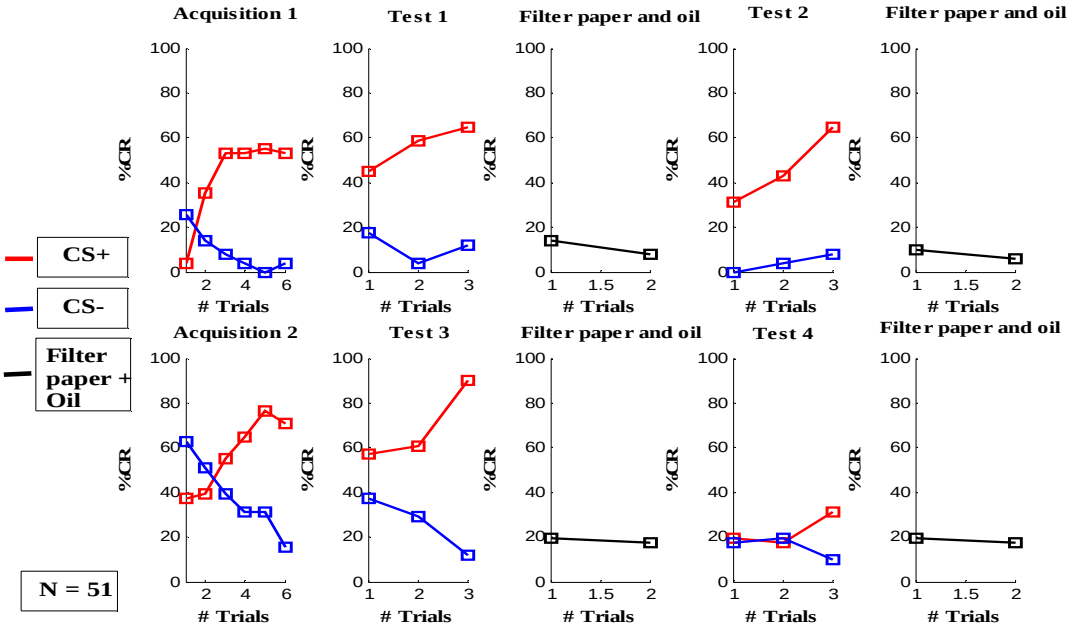


Fig. 5: Overall olfactory learning and memory performances of honeybees from colony (genotype) 98: The learning and memory performances of honeybees from colony 98 during the cumulative conditioning assay were represented here with the 8 sub-plots. The axes and the sub-plots represented the same variables and the performance graphs during the same phases of the assay as described in Fig. 3.

also visible (in the post hoc probabilities; Fisher LSD test) when the ANOVA teat formerly investigated the interaction between the group, stimulus and the conditioning trial. Hence, the conditioning data of the two odor groups (1 and 2) of the colony 73 were pooled for the analysis of the learning and memory performances (Fig. 4 - 6). All differences between the conditioned responses to the different concentrations of the CS+ and CS- stimuli during the memory retention tests were found significant with the Wilcoxon matched pairs test; the results were shown below in the tables (table: 1 – 3).

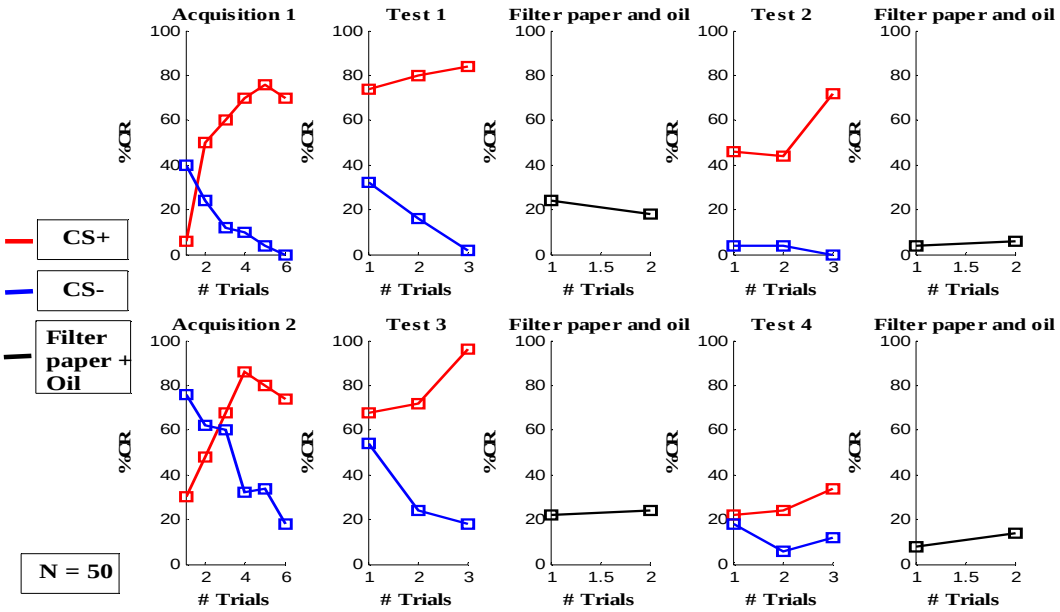


Fig. 6: Olfactory learning and memory performances of honeybees from colony (genotype) 299: The overall learning and memory performance graphs of the honeybees from the colony 299 were represented here with the x and y axes represented the same parameters as in Fig. 3. The 8 sub-plots were also represented the performances during the same phases of conditionings and memory retention tests as described in Fig. 3.

Table 1: Results of the Wilcoxon matched pairs test (WMP test) for the 3 colonies compared the conditioned responses to the lowest concentration (10^{-3}) of the CS+ and CS- odors used during the retention tests were shown here in table-1. The Z and p values were given in the table and all p values were found significant.

Colony Number	Z value	p value
73	6.53	0.000000
98	5.51	0.000000
299	6.14	0.000000

Table 2: Results of the Wilcoxon matched pairs test (WMP test) for the 3 colonies compared the conditioned responses to the second lower concentration (10^{-2}) of the CS+ and CS- stimuli during the 4-retention tests were shown here in table-2. The Z and p values were given in the table and all p values were found significant.

Colony Number	Z value	p value
73	8.78	0.000000
98	7.22	0.000000
299	8.33	0.000000

Table 3: Results of the Wilcoxon matched pairs test (WMP test) for the 3 colonies compared the conditioned responses to the training concentrations (pure odors) of the CS+ and the CS- odors during the retention tests were shown here in table-3. The Z and p values were given in the table and all p values were found significant.

Colony Number	Z value	p value
73	10.59	0.000000
98	10.04	0.000000
299	10.65	0.000000

3.6.2 Variability in the learning and memory performances of the individuals

After the colony-wise analysis, another population-based analysis was performed here to check for the possible existence of the different scorer or performer classes for the different features associated with the olfactory learning and memory. Multiple scorer classes were found in the score histograms of the eight quantified features (Fig. 7) except for the feature 'odor sensitivity'. The feature of sensitivity had only 3 different scores (0, 0.5 and 1.0) as found in the histogram because of the way it was quantified. Theoretically for each of the other 7 features, 10 possible score categories were possible. However, in place of 10, maximum 6 -7 different categories of scorer bees were found. This already showed that bees had high variability in performance scores in features like the rate of CS+ learning or odor discriminability during the conditioning and the retention tests and

so on. Major number of scores of the feature which quantified the animal's responses to the filter paper and paraffin oil were found adjacent to the minimum value of '0' apart from the few other score types. The overall lower number of responses of the bees to the filter paper and paraffin oil indicated the high specificity in their PER responses to the learned odor stimuli during the tests. Scores of individual features from the three backcrossed colonies were pooled for this analysis since no significant differences were found between the mean scores of the different features for the 3 colonies (No figure shown here: Acq1: Kurskal-Wallis ANOVA: H = 3.48 p = 0.17, Acq2: H = 2.21 p = 0.33, Disc1: H = 2.65 p = 0.26, Disc2: H = 0.57 p = 0.75, T 1, 2: H = 6.03 p = 0.04, T 3, 4: H = 0.82 p = 0.66, response to filter paper + oil: H = 0.15 p = 0.92), except for the feature of 'sensitivity'. Kurskal- Wallis ANOVA or the multiple comparisons of means revealed a significant interaction between the colony (or genotype) and the sensitivity score (Kurskal-Wallis ANOVA: H = 8.92 p = 0.01). However, for 'odor sensitivity', no significant difference was found while the Kurskal-Wallis ANOVA test was performed

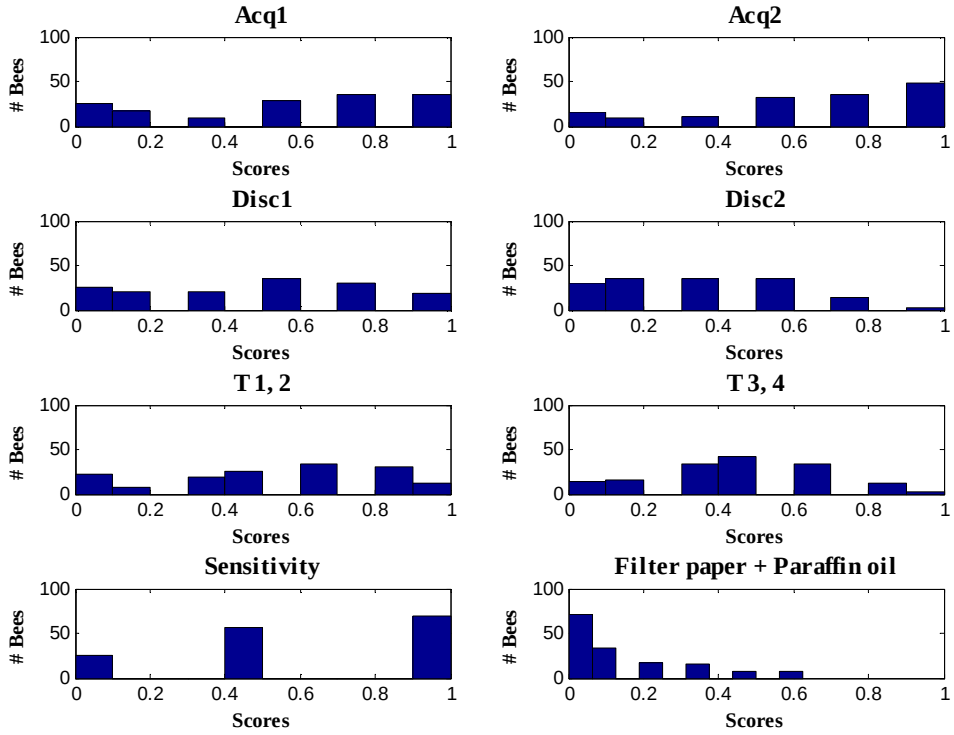


Fig. 7: Score histograms of the different features related with the olfactory learning and memory quantified for the cumulative conditioning assay: The 8 sub-plots in the figure represented the score histograms of the quantified features related with the olfactory learning and memory as described in the materials and method ('scoring scheme'). The abbreviations of the individual features (such as Acq1 or Disc2) were written on top of the respective histograms (sub-plots). The scores were plotted on the x-axis and the number of bees was represented on the y-axis. Multiple score classes were found for the individual features (in between the score range of 0 and 1) except for the feature of sensitivity, which showed only three possible scores of 0, 0.5 and 1.0 due to the way this feature was quantified. All histograms were found significantly different ($p < 0.05$; Kolmogorov Smirnov test and Liliefors test for normality) from the typical Gaussian distribution.

using the medians in place of the means. Hence, scores in the 8 quantified features from the three colonies were pooled to perform the population based analysis. For the cumulative or total performance score, although the mean score of the colony 98 was found lower than the scores of the other two colonies (Fig. 8) however; these differences were found non-significant (Kurskal Wallis ANOVA: $H = 5.02$ $p = 0.08$). Additionally, no significant differences were found in the mean cumulative performance scores along the different time point of the season (summer and autumn); reported in the 'seasonal effect' in appendix-1. Hence, the cumulative scores from the 3 colonies were pooled to analyze the variability in the gross performances of the individuals. The histogram of the cumulative score (Fig. 9), varied significantly form the typical Gaussian distribution (Kolmogorov-Smirnov test: $p < 0.05$; Lilliefors test for normality: $p < 0.01$; Shapiro-Wilk's W test: $p = 0.000$, $W = 0.938$) and showed the existence of the multiple scorer classes without any particular bias to the specific scores. The absence of score cluster was also found in the principal component analysis (Appendix-1; Fig. 10). *The non-existence of the score clusters or high heterogeneity in the combinations of the PER (1s) and the no-PER (0s) responses among the individual's was found to be the most salient feature of the data.* This result was similar to the previous finding that honeybees trained identically showed high variability in their learning performances in the different PER conditioning paradigms (Pamir *et al.*, 2011). Two different worker genotypes were used in the cumulative conditioning assay; one with two copies of the hygienic alleles and the other with one copies each of the hygienic and the non-hygienic alleles in their genome. In addition to the two main genotypes, different allelic combinations inside the individual genotypes (homozygous and heterozygous individuals) might influence the ability of the

individual bees to perform the olfactory learning and memory tasks differentially, which eventually contributed to the population heterogeneity as found in the cumulative score histogram. However, relative effects of the hygienic and the non-hygienic alleles on the olfactory learning of the backcrossed worker bees were untested in our experiments. Hence, it remained unknown from these results as how many bees of the two genotypes contributed to the total number of the lower, intermediate and the higher cumulative scorers. It was possible however, that both genotypes had their own distribution of the cumulative scores with different means and standard deviation values, hence, the cumulative score histogram showed here (Fig. 9) could just be the integrated form of the cumulative score distributions of the two genotypes.

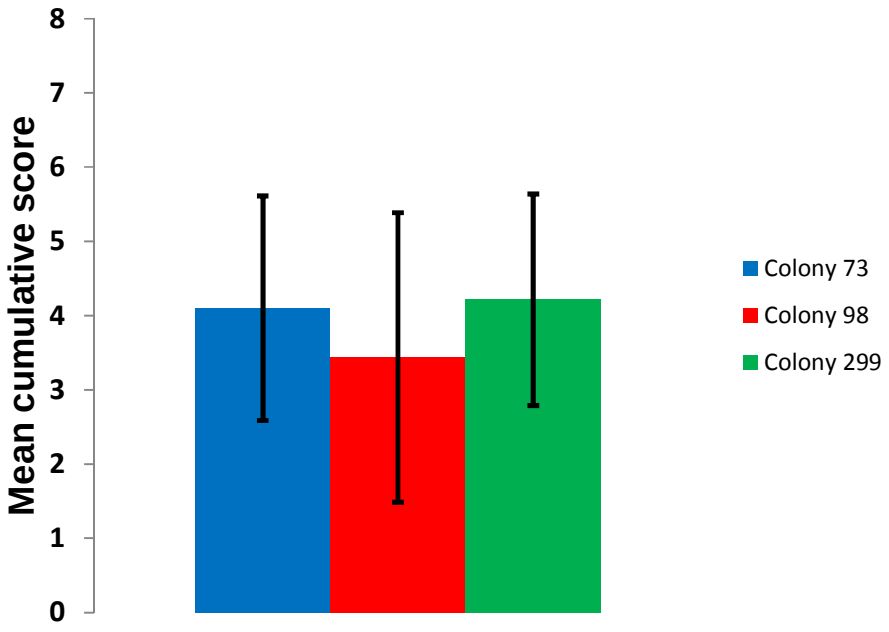


Fig. 8: Mean cumulative performance scores of the 3 backcrossed colonies: The color coded bars in this figure represented the mean (mean ± standard deviation) cumulative performance scores of the 3 backcrossed colonies (blue: colony73, red: colony 98 and green stood for colony 299). The y-axis represented the mean cumulative scores. Mean score of the colony 98 was found lower than the other two, however, the differences were not found significant (Kurskal Wallis ANOVA test; $p > 0.05$).

3.6.3 Selection of the best and the poor cumulative scorers

After the population analysis, individual's performances were analyzed to select for the groups of honeybees belong to the specific performance classes. In the first place the overall good (the best) and bad (the poor) performers were selected respectively with the higher and lower cumulative scores. Under the condition of high heterogeneity in individual's performances, these two extreme categories of the learning and memory performers were selected with the simple criteria (not statistical) of the cumulative or overall performance scores. *This arbitrary selection procedure was supported by the fact that extreme scorer categories were found in the score histograms of the individual quantified features related with the learning and memory* (Fig. 7). To select for the best cumulative performers, the cut off score of 5.6 (cumulative score > = 5.6 out of the maximum value8.0) was set. This criterion selected the honeybees with an overall performance score of at least 70% or more of the maximum cumulative score.

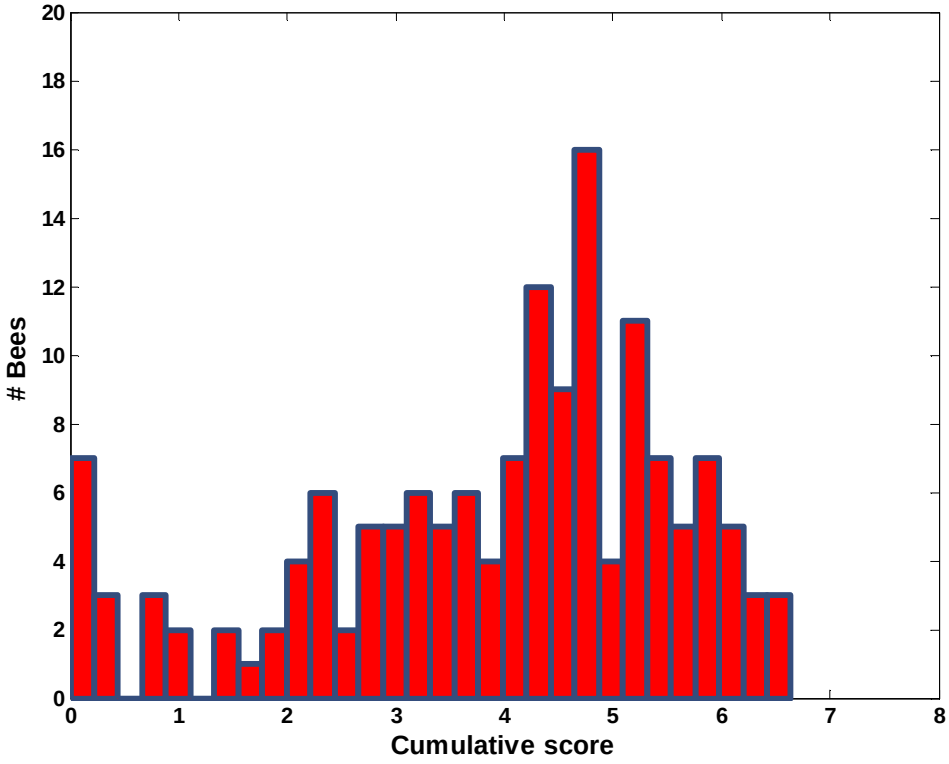


Fig. 9: Cumulative score histogram of the pooled population of honeybee: The heights of the red bars with the blue edges in the figure represented the number of honeybees correspond to the different cumulative scores in the histogram of the pooled population of honeybees trained in the cumulative conditioning assay (N = 152 bees). The x and y axes respectively represented the scores (range from 0 to 8) and the number of bees. No specific bias was found for any particular or more than one score as bees had the cumulative scores throughout the entire range. The distribution of the cumulative score was found significantly different ($p < 0.05$; Kolmogorov Smirnov test, Shapiro-Wilk's W test and Liliefors test for normality) than the Gaussian distribution.

Twenty-two bees out of 152 (14.47%) satisfied this criterion which indeed were found to perform consistently good throughout the assay as shown in their overall performance graphs (Fig. 11). All three backcrossed colonies contributed to the population of the best cumulative scorers with the decreasing number of bees found in the order; colony 98 (9 bees) > colony 299 (8 bees) > colony 73 (5 bees). Faster and reliable learning of the CS+ stimuli showed by these bees was associated with the strong discrimination between the

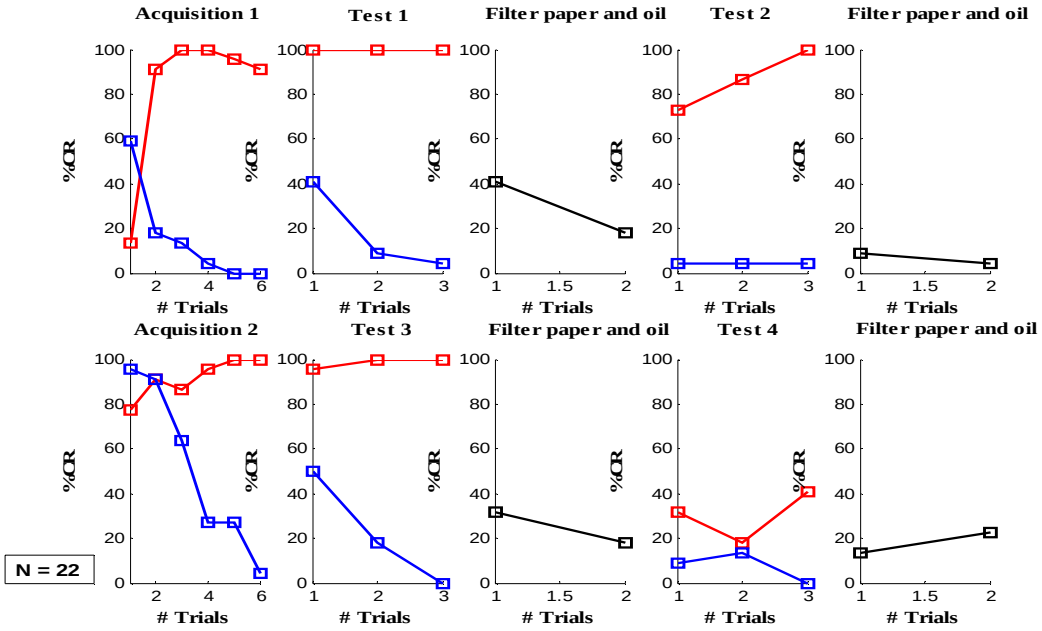


Fig. 11: Olfactory learning and memory performances of the best cumualtive scorers: Overall olfactory learning and memory performances of the best cumulative scorers were shown in this figure with the 8 sub-plots represented the performances during the same phases of the conditionings and the memory retention tests as mentioned in Fig. 3. The x and y axes

respectively represented the number of conditioning or test trials and the percent conditioned responses during the two phases of the cumualtive conditioning assay. Conditioned responses to the CS+, CS-, filter paper and oil were represented respectively by the red, blue and the black lines. The best cumulative scorers showed good performances throughout the assay as seen in their high rate of CS+ learning, high odor discriminability (both during the conditioning as well as the retention tests) and high odor sensitivity. However, they also responded more to the the filter paper and paraffin oil during the 1st compared to the 2nd test (sub-plot 3 and 5 in the 1st row, 8 and 9 in the 2nd row). Responses to the CS- stimuli were also found to decrease during the test 2 compared to the test 1 in the pairs of retention test conducted during both phases of the assay. 'N' represented the number of honeybees found in this category.

CS+ and CS- odors both during the conditioning trials and the memory retention tests, as well as high sensitivity in responses were shown to the different dilutions of the CS stimuli. These bees also showed strong responses to the filter paper and the paraffin oil during the 1st of the two tests which were found to decrease during the successive 2nd test in each of the two phases of the assay. However, the feature quantified the responses to the filter paper and oil showed low values of correlation with all other features. The consistent good performances of these individuals during the assay led to the high scores

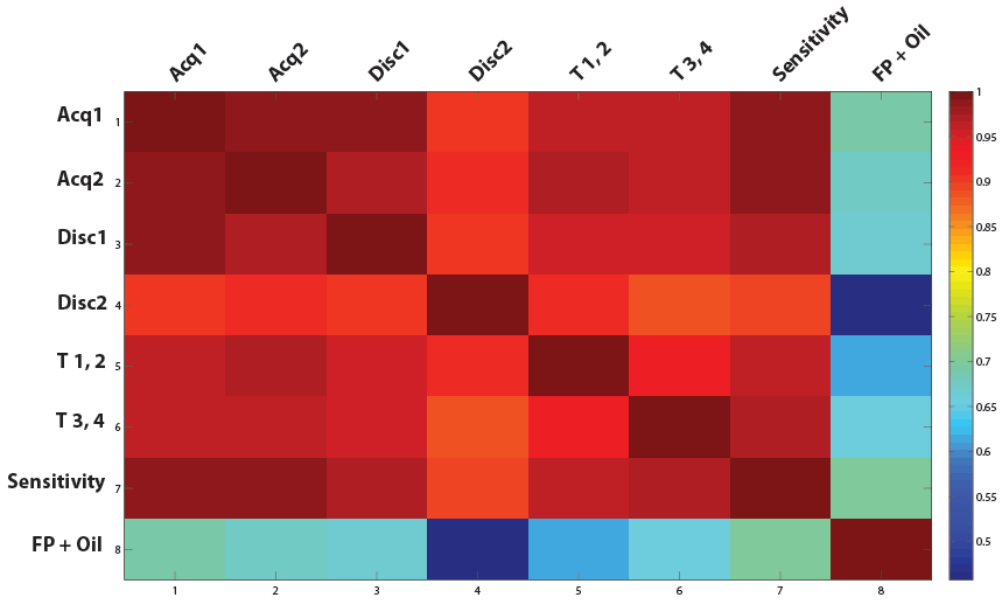


Fig. 12: Color coded correlation plot of the 8 features of the best cumualtive scorers: This plot reperesented the color coded linear or Pearson's correlation coefficients between the 8

features of the best cumualtive scorers (the color scale shown in the right side). The matrix is symmatrical on either side of the diagonal, which conveyed no information. The good cumulative performers showed very high correlations between the different features except for the feature-8 (FP + Oil), which quantified the responses to the filter paper and paraffiin oil. High correlations between the features were explained by their superior performances (throughout the assay) or high scores in different features.

in the different features and high correlations between them as shown in Fig. 12. As opposed to the best cumulative performers, the poor or bad performers were selected with the cut off score of 2.0 (cumulative score < = 2.0 out of the scale-maxima of 8.0). Bees which were selected with this criterion had the cumulative score 25% of the maximum cumulative score. A total of 20 bees (20 out of 152: 13.15%) were short-listed which showed the poor overall performances in the cumulative conditioning assay (Fig. 13). The contribution of the individual colonies to this population was found to follow the decreasing order of colony 98 (12 bees) > colony 73 (5 bees) > colony 299 (3 bees). Hence, colony 98 was found to contribute more bees to the populations of both the best and the poor performers than the other two colonies. These bees exhibited the consistent poor rate of CS+ learning, poor odor discriminability during the conditionings and the retention tests along with the overall weak responses showed to the CS stimuli during the

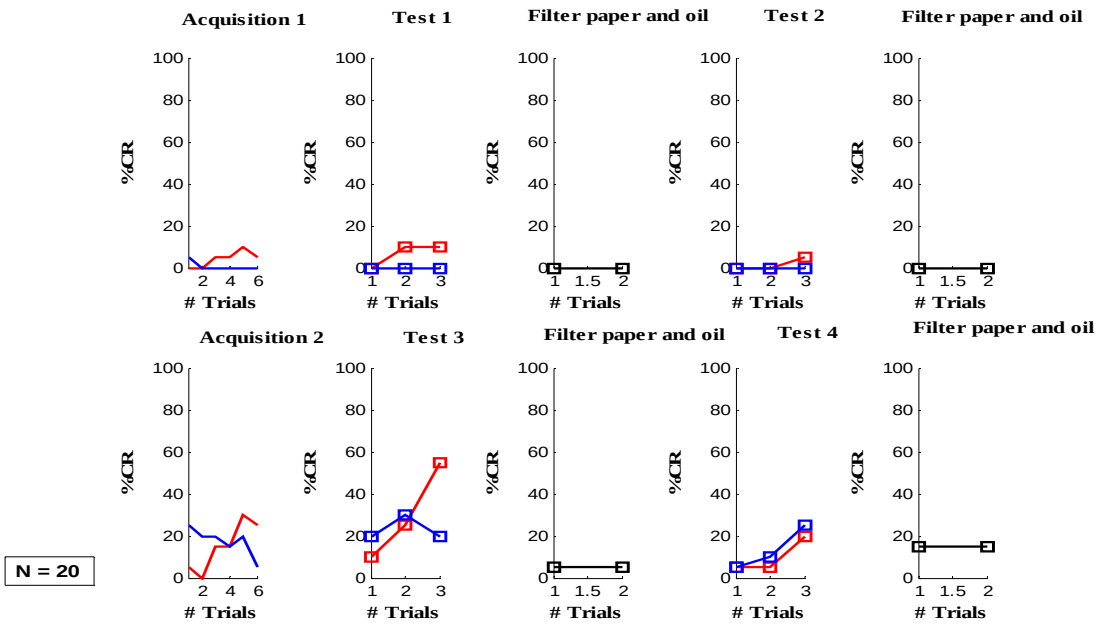


Fig. 13: Olfactory learning and memory performances of the poor cumulative scorers: Overall olfactory learning and memory performances of the poor cumulative scorers in the cumulative assay were shown in this figure. The x and y axes were respectively represented the number of conditioning or test trials and the percent conditioned responses during the two phases of the cumualtive conditioning; the 8 sub-plots represented the performances during the same phases of the conditionings and the memory retention tests as described in Fig. 3 (same color codes for the different CSs). These bees showed consistent poor rate of CS+ learning and discriminability (both during the conditioning as well as the retention tests) as well as poor odor sensitivity during the assay. Additionally, they showed low overall responses to all kinds of CSs throughout the assay. 'N' represented the number of honeybees found in this category.

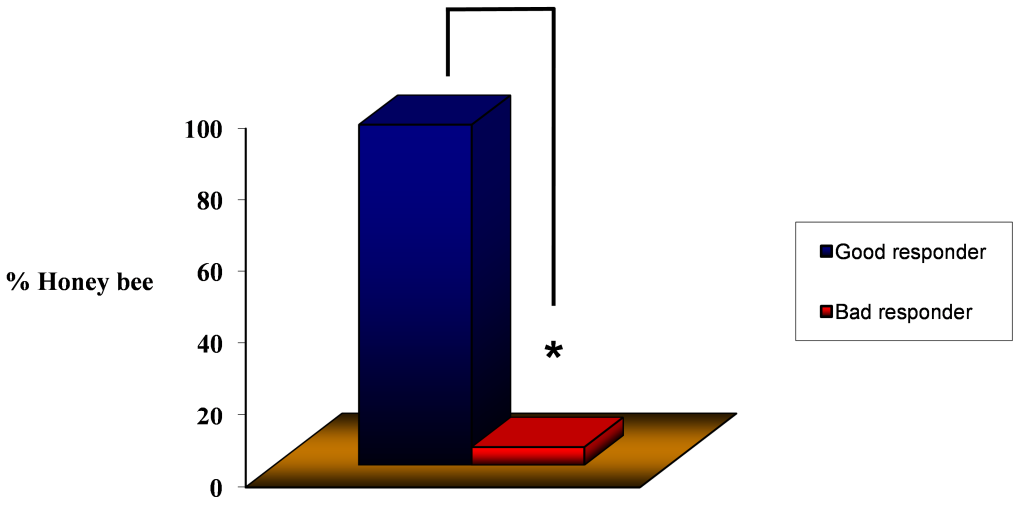


Fig.14: The US-responder categories among the bad cumulative scorers: Among the bad cumulative scorers, 95% showed consistent PER to the sucrose stimulation of the antenna, which were called as the 'good responder' (represented with the blue bar) and the rest 5% bees which showed inconsistent responses during the conditionings were called as the 'bad responder' (represented with the red bar). χ^2- test showed significant difference in number of bees between these two categories (significant difference was denoted with the asterics). The heights of the bars correspond to the percentage of honeybees found in the two categories as represented on the y-axis.

assay. The low number of PER responses to the conditioned odors throughout the assay probably indicated the general deficit of the poor cumulative performers to respond to the sucrose-US or they merely were not hungry during the assay. However, in this assay it was rather unlikely that bees did not get hungry during the prolong sessions of training and tests, started with the feeding status of overnight-satiation. While I checked for the sucrose responses of the poor scorers, 19 out of 20 i.e., 95% of the bees ('Good responder') were found to show the consistent responses to the US during the two rounds of differential conditioning (Fig. 14). Only one animal (5%) showed ('Bad responder') the inconsistent PER during conditioning however; this particular animal like others also responded to the sucrose while the PER was tested at the end of assay to check for the overall fitness. Hence, we concluded that overall poor learning and memory performances, rather not the compromised responses to the sucrose resulted in the low cumulative scores in these bees. The weak performances of these bees were also visible in the weak correlations between the different features as shown in the color coded correlation plot (Fig. 15). The poor cumulative scorers often scored '0's for the different features which contributed to the low correlation values between the pairs of features.

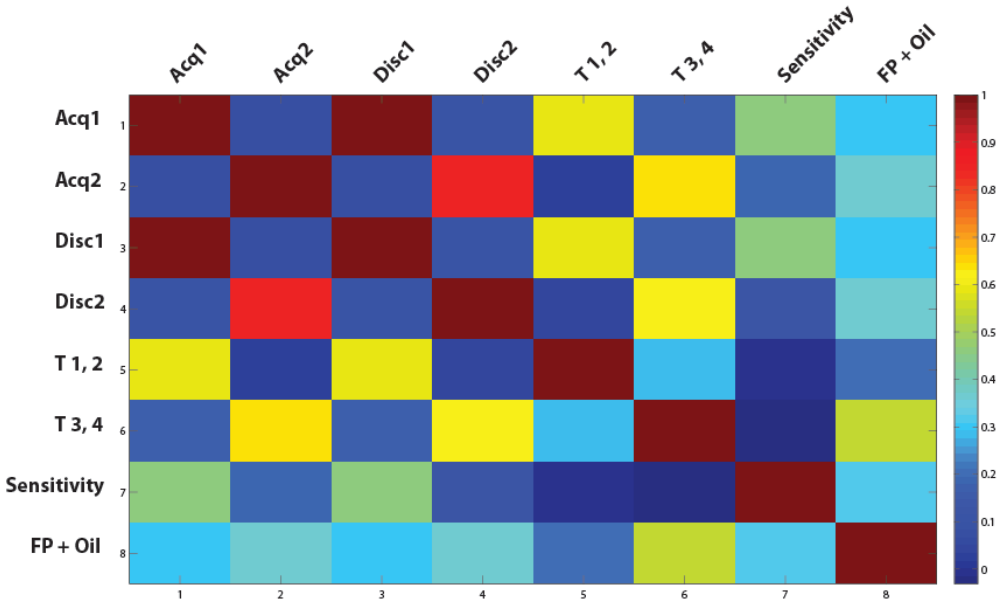


Fig. 15: Color coded correlation plot of the 8 features of the poor cumualtive scorers: This plot represented the color coded correlation coefficient values between the 8 different features of the poor cumulative scorers (the color scale shown in the right side). The bad cumulative performers showed overall lower values of correlation between the features compared to the best scorers except between the pairs of features; between Acq1 and Disc1, between the Acq2 and Disc2. Consistent poor rates of CS+ leraning and poor performances in the discrimination tasks during the two phases of assay led to the high correlations between these two pairs of feature.

However, high values of correlation were found between the features like Acq1 and Disc1 and between the Acq2 and Disc2; represented the rate and reliability of the CS+ learning (Acq1 and 2) and discriminability between the CS+ and CS- stimuli (Disc1 and 2) during the 1st and 2nd differential conditioning. These high correlations were rather obvious since the lack of responses to the CS+ (low scores in Acq1 or Acq2) also reduced the scores of odor discriminability (Disc1 and 2) during the two rounds of differential conditionings. Poor discriminability led to the poor performances during the short-term memory retention tests. This eventually reduced the correlations of odor discriminability during the retention tests (T 1, 2 and T 3, 4) both with the speed of odor learning and odor discriminability during the differential conditioning.

In addition to the cumulative differences in the learning and memory performances between the best and the poor cumulative scorers, absence of the effect of seasonal variation on the olfactory learning (details given in appendix-1; 'seasonal effect') indicated that honeybees selected with the arbitrary criteria were most likely represented the two opposite classes of learning performers present in the natural population. The two extreme types of cumulative performers however, only made up 27% of the pooled population; the rest 73% of the population was comprised of the bees with cumulative performances nearly as good as the bests or as poor as the worst performers, and in between. These bees however, were incorporated in the bivariate history analysis later in this chapter to understand the relationships between the performance scores of the different learning related features in the pair-wise manner.

In the next step I wanted to find out whether the higher or lower scores of any one or more of the quantified behavioral features were able to select for these two types cumulative scorers with higher probabilities. However, before investigating this particular issue, it was important to look at the overall correlations between the eight

features for all bees in the pooled population of the three colonies. All correlations except one for the feature-7 (sensitivity) and three for the feature-8 (responses to the filter paper and paraffin oil) were found to be statistically significant irrespective of the higher or lower values of the Pearson's correlation coefficients (statistical data not shown). However, the speed of CS+ learning (Acq1) and odor discriminability (Disc1) during the 1st differential conditioning like before, showed the highest value of correlation as found in table-4 and Fig. 16. This clearly demonstrated that only during the 1st differential conditioning, faster or slower learning of the CS-US association was correlated strongly with the superior and inferior performances in the task of odor discrimination. In other words, the rate of CS+ learning was correlated well with the rate of CS- learning during the 1st DC. However, the same two features during the 2nd phase of the assay, Acq2 and Disc2 showed lower correlation values for the entire population of bees as opposed to the small populations of the best and the poor cumulative performers. Hence, unlike the 1st DC, bees in the 2nd differential conditioning showed less correlated increase or decrease between the speed of CS+ and CS- learning. Higher olfactory generalization between the CS+ and CS- stimuli during the 2nd compared to the 1st DC was the reason behind this decrease (details given in the 'performance history analysis' later in this chapter). Correlation coefficient values of the feature-5 (discriminability during retention test 1, 2) with the Acq1 or Disc1 were also found to decrease for the whole population as opposed to the values found for the best cumulative scorers. But the

Table 4: Table 4 showed the Pearson's linear correlation coefficient values between all of the 8 features for the pooled population of honeybees. These values were represented as the 8×8 symmetrical matrix (the diagonal represented the maximum correlation of 1 between the same features) with the highest correlation (numbers denoted with the red color) found between the features Acq1 (speed and reliability of CS+ learning during the 1st differential conditioning) and the Disc1 (odor discriminability during the 1st differential conditioning).

	Acq1	**Acq2**	**Disc1**	**Disc2**	**T 1, 2**	**T 3, 4**	**Sensitivity**	**Fp+ Oil**
Acq1	1	0.55	**0.90**	0.19	0.61	0.38	0.57	0.23
Acq2	0.55	1	0.47	0.48	0.48	0.40	0.52	0.25

Disc1	**0.90**	0. 47	1	0.28	0.59	0.37	0.45	0.14
Disc2	0.19	0.48	0.28	1	0.23	0.38	0.12	-0.13
T 1, 2	0.61	0.48	0.59	0.23	1	0.39	0.68	0.26
T 3, 4	0.38	0.40	0.37	0.38	0.39	1	0.38	0.031
Sensitivity	0.57	0.52	0.45	0.12	0.68	0.38	1	0.32
Fp+ Oil	0.23	0.25	0.14	-0.13	0.26	0.03	0.32	1

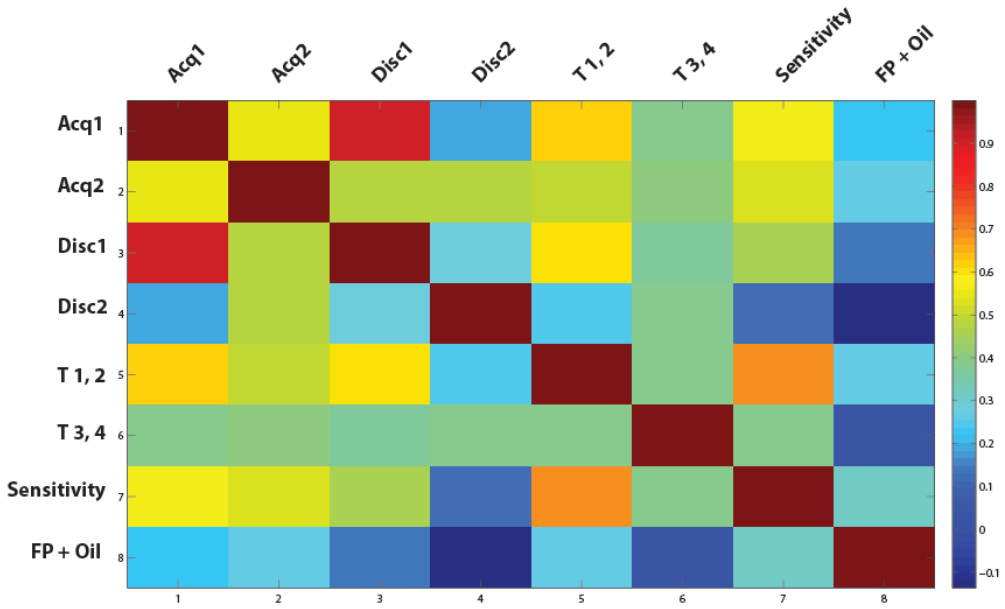


Fig. 16: Color coded correlation plot of the 8-quantified features for all bees in the pooled population: This color coded plot represented the same correlation coefficient values as given in the table 4 (shown above) for the pooled population of bees. As shown in table 4 the highest corelation was found between the Acq1 and the Disc1. The feature of sensitivity also showed good correlation with odor discriminability during the memory retention tests of the 1st phase of the assay (T 1, 2). The feature of FP + Oil, which quantified the responses to the filter paper and paraffiin oil had low correlations with others.

correlation values were similar with the values found for the poor cumulative performers. Additionally, the low correlation between the Acq2 and Disc2 was associated with the low correlations with the feature T 3, 4 (odor discriminability during test 3 and 4) due to the higher odor generalization or less discrimination during the 3rd and 4th retention tests of the assay. The feature of 'odor sensitivity' (feature-7) showed high correlation with odor discrimination during the memory retention tests (T 1, 2) of the 1st phase of the assay, albeit a low correlation was found with the feature T 3, 4 (odor discrimination during the memory retention tests; phase-2). Since, odor sensitivity was quantified using the PER responses of the 1st and 3rd retention tests, lack of correlation indicated that high odor generalization in conditioned responses to the CS+ and CS- stimuli during the 3rd and 4th retention tests reduced the scores of the feature T 3, 4, keeping the responses of the bees intact to the lowest dilutions of the CS+ odors during the 3rd test (sensitivity; see Fig. 3). The reduction in correlation between these two features was also visible in the cumulative performance graphs of the highly sensitivity bees (Fig. 17; selection criterion: score > = 0.9 for the feature 'sensitivity'). The highly sensitive bees scored well (Fig. 17) in other features like the speed of learning of the CS+ stimuli and odor discriminability during the conditionings and tests, however; the performances were not as good as found

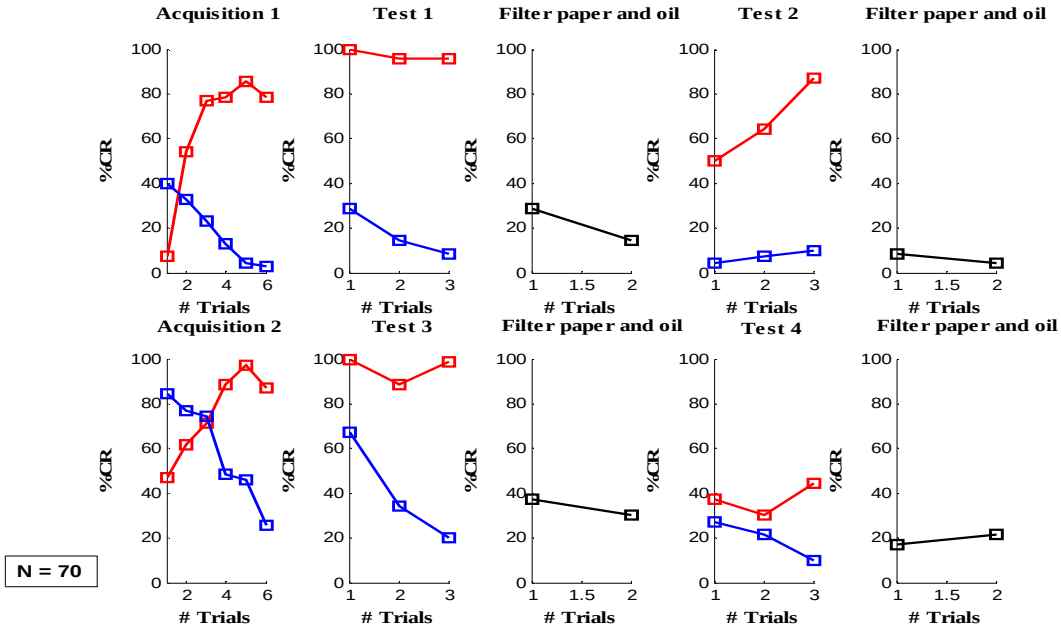


Fig. 17: The overall learning and memory performances of the honybees with high scores in 'odor sensitivity': This figure represented the overall learning and memory performances of the honeybees which were able to respond consistently to the lowest dilution of the CS+ stimuli during the test 1 and 3 or highly sensitive. The x and y axes represented the same parametrs as in Fig. 3. The 8 sub-plots were also represented the performances during the same phases of the conditionings and the memory retention tests as mentioned in Fig. 3. Conditioned responses to the different stimuli were also represented with the same color codes as in Fig. 3. The 100% CRs to the lowest CS+ dilution (the first data point in the CS+ curve during the test 1 and 3) in the two tests represented the selection points. These bees showed the switch-like CS+ learning as well as discriminated well between the CSs during the two rounds of differential conditioning and the four retention tests however; their performances were not as good as found for the best cumulative scorers (Fig. 11). They showed strong responses to the filter paper and paraffin oil during the 1st of the 2-tests which like the best cumulative scorers decreased during the 2nd test. The number of responses to the CS- stimuli also went down during the 2nd compared to the 1st test in the pair of memory retention tests.

for the best cumulative scorers (Fig. 11). In fact the best cumulative scorers were a subset of the highly sensitive bees (30% of the sensitive bees were best scorers); rest of the 70% had substantial variability in their learning speed or discriminability, which explained the lower correlation between these features (Acq2, Disc1, Disc2) with the odor sensitivity. Hence, higher odor sensitivity did not seem to dictate or strongly influence the superior overall performance of the bees in the cumulative conditioning assay. Responses of these bees to the filter paper and paraffin oil (feature-8) however, were found to decrease during the 2nd compared to the 1st test like the best cumulative scorers.

3.6.4 Selection of the best and poor cumulative scorers using the scores of the learning and memory related features

After the behavioral characterization of the best and the poor cumulative performers, it was investigated whether any one or more of the quantified features related with the olfactory learning and memory were able to select these two classes of honeybees reliability or in other words with higher probabilities than the others. To this end, first I looked at the correlation between the individual features with the cumulative score to find out one or more of these features with higher correlation values. Higher correlation value indicated that how well or poorly the change in scores of a particular feature was related

with the change in the cumulative scores of the bees. Hence, features with the higher correlation values may be able to select with higher probabilities the best and the poor cumulative performers respectively with their higher and lower scores. Acq1 showed the highest correlation with the cumulative score (table-5) which was followed by the Disc1 (table-5: numbers in bold-red font). These two features were in fact found to have the highest correlation amongst all the bees (Fig. 16), for the individual colonies (appendix-1, Fig. 18), for the best (Fig. 12) and the poor (Fig. 15) cumulative scorers, and in any other group of bees selected with a specific criteria (data not shown). The highest correlation coefficients of the Acq1 and Disc1 with the cumulative score and between themselves indicated the possibility that these two features individually or together were able to select for the best and the poor cumulative performers with higher probabilities than the other features. Other features such as the rate of learning during the 2nd differential conditioning (Acq2), odor discriminability during the retention tests 1 and 2 (T 1, 2) or odor sensitivity (Fig. 17) also showed high correlations but were found less effective while selecting the two types of extreme performers than the feature like Acq1 or Disc1 (data not shown). Henceforth, all further analyses in this section were performed with the Acq1 and Dics1.

Table 5: Table 5 represented the linear correlation coefficients between the 8 individual features with the cumulative score which was the summation of scores in all features.

	Cumulative Score
Acq1	**0.84**
Acq2	0.77
Disc1	**0.80**
Disc2	0.46
T 1, 2	0.79
T 3, 4	0.59
Sensitivity	0.76

Fp+ Oil	0.33
Cumulative Score	1

Best cumulative performers

Previous analysis found the 22 best cumulative performers in the pooled population of the honeybees. The analysis performed here, found that the higher range of scores in Acq1 (score >= 0.9, scale maxima of 1.0) were able to select 17 out of the 22 (77.27%) best performing bees. However, this criterion selected a total of 35 bees; hence, high scores in Acq1 (speed and consistency of learning of the CS+ stimuli during the 1st differential conditioning) selected the best cumulative performers with the probability of 0.48 (17/35).

The identical higher range of scores (score >= 0.9, scale maxima of 1.0) of Dics1 (odor discriminability during the 1st differential conditioning) selected 13 best bees out of the 22 (59.09%). However, a total of 19 bees were selected with this criterion, which raised the probability of finding the best cumulative performers with the high scores in Disc1 up to (13/19) 0.68. Since all of the 19 bees selected in this case, were also selected with the high scorers in Acq1 (19 out of the 35 high Acq1 scorers; 54%), hence, high scores in Acq1 and Disc1 together also selected the best cumulative scorers with the same probability of 0.68. All superior performers in the odor discrimination task were found tolearn the CS+ stimuli faster although amongst the fast learners of the CS-US association, only 54% of the bees showed the superior discriminability. The first relationship was trivial but not the second since, the faster and consistent responses to the rewarded odors (CS+) were found to be insufficient for the faster and consistent learning of the CS- or in other words odor discrimination. This indicated that learning speed or dynamics of the CS+ and the CS- stimuli varied among the bees with fast and reliable learning of the CS+ stimuli. This variability also explained the low probability selection of the best cumulative scorers with the high scores in Acq1 (many high Acq1 scorers ended up with cumulative scores below the cut off score set for the selection of the best cumulative scorers). It was concluded that *concomitant learning of identities of the rewarded and the unrewarded odor stimuli in the cumulative assay was found as the*

better criterion than the fast learning of the rewarded odors for the bees to achieve the overall performance score equal to or higher than the cut off score set for the selection of the best performers.

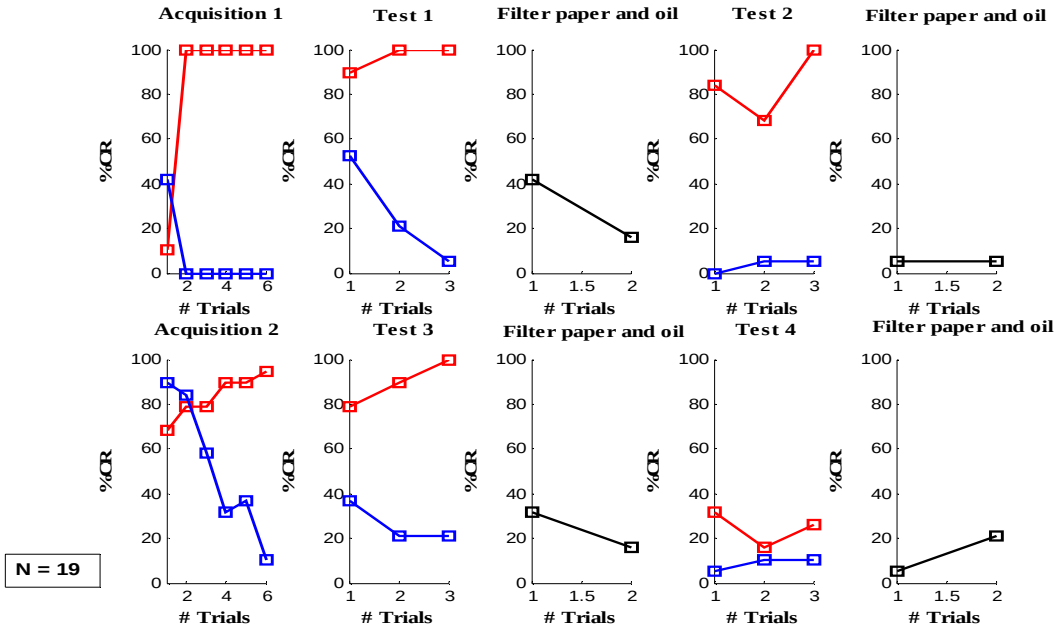


Fig. 19: The overall performances of honeybees with high scores in Disc1: This figure represented the overall learning and memory performances of bees in the cumulative conditioning assay which scored high in the feature Disc1. The x and y axes respectively represented the number of conditioning or retention test trials and the percent conditioned responses to the CS stimuli during the two phases of the cumualtive conditioning. The 8 sub-plots also represented the performances during the same phases of the conditionings and memory retention tests as mentioned in Fig. 3. These bees not only discriminated strongly between the CS+ from the CS- odors during the 1st differential conditioning, but also showed high discriminability during the following retention tests as well as during the 2nd differential conditioning. Additionally, these bees showed the high odor sensitivity and strong responses to the filter paper and paraffin oil during the 1st of the 2 tests which like the best cumulative scorers declined during the 2nd test. The number of responses to the CS- stimuli also decreased during the 2nd compared to the 1st retention test. 'N' represented the total number of honeybees found in this scorer category.

Poor cumulative performers

Lower range of scores in the cumulative performances previously selected 20 bees from the pooled population of the three colonies and amongst them 17 (85%) were selected with the criterion of the lower scores (score <= 0.1, scale maxima of 1.0) of the Acq1.

This particular criterion selected a total of 26 bees, hence, low scores in Acq1 selected the bad cumulative performers with the probability of (17/26) 0.65.

The lower range of scores (score <= 0.1, scale maxima of 1.0) of Disc1 shortlisted the same 26 bees in total as found with the Acq1. Hence, unlike the best cumulative performers, the poor performers were selected with the same probability of 0.65 using the low scores in either of the two features; Acq1 or Disc1. It was obvious that low scores in Acq1 contributed poorly to the scores of the Disc1. Since, Disc1 selected the best cumulative scorers with higher probability than the Acq1 and the bad performers with the same probability as the Acq1; hence, learning the discrimination between the CS+ and CS- odors popped out again as the best criterion or the strongest amongst the eight features to select the two extreme types of cumulative scorers with highest probabilities.

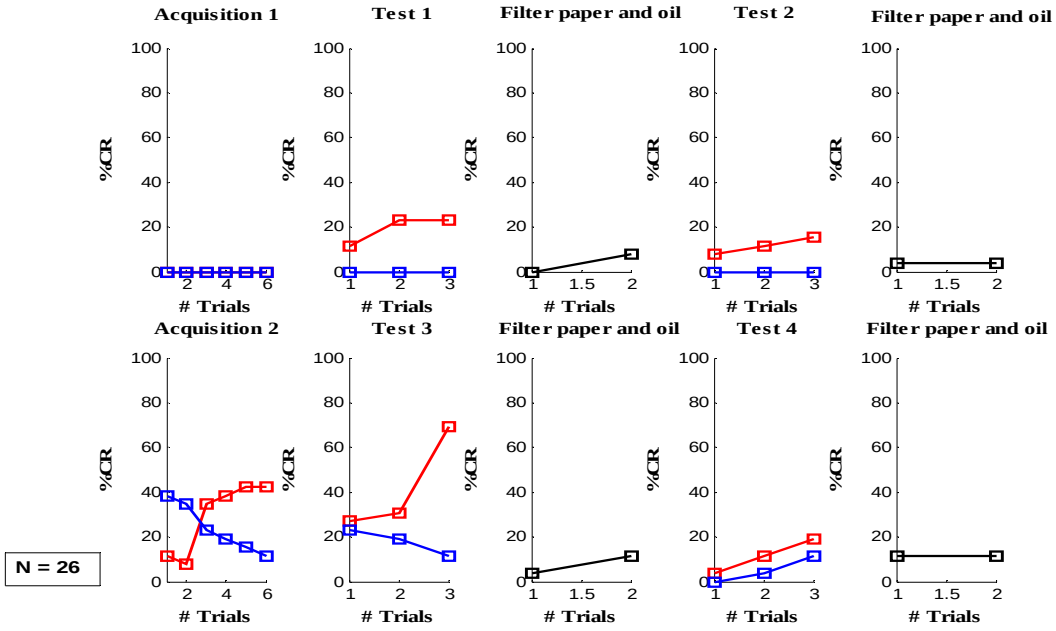


Fig. 20: The overall olfactory learning and memory performances of the low scorers in Disc1: This figure showed the overall learning and memory performances of bees in the cumualtive assay which scored low in Disc1. Their overall performance during the 2nd differential conditioning and retention tests were better than the 1st however; the overall performances were poor throughout the entire assay like the poor cumulative scorers. 'N' represented the total number of honeybees found in this scorer category.

Honeybees selected with the high (Fig. 19) and low scores (Fig. 20) of Disc1 showed respectively the superior and inferior overall performances in the cumulative assay like the best and the poor cumulative scorers. However, these two cumulative scorer classes were selected by the scores in Disc1 only with the probability values nearly 70%, rather not high as 90% or more to be adequately sure about the usefulness of the feature, Disc1 to select for the best and the worst cumulative performers. This clearly indicated the individualistic variability in the learning and memory performances during the cumulative conditioning assay such that the lower performance scores in one or more of the features in an individual bee were compensated by the higher scores in one or others which resulted in the failure of selecting these bees with very high probabilities using the scores in the single features.

3.6.5 Analysis of performance histories of the different selected groups of performers

The analysis until now was focused on the behavioral characterization of the good and the bad cumulative performers. In this section I discussed the performance histories of the different performer classes or group of bees selected with the specific criteria from the pooled population of the three colonies. Initially the learning dynamics of the rewarded (CS+) and the unrewarded (CS-) odor stimuli and the phenomena of olfactory generalization during the differential conditioning were discussed for the high, intermediate and low cumulative scorers. The low scorers were selected with the score range higher than the poor cumulative scorers (see previous section) since the poor performers provided least information about their learning related performances due to the consistent low level of responses to all kinds of CSs used in the assay. Learning (speed) dynamics of the CS+ and CS- stimuli were also discussed for the bees with different range of performance scorers of the feature Acq1, represented the speed and reliability of the CS+ learning during the 1st DC. These analyses were performed with the bees which did not receive any prior odor training, hence, the PER conditioning data generated only during the 1st differential conditioning was used.

Honeybees with the high or best cumulative scores (score > = 5.6 or >= 70% of the maximum score) showed significantly high responses to the CS- (Fig. 21) compared to the CS+ stimuli during the 1st CS- conditioning trial (RM-ANOVA showed significant stimulus (CS+/CS-) × trial effect: $F_{5, 210} = 42.60$, $p = 0.0000$; followed by the Fisher LSD post hoc test for the 1st trial CS+ *vs.* CS-: $p = 0.000000$). In fact the CS- responses of these bees during the 1st trial were found significantly higher than the other two classes of cumulative scorers (Fig. 22) used in this analysis (RM-ANOVA showed significant response × group effect: $F_{10, 410} = 2.94$, $p = 0.0013$; followed by the Fisher LSD post hoc test: the 1st CS- trials between the best and intermediate scorer $p = 0.000462$, the 1st CS- trials between the best and low scorers $p = 0.000015$). High initial responses (60% CR) to

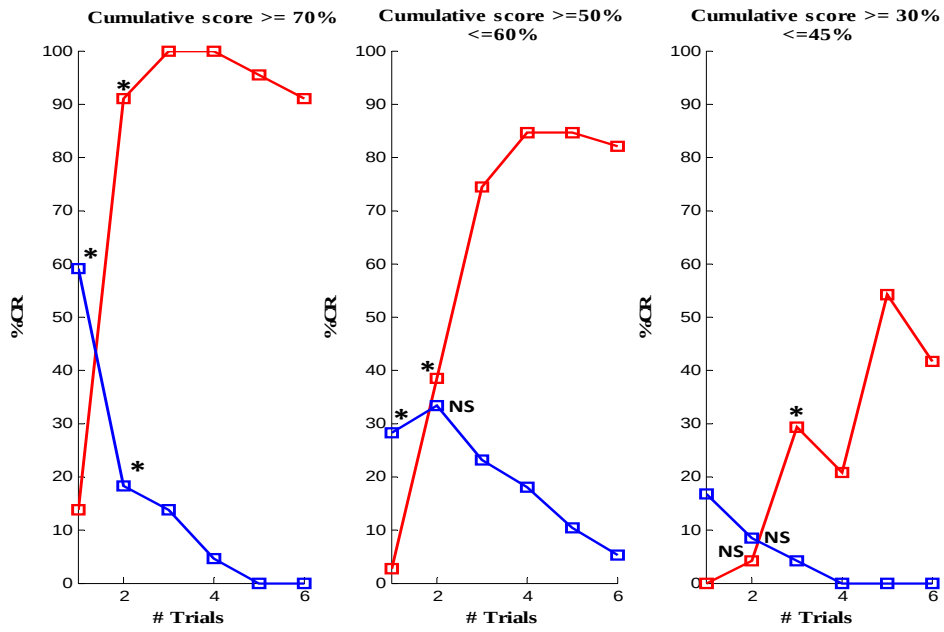


Fig. 21: Learning dynamics of the CS+ and CS- stimuli of the high, intermediate and low cumulative scorers: Rewarded (CS+; represented by red lines) and unrewarded (CS-; represented by blue lines) odor stimuli were learned with different rates by the three cumulative scorer classes during the 1st differential conditioning. The x and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the CS stimuli. The 1st sub-plot in this figure showed the concomitant learning of the CS+ and CS- stimuli of the good cumulative scorers (N = 22 bees). These bees showed significantly higher initial responses to the CS- compared to the CS+ stimuli (significantly higher responses found in the Fisher LSD post hoc test $p < 0.05$; denoted with the 1st asterics on the blue line) possibly due to the strong

effects of odor generalization and the sucrose mediated arousal apart from the spontaneous responses. However, fast learning of the CS- stimuli from the 2nd conditioning trial overshadowed these effects as found in the significant lowering of the CRs (denoted with the 2nd asterics on the blue line) to the CS- stimuli. The 2nd sub-plot showed the abrupt learning of the CS+ stimuli from the 1st to the 2nd trial (significant increase in CR, denoted with the asterics on the red line) of the intermediate cumulative scorers (N = 39). These bees did not learn the CS+ and CS- together due to the slower learning rate of the CS- (non-significant change in the CRs form the 1st to the 2nd CS- trial; denoted with 'NS' on the blue line). However, like the best scorers these bees also showed the significantly higher initial responses to the CS- than the CS+ which were probably associated with the effects of odor generalization and the sucrose mediated sensitization (significantly higher CS- responses in the 1st trial compared to the CS+; denoted with the asterics on the blue line). The third sub-plot represented the slower (non-significant change in the CRs form the 1st to the 2nd CS+ trial; denoted with 'NS') and unstable CS+ learning curve of the low cumulative scorers (N = 24 bees). These bees learned the CS- stimuli more steadily although with a slower rate (non-significant change in the CRs form the 1st to the 2nd CS- trial; denoted with 'NS' on the blue line). The overall responses to the two types of CS stimuli in this scorer category were found lower compared to the other 2 scorer classes.

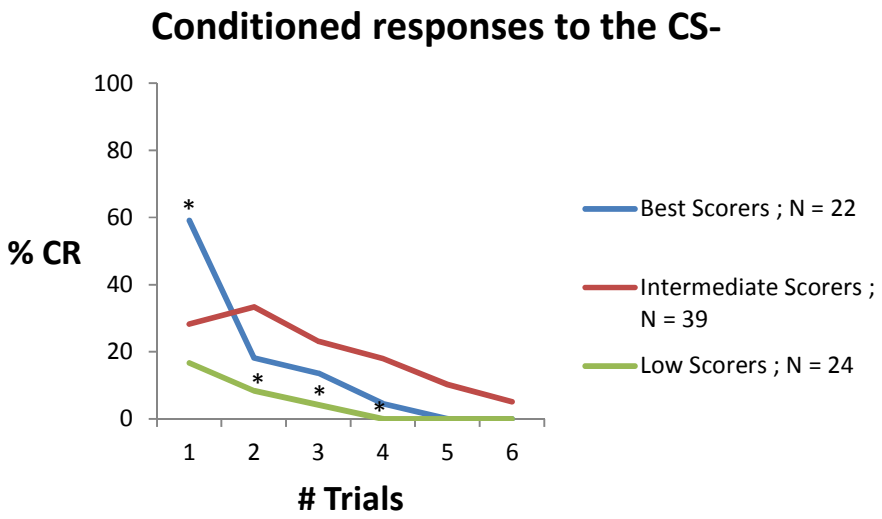


Fig. 22: Comparison of the conditioned responses to the CS- stimuli between the high, intermediate and low cumulative scorers: This figure was an extension of the Fig. 21, highlighted the differences in learning dynamics of the CS- stimuli between the three types of cumulative scorers during the 1st differential conditioning. The x and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the CS- stimuli. All three classes of bees learned the CS- stimuli as evident from the 3 acquisition functions (best scorer: blue line, intermediate scorer: dark brown line, low scorer: green line). However, the best cumulative scorers showed significantly higher initial responses to the CS- (denoted with the 1st asterics on the blue line) compared to the other two classes (possibly due to higher odor generalization and sucrose arousal), although these bees quickly learned not to respond to the CS- during the successive conditioning trials. More persistent effects of odor

generalization or sensitization were found in the intermediate scorers as they showed significantly higher CRs during the 2nd, 3rd and the 4th conditioning trials compared to the low cumulative scorers (denoted with the last 3 asterics on the green line). The low scoring bees rather showed minimum odor generalization and sensitization effects among the three scorer classes however, they showed the lowest overall responses to the two CS stimuli. 'N' represented the number of honeybee found in the three scorer categories.

the CS- stimuli did not only reflect the component of the spontaneous responses but also strongly indicated the effect of higher generalization between the CS- and the preceding CS+ odor stimuli in the best cumulative scorers compared to the other two classes. Additionally, there was a component of arousal or sensitization mediated by the sucrose (US) of the 1st CS+ conditioning trial previous to the 1st CS- trial. The combined effects of the spontaneous response, odor generalization and the sucrose mediated sensitization although were strong initially, but decreased significantly during the next CS- trial when the conditioned responses (CRs) declined sharply (Fisher LSD post hoc test 1st CS- trial *vs.* 2nd CS- trial: $p = 0.000003$) followed by the continuous decrease during the further trials until the CRs dropped down to '0'. The steep decline in the CS- acquisition function was definitely due to the learning of the CS- odors which opposed the effects of the odor generalization and sucrose-arousal. However, a component related with the US habituation might also involve with the decrease in the CRs to the CS- odors. The significant decrease in the CRs during the 2nd CS- trial was associated with a sharp and significant rise in the CRs to the CS+ odors (Fisher LSD post hoc test 1st CS+ trial *vs.* 2nd CS+ trial: $p = 0.000000$) which ultimately followed the stable asymptote. These results although confirmed the fast, switch-like and reliable learning of the CS+ stimuli in this particular group of bees but were unable to disentangle between the effects of CS- learning and the US habituation. In absence of understanding about the strength of the habituation effect, it was concluded that independent of the possible effects of the US habituation, the best cumulative scorers learned the CS- stimuli concomitantly with the CS+ stimuli during the 1st differential conditioning.

The intermediate cumulative scorers were selected with the criterion of bees scoring in between 50% – 60% (cumulative score range >= 4 but <= 4.8) of the maximum cumulative score also showed the significantly high responses to the CS- (Fig. 21) during the 1st training trial compared to the CS+ stimuli (RM-ANOVA showed

significant stimulus × trial effect: $F_{5, 380} = 26.79$, $p = 0.0000$; Fisher LSD post hoc test for the 1st trial CS+ *vs.* CS-: $p = 0.0036$). During the next conditioning trial although a sharp rise in the CR was found for the CS+ (Fisher LSD post hoc test 1st CS+ trial *vs.* 2nd CS+ trial: $p = 0.000014$), however, the responses to the CS- did not decline significantly (Fisher LSD post hoc test 1st CS- trial *vs.* 2nd CS- trial: $p = 0.52$) and even remained higher than the best (statistically non-significant) and the low cumulative scorers (Fisher LSD post hoc test after RM-ANOVA: between the intermediate and low scorers; 2nd CS- trials: $p = 0.0035$, 3rd CS- trials: $p = 0.026$, 4th CS- trials: $p = 0.035$) for rest of the conditioning trials (Fig. 22). Conditioned responses of this scorer class of bee to the CS- stimuli were only declined gradually with no significant decrease found in the CRs between the consecutive CS- trials. Hence, like the best scorers, the intermediates also showed the high initial responses to the CS- which were associated with the components of the spontaneous response, odor generalization and the sucrose mediated arousal, as well as the switch-like learning of the CS+ odors however*;* unlike the best scorers this group of bees did not show the concomitant learning of the CS+ and CS- stimuli due to slower learning rate of the CS- compared to the CS+.

The low cumulative scorers were selected (Fig. 21) with the criterion of bees scoring in between 30% and 45% of the maximum cumulative score (cumulative score >= 2.4 but <= 3.6). These bees like the other two scorer classes showed the initial higher responses to the CS- compared to the CS+ (Fig. 23), but unlike the others this difference was not found significant (RM-ANOVA showed significant stimulus × trial effect: $F_{5, 230} = 8.75$, $p = 0.00000$; followed by the Fisher LSD post hoc test for the 1st trial CS+ *vs.* CS-: $p = 0.069$). Conditioned responses to the CS- indeed were found significantly lower in this category of bees than the high and the intermediate cumulative scorers (results shown before). This indicated that the CRs to the unrewarded odor stimuli were least affected by the effects of odor generalization and the US mediated arousal, albeit the overall responsiveness to both type of CS stimuli was also found lower in these bees compared to the other two categories. No significant increase in the CRs was found between the 1st and the 2nd CS+ conditioning trials (Fisher LSD post hoc test 1st CS+ trial *vs.* 2nd CS+ trial: $p = 0.64$) until the first jump in responses took place during the 3rd CS+ trial (Fisher LSD post hoc test 2nd CS+ trial *vs.* 3rd CS+ trial: $p = 0.006$). The CS+ learning curve of

the low cumulative scorers neither showed the abrupt or switch-like increase in the CRs (rather gradually rising CRs) like the other two scorer classes nor had the stable asymptote, rather inconsistencies in CRs were found along the subsequent conditioning trials (decrease during the 4^{th} compared to the 5^{th} trial: $p = 0.00028$, decrease again during the last trial). The reason for the instability of acquisition function of the CS+ stimuli although was unclear however; probably indicated the poor CS+ learning (response noises or non-specific responses during the conditioning trials). The smaller population size of these bees compared to the other two classes might also contribute to the responses instability to the CS+ stimuli. On the other hand, learning of the CS- stimuli although was followed the slow, gradual dynamics (no significant differences in the CRs were found between any two consecutive CS- conditioning trials) but was more consistent than the CS+ as the CRs were gradually dropped down to '0' along the successive conditioning trials. Hence, like the intermediates, the low cumulative scorers also showed the gradual learning of the CS- which was not concomitant with the learning of the CS+ however*;* unlike the other two scorer categories, the CS+ learning was found slower and inconsistent along the conditioning trials. It seemed that the low cumulative scorers rather learned the CS- stimuli earlier and stably than the CS+.

In addition to the analysis of the learning dynamics of the CS+ and CS- stimuli, the possible differences in the final or asymptotic levels of the CS+ learning between the three scorer categories of honeybees were also investigated. Quantification of the asymptote values from the CS+ learning curves, in this case was not found to be straight forward since, the asymptote was unstable in the low cumulative scorers. Hence, the mean conditioned responses during the last three conditioning trials were directly compared between the three types of cumulative scorers to find out the possible differences in CRs to the CS+ stimuli during the end of conditioning. Repeated measurement ANOVA showed the significant group × response interaction ($F_{2, 82} = 50.60$, $p = 0.00000$) which further revealed the significant gradual decline in the mean responses during the last three conditioning trials from the best until the low cumulative scorers (Fisher LSD post hoc test: between best and moderate scorers $p = 0.03$, between moderate and low scorers $p = 0.000000$, between best and low scorers $p = 0.000000$). It was concluded that in addition to the concomitant learning of the CS+ and CS- stimuli,

the best cumulative scorers also showed the higher mean CR to the CS+ odors during the end of the differential conditioning (or final level of CS+ learning) compared to the other two classes of scorers, although the effects of odor generalization and sucrose mediated arousal were initially found higher in these bees than the others. The moderate scorers were better than the low cumulative performers in terms of the speed and final level of the CS+ learning however*;* these bees did not show concomitant learning of the CS+ and CS- stimuli. The low cumulative scorers showed the unstable and minimum final level of the CS+ learning amongst the three scorer categories along with the overall low responsiveness to the CS stimuli during the differential conditioning. *It was not surprising that eventual lowering of the cumulative scores selected the groups of bees with inferior levels of odor learning however; the differences found in the learning dynamics of the CS+ and CS- stimuli* as well as in the *final levels of the excitatory CS+ learning* during the differential conditioning were never reported systematically for the different types of learning and memory performers as were illustrated here.

One of the noticeable features in olfactory learning of the best cumulative scorers was the higher average score of 0.95 in the feature Acq1 compared to the moderate (0.72) and low (0.30) cumulative scorers. The number of PER responses to the CS+ stimuli during the 1st differential conditioning was quantified by the feature Acq1, and a high average value meant the early and consistent responses to the CS+ stimuli throughout the 1st differential conditioning.

Here, it was analyzed whether the early and consistent learning of the CS+ stimuli influenced the dynamics of the CS- learning during the conditioning. Three different categories of bees were selected like before for this analysis with the high (score >= 0.9; maximum score 1.0), intermediate (score >= 0.5 and <= 0.6) and low range of scores (score >= 0.3 and <= 0.45) for the Acq1. Honeybees with the earlier (faster) and consistent responses to the CS+ (high Acq1 scorers) showed significantly higher responses to the CS- stimuli during the 1st conditioning trial (Fig. 23) compared to the CS+ (RM-ANOVA showed significant stimulus × trial effect: $F_{5, 340} = 99.98$, $p = 0.0000$; followed by the Fisher LSD post hoc test for the 1st trial CS+ *vs.* CS-: $p = 0.000000$). This meant that the high Acq1 scorers like the best cumulative scorers also showed the high initial responses to the CS- stimuli which were associated with the components of

spontaneous response, odor generalization and the sucrose mediated arousal. However, responses to the CS- in the successive conditioning trials were declined significantly (Fisher LSD post hoc test 1st CS- *vs.* 2nd CS- trial p = 0.012, 2nd CS- *vs.* 3rd CS- trial p = 0.00049, 3rd CS- *vs.* 4th CS- trial p= 0.00049) until the stable asymptote was reached. This sharp and continuous reduction in the CRs was definitely associated with the learning of the CS- odors (which reduced the effects of the generalization and arousal) apart from the possible involvement of the effect of US habituation. On the other hand CRs to the CS+ stimuli as expected (selection criterion of high score in Acq1) showed the steep jump during the 2nd trial from the initial level (~10% CRs) until the maximum (100%) and remained stable for rest of the conditioning trials. Hence, the early and stable CS+ learning of these bees took place together with the learning of the CS-, although the later

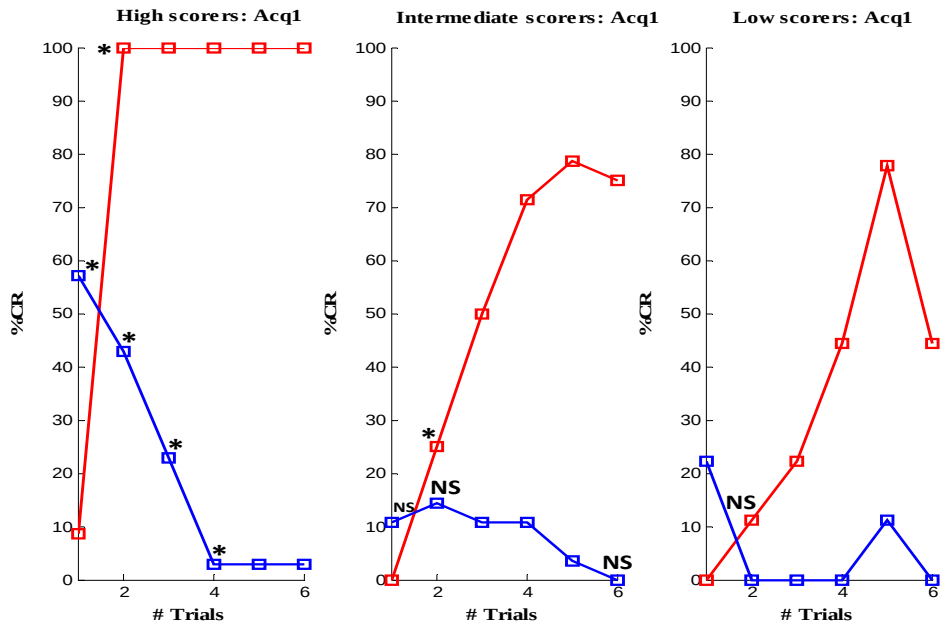


Fig. 23: Learning dynamics of the CS+ and CS- stimuli of the high, intermediate and low scorers of the Acq1: The high, intermediate and the low scorers in Acq1 learned the rewarded (CS+; represented by red lines) and the unrewarded (CS-; represented by blue lines) odor stimuli with different dynamics. X and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the two CS stimuli. High scorers in Acq1 learned the CS+ stimuli fast and respond consistently to the CS+ throughout the conditioning. The 1st sub-plot in this figure showed the concomitant learning of the CS+ and CS- stimuli of the high Acq1 scorers (N = 35 bees). These bees showed the initial significantly higher responses to the CS-

compared to the CS+ (denoted with the 1st asterics on the blue line) due to the effects of odor generalization and the sucrose-arousal. However, fast learning of the CS- from the 2nd trial overshadowed these effects as the CRs decreased significantly during the next 3 successive CS- trials (denoted with the 2nd, 3rd and 4th asterics on the blue line). The 2nd sub-plot showed the abrupt or switch-like learning of the CS+ stimuli from the 1st to the 2nd trial (significant increase in CRs, denoted with the asterics on the red line) of the intermediate scorers (N = 28 bees). These bees did not learn the CS+ and CS- together due to the slower rate of learning of the CS- odors (non-significant change in the CRs form the 1st to 2nd and from the 4th to 5th CS- trial; denoted with the 'NS' on the blue line). However, unlike the intermediate cumulative scorers (Fig. 21) these bees showed no significant increase in responses to the CS- compared to the CS+ during the 1st trial (denoted with the 1st 'NS' on the blue line). The 3rd sub-plot represented the slower and unstable CS+ learning curve of the low Acq1 scorers (N = 9 bees). These bees showed the least number of responses to the two type of CS stimuli in general amongst the three scorer classes (non-significant difference in the CRs between the 1st CS+ and CS- trial; denoted with 'NS' on the blue line), albeit the number of bees (sample size) was also lowest in this category.

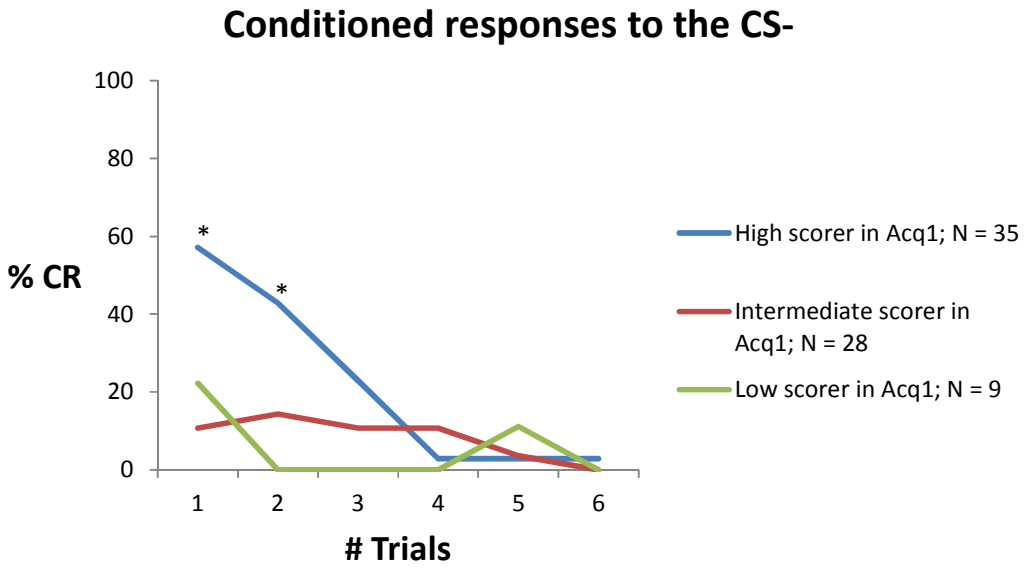


Fig. 24: Comparison of the conditioned responses to the CS- stimuli between the high, intermediate and low Acq1 scorers: This figure was an extension of the Fig. 23, highlighted the differences in dynamics of learning of the CS- stimuli between the three Acq1 scorer classes during the 1st differential conditioning. The x and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the CS- stimuli. All three scorer classes learned the CS- stimuli as evident from their acquisition functions (best scorer: blue line, intermediate scorer: dark brown line, low scorer: green line). However, the best Acq1 scorers showed significantly higher initial responses to the CS- (denoted with the 2 asterics on the blue line) compared to the other two classes due to the higher odor generalization and effects of sucrose arousal. CRs to the CS- odors between the intermediate and the low Acq1 scorers were not found to differ significantly throughout the conditioning unlike the two similar cumulative

scorer categories as shown in Fig. 22. 'N' represented the number of honeybees found in the three scorer categories.

one might incorporate an US habituation component. The concomitant learning dynamics of the CS+ and CS- odors were mimicking the scenario of the best cumulative scorers; in fact 48.5% of the high scorers in Acq1 were also found to be the best cumulative scorers.

The moderate or intermediate scorers showed the slow dynamics (Fig. 23) of the CS- learning with no significant differences found in the CRs between any of the two successive CS- conditioning trials, albeit these bees showed significantly lower responses to the CS- during the 1st and 2nd conditioning trials (RM-ANOVA group × stimulus: $F_{2, 69}$ = 5.71, p = 0.0050; followed by the Fisher LSD post hoc test for the 1st CS- trials between high and intermediate Acq1 scorers p = 0.000000, for the 2nd CS- trials between the same two groups p = 0.00039) compare to the high Acq1 scorers (Fig. 24). This meant that for the intermediates scorers, the CRs to the 1st presentation of the CS- stimuli suffered less than the high Acq1 scorers with the effects of odor generalization and the sucrose arousal apart from their possible low spontaneous responses. However, unlike the high Acq1 scorers fast discrimination between the two CSs was not found in the intermediate scorers; as revealed by the non-significant difference between the CRs of the two stimuli during the 2nd conditioning trials (RM-ANOVA showed significant stimulus × trial effect: $F_{5, 270}$ = 13.02, p = 0.00000; followed by the Fisher post hoc test: 2nd trial CS+ *vs.* CS- p = 0.25). The learning curves of the CS+ and CS- of these bees were mimicking the intermediate cumulative scorers, with an overlap of 35.8% bees in between these two classes.

The low Acq1 scorers like the intermediate category showed significantly lower responses to the CS- (Fig. 24) compared to the high Acq1 scorers during the initial conditioning trials (Fisher LSD post hoc test for the 1st CS- trials between high and low Acq1 scorers p = 0.0032, for the 2nd CS- trials between the same two groups p = 0.00031), but this was associated with a slow odor discrimination (Fig. 23) with no significant differences found between the CRs to the CS+ and CS- stimuli even during the 2nd and 3rd conditioning trials (RM-ANOVA showed significant stimulus × trial effect: $F_{5, 80}$ = 3.32, p = 0.0088; followed by the Fisher post hoc test for the 2nd trial of

CS+ *vs.* CS-: p = 0.48, 3rd trial CS+ *vs.* CS-: p = 0.16). The differences between CRs during the successive CS- training trials were also found non-significant (results were not shown; same set of statistical tests) with a further increase in the CR found during the 5th conditioning trial. Interestingly, like the low cumulative scorers, the low Acq1 scorers also showed an unstable asymptote for the CS+ odors, although only 16.6% of the bees were found in common between these two categories. The lower sample number (only 9 bees) in this particular category in Acq1 scorer compared to the other two possibly contributed to the instability of the CS+ learning curve.

These analyses revealed that learning dynamics of the CS+ and CS- stimuli during the differential conditioning had similarities between the two groups of honeybees; one with the superior overall learning and memory performances and the other which learned the CS+ stimuli fast and showed consistent PER to the CS+ throughout the differential conditioning. The CRs to the CS- stimuli in both group of bees although initially were affected with the strong effects of odor generalization and the sucrose mediated arousal, however; concomitant learning of the CS+ and CS- stimuli found only in these bees quickly overshadowed these effects. The intermediate scorers of the feature Acq1 or the cumulative performance showed faster learning of the excitatory CS+ stimuli compared to the CS- stimuli. The low scorer category showed the slower learning rate for both CS stimuli compared to the other two scorer classes with an unstable asymptote found for the excitatory CS+ stimuli.

It is never easy to understand from the population learning graphs whether the rewarded stimulus is learned together with the unrewarded stimulus during the differential conditioning or whether they are learned by the bees with differential dynamics dependent of their learning capabilities. *The results in this assay clearly demonstrated the fact that the rates of learning of the CS+ and CS- stimuli during the differential conditioning vary in the population, and depend on the overall learning performance of the individuals.*

At the end it was also checked whether the criterion of the fast learning of the CS+ stimuli during the differential conditioning was alone sufficient for the concomitant learning of the CS- stimuli or the condition of response consistency during the

conditioning was also required, since both features were quantified by the Acq1. Honeybees started to respond to the CS+ stimuli respectively from the 2nd, 3rd and 4th CS+ conditioning trials irrespective of the consistency of further PERs during the 1st differential conditioning were selected for this analysis. The results were given in appendix-1 (Fig. 27 and 28), however, it was found that bees which start responding early (fastest) to the CS+ showed higher odor generalization and effects of sucrose arousal compared to the two types of delayed responders. No differences were found in the CS+ learning between these three categories of bees, but the CS- learning (sharp decrease in conditioned responses along with the conditioning trials) was found concomitant with the CS+ only for the earliest (fastest) responders. It was concluded that bees which learned the CS-US association early (and respond early) showed the initial high spontaneous responses to the CS- in combination with the effects of odor generalization and sugar arousal however; only the fast learning bees were able to learn the rewarded and the unrewarded odors together. These learning features were similar as found for the high Acq1 and cumulative scorers. The delayed responders rather learned the CS- stimuli with a slower rate, like the intermediate or the low scorers in Acq1. Additionally, ~ 50%, of these fast learning bees were found to overlap with the population of the best cumulative performers. These fast learners showed an overall good performance in the cumulative conditioning assay (data not shown) and also found to be an interesting class of bees by another recent analysis performed by Evren Pamir and colleagues (unpublished data). They found that fast learners of the CS+ stimuli can develop the similarly strong CS+ memory while trained with smaller number of conditioning trials, with the bees conditioned with higher number of trials.

Increase in conditioned responses between the differential conditioning and the short-term memory retention test

Before proceeding to the next section analyzing the performance histories of the different types of performers, one other interesting observation regarding the short-term memory was documented here. Short-term memory (STM) in the honeybee was reported to have the early (in seconds) and the late (in minutes) phases which last for about 15 min after

the one-trial olfactory PER conditioning. Within this time period, experimental procedures such as cooling the whole animal or the selective brain neuropiles were reported to interrupt the consolidation of the STM (late-STM) in honeybees (Menzel 1999; Erber *et al.*, 1980). Like olfaction, free flying bees during the color learning also showed the same 15 min time span of the STM after the single conditioning trial when the memory was found susceptible to the impairment but no longer after the 15 min (Erber 1975; Erber 1976). Honeybees trained with the one-trial odor conditioning protocol showed differences in odor generalization while tested shortly after (30 sec) the training compared to the group tested after longer time delay (15 – 30 min). The author concluded that change in the content of form of the olfactory memory (some form of consolidation) within the shorter time scale (in the range of minutes) led to the change in responses of bees to the other untrained odors used to test the effect of generalization (Smith 1991). The term 'memory consolidation' in honeybee was used before in the context of the short-term memory (Smith 1991) as well as for the longer time (> 2 hr.) processes (Müller *et al.,* 2003) which was reported to transform the memory from the vulnerable state into the state with higher resistance to the process of extinction or other kinds of interferences (Menzel 1990, 1979; Menzel *et al.,* 1993). However, bees in these protocols were trained in the absolute PER conditioning paradigm either using the one or multiple training trials unlike the protocol of the cumulative conditioning assay performed here, where the bees received multiple (6 rewarded or CS+ and 6 unrewarded or CS-) training trials in the differential conditioning paradigm and were tested after 20 min of the conditioning. Hence, the cumulative protocol probably created a different form of late short-term memory (Menzel *et al*., 1999) which was consolidated over the period of 20 min before being tested. The stability of the 20 min memory after the extensive (for 96 min) differential conditioning was although not tested with any memory impairment protocol however; compared to the previously reported STM, the STM of the cumulative assay could also be different in this regard. Nevertheless, unlike the definition of 'memory consolidation' as the process which changes the memory from the vulnerable into the stable state, consolidation of odor memory in the cumulative conditioning paradigm was defined as the process which *operated within the shorter time scale* (min range) and manifested through the *increased CRs only to the rewarded odors* (CS+) from

the average CRs of the last two conditioning trials into the 1st memory retention test (1st of the 2 tests) while the bees were undisturbed in-between. Bees which did not show the learned responses during conditioning need to contribute to the population CRs of the retention test for this increase. This particular group of bees along with the bees responded constantly during the training and test were together considered to consolidate the CS+ memory within the 20 min time span which stabilized their responses during the retention test. The definition of 'memory consolidation' given here was not based on the reports showed the dependence of the different memory phases and the corresponding consolidation processes on the process of *de novo* protein synthesis (Wittstock *et al*., 1993; Wüstenberg *et al*., 1998; Menzel 1999). This definition compared the CRs of the conditioning trials only with the retention test trial where the highest or training concentrations of the CS+ odors were tested. Conditioned responses were found to decrease during the last conditioning trial compared to the trial before in the different selected group of bees hence; the averaging of the last two trials was performed to avoid any potential response-bias in the analysis. However, the definition of 'consolidation of the short-term memory' given here posed one limitation that bees with high levels of conditioned responses to the CS+ odors during the conditioning could not be followed for the further increase during the test due to the 'ceiling effect'. On the other hand bees with the lower range of cumulative-performance scores (score >= 30% and <= 45% of the maximum cumulative score) showed the increase in conditioned responses (CRs) from the 1st differential conditioning to the 1st retention test of the assay, although this difference was not found significant. However, during the 2nd differential conditioning these bees showed the significant increase (G test: G = 4.57, p = 0.03) in the CRs from the training (77% CR) to the retention test (96% CR). Honeybees selected with the other criterion such as the moderate or intermediate cumulative scorers (score >= 50% and <= 60%) also showed the significant increase in CRs to the CS+ stimuli between the conditioning and test during the 2nd phase of the cumulative assay (G test: G = 4.31, p = 0.03). The intermediate scorers in Acq1 (score >= 0.5 and <= 0.6) showed the similar significant rise during the 3rd retention test compared to the mean CR of the last two conditioning trials of the 2nd DC (G test: G = 5.81, p = 0.01). The low Acq1 scorers also showed the similar increase in the CRs but the differences during both phases of the

assay were not found significant, probably due to the smaller sample number found in this category (only 9 bees). Honeybees selected with the criterion of scoring high in the feature of odor sensitivity also showed (Fig. 17) the significant rise in the CRs to the CS+ odors between both differential conditioning and the following 1st retention tests (G test during the 1st phase between conditioning and test: G = 12.8, p = 0.0003, G test during the 2nd phase: G = 21.17, p = 4.2×10^{-6}).

One might find more number of such differences with bees selected with the other criteria however; for the present purpose only these groups of honeybees were reported. These results confirmed that consolidation of the short-term olfactory memory in the time range of 20 min, post-conditioning was visualized in the increased conditioned responses to the CS+ during the retention tests. It was also important in this context to mention that during the retention tests bees were exposed with increasing concentrations of the CS+ and CS- odors, and the highest or training concentrations were presented only at the end in the series of odor dilutions. However, multiple presentations of the odor dilutions probably did not incorporate any extinction component in the animal's responses to the training concentrations of the CS+. Alternately, the possible extinction effect was suppressed by the highest concentrations of the CS+ odors (due to some form of 'concentration effect').

Until now it was shown that performance histories of the bees were able to predict or drive the future performances such as the fast learners or the good discriminators scored well in the different learning and memory related features. Here at the end of the result section, the relationships between the response histories to the different CS stimuli during the phases of conditioning and retention tests were analyzed with the bivariate histogram analysis using the original binary data of the pooled population. This analysis revealed many interesting aspects about the performance or response probabilities between the different pairs of behavioral features or the CS stimuli.

3.6.6 Bivariate histogram analysis revealed the pair-wise relationships of the features

Responses to the CS+ and CS- odors during the 1st and the 2nd differential conditionings

Bivariate histogram analysis showed that conditioned responses to the two CSs were more specific during the 1st differential conditioning (DC) (Fig. 27; 1st plot) compared to the 2nd, however, more bees were found to show no responses to either of the two CS stimuli during the 1st compared to the 2nd conditioning. During the 2nd DC bees became hungry after being trained and tested for 3 hours and probably also suffered with some form of arousal effect due to the repeated sucrose feeding events of the 1st DC. This was one of the possible reasons which led to the more number of incorrect responses between the two CSs (more generalization) as found during the 2nd DC.

Discrimination between the CS+ and CS- during the test 1 and test 2

During the 1st and 2nd memory retention tests bees discriminated well between the two dilutions and the training concentrations of the CS+ and CS- however; more responses to the CS- (Fig. 28) were found during the 1st compared to the 2nd test.

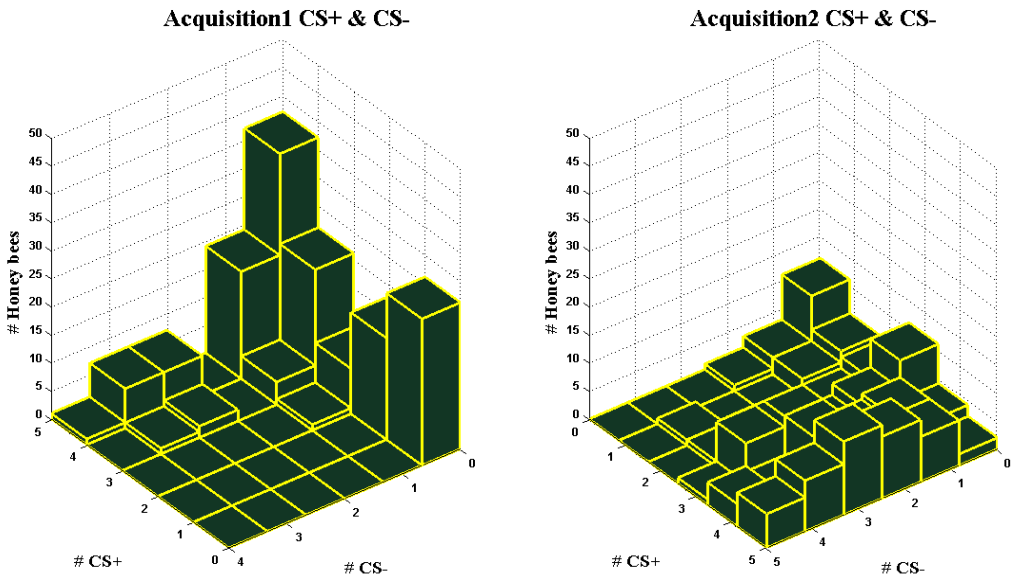


Fig. 27: Bivariate histogram analysis between the PER responses to the CS+ and CS- stimuli during the two phases of differential conditioning: The x, y and z axes in both sub-plots of this figure respectively represented the number of PER responses shown to the CS-, CS+ (binary data) and the number of honeybees. Each bar inside each of the sub-plot represented a certain number

of bees with the specified number of PER responses to the CS+ and CS- stimuli. During the 1st differential conditioning (Acquisition1; 1st sub-plot) bees learned the discrimination task well between the two CS stimuli with minimum number of responses shown to the CS-. However, during the 2nd differential conditioning (Acquisition2; 2nd sub-plot), bees showed more generalization and less discrimination between the two CSs with more number of responses shown to the CS- odors.

Discrimination between the CS+ and CS- stimuli during the test 3 and test 4

During the 3rd retention test (Fig. 29) bees showed more generalization in responses between the different concentrations of the CS+ and CS- odors compared to the retention test 1 and 2. During the 4th and the last retention test an overall reduction in the PER responses to the CSs was associated with the least odor discrimination (evident form Fig. 3). The lack of responsiveness during the 4th-retention test probably indicated an overall decline in the motivational state of the bees to respond to the odor stimuli after being trained and tested for more than 6 hours. During the test 3 bees were able to discriminate well between the training concentrations of the CS+ and CS- odors but generalized more while the dilutions were presented (Fig. 3). Hence, high number of responses to the

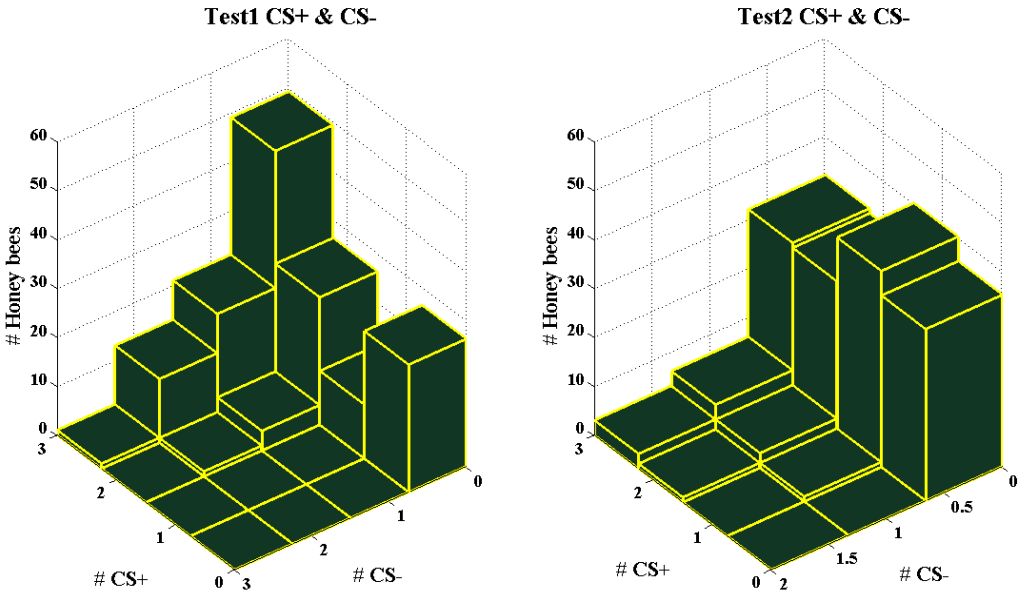


Fig. 28: Bivariate histogram analysis between the PER responses to the CS+ and CS- stimuli during the memory retention test 1 and test 2: Bees showed good discrimination between the dilutions of the CS+ and CS- odors during both retention tests, but during the test 1 (1st sub-plot) they responded little more to the CS- compared to the test 2 (2nd sub-plot). The x, y and z axes in both sub-plots of this figure respectively represented the number of PER responses shown to the CS-, CS+ and the number of honeybees.

dilutions of the CSs probably did not reflect the possible deficit in learning rather they indicated the effects of hunger and arousal in bees. Together these effects contributed to the higher number of incorrect responses while the dilutions of the CS stimuli were presented, but exposure to the highest concentrations restored the specific higher responses to the CS+ compared to the CS- stimuli (some form of 'concentration effect').

CS+ responses between the test 1 and test 2 and between the test 3 and test 4

In the first pair (test 1 and 2) of the memory retention test more number of bees was found unresponsive to the respective CS+ stimuli compared to the test 3 and 4. Honeybees which showed strong responses (3 responses) during the test 1 and 3 also

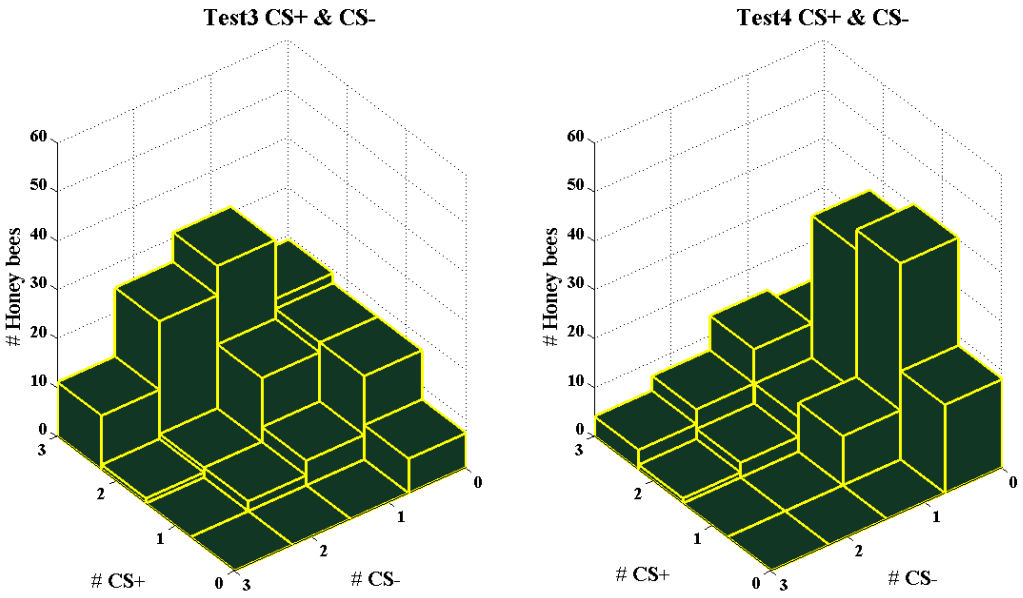


Fig. 29: Between the PER responses to the CS+ and CS- during the retention test 3 and test 4: Honeybees generalized more between the dilutions of the CS+ and CS- odors during the retention test 3. Bees which showed strong responses to the CS+ were also found to show more responses to the CS-. During the test 4 majorities of the bees did not show responses to either of the two CSs along with the response generalization found between the dilutions of the CS+ and CS- odors amongst the responding bees. The x, y and z axes represented the same variables as mentioned in Fig. 28.

showed strong responses during the following retention test 2 and 4 (Fig. 30) although, some of the strong responders of test 1 and 3 reduced their number of CS+ responses (1 or 2 PER responses) during the test 2 and 4. The weak responders (in both pair of retention tests) during the 1st of the two retention tests responded weakly during the following test.

CS+ responses between the two differential conditionings and between the retention test 1 and test 3

Honeybees which respond strongly to the CS+ odors during the 1st round of differential conditioning did not response consistently during the 2nd conditioning (Fig. 31). The population of strongly responding bees (5 responses) of the 1st DC was distributed into the populations with 5, 4 or 3 responses during the 2nd DC. Hence, the response consistency to the CS+ odors was not maintained between the two rounds of differential conditioning. However, bees which showed higher total number of responses to the CS+ during the test 1, also responded strongly to the CS+ stimuli during the test 3 (Fig. 31).

CS+ responses between the 1st differential conditioning and test 1 and between the 2nd differential conditioning and test 3

During both rounds of the differential conditioning bees with higher number of responses (Fig. 32) to the CS+ odors were found to show more frequent responses to the CS+ presentations during the following tests (test 1 and test 3) compared to the bees with

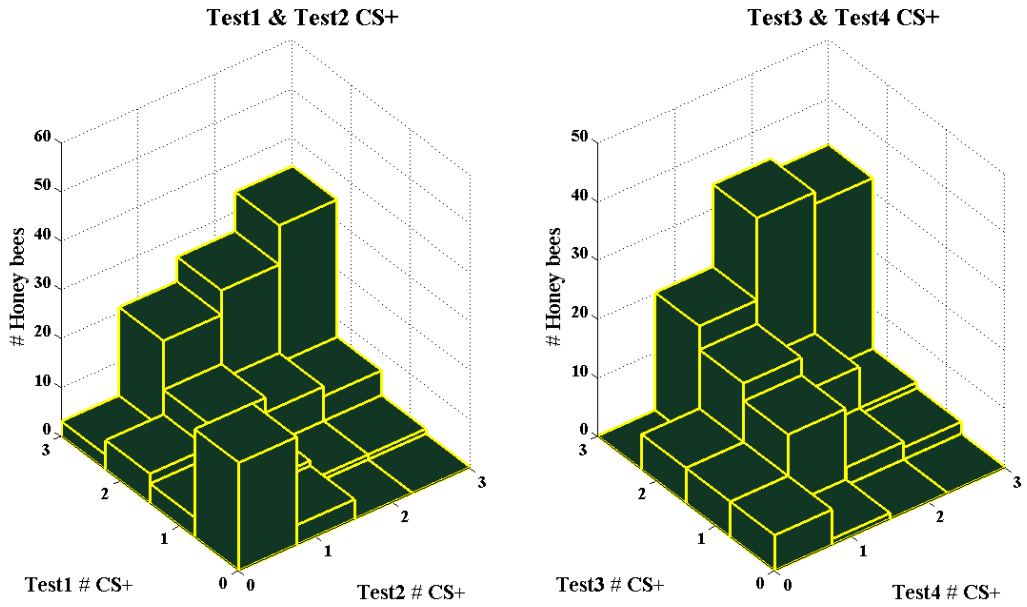


Fig. 30: Between the CS+ responses of the retention test 1 and 2 and between the test 3 and 4: Bees with higher number of responses to the CS+ both during the test 1 and 3 also showed stronger responses to the CS+ stimuli respectively during the test 2 and 4. However, bees with weaker responses in test 1 or 3 continued reponding weakly during the test 2 and 4. The x, y and z axes represented the same variables as described in Fig. 28.

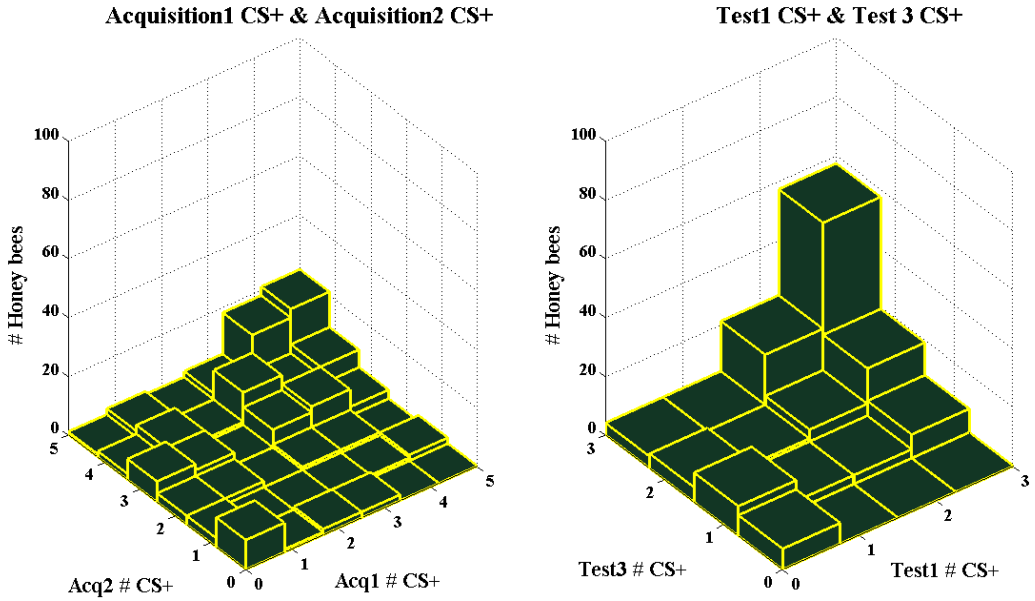


Fig. 31: Bivariate histogram analysis between the CS+ responses of the two differential conditionings and between the responses during the test 1 and 3: Bees that responded strongly to the CS+ during the 1st differential conditioning (Acquisition1) did not respond so well during the 2nd (Acquisition2). However, bees with higher number of responses to the CS+ during the test 1 also showed strong responses during the test 3. The x, y and z axes represented the same variables as mentioned in Fig. 28.

lower number of responses during the conditioning trials. This effect was particularly prominent for the 2nd DC and test 3 due to the higher number of bees responded to the CS+ during the 2nd compared to the 1st differential conditioning. *Hence, bees with strong responses to the CS+ stimuli during the 1st differential conditioning although just failed to maintain their strong responses during the second DC (Fig. 31) however; they were able to maintain their good-response levels to the CS+ odors during the following memory retention test.*

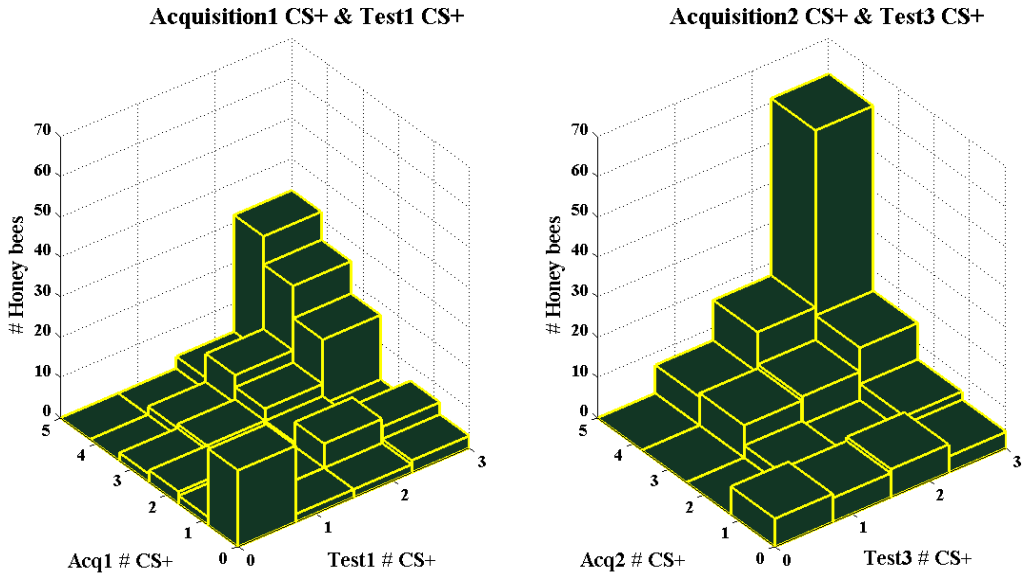


Fig. 32: Between the CS+ responses during the 1st conditioning and the test 1 and between the 2nd conditioning and the test 3: Bees with more number of responses to the CS+ stimuli during the two phases of differential conditioning (Acquisition 1 and 2) were also able to show more number responses to the CS+ stimuli during the following retention tests (test 1 and 3). The x, y and z axes represented the same variables as mentioned in Fig. 28.

Between CS+ responses during the differential conditionings and to the lowest CS+ dilution (odor sensitivity) during the following retention test

Bees with strong (higher number) responses to the CS+ stimuli during both rounds of the differential conditioning were found to respond strongly to the lowest dilution (-3) of the CS+ odors during the following retention test 1 and 3 (Fig. 33) and *vice versa*. This meant that bees were able to transfer the learned information (knowledge) about the meaning of the pure concentrations of the CS+ stimuli to respond correctly to the same stimuli in lower concentration.

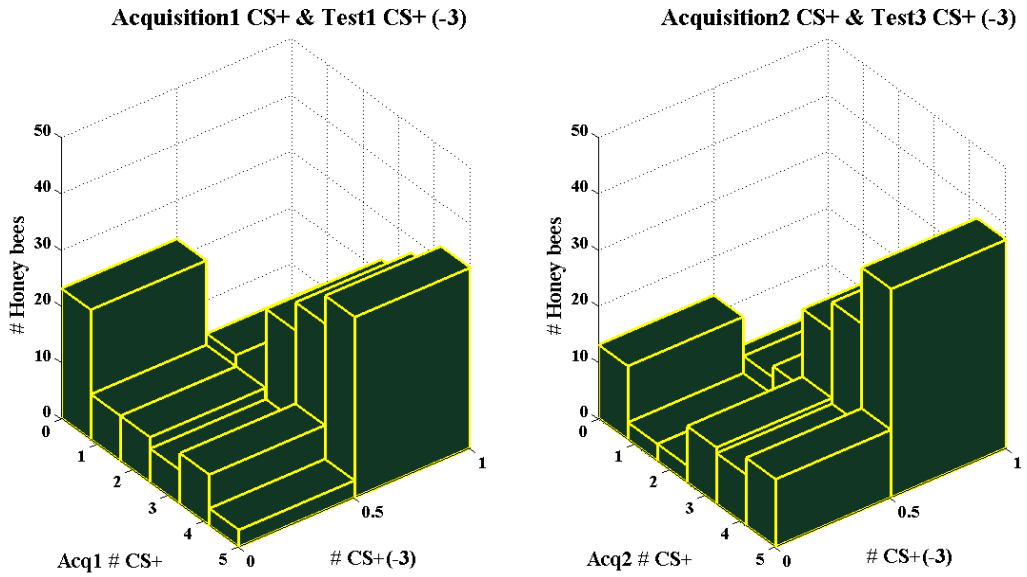


Fig. 33: Between the CS+ responses of the differential conditionings and responses to the lowest dilution (10^{-3}) during the retention test 1 and 3: Bivariate histogram analysis showed that bees with stronger responses to the CS+ during the acquisition phase-1 and 2 (conditioning 1 and 2) also responded more often to the lowest dilution of the CS+ stimuli during the following memory retention tests compared to the bees with the weaker CS+ responses during the time of differential conditioning. The x, y and z axes represented the same variables as described in Fig. 28.

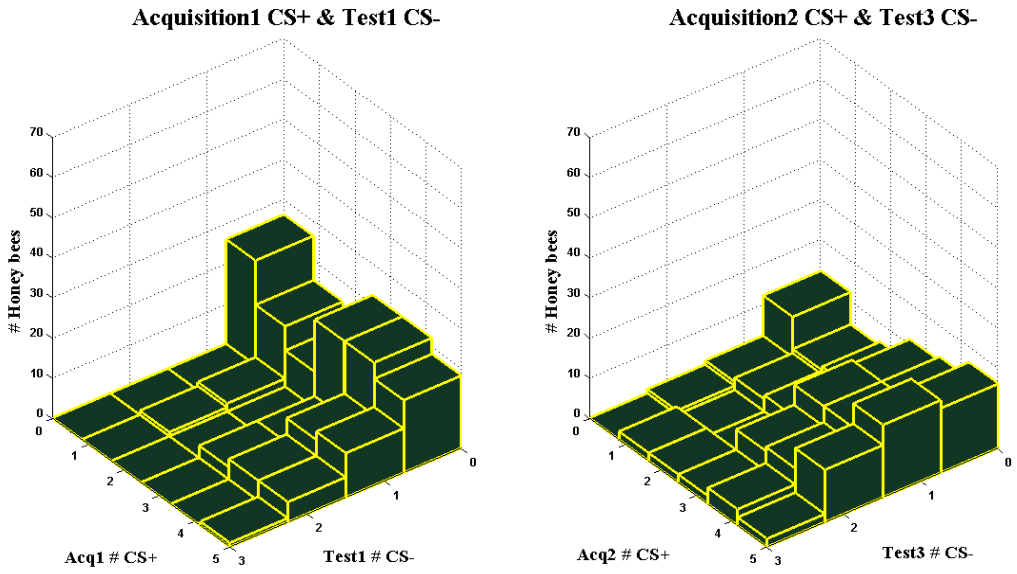


Fig. 34: Between the CS+ responses during the differential conditionings and the CS- responses during the test 1 and 3: Bivariate histogram analysis showed that bees overall had lower number of CS- responses during the test 1 compared to the test 3. Honeybees with higher number of CS+ responses during the 2nd differential conditioning showed more number of responses to the different concentrations of the CS- odors during the test 3compared to the bees with the lower number of CS+ responses. During the test 1 only the stronger CS+ responders of the 1st conditioning showed few responses to the CS- dilutions. The x, y and z axes represented the same three variables as mentioned in Fig. 28.

Between CS+ responses during the two conditionings and CS- responses during the retention test 1 and test 3

Bees in general (lower or higher number of the total CS+ responses during the 1st differential conditioning) showed least responses to the dilutions as well as to the training concentrations of the CS- stimuli while the short-term memory was retrieved during the test 1 (Fig. 34). However, bees with strong responses to the CS+ during the 2nd differential conditioning showed higher number of responses to the CS- compared to the weak responders during the same conditioning. The total number of responses to the CS- stimuli was found higher during the test 3 compared to the test 1, explaining the higher odor generalization as found before during the test 3 (Fig. 29).

CS- Responses between the two conditionings and the tests 1 and test 3

As mentioned before, that many bees did not response to both CSs during the 1st DC which was found here again as bulk of the population showed no responses to the CS- odors during the conditioning as well as to the pure concentrations during the test 1 (Fig. 35). However, due to the effects of odor generalization, the number of conditioned responses to the CS- was found to increase during the 2nd differential conditioning and test 3. Similar scenarios were found while the numbers of responses were measured to the lowest dilution of the CS- odors (10^{-3}) during the test 1 and 3 (Fig. 36). Bees with higher number of responses to the CS- during the 1st conditioning showed nearly no response to the lowest dilution of the CS- during the test 1. On the other hand bees with stronger or weaker responses to the CS- odors during the 2nd differential conditioning showed stronger responses to the lowest CS- dilution during test 3 (Fig. 36). However, exposure to the training concentrations of the CS- reduced the number of responses during the test 3 (Fig. 35).

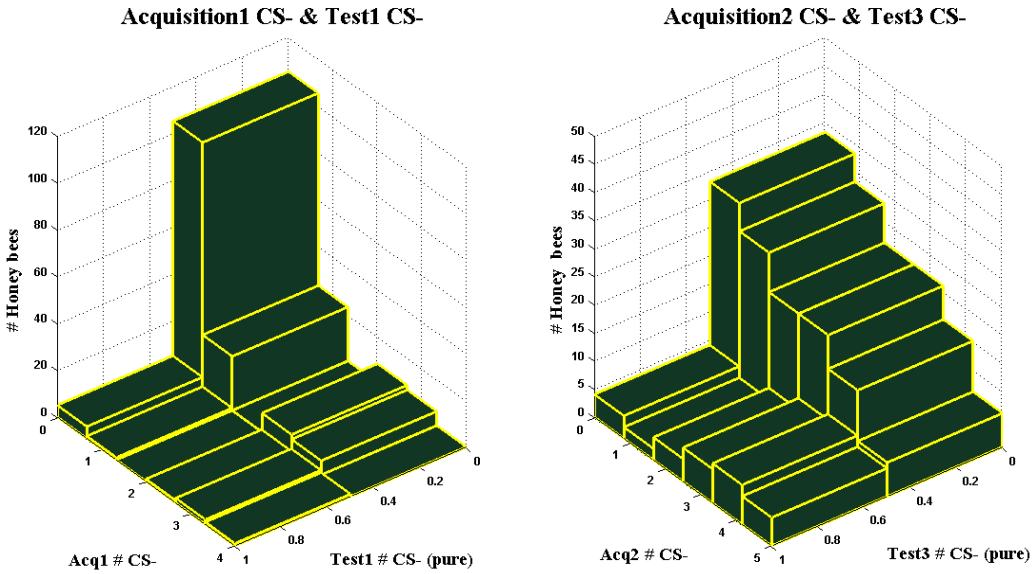


Fig. 35: Between the CS- responses during the differential conditionings and to the pure concentrations of the CS- stimuli during the test 1 and 3: During the 1st differential conditioning (Acquisition1) bees showed least responses to the CS- and only shown fewer

responses to the pure concentrations (training concentrations) of the CS- odors during the test 1. However, during the 2nd differential conditioning (Acquisition2) more number of bees showed stronger responses to the CS- as well as more responses were found during the test 3 to the training concentrations of the CS-. The x, y and z axes represented the same variables as mentioned in Fig. 28.

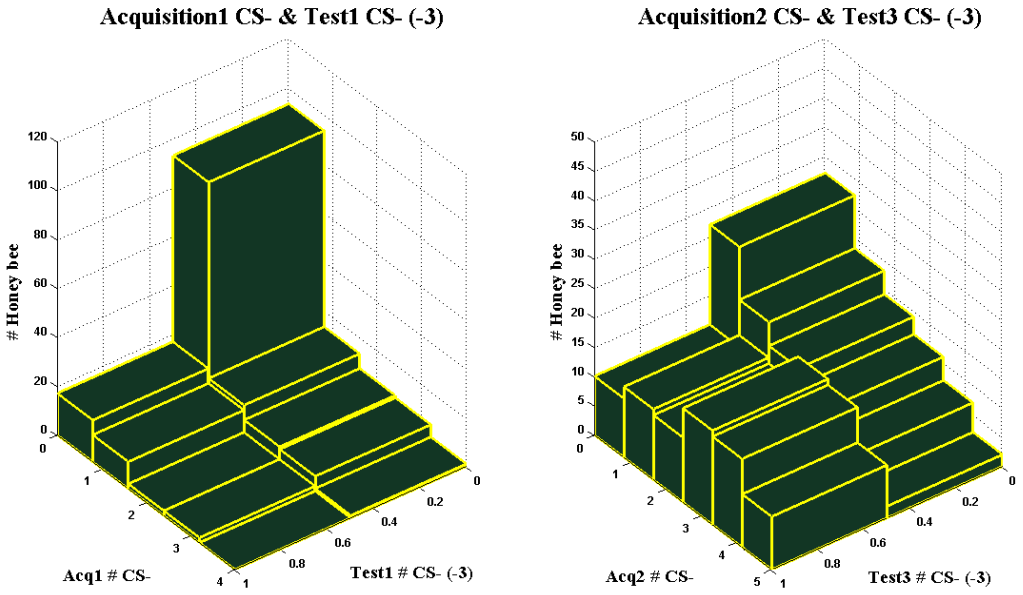


Fig. 36: Bivariate histogram analysis between the CS- responses during the differential conditionings and to the lowest concentration (10^{-3}) of the CS- stimuli during the test 1 and 3: Similar to the Fig. 35 bees showed fewer responses to the lowest dilution of the CS- stimuli during the test 1, however, during the test 3 more number of responses to the lowest CS- dilution were found in the bees which showed either higher or lower number of CS- responses during the 2nd conditioning trials.

Between CS- responses during the two conditionings and the CS+ concentrations during the test 1 and test 3

Honeybees with higher number of CS- responses or higher generalization between the CS+ and CS- odors during the 1st round of DC were found to show strong responses to the lowest CS+ dilution (10^{-3}) during the test 1 (Fig. 37) although, the total number of bees found in this category was low. These bees also showed strong responses to the training concentrations (pure) of the CS+ stimuli during the test 1 (Fig. 38). Nearly half

of the bees with no responses to the CS- during the 1st DC were found to show higher sensitivity as they respond to the lowest CS+ dilutions (Fig. 37). The number of bees in this category was found little higher while the memory retention test was conducted with the training concentrations of the CS+ odors (Fig. 38). *Results until now showed that honeybees with no responses or higher number of responses to the CS- odors during the 1st differential conditioning actually showed strong responses to the CS+ concentrations during the test 1; hence, they learned the meaning of the CS+ stimuli sometime during or after the conditioning.* Similar scenario found during the test 3 for the responses to the highest and lowest CS+ concentrations as honeybees either with higher or lower number of CS- responses during the 2nd DC showed strong responses to the different concentrations of the CS+, although the number of responses to the pure concentrations were higher than the lowest dilution.

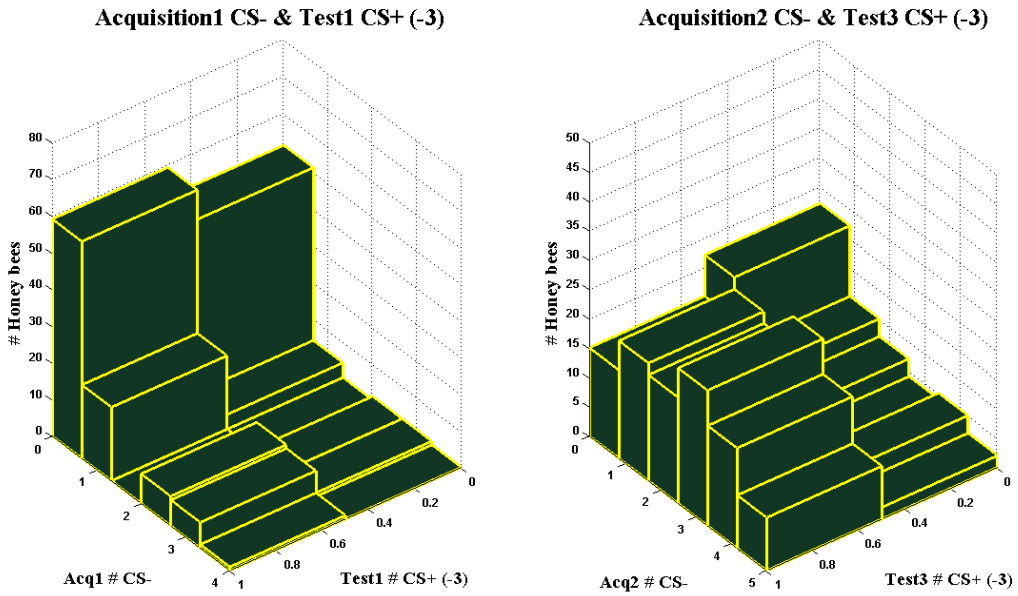


Fig. 37: Bivariate histogram analysis between the CS- responses during the differential conditionings and responses to the lowest concentration (10^{-3}) of the CS+ during the test 1 and 3: Bees which showed stronger responses to the CS- during the 1st differential conditioning responded to the lowest CS+ dilution (10^{-3}) during the test 1. Bees with lower number of CS- responses and half of the bees which showed no CS- responses also responded to the CS+ dilution during the test 1. During the test 3 (similar to the scenario with the lowest CS- dilution)

bees with either higher or lower number of CS- responses during the 2nd differential conditioning showed strong responses to the lowest CS+ dilution.

Between responses to CS+ during conditionings and the filter paper and paraffin oil during test

The number of responses to the filter paper and paraffin oil were found to decrease during the 2nd retention test compared to the 1st in the two pairs of tests conducted in each of the two phases of the cumulative conditioning assay (Fig. 3). However, it was found that during both phases of differential conditioning bees with higher number of responses to the CS+ odors also showed more frequent responses to the filter paper and paraffin oil compared to the bees with lower number of CS+ responses (Fig.39). *This meant that bees which learned the CS+ faster and respond consistently during the conditioning also showed higher responsiveness to the novel CS stimuli (filter paper and oil) compared to the slow learners and / or inconsistent responders to the CS+ odors.*

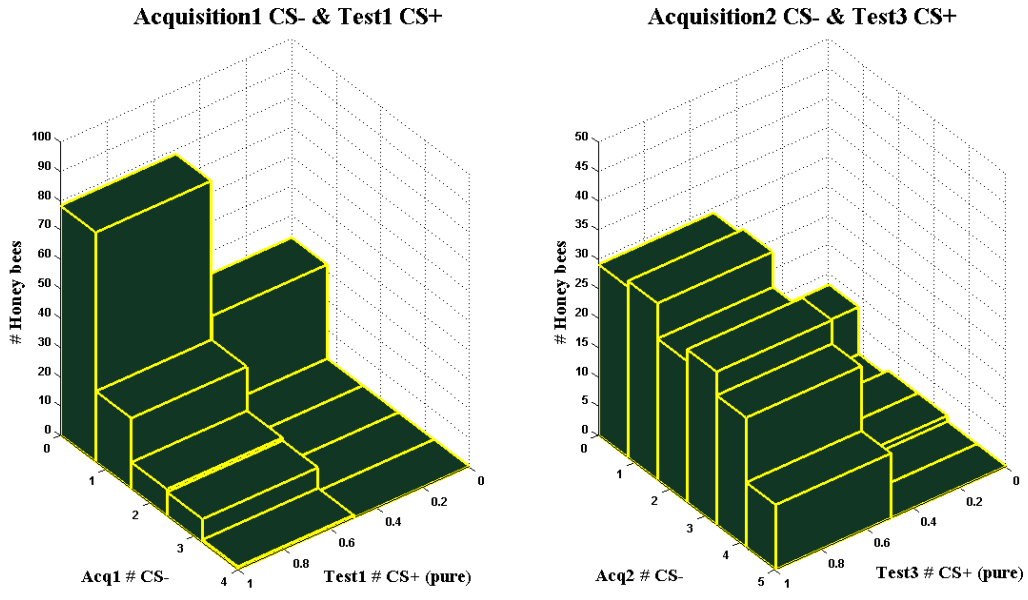


Fig. 38: Bivariate histogram analysis between the CS- responses during the differential conditionings and to the training concentrations of the CS+ odors during the test 1 and 3: Bees with no responses or weaker responses to the CS- during the conditioning responded

strongly to the pure concentrations of the CS+ stimuli during the test 1. During the test 3 bees also showed higher responses to the CS+ compared to the lowest dilution (Fig. 37).

Between responses to CS- during the conditionings and to filter paper and oil during the test

Majority of the honeybees showed no responses to the CS- during the 1st conditioning; amongst them the majority showed no responses to the filter paper (FP) and paraffin oil during the following test (Fig. 40). Additionally, fewer bees which showed more responses to the CS- during the 1st DC also did not extend their proboscis to the stimuli like FP and oil. During the test, after the 2nd conditioning more number of bees was found to respond to the filter paper and oil; especially those which showed higher number of CS- responses during the 2nd differential conditioning.

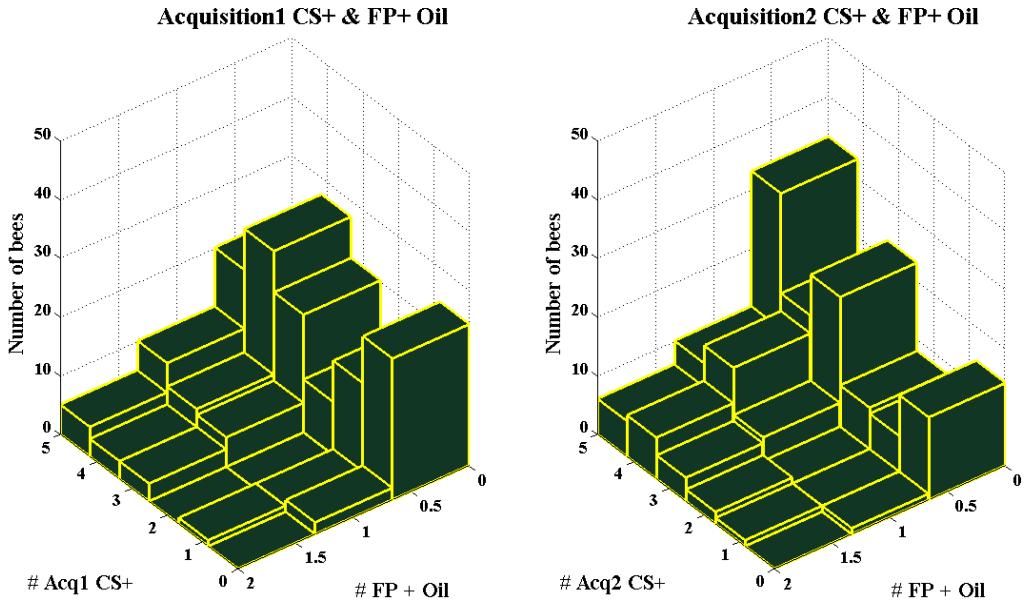


Fig. 39: Between the responses to the CS+ during the conditionings and to the filter paper (FP) and paraffin oil: Bees with stronger responses to the CS+ stimuli during both phases of the differential conditioning also showed more frequent responses to the filter paper and paraffin oil than the bees which responded weakly during the conditioning. However, the overall number of responses to the filter paper and oil were found low in both cases.

3.7 Discussion

3.7.1 Heterogeneity in learning and memory performances inside the population of honeybee

Previously we showed (Pamir *et al.*, 2011) that honeybees trained identically in the different olfactory PER conditioning paradigms (absolute, differential conditioning and extinction learning) varied from each other in their learning probabilities during the conditioning trials. There were bees which never learned the association between the CS and the US stimuli during conditioning and consequently developed no long-term memory with high probability. On the contrary, bees which learned the association or no association (CS- learning during the differential conditioning and extinction learning) between the CS and the US stimuli were found to remain stable in the learned state throughout the training trials and later also developed the long-term memory with high probability. These two groups of bees, found consistently in the different experimental data sets confirmed the existence of heterogeneity in individual's learning inside the population of the honeybee. Current results of the cumulative olfactory learning assay also showed the heterogeneity in performances among the individuals for the different

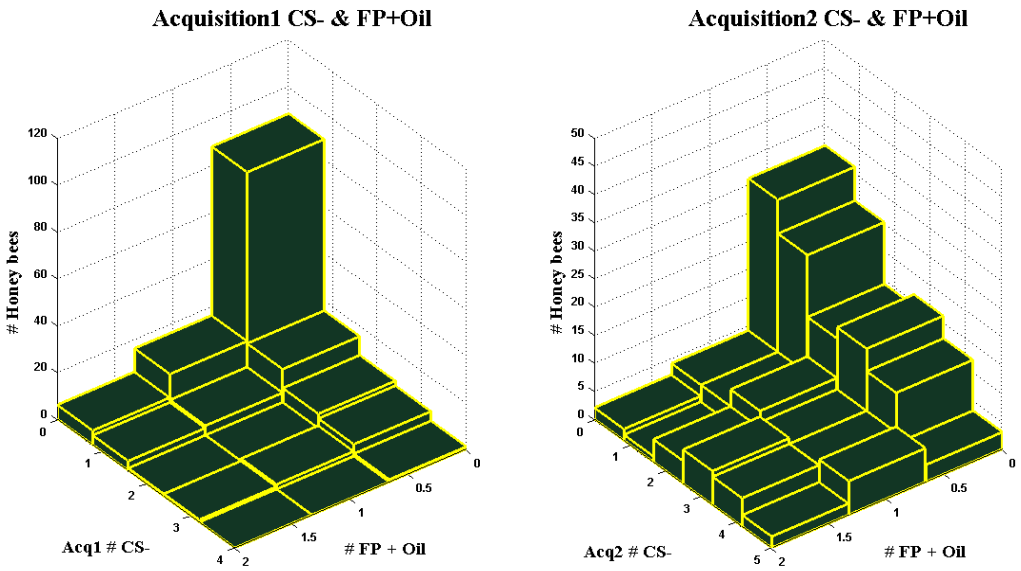


Fig. 40: Bivariate histogram analysis between the responses to the CS- during the conditionings and to filter paper and paraffin oil: Very low number of responses to the filter paper and paraffin oil showed by the bees with either lower or higher number of CS- responses during the 1st differential conditioning. The total number of responses to the filter paper and paraffin oil was increased after the 2nd conditioning, with more contributions came from the bees with stronger CS- responses during the conditioning.

learning related features such as the rate and reliability of the CS+ odor learning, discriminability during the conditioning and the retention tests, odor sensitivity and the overall or cumulative performance. This heterogeneity was consistently found in the individual backcrossed colonies both throughout the whole experimental season (summer and autumn) as well as from one to another day (data not shown here). The high variability between the individuals prohibited the formation of any detectable performance cluster of bees in the score histograms of the quantified features. Hence, variability in the performance level of the individual bees was found as the strongest (most salient) feature in the data set generated with the cumulative olfactory conditioning assay. Other results in honeybee also showed the variability in the learning performance of the individual bees (Mota and Giurfa 2010). The authors in this study conditioned the bees in the multiple-olfactory-reversal learning paradigm and found the existence of at least three different performer categories. One of the three kinds with higher odor discriminability was able to reverse the odor contingencies most efficiently; the other type showed the reversal capability, however, was unable to discriminate between the alternate CS+ and CS- stimuli due to the lack of extinction of the first CS+ memory. The third and last category of bees performed the discrimination and reversal tasks poorly throughout the whole assay. Two other recent studies correlating behavior with the neuronal responses also found differences in the odor evoked calcium responses in the antennal lobe neuropil between the so called learners and the non-learners, selected with the heuristic behavioral criteria (Roussel *et al.,* 2010; Rath *et al.,* 2011). Honeybees (Rath *et al.,* 2011) which were able to discriminate between the CS+ and CS- odors also showed the significant increase in the distance or distinctness of the glomerular response patterns (measured through the Euclidean distances) between these two odors after the learning compared to the bees which failed to learn the discrimination.

These individual evidences illustrated the fact that variability in individual's learning behavior is a commonly encountered event in the natural populations of honeybee which is not adequately described by the group averaged measurements, performed frequently in the past. However, understanding of the individual's learning behavior is important to comprehend our knowledge about the learning related plasticities (functional and anatomical) in the neural networks as well as in the molecular machineries of individual's brain. Hence, more rigorous research in this direction is required apart from the population approaches to understand the phenomena of 'learning'. It is important to remember in this context that honeybees do not have any genetic component to perform good or bad in the olfactory PER conditioning assay, since it is an artificial method. However, this procedure can test the effectiveness of the genetic repertoire controlling the natural learning abilities of olfactory information in honeybees, under conditions of less parametric variability. Hence, variability found in the individual's olfactory learning and memory performances in the cumulative conditioning assay was probably reflecting the variability in the natural population of the forager bees due to their differences in the genetic background as well as in the history of odor tuning. The heterogeneity found in the experimental population probably had the influences from components such as the fluctuation of the day-to-day weather conditions, seasonal change, changes in the colony's food reserve, age and motivational status of the bees (Behrends and Scheiner 2010; Hadar and Menzel 2010). In the cumulative assay I tried to control for the experimental age group by using only the forager bees, their bowl content (through overnight satiation) as well as kept monitoring for the sufficient food reserve of the colonies throughout the summer and autumn. Effects of the fluctuations in weather condition on the olfactory learning although was uncontrolled, but analysis showed no significant effect of the seasonal change on the olfactory learning and memory performances of the bees used in the assay ('seasonal effect'; appendix-1). Hence, individual's variability found in this assay was most likely reflecting the natural heterogeneity in the learning and memory performances of the individual honeybees of the three backcrossed colonies.

3.7.2 Individual's analyses of learning and memory picked up the different performer classes from the performance heterogeneity

In the middle of heterogeneity in the learning and memory performances, different performer classes were recognized. First, with the best and the poor cumulative performers were selected from the pooled population of the backcrossed colonies using the arbitrary criteria of higher and lower ranges of the cumulative score. The best cumulative showed superior learning and memory performance throughout the assay, with high performance scores in the different features. This eventually led to the higher correlation between the scores in the different features in these bees. On the other hand, the worst or poor cumulative scorers nearly did not learn the meaning of the stimuli throughout the entire assay and scored low in the different features. This resulted in the fewer high correlations found between some of the features for these bees. *The opposite type of cumulative performance of these two classes of bees was not surprising since they were selected with the criteria of extreme scores*. However, the prolong conditioning and test protocol of the cumulative assay, absence of seasonal effect on the olfactory learning (no effect on the learning rate, discriminability and odor sensitivity) and intact sucrose responsiveness of the poor performers during the assay strongly indicated that arbitrary selection shortlisted the true-opposite types of learning performers from the randomly caught foragers of the three backcrossed colonies.

Further analysis showed one commonality in these two groups of bees; high correlation between the scores in Acq1 (the speed of CS+ learning) and Disc1 (odor discriminability) during the 1st differential conditioning (DC). These two features in fact were found to show the highest correlation in the pooled population as well as in other selected groups of bees. It was evident from the bivariate histogram analysis that bees overall showed lower number of responses to the CS+ and CS- stimuli during the 1st compared to the 2nd DC. As a result, bees scored higher (data not shown) in the feature of learning-speed (Acq2) during the 2nd differential conditioning compared to the 1st (Acq1), albeit showed decreased correlation between the Acq2 and odor discriminability (Disc2) due to the higher number of CS- responses and lower scores in the Disc2. Apart from their high correlation Acq1 and Disc1 also showed the high correlation with the cumulative performance score of the bees. This indicated that performance scores in these two

features strongly influenced the cumulative performance of the bees. However, performance heterogeneity of the different scorer categories including the best and the poor cumulative scorers during the assay resulted in the scenario where the scores in the single features or in combination with others were able to select the different performer classes with only lower probabilities, except for the feature of odor discrimination during the 1st DC (Disc1). Disc1 was found to be the strongest amongst the learning related features to predict the overall or cumulative performance levels of the honeybees. Higher and lower scores in this feature were able to select the best and the poor cumulative performers respectively with the probabilities of 0.68 and 0.65. Similar result was found in the recent work of Theo Mota (Mota and Giurfa 2010) where the best performing bees amongst the three different performer classes showed the highest olfactory discrimination throughout the different phases of the multiple-reversal learning assay. Hence, fast and consistent learning of the identity of the rewarded (CS+) and the unrewarded (CS-) odor stimuli was found to be the important or necessary qualification for the bees to perform superiorly throughout the cumulative conditioning assay. However, it is possible that other olfactory learning assays in the honeybee may also find odor discriminability or any other behavioral feature as the strongest predictor of the individual's overall performance levels.

Apart from the consistently good cumulative performances the best cumulative scorers and the high scorers in Acq1 showed the concomitant learning of the CS+ and CS- odors during the differential conditioning (DC) which was not seen in the poor Acq1 and cumulative scorers. The early but not the delayed learners and / or responders to the CS+ stimuli also showed the 'together-learning' dynamics of the CS+ and CS- odor stimuli during the DC. This revealed the fact that learning of the rewarded and the unrewarded odor stimuli *do not follow any common or general dynamics in the bees, rather the learning dynamics depend on the type of learning performers*.

However, the high scorers in cumulative performance, the fast and consistent CS+ learners and also the high scorers in Disc1 all showed the stronger effects of odor generalization and the sucrose mediated arousal compared the low Acq1, Disc1 or cumulative scorers during the 1st differential conditioning. The initial high level of conditioned responses to the CS- during the DC however, was suppressed by the fast

learning of the CS- stimuli in the high scorers, which led to the concomitant learning of the CS+ and CS- stimuli. The underlying reason for the high odor generalization or sucrose mediated arousal found in the high scoring bees was not understood but previous research (Brandes *et al.,* 1988) in honeybee found the stronger effects of sensitization in the good olfactory learners compared to the bad learners while PER responses of the bees to an odor were counted 30 sec after the sucrose stimulation of the antennae. This non-associative form of sensitization or arousal effect on the odor response was found to disappear after 1 min of the sucrose stimulation both in the good and the bad learners (Brandes *et al.,* 1988). However, unlike the finding of Brandes and colleagues, the best cumulative scorers in our experiments did not show the fast reduction in sensitization within 1 min. Differences in the two experimental protocols probably led to the differences in results. Bees in Brandes's study received only the antennal stimulation of sucrose whereas; in the cumulative conditioning assay sucrose was feed to the bees apart from the antennal stimulation during the time of conditioning with the odor-sucrose paired protocol. Hence, the nature or form of sensitization induced in these two groups of bees was probably different due to the differences in the way the sucrose was delivered. Possible higher overall responsiveness to the olfactory and gustatory stimuli of the high scoring bees than the corresponding low scorers led to the initial high odor generalization and arousal in these bees which later became suppressed by the effects of stable CS-learning.

However, irrespective of the arousal or odor generalization effects, the high Acq1 scorers or the fast and consistent learners of the rewarded odor stimuli were found to be an interesting class of bees as they showed superior overall performance during the cumulative assay (performance graphs not shown). In an independent recent study conducted by Evren Pamir and colleagues (unpublished) it was also found that in the absolute PER conditioning paradigm once the fast learning bees started showing the conditioned responses to the odor stimuli, they did not require any additional training trial to develop the odor memory similarly strong as showed by the slow learning bees conditioned with more number of trials. The reasons behind the superior performance of the fast learners were unclear, but results of the cumulative assay suggested that behavioral mechanisms regulating the rate of odor learning was also regulating the odor

discriminability and sensitivity which all-together contributed to the overall performance of the bees in the cumulative olfactory learning and memory tasks. Further research in this direction is required to understand the cellular and molecular basis of the behavioral superiority of the fast learners as well as the common regulating mechanisms.

In addition to the learning speed and discriminability, the low scores in odor sensitivity was also found to select the poor cumulative scorers with high probability ($p = 0.72$; analysis not shown) but the high scores were only able to select the good cumulative scorers with the low probability of 0.3. Hence, large fraction of bees selected with the criterion of scoring high in 'odor sensitivity' did not perform as good as the high Acq1 or cumulative scorers in the different learning and memory features such as the learning speed and odor discriminability, although they showed the switch-like learning of the CS+ stimuli (see Fig. 17). On the contrary, the high cumulative, Acq1 or Disc1 scorers showed higher odor sensitivity, meaning that odor sensitivity was a *weaker feature compared to the Acq1 and Disc1* to predict the overall performance levels of the bees trained in the cumulative conditioning paradigm or in other words the *performance scores in the feature 'sensitivity' were better controlled by the scores in the learning speed and odor discriminability, but not vice versa*. How the learning speed and/or discriminability control the odor sensitivity more efficiently than the other way round is not known from the perspective of neuronal processing, which is an interesting endeavor for future.

It was interesting to note that only 27% of the entire population of honeybees used in the assay was performed very well or very poorly. The rest 73% showed the cumulative performances ranging from just better than the poor until just weaker than the best performers. Meaning, that majority of the honeybee foragers participated in the assay either performed 'not so good' or 'moderately well' in the complex form of olfactory learning and memory tasks. Only, the small fraction of the foragers performed either very well or very poorly in the same set of olfactory task. Laska and colleagues previously showed in their study that honeybees in the free flying condition can discriminate between an enormous number of chemical compounds with different carbon chain lengths and functional groups (Laska *et al.*, 1999). Hence, it was unclear whether the proportions of the different types of olfactory performers found in the cumulative assay

indeed reflect the scenario of the natural colonies or the result was specific to this assay. However, one can speculate that bees during the foraging on flowers do not learn the odor information in the same way as they did in the cumulative conditioning assay. So, probably, this assay did not represent the olfactory learning ability of the average individuals, however, contributed to the selection and characterization of the different performer classes.

Bees were trained in multiple conditioning and test trials in the cumulative conditioning assay and it was found from the performance history analysis that bees in the pooled population showed more response generalization between the pure concentrations of the CS+ and CS- stimuli and dilutions during the 2nd compared to the 1st phase of the assay. The high odor generalization was also manifested in the higher number of responses to the paraffin oil and filter paper during the 2nd compared to the 1st phase. The reasons for the increased generalization although were not fully understood but prolong conditioning and test protocol of the 1st phase possibly incorporated some sensitization components (due to hunger and sucrose arousal) in the odor responses of bees which contributed to the higher number of incorrect responses to the different CSs during the entire 2nd phase of the assay.

3.8 The next step

From behavior to gene expression

Correlating the learning behavior of bees with the patterns of gene expression is a long standing interest in neuroscience. Amongst the different model systems the fruit fly *Drosophila melanogaster* contributed substantially to the understanding of genetic regulation of the learning behavior. A whole set of fly mutants defective in the processes of olfactory learning and memory consolidation such as *radish, rutabaga, amnesiac, dunce* (Keene and Waddell 2007; Quinn *et al.,* 1979; Dudai *et al.,* 1976; Livingstone *et al.,* 1984; Heisenberg *et al.,* 1985; Folkers *et al.,* 1993) helped the neurobiologists to comprehend the connections between the complex behavior and gene function. However, unlike the *Drosophila* and other popular genetic models, honeybee has no available mutant defective in the olfactory learning and memory processes. The popular ways in

the honeybee to correlate behavior with the gene function are either to intervene with the function of the gene product with the pharmacological agents or to down-regulate the gene-expression level using the RNA-interference techniques. Additionally, the strategy of studying the gross-expression patterns of the genes in the individual neuropil or globally in the whole brain with reference to the specific behavior was also employed to study the genetic networks underlying the complex behavior (Whitfield *et al.,* 2002). These approaches are benefitted from the genome sequencing of the honeybee (Weinstock *et al.,* 2006) which made the task of assigning genes and the open reading frames easier. As the next and step forward after the cumulative olfactory conditioning assay, the possible genetic signatures of the learning and memory performances were investigated in the individual bees. The best and the poor cumulative performers were selected (as described previously) for this purpose and their gross gene expression patterns were studied in the mushroom body neuropil to find out the putative correlations between their behavioral performances with the expression patterns of the learning related genes. Possible conserved differences in the learning related gene expression patterns between these two types of cumulative scorers can be used to understand the underlying genetic or molecular basis of the differential learning behavior. The expression study was focused on the mushroom body neuropil since this particular brain region was previously reported both in honeybee and *Drosophila* to be involved in the processes of olfactory learning and memory (Howse 1974; Menzel *et al.,* 1974; Erber *et al.,* 1980; Menzel *et al.,* 1988; for reviews: Menzel 1983; Heisenberg 1989; Heisenberg 2003). Analysis of the gene expression data is currently ongoing hence; findings from the behavioral analysis were only discussed in this chapter. Apart from the possible genetic differences between the best and the poor performers the results of this study also have the potential to indicate the possible interesting candidate genes generally involve with the processes of learning and memory. The roles of these candidate genes can also be tested further.

3.9 Outlook

Connecting behavior with the gene expression pattern is a difficult task due to the multiple levels of information processing in between. However, more research in this alley is required to understand the genetic networks of the individual neuropiles as well as the connectome regulating the olfactory learning behavior of the individual bees. A previous study (Brandes and Menzel 1990) in the honeybee proposed the common learning mechanisms for the olfactory and the visual stimuli when the authors found bees from multiple colonies respectively good and bad in their olfactory learning performances, to perform similarly in the visual learning task. Here, in this chapter I reported a complex and time lapsing odor training protocol which was designed to investigate the individual's performances in place of the population. The same assay or any other complex form of olfactory learning protocol merged with the certain form of complex visual learning assay can be used to envisage the nature of the common behavioral machinery regulating the learning of these two sensory modalities in the individual honeybee. This will also enable us to find out the possible correlations between the behavioral performances of the different performer classes with their gene expression patterns to get a hand over the genetic components of the common machinery.

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Appendix-1

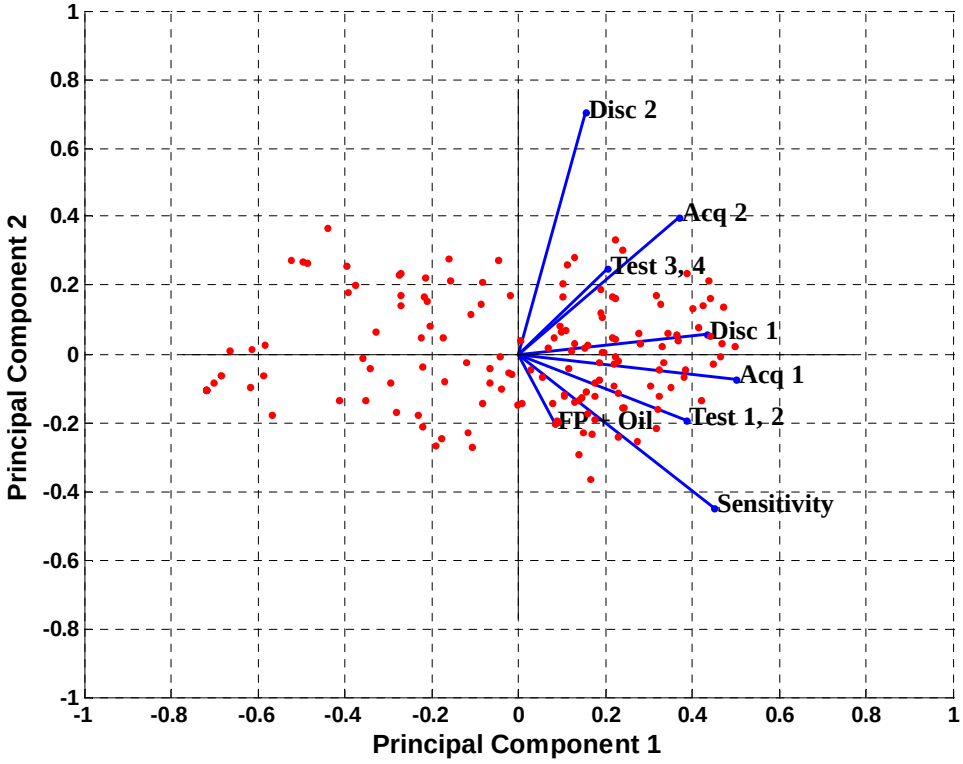

Fig. 10: Biplot showing the contributions of the different learning and memory related features to the variability explained by the first 2 principal components: The biplot represented the 1st principal component (PC) on the horizontal axis and the 2nd PC on the vertical axis. Performance scores in the 8-quantified learning and memory related features were used as the input in the PCA which were then transformed into multiple sets of 8 coefficients as represented by the different principal components. Each of the 8 features or variables was represented by the 8 different vectors with directions and lengths determining their relative contributions to the specific PC. The 1st PC (Principal component 1) had positive coefficients (meaning that all features contributed positively to the total variability explained by the 1st PC) for all of the 8 features (denoted with the blue lines) with no apparent cluster of bees (each red dot represented one animal) found in the principal component space, indicated the high variability in performance of the individual bees. However, unlike the 1st PC, the 2nd PC (Principal component 2) had the negative coefficients for the features such as the Acq1, T 1, 2, sensitivity and response to the FP and paraffin oil and positive coefficient values for the rest. The 2nd PC separated the honeybees with low scores in all of these four features and high scores for the others such as the Acq2, Disc1, Disc2 and T 3, 4 and *vice versa*. Importantly, nearly equal

number of bees were separated with the 2nd PC, meaning that majority of the bees which performed well during the first phase of the cumulative assay performed poorly during the 2nd phase and *vice versa*; explained the performance heterogeneity found in the pooled population (as showed previously in Fig. 9). These two PCs together explained nearly 70% of the total variability of the data.

Seasonal Effect

Seasonal effects on the olfactory learning and memory performances of the honeybees were reported previously (Blažytė-Čereškienė and Skirkevičius 2006; Hadar and Menzel 2010). Čereškienė and colleagues in their study reported that worker honeybees (*Apis mellifera*) achieved the highest learning speed during the time of autumn (September - November) and lowest during the spring (from March to July) in an olfactory PER paradigm with the odors extracted from the queen. In the cumulative conditioning assay all experiments were conducted between July (July – August: summer) and October (September – October: spring) 2010. To investigate the possible seasonal effect on the olfactory learning, first a month-wise analysis was performed where the data from all three colonies were pooled and the differences in scores in the behavioral features (e.g. the speed of odor learning, discriminability, sensitivity and the cumulative performance) along the individual months were analyzed. In most of the cases a significant decrease in the mean scores in the features was found for September (data not shown: Kurskal Wallis ANOVA and multiple comparisons of the mean) along with an increase found for the month of October. However, due to the smaller sample sizes of July and October no conclusions were drawn from this analysis. Colony-wise analysis during the individual months also suffered the same problem of weak sample size, hence, I pooled the scores in individual features and for the individual colony generated during the time of summer (data: July + August) and compared them with the pooled data of the autumn (data: September + October). Mann-Whitney U test was performed for the individual colony to compare the total scores for the rate of CS+ learning (adding the scores in Acq1 and Acq2), total scores for odor discriminability (adding the scores in the four features: Disc1 + Disc2 + T 1, 2 +T 3, 4), odor sensitivity and the cumulative performances between the time of summer and autumn. Mean scores in the different features did not show any significant difference for the two time points of the season except in one case; colony 73

showed the significantly higher mean value of odor discriminability during the summer time compared to the autumn (Mann-Whitney U Test; U = 202.5, Z = 2.263, p = 0.022, 2-sided p value). No significant difference in the mean scores in the same four features was found between the summer and autumn while the scores were pooled for all bees from the three backcrossed colonies (data not shown). However, an increase (not significant) in the total number of PER responses to any type of CS stimuli (overall responsiveness) was observed for the individual colonies during the autumn compared to the summer. It was concluded that honeybees used in the cumulative conditioning assay did not show any significant effect of seasonal variation on olfactory learning and memory.

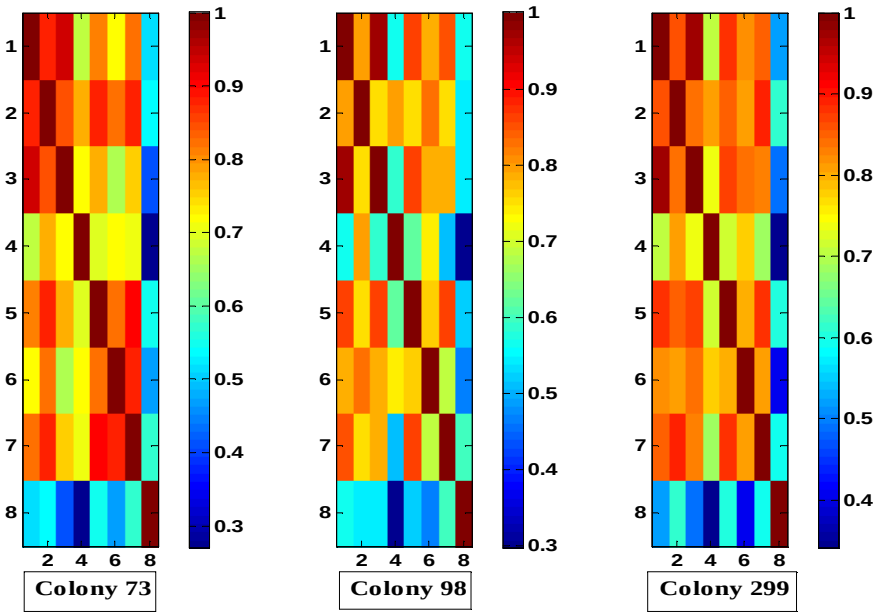

Fig. 18: Color coded correlation plot between the 8 features of the individual backcrossed colonies: The three sub-plots in the figure represented the color coded correlation plots between the 8 features for the three backcrossed colonies (colony 73, 98 and 299). Correlation values were found higher for the individual colonies compared to the pooled population of the honeybee (Fig. 17), and for all colonies the highest correlation was found between the Acq1 and the Disc1 like in Fig. 12, 15 and 16. Responses to the filter paper and paraffin oil showed the least correlations with the other features. Name of the individual colony was mentioned under each sub-plot.

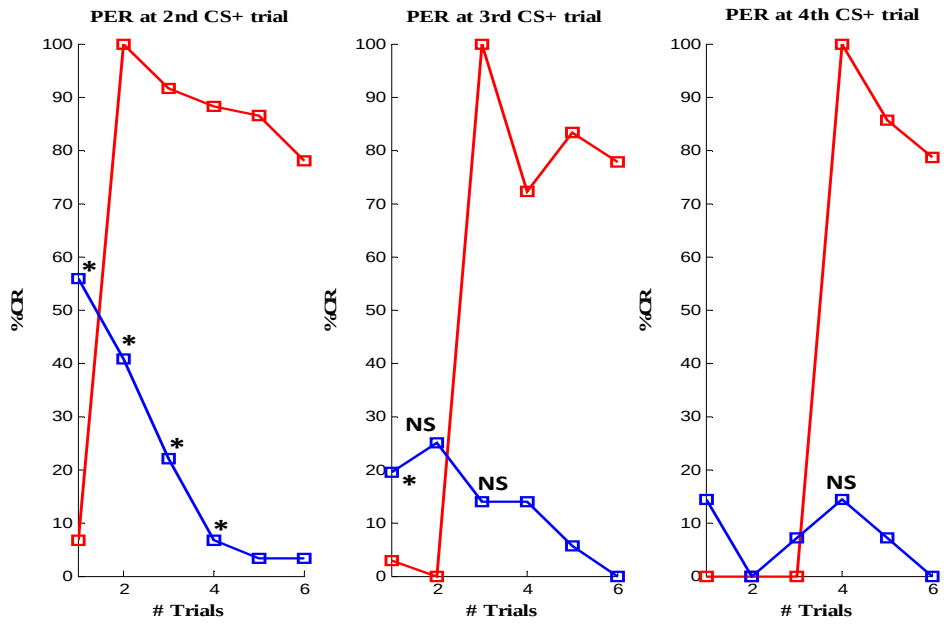

Fig. 25: Learning dynamics of the CS+ and CS- stimuli of the early and delayed responders of the CS+ stimuli: Learning dynamics of the rewarded (CS+; represented by red lines) and the unrewarded (CS-; represented by blue lines) odor stimuli were found to vary among the bees which started responding to the CS+ stimuli respectively from the 2nd, 3rd and 4th conditioning trials during the 1st differential conditioning. The x and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the CS stimuli. The selection points (selection criteria) of these bees were the 100% conditioned responses to the CS+ stimuli respectively during the 2nd, 3rd and 4th conditioning trials. Bees which started to respond (1st plot) from the 2nd CS+ trial (N = 59 bees) showed significantly higher initial responses to the CS- compared to the CS+ stimuli (significant difference was denoted with the 1st asterics on the blue line) possibly due to the strong effects of odor generalization and sucrose mediated arousal apart from the spontaneous responses. However, the fast learning of CS- stimuli led to the significant reduction in the CRs during the successive conditioning trials (denoted with the last 3 asterics on the blue line). The 2nd plot represented the learning dynamics of the bees which started responding to the CS+ from the 3rd trial (N = 36 bees). The learning of CS- stimuli in this category was found slower compared to the 2nd CS+ trial responders (non-significant change in the CRs form the 1st to 2nd and from the 2nd to 3rd CS- trial; denoted with the 'NS' on the blue line). However, these bees like the former type also showed significantly higher initial responses to the CS- compared to the CS+ during the 1st trial (denoted with the asterics on the blue line), due to the possible effects of odor generalization and sugar arousal. The CS- learning curves of the 2nd-trial and the 3rd-trial CS+ responders looked more similar respectively with the high and intermediate cumulative scorers (Fig. 23). The 3rd plot represented the learning dynamics of the bees started responding to the CS+ stimuli from the 4th conditioning trial (N = 14 bees). The slow rate and instability in the CS- learning of these bees reflected the scenario of the low Acq1 scorers (Fig. 24). However these 3 types of CS+ responders had overlapping bees with the three cumulative or Acq1 scorer categories.

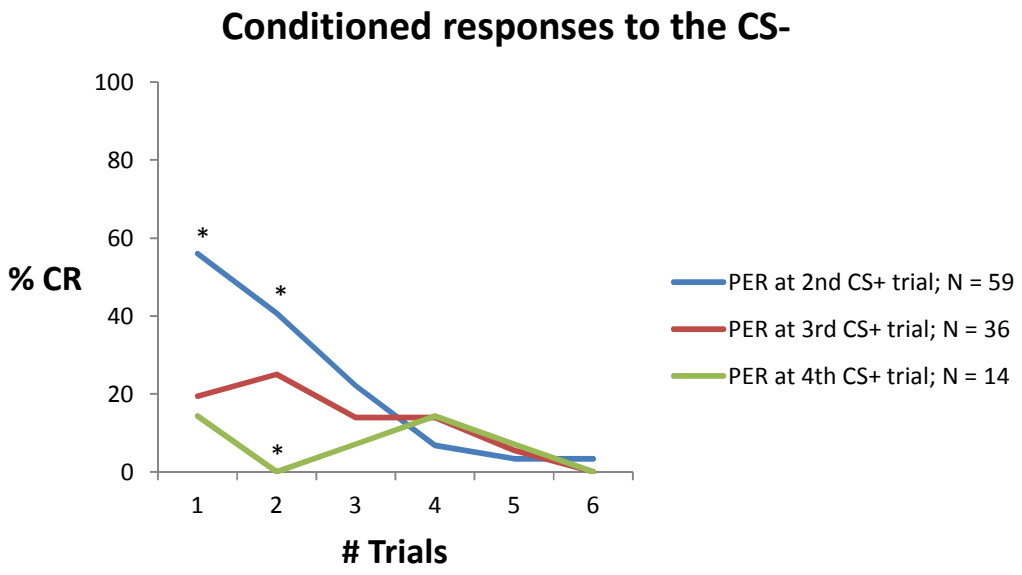

Fig. 26: Comparison of the conditioned responses to the CS- stimuli between the early and delayed responders of the CS+ stimuli: This figure was an extension of Fig. 25, highlighted the differences between the conditioned responses to the CS- stimuli of the three groups of bees which started responding to the CS+ stimuli respectively from the 2nd, 3rd and 4th conditioning trials during the 1st differential conditioning. The x and y axes respectively represented the number of conditioning trials and the percent conditioned responses (CRs) to the CS- stimuli. All three classes of bees learned the CS- stimuli as evident from their leaning curves (blue, dark brown and the green lines respectively represented the bees started responding from the 2nd, 3rd and 4th CS+ trial). However, the early CS+ responders (2nd trial) showed significantly higher responses to the CS- during the 1st and 2nd conditioning trials compared to the 3rd and 4th CS+ trial responders (significant differences were denoted with 2 asterics on the blue line). Bees which started responding from the 3rd CS+ trial showed significantly higher responses compared to the 4th trial responders only during the 2nd CS- conditioning trial (denoted with the single asterics on the green line). 'N' represented the number of honeybees found in the three selected categories.

Honeybees which started responding early (from the 2nd trial; they were called as 2nd trial responders) to the CS+ stimuli showed significantly higher responses (Fig. 25) to the CS- compared to the CS+ during the 1st presentations of these two stimuli (RM-ANOVA found significant stimulus ($F_{1, 116}$ = 248.84, p = 0.00), trial ($F_{5, 580}$ = 22.85, p = 0.00) and stimulus × trial effects ($F_{5, 580}$ = 80.73, p = 0.0000); followed by the Fisher LSD post hoc test between the 1st CS+ and CS- trial p = 0.000000). Strong effects of odor generalization and sucrose mediated arousal (due to the preceding CS+ trial) along with the component of spontaneous responses led to the high responses to the 1st presentation

of CS- stimuli in these bees. In fact, like the high scorers in Acq1 and the best cumulative scorers this group of bees showed significantly higher CS- responses during the 1st trial compared to the other two groups (Fig. 26) of delayed CS+ responders (RM-ANOVA showed significant stimulus ($F_{5, 530}$ = 9.16, p = 0.000000), group ($F_{2, 106}$ = 4.47, p = 0.01) and stimulus × group effects ($F_{10, 530}$ = 4.62, p = 0.000002); followed by Fisher LSD post hoc test between the 1st CS- trials of the 2nd and 3rd trial responders p = 0.000001, between the 2nd CS- trials of the 2nd and 3rd trial responders p = 0.03, between the 1st CS- trials of the 2nd and 4th trial responders p = 0.00005 and between the 2nd CS- trials of the 2nd and 4th trial responders p = 0.00007). Hence, the strongest effects of generalization and arousal were found in the early CS+ responders amongst the three selected categories. However, these bees showed the fast learning of CS- stimuli as the conditioned responses were found to decrease significantly during the successive CS- trials after the 1st one, and overshadowed the effects of generalization and arousal (Fisher LSD post hoc test: between the 1st and 2nd CS- trial p = 0.007, between the 2nd and 3rd CS- trial p = 0.001, between the 3rd and 4th CS- trial p = 0.007). Conditioned responses to the CS+ stimuli however were not found different between the three groups of bees. These results showed that the early CS+ learners also learned the CS- stimuli early and concomitantly with the CS+. Hence, it was concluded that bees which fulfilled the criterion of the early learning of rewarded stimuli already showed the similar dynamics of CS- learning as the high cumulative and high Acq1 scorers.

Amongst the delayed responders, bees which started to respond to the CS+ stimuli from the 3rd conditioning trial (they were called as 3rd trial responders) also showed significantly higher responses to the CS- during the 1st trial (Fig. 25) compared to the CS+ stimuli (RM-ANOVA showed significant stimulus ($F_{1, 70}$ =112.82, p = 0.000000), trial ($F_{5, 350}$ = 29.11, p = 0.000000) and stimulus × trial effects ($F_{5, 350}$ = 53.53, p = 0.000000); followed by Fisher LDS post hoc test: between the 1st CS+ and CS- trial p = 0.02), which probably involved the initial effects of odor generalization and arousal apart from the spontaneous responses. These bees however, behaved like the intermediate scorers in Acq1 or cumulative performances since they learned the CS- stimuli rather gradually with no significant differences found between the conditioned responses along the successive CS- conditioning trials (results of the statistics were not shown). Hence,

they learned the CS+ stimuli faster than the CS-. Similar faster CS+ learning was observed for the bees started responding to the CS+ stimuli from the 4th conditioning trial (they were called as 4th trial responders). These bees showed lower responses to the CS- stimuli (Fig. 25) compared to the other two categories during the 2nd CS- conditioning trial (RM-ANOVA followed by the Fisher LSD post hoc test between the 2nd CS- trials of the 3rd and 4th trial responders $p = 0.02$) along with the overall low and irregular responses to the CS- stimuli (Fig. 26). The learning dynamics of the CS- odors in these bees mimicked the low Acq1 scorers.

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Chapter-4

Olfactory adaptation changes the glomerular response strengths and representations of odors in the honeybee antennal lobe

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4.1 Abstract

Odors are coded as the specific combinatorial activity patterns of glomeruli in honeybee antennal lobe (AL). Based on this knowledge I investigated the possible changes in the response strength and representation pattern of odors in the AL glomeruli when they were adapted with the background odor stimulus. Olfactory adaptation either with the odor mixture of unknown quantitative complexity such as the odor of honeybee colony or with the synthetic mixture of known composition were found to increase the average response strength of the AL glomeruli to the test odors during adaptation compared to the un-adapted condition. Analysis of the individual glomeruli in most of the cases also showed the adaptation induced increase in the strength of odor responses although, we found glomeruli which showed the adaptation induced decrease in the odor response strength. Amongst the different test odors, only three showed the common pattern of change in the glomerular response strength with both adaptation stimuli; floral odor 1-hexanol and the sting pheromone odor isoamyl acetate showed the increase and 1-octanal showed the decrease in glomerular response strength during the adaptation. However, for the test odors common increase in distances between their glomerular representation patterns was found due to the introduction of background adaptation stimuli compared to their removal. Adaptation-induced changes in the glomerular response strength to the test odors possibly enhanced the specific forms of odor discrimination in the glomerular coding space which was manifested in the increased Euclidean distances. Additionally, these changes were found to persist even after 5 min of removal of the adapting stimuli.

Author's contribution: This is a manuscript which will be submitted for publication in an international peer reviewed journal. Please refer to page number iii of this dissertation for details about the author's contribution.

4.2 Introduction

Antennal lobe (AL) of the invertebrate brain is the structural and functional analogue of the vertebrate olfactory bulb (OB) and is the first processing station of the olfactory information in the central brain area (Boeckh *et al.,* 1990). Antennal lobe, like the olfactory bulb has the genetically determined convergence of receptor neuron afferents in the olfactory glomeruli which are the anatomical and functional units of this neuropil (Mombaerts *et al.,* 1996; Mori *et al.,* 1999; Gao *et al.,* 2000; Galizia *et al.,* 1999b; Vosshall *et al.,* 2000). In the European honeybee *Apis mellifera* about 60,000 receptor neurons of the antennal sensilla (Esslen and Kaissling 1976) convey the input olfactory information through the four antennal nerve tracts (T1-T4: Suzuki 1975) into the AL. This initial package of information is then processed within the 165 olfactory glomeruli where the axon terminals of the receptor neurons form the densely packed synaptic connections (Gascuel and Masson 1991) with the 4000 local interneurons (LNs: Witthöft 1967) and the 800 projection neurons (PNs: Bicker 1999). The typical construction of the antennal lobe like the olfactory bulb permits the processing of input information from the higher number of receptor neurons, which then is transferred by the fewer number of output projection neurons (PNs) to the higher order neuropiles such as mushroom body and lateral horn for further processing. Receptor neurons expressing the same receptor molecule in the insect AL and in the vertebrate OB either have the broader response specificity to odors (Honeybee: Sachse *et al.,* 1999; *Drosophila*: Vosshall *et al.,* 1999; Zebrafish: Friedrich and Korsching 1997; Salamander: Cinelli *et al.,* 1995; Rat: Duchamp-Viret *et al.,* 1999; Both AL and OB: Boeckh *et al.,* 1990) and / or they innervate the distal glomeruli (except the olfactory bulb in rat: Uchida *et al.,* 2000) which leads to the specific spatio-temporal activity pattern in the glomeruli to the presentation of odors as visualized in the optical measurements of calcium activities (Joerges *et al.,* 1997; Sachse and Galizia 2002; Friedrich and Korsching 1998; Cinelli *et al.,* 1995; Meister and Bonhoeffer 2001). The specific combinations of the glomerular ensemble activated by the odors constitute the spatio-temporal component of the identity code of odors in these neuropiles. Electrophysiological and optical recordings in honeybee AL until now enriched our understanding of the spatio-temporal coding schemes of this neuropil at the input and output processing levels (Sachse *et al.,* 1999; Sachse and Galizia

2002; Galizia and Kimmerle 2004). Intracellular recording from the lateral-ACT and the median-ACT (ACT: antennocerebral tract) tracts of the projection neurons in different studies (Müller *et al.,* 2002; Krofczik *et al.,* 2009) although showed differences in results for parameters such as the response latency and temporal pattern of responses however, confirmed the fact that odor identity in the population of PNs is coded by the specific spatio-temporal patterns of firing and response latencies. Additionally, it was reported that networks of the local interneurons, inhibiting the PN-circuits play the important role to shape up the odor representation patterns both in space and time (Sachse and Galizia 2002; Krofczik *et al.,* 2009).

Subsequent recordings of the electrical and optical signals in honeybee AL showed the correlated increase and decrease in spiking frequencies and intracellular calcium activities (concentrations) in the uniglomerular projection neurons (PNs) and some of the heterogeneous local interneurons (Galizia and Kimmerle 2004). However, calcium responses were often found to outlast the electrical recordings. Resembling the spike activities of neurons, these long lasting calcium responses in the dendrites and especially in the cell bodies of PNs indicated that intracellular calcium concentration of the postsynaptic PNs is the function of local membrane potential. Additionally, this suggested the possible important roles of calcium ions for the induction and / or maintenance of the long-term cellular plasticities in the AL network. In other words, measurements of calcium responses can be used to understand the different forms of plasticity in the AL neurons.

In fact, early report by Till Faber and colleagues already showed (Faber *et al.,* 1999) the changes in calcium responses of the AL glomeruli as a result of differential olfactory conditioning when they recorded the calcium signals form the entire neuronal network of the AL. Response strength of the AL network showed the increase to the presentation of the rewarded or CS+ odor after the conditioning compared to before. However; the activity of the AL network remained same for the unrewarded or CS- odor between the conditions of before and after conditioning. *In vivo* calcium imaging of the projection neuron of honeybee AL later on (Rath *et al.,* 2011) also reported the similar network plasticities as a result of the differential olfactory conditioning. These results altogether

contributed largely to the understanding of the phenomena of population odor coding and olfactory learning in honeybee antennal lobe.

In contrast to the processes of olfactory learning and discrimination, little is known about the adaptation-related physiological plasticities in honeybee AL. Adaptation is an important memory process or form of plasticity that reduces the responses of the neural system to the static and possibly irrelevant background stimuli (sensory) due to prolong exposure. Adaptations of the visual (Honeybee: Kindermann and Hertel 1986; *Formica polyctena*: Menzel and Knaut 1973; Arctiid moth: Grünewald and Wunderer 1996) and olfactory stimuli (*Drosophila*: Störtkuhl *et al.,* 1999; Silkworm moth: Kaissling *et al.,* 1987; Housefly: Kelling *et al.,* 2002) were studied before in the different insect species. Olfactory adaptation although reduced the behavioral responses of the adult *Drosophila* flies to the adapting odor but increased the response amplitude of the olfactory receptor neurons in the houseflies to the lower dilutions of the test odors. Similar to the *Drosophila*, reduction in the chemotaxis behavior to the pre-exposed (adapting stimulus) odor stimuli was also found in the model system of *Caenorhabditis elegans* (Colbert and Bargmann 1995). Apart from the behavioral evidences results of the neurophysiological studies implicated an important role of the calcium influx in the neurons to impart the state of adaptation. In the vertebrate olfactory receptor neurons, a form of adaptation with the time scale of minutes was identified which was mediated by the cGMP messenger (Zufall and Leinders-Zufall 1997). It was proposed that cGMP mediated activation and opening of the cyclic nucleotide-gated channels resulted in the influx of the calcium ions which in turn nucleated the feedback regulatory circuits to induce the adaptation process.

Single unit recordings of the rat olfactory bulb (OB) neurons showed that neurons differ in their excitability when they were adapted with the brief (2 sec) pulses of the adaptation odor (Mair 1982). Some OB-neurons were found to enhance and some suppressed their responses (spikes per second) in the adaptation state which were found to be independent of the concentration and identity of the adaptation odor. The strict change in the patterns of neuronal firing in the adaptation state was further manifested when a second odor stimulus was tested after adaptation with the first odor. It was found that neurons which showed the facilitative self-adaptation (increased firing rate to the adaptation odor) never showed any decrease in responses to the second odor in the adapted state (cross-

adaptation) compared to the un-adapted state. On the other hand, neurons with the suppressive self-adaptation never showed any facilitative cross-adaptation responses to the second test odor. The identities of the recorded neurons as well as the reasons behind the excitatory and inhibitory adaptive responses of the bulb neurons were not investigated in this study. However, modulations in spiking responses due to short-term olfactory adaptation were believed to be mediated by the intrinsic properties of the bulb neurons. The potential influences of the receptor neurons were omitted since, olfactory receptor neurons in the tiger salamander were found to show the response suppression during both self and cross-adaptation (Baylin and Moulton 1979). However, receptor neurons in frog were found to resist the process of adaptation robustly as the complete adaptation recovery for the test odors was found to happen within 25 sec even if the adaptation was induced with the higher concentrations of the stimulus (Van Boxtel and Köster 1978). Hence, olfactory adaptation did not decrease the responses of the receptor neurons or the second order olfactory bulb neurons monotonically.

In the insect models, Kaissling and colleagues reported that olfactory adaptation of receptor neurons of the antennal sensilla reduced the amplitudes of the receptor potential to the different concentrations of the test stimuli (Silkworm moth *Antheraea polyphemus*: Kaissling *et al.,* 1987). Effects of the self-adaptation however, were found stronger than the cross-adaptation responses. In all these experiments the common procedure of brief (in seconds) and multiple-exposure of the neuronal populations to the adapting stimulus was used to achieve the olfactory adaptation. Similar protocols were popularly used to study the adaptive responses of neurons in shorter time scale. A common feature of excitatory spiking neurons of the different processing pathways namely the ‘spike frequency adaptation’ also represents the adaptation process within the time scale of milliseconds and seconds (Bear *et al.,* 2006; Benda and Herz 2003; Fuhrmann *et al.,* 2002).

In comparison, studies employed constant and prolong (in the time scale of minute) exposure protocols to achieve the neuronal adaptation and further investigation of their odor response properties were limited in number. Best and Wilson in the anesthetized rats used the 50 sec constant exposure protocol to study the olfactory adaptation in pyramidal cells of the piriform cortex. Fast synaptic depression of the input signals of the

mitral/tufted cells to the pyramidal cells (PRCs) was found to mediate the quick (within 50 sec) response adaptation of the PRCs to the static background odor (Best and Wilson 2004). Longer exposure protocol (single unit recordings) employed by Chaput and colleagues showed the gradual reduction in responses (decreased rate of firing) of the projection neurons in the awaked rabbit when the animals were exposed constantly for 1 hour to the background odor stimulus (Chaput and Panhuber 1982). In the housefly *Musca domestica* olfactory receptor neurons were adapted with the 15 min constant exposure to the background stimuli (using the synthetic odors and the natural habitat-odor of chicken manure) by Kelling and colleagues (Kelling *et al.,* 2002). In this study the long-term adaptation achieved with the higher but not with the lower concentrations of the synthetic odor stimuli were found to influence the odor sensitivity of the receptor neurons. Adaptation increased the responses of the receptors (measured in the electro-antennogram recordings) to the lower concentrations of the test odor stimuli and decreased the EAG responses to the higher concentrations. Among the different cell types of the antennae stronger effects of adaptation was found in the receptor cells responded with the tonic increase in firing frequency compared to the phasic responders. Hence, in the housefly long-term adaptation was found to affect the responses of the receptor cell types differentially and non-monotonically for the different intensities of the test odor stimuli.

In honeybee, anatomical traces of the adaptive changes were reported in the mushroom body lip region by Krofczik and colleagues when they investigated the number and volume of the microglomerular structures constituted by the connections between the pre-synaptic boutons of the projection neurons and the post-synaptic dendritic spines of the Kenyon cells (Krofczik *et al.,* 2008). They found that manipulations of social behavior and sensory experience led to the decrease in number of the microglomerular structures but increase in the volume of the boutons. Such types of anatomical change, signifying the specific forms of plasticity probably are also operating in the other parts of the brain in response to the process of sensory adaptation. Apart from the anatomical evidence, behavioral experiments confirmed that honeybees were able to learn odor stimulus (isoamyl acetate) in the PER conditioning paradigm when they were adapted with the continuous background of the colony odor (see ‘Results’ in chapter-2 of this dissertation).

However, learning in the adapted state was found compromised compared to the un-adapted state. The signal processing of the olfactory neurons for both the conditioned and adaptation stimuli in honeybee brain was not investigated in this dissertation; neither there was any previous report. However, understanding the phenomena of odor perception and learning in the adapted state of the olfactory system is extremely important since animals e.g. insects in general recognize or learn the biologically meaningful odor stimuli while encountering the constant odorous backgrounds which act as the adaptation stimuli. Hence, filtering the information of the static background odor from the incoming target odor cues is an essential task of the olfactory system. The process of adaptation in this regard possibly plays an important role by reducing the bee's responses to the unchanging background and enhancing the discrimination between the target and the background (untested in insect). In honeybee, much information is available on the glomerular odor coding (spatio-temporal) in the antennal lobe but the potential effects of olfactory adaptation on the glomerular odor coding are least known.

4.3 Aim of the investigation

The goal was to investigate the possible changes in the odor response strength of the antennal lobe glomeruli induced by the long-term adaptation with the constant background of odor stimulus. Euclidean distances between the glomerular responses to the odors were quantified during the conditions of before, during and after adaptation. These linear distances were used to measure the possible changes in the glomerular representation patterns of the odors due to olfactory adaptation. We measured the possible changes in odor response strengths and representation patterns in the glomeruli to confirm the change in odor coding of the antennal lobe neuropil due to olfactory adaptation. To achieve the goal, we measured the odor evoked calcium responses of the l-ACT subset of projection neurons innervating the surface glomeruli of the AL. Responses of the few surface glomeruli innervated by the m-ACT PN tract were also recorded due to the common backfilling of the calcium sensor dye. Two different odor-mixtures viz. the odor of honeybee colony (mixture of odors with unknown complexity) and the equal volume mixture of four pure odors (odor mixture of known complexity) were used for the adaptation of AL glomeruli. Honeybees were exposed constantly for ~ 20 min to the

background adaptation stimulus to achieve the glomerular adaptation. Responses to the set of test odors were measured in absence (before), in presence (during) and after the withdrawal (after) of the background adaptation stimulus to understand the potential effects of adaptation and its removal on the glomerular odor responses with respect to the initial condition of no-adaptation (before).

4.4 Materials and methods

4.4.1 Preparation of honeybees for the backfilling of projection neurons

Honeybee (*Apis mellifera*) foragers were used for the calcium imaging experiments. One whole round of experiment consumed two days to complete; on the first day the captured foragers were anesthetized on ice and fixed in the Plexiglas recording chambers using the low temperature melting wax as described previously (Joerges *et al.,* 1997; Sachse and Galizia 2002; Szyszka *et al.,* 2005; Hähnel *et al.,* 2009). Bees were fed with 2-3 drops of the 30% (W/V) sucrose solution and kept inside a Styrofoam box which was adequately moistened with the water-soaked towels. Honeybees were prepared after 2-3 hours for the intracellular backfilling of the projection neurons (PNs) running through the lateral and the median antenno-protocerebral tracts (l-ACT and m-ACT projection neurons). To start with, a rectangular piece of cuticle of the head capsule between the eyes was cut-opened and then the glands and trachea were removed (with forceps) carefully from the right half of the brain to make the antennal lobe (AL) and the mushroom body (MB) neuropiles clearly visible. For backfilling (injection), a glass capillary was pulled with the approximate tip diameter of 10 µm. At the tip of the capillary tube, the dye mixture was coated for injection, which consisted of the calcium sensor dye Fura-2 dextran (10000 MW, Molecular Probes, Eugene, OR, USA) and the lysine fixable dye tetramethylrhodamine-dextran (10000 MW, Molecular Probes, Eugene, OR, USA) dissolved in the distilled water. During experiments we only injected the calcium sensitive dye in the right half of the brain and imaged the right-AL. All injections of the dye mixture were performed in the area between the lateral and medial calyces of the MB as shown in Fig.1A. During the injection the entire body of the bee was pressed against the Plexiglas walls with a small piece of sponge to stop the brain movement and pumping

of haemolymph inside the brain. This procedure was helpful for performing better injections of the sensor dye at the correct location as well as allowed the dye to diffuse inside the brain for longer time (rather pumped out sooner). After the injection the piece of the cuticle was placed back on top of the head capsule and sealed with the *n*-eicosan wax (Sigma). Bees were fed until satiation thereafter with the 30% sucrose solution, and kept inside the same Styrofoam box for overnight at 17-20^{o}C. On the second day all bees were fed again in the morning with 2-3 drops of the 30% sucrose solution, at least 45 min to 1 hour before the imaging-experiment. Then the legs, tip of abdomen, mandibles and the proboscis of the bee were fixed with the same low melting wax against the Plexiglas walls and the whole body was pressed like before with the piece of sponge and fixed it with a sticky tape for complete immobilization. This step was crucial as it nearly stopped the brain movement during the calcium recording. In the next step antennae were fixed with the *n*-eicosan on top of the head capsule which made the bee ready for the final manipulation. In the final phase, the piece of the cuticle was opened again to expose the antennal lobe, all gaps in the recording chamber surrounding the bee were closed with the Vaseline (local drugstore), the chamber was bathed with the bee ringer solution (composition in mM: 130 NaCl, 7 $CaCl_2$, 6 KCl, 2 $MgCl_2$, 160 sucrose, 25 glucose and 10 HEPES, pH 6.7, 500 mosmol; Yamagata N *et al.*, 2009) and the bee was taken to the microscope for the *in vivo* calcium imaging. Only bees with the Fura-2 dextran staining over all the surface glomeruli of the antennal lobe (Fig. 1B) were used for the imaging.

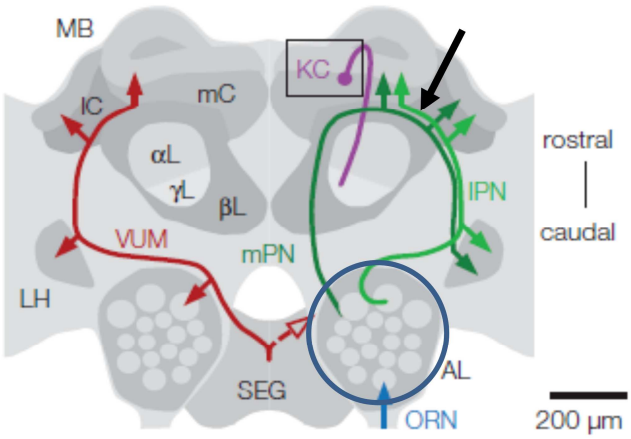


Fig. 1A: olfactory system of the hoeny bee and the site of Fura-2 dye injection for backfilling of the projection neurons: This figure depicted the frontal view of honeybee olfactory system (modified from Szyszka *et al.,* 08) with the three neuronal populations processing the olfactory information viz. the receptor neurons (ORNs; blue arrow), projection neurons (PNs; green tracts) and the mushroom body (MB) intrinsic Kenyon cells (KCs; magenta) were shown. The black arrow indicated the area between the lateral and the median calyces of the MB where the calcium sensor Fura-2 was injected for intracellular backfilling of the PNs. Calcium signals of the PNs to the odor stimuli were recorded from their dendritic branches arborizing in the antennal lobe (AL) neuropil. AL glomeruli innervated by the lateral-antennocerebral tract (l-ACT) of the PNs were majorly imaged in this study as this PN subpopulation arborizes exclusively on the AL surface (ventro-rostral; oval shaped area with blue boundary which was imaged). Additionally, few glomeruli innervated by the median antennocerebral tract (m-ACT) of the PNs were also imaged. The reward sensing VUM neuron (red) also shown here, connecting the sub-esophageal ganglion with the AL, MB and the lateral horn of the brain.

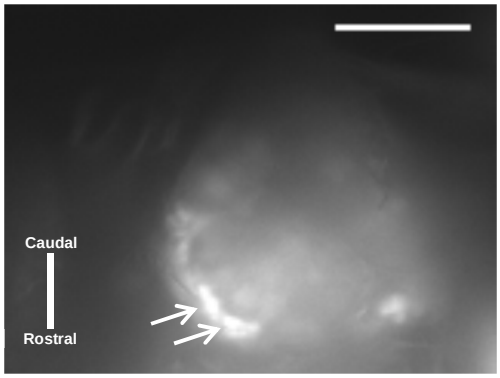


Fig. 1B: Fura-2 dextran fluorescence image of the ventro-rostral glomeruli of the right antennal lobe captured with the 380 nm wavelength: Antennal lobe (AL) imaging was performed with bees of this kind where all the surface glomeruli (scale bar 100 µm) were stained with the calcium sensor dye Fura-2. Glomerular boundaries in this picture were also visible in some of the cases (blob-like structure). The brighter areas (indicated by the white arrows) at the periphery of the AL represented the cell bodies of the projection neurons with the saturated pixel intensities.

4.4.2 Experimental protocol

Two sets of experiments were performed with the same protocol but using the different adaptation stimuli. In one group, bees were adapted with the odor mixture of honeybee colony and in the other group the adaptation was achieved with the mixture of four pure odors. After the dye injection during the day-1, bees were kept overnight and on the second day AL imaging was performed. The sensor dye was allowed to diffuse for 17 –

18 hours between the time of injection (in the axons) and imaging of the AL glomeruli. For both experiments, odor responses of the glomeruli were measured during the three different conditions (Fig. 1C) or phases. During the first condition calcium responses of the un-adapted bees to the set of test odors were measured with the laboratory background. This was followed by the phase of adaptation, where bees were continuously exposed to the background odor stimulus for ~ 20 min to achieve the olfactory adaptation of the AL glomeruli. Calcium responses of the glomeruli evoked by the same set of test odors were recorded again during this condition in presence of the background adaptation stimulus (second phase). In the third and last stage of the experiment, the adaptation stimulus was switched off and bees exposed to the laboratory background were allowed to de-adapt or recover from the state of adaptation for 5 min. After this time of de-adaptation, odor evoked responses of the AL glomeruli were again measured. Hence, one full experiment was comprised of the recorded calcium signals from the AL glomeruli to the set of test odors during the conditions of before, during and after adaptation (Fig.1C). A set of eight pure odors viz. 1-hexanol (1-6ol), 1-nonanol (1-9ol), Isoamyl acetate (IAA), geraniol (Ger), 1-octanal (1-8al), 2-heptanone (2-7on), linalool (Lina) (all purchased from Sigma Aldrich) and 1-octanol (1-8ol; Roth Gmbh) used in the experiments were always presented to the bees with this specific sequence; 1-6ol, 1-9ol, IAA, geraniol, 1-8ol, 2-7on, linalool and 1-8al. Odors were delivered through the 150 mm long Pasteur pipette connected with the odor delivery channels of the custom built olfactometer controlled by the solenoid valves (Galizia *et al.,* 1997; Yamagata *et al.,* 2009) operating (switching on and off) through the programs written in the image acquisition software, Till Vision (Till photonics, Germany). An amount of 10 µl of pure odor soaked on a piece of filter paper (1 cm^2) was used as the stimulus source when the odor-evoked responses were measured. The constant air flow of the olfactometer was kept at 1 liter with every time one of the odor channels were delivering 100 ml. of the odor containing air into the system, without changing the total amount of air-delivered to the bees.

Adaptation with the colony odor

The odor of a functional honeybee colony (a 4-frame colony; length, height and inner width of 52, 32.5 and 28 cm) was used for adaptation during this experiment. A round shaped hole was made at the roof-top of the colony which was covered with a piece of nylon-net to restrain the bees from flying away. During the experiment, the entrance of the colony was enclosed with a piece of tape and a metal exhaust pipe (length 110 cm) fitted on top of the round hole and attached with an exhaust fan was used for sucking the air from the colony and delivered to the bees.

Adaptation with the mixture of four pure odors (synthetic odor mixture)

An equal volume mixture (1:1:1:1 V/V) of four pure odors viz. 1-hexanol, 1-nonanol, 2-octanone (Aldrich) and limonene (FERAK-Berlin) was used as the adapting stimulus in this experiment. Two out of the four odors (1-hexanol and 1-nonanol) were common to the original bouquet of eight test odors. An amount of 6 ml of the odor mixture kept in a glass petri dish and placed inside a Styrofoam box (28.5 cm^2; height 23.5 cm) with a hole made on top it was used during the experiment. Again a similar arrangement of metal pipe fitted (36 cm long) with an exhaust fan was used to deliver the odor mixture to the bees to achieve olfactory adaptation.

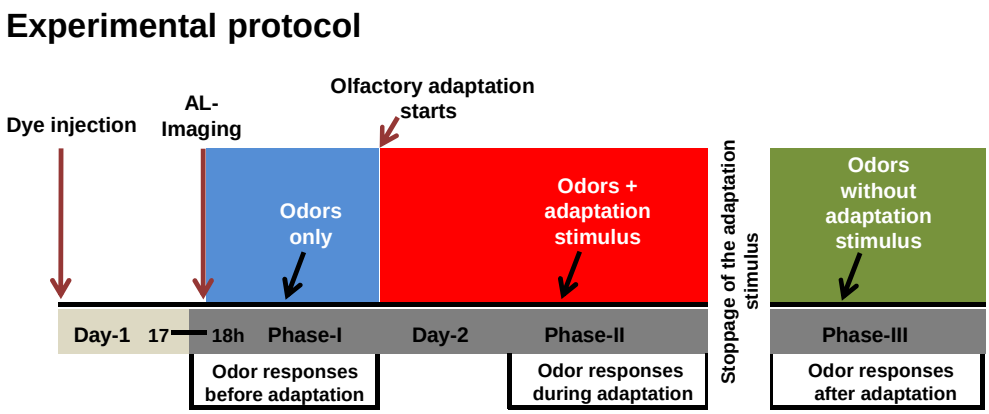


Fig. 1C: Common experimental protocol of the adaptation experiments: Honeybees were injected with the sensor dye (indicated by the dark red arrow) on day-1 (light grey area of the horizontal stripe) and were kept overnight (17 – 18 hours) before the AL imaging (the second dark red arrow indicated the time point of imaging) was performed on the next day (Day-2; dark grey area of the horizontal stripe). Imaging of the odor responses of the AL glomeruli had three

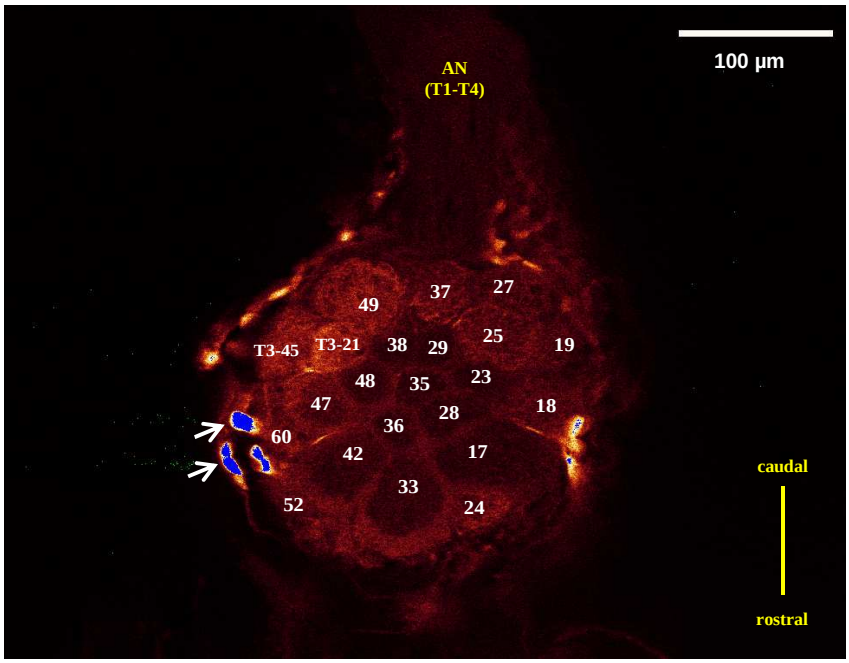
different phases which were represented in the figure with three different colors. During the first condition or phase-I ('before' adaptation; blue color zone) odor responses of the bees were measured under the background condition of the laboratory. This was followed by phase-II, where ('during' adaptation; red color area) bees were first exposed with the background odor stimulus for ~ 20 min to achieve the adaptation of the glomeruli. The start (switch on) of the adaptation background was denoted with the dark red arrow at the boundary of the blue and red colored zones. Glomerular responses to the same set of 8 test odors were measured again during the adaptation keeping the background odor 'on'. The end of this phase was followed by the stoppage of the adaptation background as represented by the vertical white stripe, which lasted for 5 min for the adaptation recovery of bees. The last condition or phase-III ('after' adaptation; green color zone) was commenced then with the recording of the PN's responses again to the same set of test odors without the adaptation background.

Calcium imaging of the antennal lobe glomeruli

Calcium measurements of the PN dendrites innervating the AL glomeruli were performed with the constant temperature of 25^{o}C using the imaging set-up of Till photonics mounted on an upright fluorescence microscope (Zeiss Axioskop, Germany). Ratiometric imaging of the Fura-2 was performed with the alternate measurements taken with the excitation wavelengths of 340 and 380 nm. Images during the odor stimulation were captured with the frame rate of 5 Hz for 10 sec (total of 50 image frames were captured: odor measurement or odor movie) with the interstimulus interval of 1 min. Measurements of the calcium signals started 2.8 sec before the odor onset, continued during the odor stimulation for the next 3 sec and for the last 4.2 sec after the odor offset. However, signal recording during the adaptation was performed with a 10 fold slower frame rate (capturing rate of the CCD camera) of 0.5 Hz for longer period of time (100 sec). Multiple movies (measurements) were taken during the time of adaptation with the inter-recording interval of 2 min for ~ 20 min. The first adaptation-movie (for both adaptation experiments) only had the images 20 sec before the onset of the adaptation stimulus, but for the remaining movies the background adaptation stimulus was never switched off. Images were acquired with the 20x water-dip objective of NA 0.95 (Olympus), the 410-nm dichroic mirror, and the long pass 440 nm filter arranged with the Till Imago CCD camera (640 × 480 pixels; 4 × binning on chip to 160 × 120) which allowed to achieve the spatial resolution of 4.53 μm.

4.4.3 Confocal Microscopy to check for the staining of the PNs and glomerular anatomy

After the measurement of calcium activity, the heads were collected and immersed in the 4% paraformaldehyde solution for overnight at 4^{o}C and then the brains were taken out of the head capsules for further processing. Brains were thoroughly rinsed in the PBS buffer and dehydrated with the increasing concentrations of ethanol (50%, 70%, 90%, 99% and

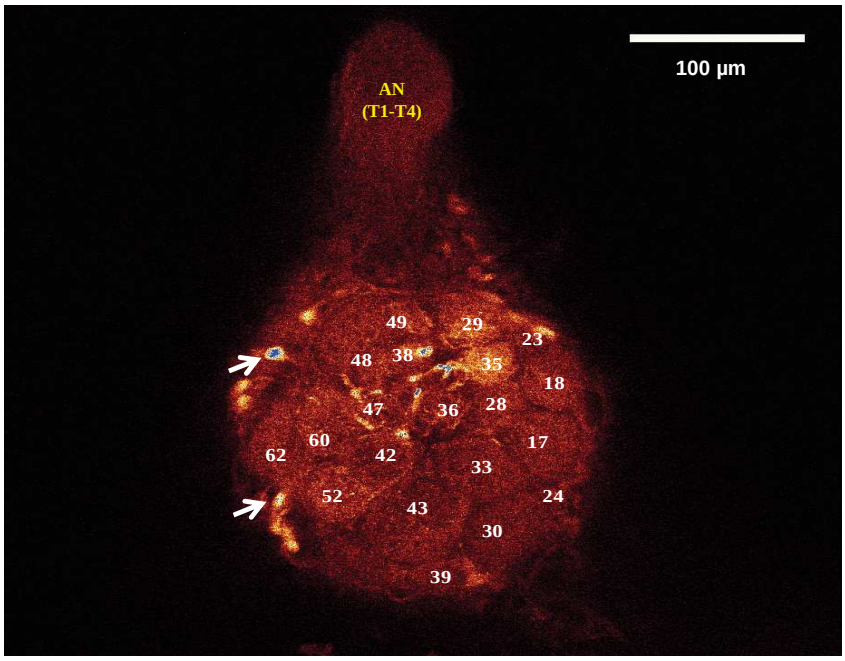


D-1

100%). The dehydrated brains were then cleared with the methyl salicylate treatment. The cleared brains were placed on the glass slides, immersed in the methyl salicylate solution and taken to the confocal laser-scanning microscope (Leica TCS SP2; Leica, Wetzlar, Germany) for capturing images (Fig. 1D). Brains were excited with the wavelength of 543 nm of the Green HeNe laser and scanned through the 20x oil objective with the NA of 0.70 (Olympus, Tokyo, Japan) to check for the staining of the projection neurons and glomerular anatomy (Fig. 1E) on the surface of the AL.

4.4.4 Data processing

Calcium-response data of the glomeruli were analyzed using the custom-written programs in the IDL (RSI, boulder, CO, USA). Imaging of the Fura-2 was ratiometric as the measurements were taken simultaneously for the wavelengths of 340 and 380 nm. Hence, at first the ratio of Ca^{2+} signal (absolute values; F-340/ F-380) for each pixel of the individual frames of all recordings (movies) was calculated. The ratiometric measurements were corrected for dye-bleaching through the subtraction of an exponential

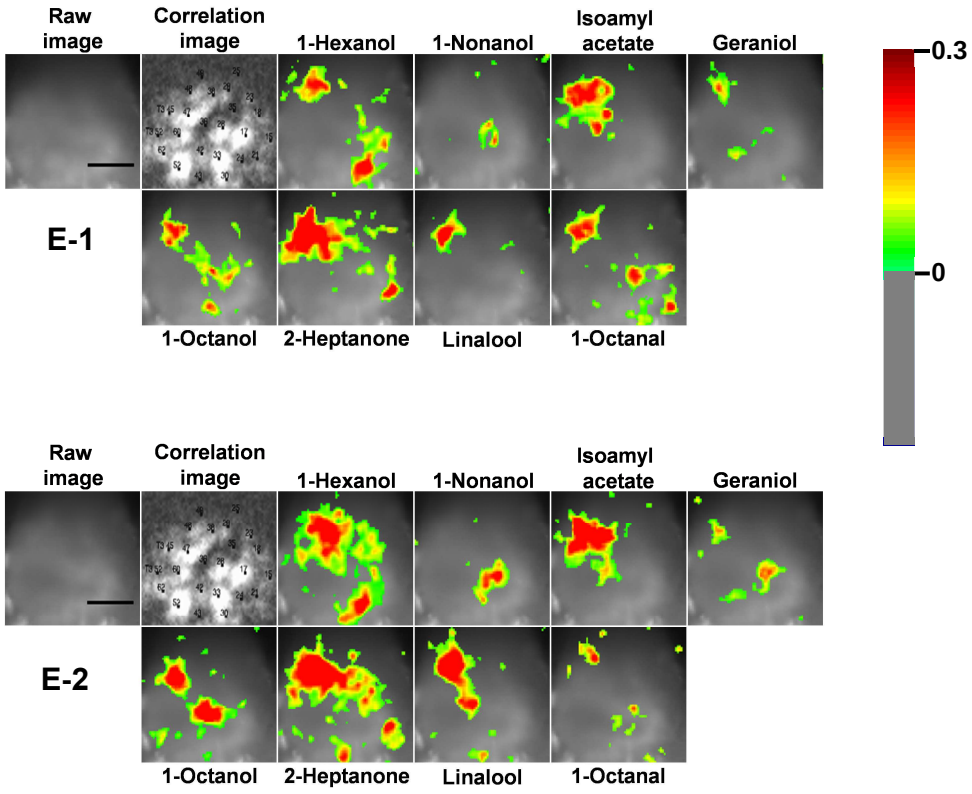


D-2

Fig. 1D: Representative confocal images of the antennal lobe of two honeybees for the fixable fluorescent dye rhodamine-dextran: The two sub-figures (D-1 and D-2) displayed the arrangement of the surface glomeruli innervated by the l-ACT and m-ACT tracts of the projection neuron stained with the rhodamine-dextran dye and imaged with the 543 nm wavelength of the HeNe laser. These images along with the raw fluorescence images and the odor response patterns of the glomeruli were used to assign the surface glomeruli in bees. The white arrows in both subfigures indicated the cell bodies of PNs which appeared as bright (blue color) spots with the saturated pixel intensities (also found in Fig. 1B).

decay function of the mean brightness over all the image frames. Background fluorescence was calculated through averaging of the pixel intensities over 10 frames (frame number 4 until 13) of each of the movies which then was subtracted from each of

the ratiometric measurements to calculate the delta-F (dF). Next, the percent changes of the delta-F values were calculated to produce the gray scale delta-F images. These images then were transformed into the false-colored images for visualization of the odor evoked spatial activity patterns across the AL-glomeruli. Ca^{2+} activity patterns were overlaid on top of the raw fluorescence images for direct visualization of the glomerular anatomy and their calcium response patterns to the test odors (Fig. 1E). Movement (within one measurement) and shift corrections (between successive measurements) were performed manually on the morphological images using the IDL programs. The final data set only incorporated bees which showed clear staining of the surface glomeruli and consistent responses to the test odors throughout the three phases (before, during and after adaptation) of experiment. Glomerular assignment was performed by comparing the relative positions and sizes of the glomeruli found in the confocal and in the raw fluorescent images with the digital atlas of the honeybee antennal lobe (Galizia *et al.*, 1999a) as well as through the comparison of glomerular responses (for odors e.g., 1-nonanol, 1-hexanol, Isoamyl acetate, and 1-octanol) with the physiological atlas of AL



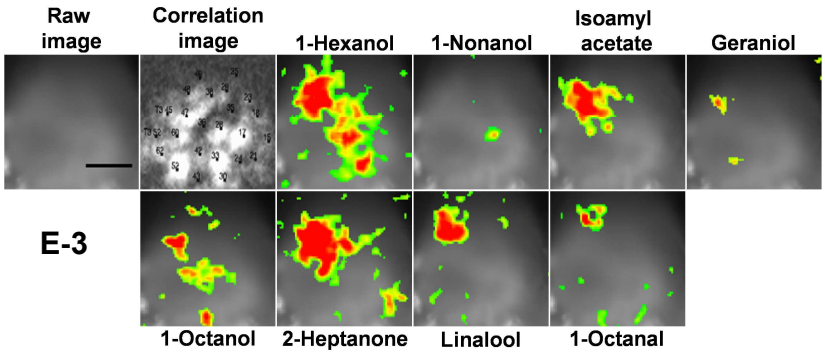


Fig. 1E: Odor evoked spatial activity patterns of the antennal lobe glomeruli during the conditions of before, during and after adaptation with the synthetic odor mixture: The set of three subfigures (E-1 to E-3) represented the response patterns of the AL glomeruli to the test odors (1-hexanol, 1-nonanol, isoamyl acetate, geraniol, 1-octanol, 2-heptanone, linalool and 1-octanal) respectively during the three experimental conditions of 'before', 'during' and after adaptation. These images were made from one representative bee, adapted with the background of the synthetic odor mixture (equal volume mixture of 4 pure odors). For each of the sub-figures, the first two images of the first row respectively represented the raw fluorescence image (captured at 380 nm) and the correlation image (showed the assigned glomerular assignment; made in IDL) of the AL. The following 8 images including the 2nd row showed the spatial patterns of calcium signals of the AL glomeruli to the 8 test odors (scale bar 100 µm) overlaid with the raw fluorescence images of the AL. The pixel intensity values of the glomeruli during the odor evoked responses were represented with the false colors (color scale was given on the right side of the 1st sub-figure) setting the gray scale values below the scale minimum '0'. It was apparent in these images that odor evoked spatial activity patterns of the AL glomeruli changed between the three experimental conditions.

(Galizia *et al.*, 1999b; Sachse *et al.*, 1999; Sachse and Galizia 2002). Temporal traces of calcium responses of the individual glomeruli were obtained through the integration of fluorescence signals inside a square area of 3×3 pixels, selected in the middle of the identified glomeruli using the IDL program. For each honeybee the percent delta-F (from the background) values of the odor-measurements were normalized with the highest odor response of that bee (set as the 100%) to discard the variability in highest responses and background fluorescence (due to the differences in staining) between the different honeybees. Hence, all quantifications were performed using the normalized percent delta-F values of the individual bees. Quantitative analysis, statistical tests and figure-making were performed using the Excel (Microsoft), Matlab (Version 2007a, The Mathworks, Natick, MA, USA), Adobe illustrator (CS5) and ImageJ version 1.45. For analyses,

pooled data sets (from all bees) of the two adaptation experiments performed with the colony odor (N = 12 bees) and the synthetic odor mixture (N = 5 bees) were used.

4.4.5 Statistical analysis

Two sets of statistical tests were employed to analyze the data. In one of the two, data of the three experimental conditions ('before', 'during' and after the adaptation) were first compared together using the Friedman ANOVA test which was followed by the Wilcoxon matched pairs test (with Bonferroni correction) to compare between the pairs of conditions. The same data was analyzed again with the second set of tests employed the repeated measurement ANOVA (RM-ANOVA) and the Bonferroni post hoc test to find out the possible differences between the means (of certain variable) of the three experimental conditions. The results of these statistical tests were either reported in this chapter or in appendix-2; however, the figures given here only showed the significant differences found in the Wilcoxon matched pairs test.

Adaptation with the background odor stimuli

Multiple measurements were taken in each bee during the adaptation process of the AL glomeruli to the background odor stimuli. During the analysis, calcium response data (time traces of the 50-frame recording) of the AL glomeruli were pooled from all bees (for both experiments; adaptation with the colony odor and with the mixture of pure odors) for the two time points; during the onset of the adaptation stimulus and at the point of adaptation. Statistical tests (Wilcoxon matched pairs test and RM-ANOVA) were performed to compare between the mean responses of these two time series of responses.

Gross analysis of the glomerular response strength to the test odors

The response time traces of all glomeruli (for each adaptation experiment) to the set of test odors were pooled from all bees and for the three experimental conditions ('before', 'during' and after the adaptation). These three series of values were compared using the two sets of statistical tests to find out the differences in average glomerular response strengths between the three conditions. In addition, comparisons between the glomerular response strengths of the three conditions, separately for the time of odor stimulation (for

3 sec) and after the offset of odor stimulation (for 4.2 sec) were performed. For these analyses the integrated response intensities or areas under the curves (summation of the normalized % delta-F values of the temporal traces) were calculated and compared.

Response strength of the individual glomeruli

Response time traces of the 14 selected glomeruli to the test odors during the three conditions were pooled at first from all bees. Percent normalized delta-F values only during the time of odor stimulation were used to make three response matrices for the analysis with RM ANOVA or these matrices were converted into three column vectors for the pair-wise comparisons (between the conditions) of the WMP test.

Mean response strengths (only during the odor stimulation) of glomerulus 17, 28 and 33 to the individual test odors between the three conditions were compared next in the result-section. In this case no statistics was performed on the response data if the mean values of glomerular response strength were found below 10% of the normalized delta-F. Hence, no conclusions were drawn for these odors. The glomerular response time traces in all of these cases showed weak responses (traces not shown).

Individual odors were categorized

The time series data of all glomeruli (from all bees) to the individual test odors were pooled separately for the three experimental conditions to form the three response matrices. Only the values during the time of odor stimulation (3 sec) were used to calculate the integrated intensities or areas under the time traces which then were compared (between the three conditions) using the same two sets of statistical tests.

Calculation of the Euclidean distance

For individual odors time traces of glomerular responses during the three experimental conditions were pooled at first from all bees. Then Euclidean distances (EDs) between the pairs of conditions were calculated using the response data. Hence, for individual odors three distance matrices were generated using the responses between the pairs of experimental conditions: ‘before’ and ‘during’, between ‘during’ and ‘after’ and lastly between ‘after’ and ‘before’. These matrices were directly used to perform the RM-

ANOVA test or they were converted into three column vectors for the Friedman ANOVA test and the following Wilcoxon matched pairs test.

4.5 Results

4.5.1 Olfactory adaptation of the AL glomeruli with the background odor stimuli

To adapt the receptor neurons with the long-duration odor pulse protocol Kelling and colleagues exposed the houseflies to the background odor stimuli constantly for 15 min (Kelling *et al.,* 2002). In our experimental protocol, similar to Kelling's study honeybee subjects were exposed to the background odor stimuli (either the colony odor or the synthetic mixture of four pure odors) continuous for ~ 20 min to achieve the olfactory adaptation of the AL glomeruli. Adaptation was defined as the process that decreased the strength of calcium signals of the glomeruli with time to the adaptation stimulus (AS) from the onset of the stimulus until the point of no detectable responses; considered as the point of adaptation. Changes in calcium concentration were recorded 20 sec before the onset of the adaptation stimulus (AS) to capture the adaptation related events. However, individual bees (data not shown for individuals) were neither found to show the evoked responses during the onset of the AS nor showed the subsequent decrease in the strength of calcium responses over time to the adaptation stimulus (found both for the colony odor and synthetic mixture).

For analysis of the pooled data from all bees (separately for the two adaptation experiments), the mean glomerular response strengths both during the onset of the AS and at the point of adaptation (blue and red colored traces respectively in the Fig. 2A and 2B) were calculated and compared using the two sets of statistical tests. Friedman ANOVA showed no significant difference ($\chi^2 = 0.002$, df = 1, p = 0.9) between the mean response strengths of the glomerular ensemble of the two time points (Fig. 2A) when the odor from the honeybee colony was used for adaptation. Repeated measurement ANOVA (RM-ANOVA) also showed the non-significant difference between the two means (F (1,530) = 0.14, p = 0.7). The mean response strength of the glomerular ensemble although was found to increase during the onset of the colony odor (indicated by the black arrow in Fig. 2A), but this small change in fluorescence intensity probably

represented some noise in signal recording. Background adaptation with the mixture of four pure odors also showed the similar result as both the Friedman ANOVA (χ^2 = 1.05, df = 1, p = 0.3) and RM-ANOVA (F (1,224) = 0.01, p = 0.88) showed the non-significant difference between the mean glomerular response strengths measured during the same two time points (Fig. 2B).

These results showed that our experimental protocol was failed to capture the calcium events during the process of olfactory adaptation of the glomeruli. The reasons were not understood since the same set of AL glomeruli showed clear calcium responses throughout the 3 sec of exposure with the test odors. During adaptation we recorded the calcium signals with the frame rate of 0.5 Hz (rate of capturing) which was slower than the rate at which odor responses were measured (5 Hz). However, with the slower rate we recorded the calcium signal for longer period of time (100 sec) than the odor measurements (10 sec). It was rather unusual that we did not detect any early (during onset of AS) or late calcium signals throughout the 20 min exposure with the two adaptation stimuli. In addition, to the absence of excitatory calcium signals glomeruli also did not show any inhibitory responses during this time. Hence, constant exposure for ~ 20 min most likely adapted the glomerular responses to the background adaptation stimuli.

4.5.2 Olfactory adaptation changed the odor response strength of the glomerular ensemble

Adaptation associated calcium events although were not detected in the analysis, however, significant changes were found in the odor response strength of the AL glomeruli between the three conditions; absence, presence and the removal of the background adaptation stimulus. Data sets of the two adaptation experiments were analyzed identically and the results were discussed below.

Adaptation with the colony odor

Response time traces of the all glomeruli or glomerular ensemble (all glomeruli in the pooled data from all bees were called glomerular ensemble here) to the test odors were compared between the (Fig. 3A) conditions of before, during and after adaptation using

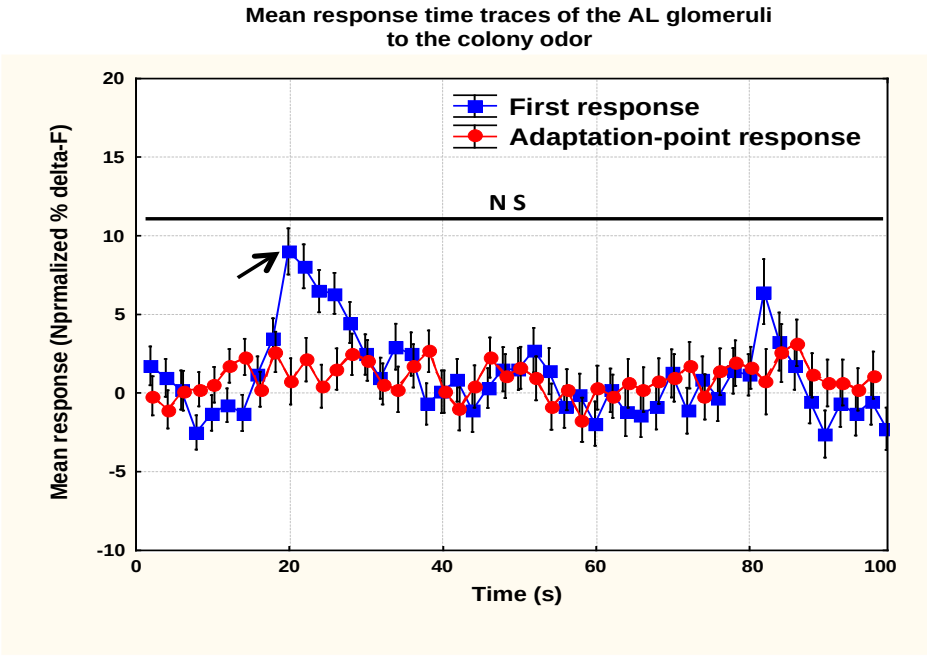

Mean response time traces of the AL glomeruli
to the colony odor
First response
Adaptation-point response
N S
Mean response (Nprmalized % delta-F)
Time (s)
20
15
10
5
0
-5
-10
0
20
40
60
80
100

Fig. 2A

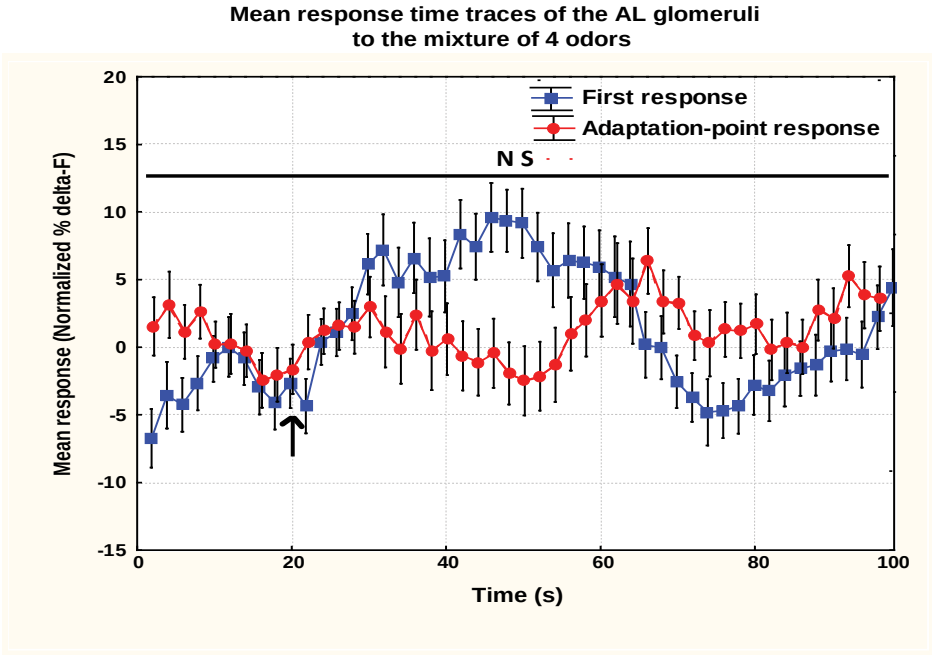

Mean response time traces of the AL glomeruli
to the mixture of 4 odors
First response
Adaptation-point response
N S
Mean response (Normalized % delta-F)
Time (s)
20
15
10
5
0
-5
-10
-15
0
20
40
60
80
100

Fig. 2B

Fig. 2A and 2B: Comparison between the glomerular response strengths during the onset of adaptation stimulus and at the point of adaptation; colony odor and synthetic odor mixture: The blue line in figure 2A represented the mean response time trace of the pooled population of glomeruli from all bees (12 bees) during the onset (represented as 'First response') of the adaptation stimulus; the colony odor. Onset of the stimulus was indicated with a black arrow, 20 sec after the beginning of image acquisition. The red line represented the mean response time trace at the point of adaptation (represented as 'Adaptation-point response'). The blue and red lines in figure 2B were respectively represented the same mean response time traces calculated from the pooled data set (5 bees) as described for Fig. 2A when bees were adapted with the mixture of four pure odors (onset was denoted with the black arrow). The horizontal black lines with NS (non-significant) written on top inside these two figures represented the non-significant differences ($p > 0.05$) between the mean responses of the two time points. The abscissa in both figures showed the total time (100 sec) of signal recording and the ordinates represented the normalized percent change in fluorescence of the glomerular ensemble. Vertical bars of the data points represented the 95% confidence intervals of the means.

the Friedman ANOVA which showed significant interaction between the glomerular response and condition ($\chi^2 = 20.48$, df = 2, $p = 0.00004$). Pairwise comparisons of the conditions using the Bonferroni corrected Wilcoxon matched pairs test (WMP test) revealed the significant decrease in mean response strength during the adaptation compared to 'before' ($Z = 2.68$, $p = 0.007$) and after ($Z = 6.2$, $p = 0.000000$) adaptation. However, the difference found between 'before' and after adaptation was not significant ($Z = 1.48$, $p = 0.13$). These results showed that adaptation with the colony odor had an inhibitory effect on the average odor response strength of the AL glomeruli which was reversible as the response strength was gone up to the un-adapted level (before adaptation) after the removal of background adaptation (Fig. 3A). Repeated measurement ANOVA also found the significant response × condition effect ($F (98, 312669) = 15.61$, $p = 0.000000$) however, the differences between mean responses of the three conditions were not found significant ($F (2, 6381) = 1.35$, $p = 0.25$). The discrepancy found between the results of the Wilcoxon matched pairs test and the RM-ANOVA indicated that the inhibitory effect of colony odor adaptation was not strong.

The gross analysis of glomerular responses throughout the 10 sec time of signal measurement was followed by the analysis to test whether the response strength of the glomerular ensemble showed any change during the time of odor stimulation (3 sec window) and after the offset of odor stimulation (4.2 sec window).

Comparison of the integrated responses intensity (strength) or the area under the response time series during the time of odor stimulation (for 3 sec) revealed the significant

difference in mean responses (Friedman ANOVA: χ^2 = 52.80, df = 2, p = 0.00000) between the three conditions (Fig. 3B) which was further tested with the Bonferroni corrected WMP test to compare between the condition-pairs. Significant increase in the mean integrated intensity was found both 'during' and after the adaptation compared to 'before' (between 'before' and 'during': Z = 2.64, p = 0.008; between 'before' and 'after': Z = 6.91, p = 0.000000) as well as after the adaptation compared to 'during' (Z = 5.28, p = 0. 000000). RM-ANOVA performed with the normalized percent delta-F values (in place of the integrated intensity values) during the time window of odor stimulation also revealed the significant response × condition (F (28, 89334) = 45.09, p = 0.000000) and the significant condition (F (2, 6381) = 6.97, p = 0.0009) effects, which in turn confirmed the results of the Friedman ANOVA that the mean integrated intensity values differed between the three experimental conditions. Bonferroni post hoc test showed the significantly higher mean value after the adaptation compared to the other two conditions (between 'before' and 'after': p = 0.0007; between 'during' and 'after': p = 0.03), but no significant difference was found between 'before' and during adaptation. While we compared the integrated response intensity after the odor offset (Fig. 3C) significant difference (Friedman ANOVA: χ^2 = 11.65, df = 2, p = 0.002) was found in the glomerular responses between the three conditions. Pair wise comparisons with the Bonferroni corrected WMP test revealed the significant decrease in the mean integrated intensities both 'during' (between 'before' and 'during': Z = 3.35, p = 0.0008) and after (Z = 2.81, p = 0.004) the adaptation compared to 'before' with no significant difference found between 'during' and after adaptation (Z = 0.42, p = 0.7). Repeated measurement ANOVA with the normalized percent delta-F values (in place of the integrated intensity values) also showed the significant response × condition (F (40, 127620) = 2.45, p = 0.000001) as well as the significant condition (F (2, 6381) = 3.86, p = 0.02) effects, which confirmed the results of the Friedman ANOVA. Bonferroni post hoc test however, revealed that only the difference between 'before' and during adaptation (p = 0.01) was significant but not between 'before' and 'after' or 'during' and after the adaptation.

Results showed that colony odor adaptation significantly increased the response strength of the AL glomeruli during the time of odor stimulation (weak effect: significance found in WMP test but not in RM-ANOVA, Fig. 3B). However, mean glomerular response

strength after the odor offset was found to decrease significantly during the adaptation compared to the condition 'before' (strong effect: significant difference found in both tests, Fig. 3C). This probably explained the overall decrease (Fig. 3A) in the mean response strength during the adaptation compared to 'before'. It was concluded that adaptation with *the odor mixture of honeybee colony influenced the odor response strength of the AL glomeruli differentially* during the time of odor presentation and after

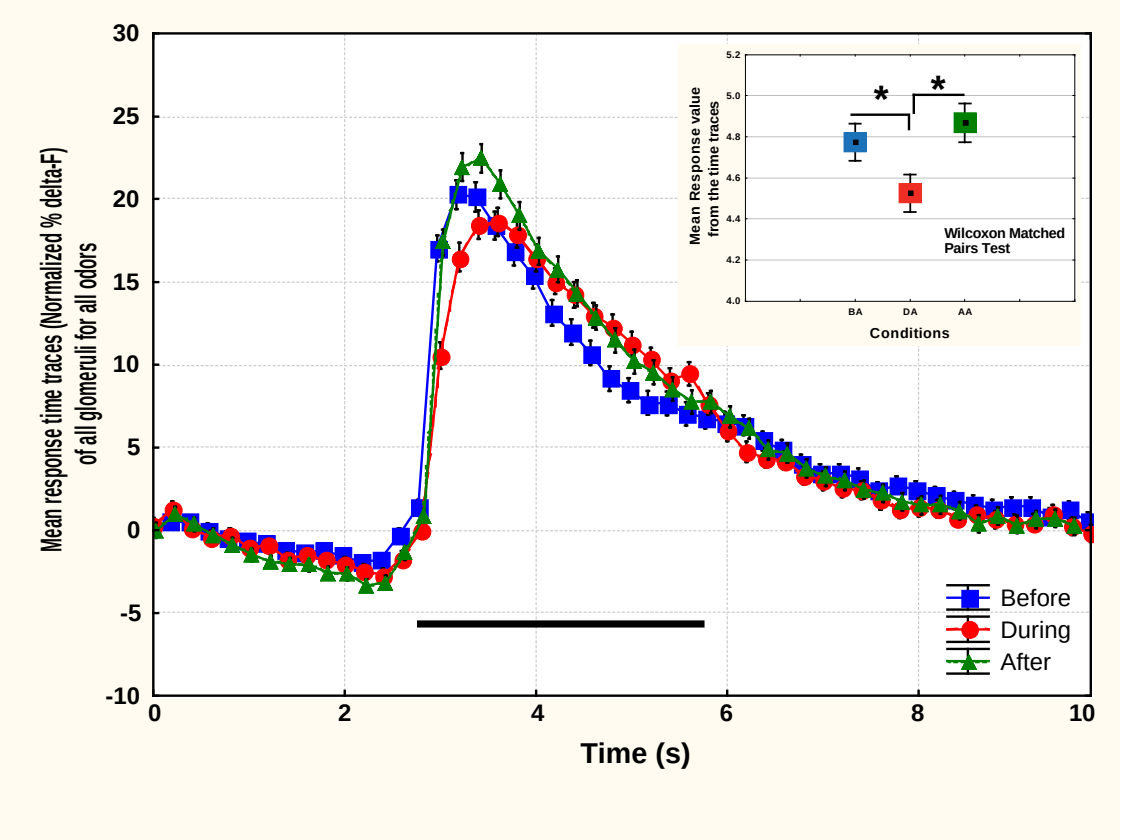


Fig. 3A: Comparisons between the mean response strengths of the AL glomeruli to the test odors of the three conditions; before, during and after adaptation with the colony odor: In this plot the mean response time series of the glomerular ensemble (266 glomeruli pooled from the 12 bees) of the three conditions were represented with the three colors; blue, red and green, respectively represented the conditions of 'before', 'during' and after adaptation with the colony odor. The x and y axes respectively represented the total recording time of 10 sec for the test odors and the normalized percent fluorescence change of the glomerular ensemble. Odors were delivered 2.8 sec after the recordings started and lasted for 3 sec as indicated by the black horizontal bar under the line graphs. Vertical bars in the line graphs represented the 95% confidence intervals of the means. Friedman ANOVA showed significant interaction ($p < 0.05$) between the glomerular response and the experimental condition. This was followed by the Wilcoxon matched pairs test (with the Bonferroni correction) which revealed the significant

decrease in mean response strength during the adaptation compared to both 'before' and after adaptation. Mean response strength after the adaptation showed no significant difference with the condition 'before'. Results of the Wilcoxon matched pairs test were shown in inset at the top right corner of the figure. Mean response strengths were calculated from the same three line graphs and represented with the box and whisker plots (y axis; mean ± standard error) and with the same color code. Significant differences in the inset plot were denoted by asterics. The x-axis of the inset-plot represented the abbreviations of the three experimental conditions; BA stood for before adaptation, DA for during adaptation and AA for after adaptation.

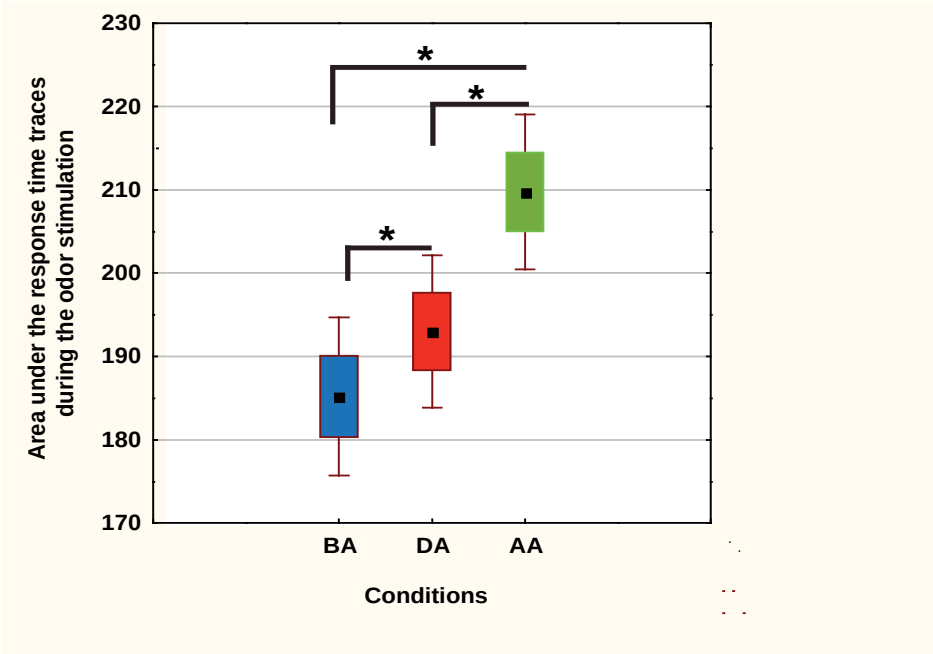


Fig. 3B: Comparisons between the mean integrated response intensities during the odor stimulation of the three experimental conditions (adaptation with colony odor): Colony odor adaptation changed the mean strength of glomerular responses (between the 3 conditions) during the time of odor stimulation (3 sec) as measured by the integrated intensity or area under the response time traces (represented on the y-axis). The mean values were represented here with the box and whisker plot (mean ± standard error) and coded with the same three colors as in Fig. 3A to represent the three experimental conditions. The x-axis showed the abbreviations of the three conditions viz. BA, DA and AA, which were same as described in Fig. 3A. Significant gradual increase in the integrated response intensity (strength) was found between the three conditions (significant differences were denoted by asterics).

the odor offset. Overall, *adaptation seemed to exert some form of inhibition on the odor responses of the glomeruli which was overshadowed during the odor stimulation* (although weak effect) *but appeared strongly in the post-offset responses* (strong effect).

Interestingly, *removal of the adaptation stimulus for 5 min did not rescue the odor evoked responses of the glomeruli back to the initial level of no-adaptation*. The glomerular ensemble showed significantly higher responses to the odors after the removal of adaptation stimulus compared to the conditions of before and during adaptation (strong effect: significant difference found in both tests, Fig. 3B). Responses after the odor offset although were gone down after the adaptation compare to before, however, this difference was not found significant in the RM-ANOVA test (but significance found in WMP test). These effects which were not visible in the gross analysis (Fig. 3A) confirmed the long lasting effects of the colony odor adaptation.

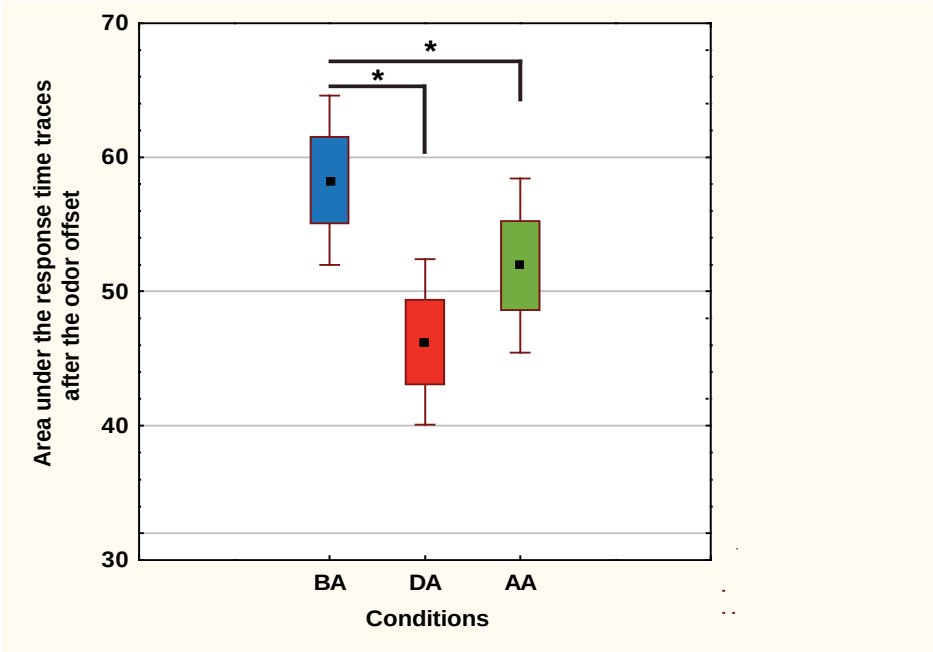


Fig. 3C: Comparisons between the mean integrated response intensities after the odor offset of the three experimental conditions (adaptation with colony odor): Background adaptation with the colony odor changed the mean strength of glomerular responses (between the 3 conditions) after the odor offset as measured by the integrated intensity or area under the response time traces (represented on the y-axis). Mean (mean ± standard error) values 'during' and after the adaptation was found to decrease significantly compared to 'before'. No significant difference was found between 'during' and after adaptation. Significant differences were denoted by asterics.

Adaptation with the mixture of four odors

Background adaptation with the synthetic mixture of four odors also changed the response intensity of the glomerular ensemble (pooled data of all glomeruli of 5 bees) to the test odors like the colony odor (Fig. 3D). Adaptation led to significant change in glomerular responses between the three conditions as found in the Friedman ANOVA (χ^2 = 183.91, df = 2, p = 0.00000) which was further tested with the Wilcoxon matched pairs test (with Bonferroni correction) to compare between the conditions. Pairwise comparisons revealed that mean response strength was significantly increased during the adaptation compared to the other two conditions (between 'before' and 'during': Z = 10.48, p = 0.000000, between 'during' and 'after': Z = 16.29, p = 0.00). Additionally, the mean response strength after the adaptation was found to decrease significantly compared to the condition 'before' (Z = 4.55, p = 0.000005). Repeated measurement ANOVA also showed the significant response × condition (F (98, 265629) = 5.62, p = 0.000000) and the significant condition effects (F (2, 5421) = 8.57, p = 0.0001). Bonferroni post hoc test confirmed the significantly higher mean response strength during the adaptation compared to both 'before' (p = 0.03) and after (p = 0.0001) adaptation. However, no significant difference was found between 'before' and after adaptation. The results of the gross analysis showed that adaptation with the background of synthetic odor mixture (mixture of known composition) unlike the colony odor (mixture of unknown composition) exerted an excitatory effect (strong effect: significant difference found in both WMP test and RM-ANOVA, Fig. 3D) on the odor responses of the AL glomeruli.

Comparison of the integrated intensity (or area under the response time series of glomeruli) during the odor stimulation showed the significant (Fig. 3E) change in responses between the conditions (Friedman ANOVA: χ^2 = 7.46, df= 2, p = 0.02). Pairwise comparisons disclosed the significantly higher value of mean integrated intensity during the adaptation compared to 'before' (Z = 3.17, p = 0.0014), although, no significant differences were found between 'before' and 'after' (Z = 2.03, p = 0.04) and between 'during' and after the adaptation (Z = 1.16, p = 0.24). RM-ANOVA also showed the significant response × condition (F (28, 75894) = 8.29, p = 0.000000) and the condition effects (F (2, 5421) = 3.17, p = 0.04) which was followed by the Bonferroni

post hoc test which confirmed the results of the WMP test (significantly higher mean value 'during' compared to before the adaptation: p = 0.03).

Integrated response strength after the offset of the odor stimuli (Fig. 3F) showed the significant interaction between the condition and response strength (Friedman ANOVA: χ^2 = 54.06, df= 2, p = 0.00000); however, no significant difference was found between 'before' and during adaptation (WMP test with Bonferroni correction: Z = 1.63, p = 0.10) unlike the last experiment (Fig. 3C). Additionally, the responses after the odor offset were found to decrease significantly after the adaptation compared to both 'before' (Z = 5.37, p = 0.000000) and during adaptation (Z = 8, p = 0.00000). RM-ANOVA showed the significant response × condition (F (40, 108420) = 2.29, p = 0.000006) and the significant condition effects (F (2, 5421) = 18.49, p = 0.000000). Bonferroni post hoc test confirmed the results of the WMP test as the significant decrease in mean responses were found after the adaptation compared to both 'before' (p = 0.000007) and during (p = 0.000000) adaptation.

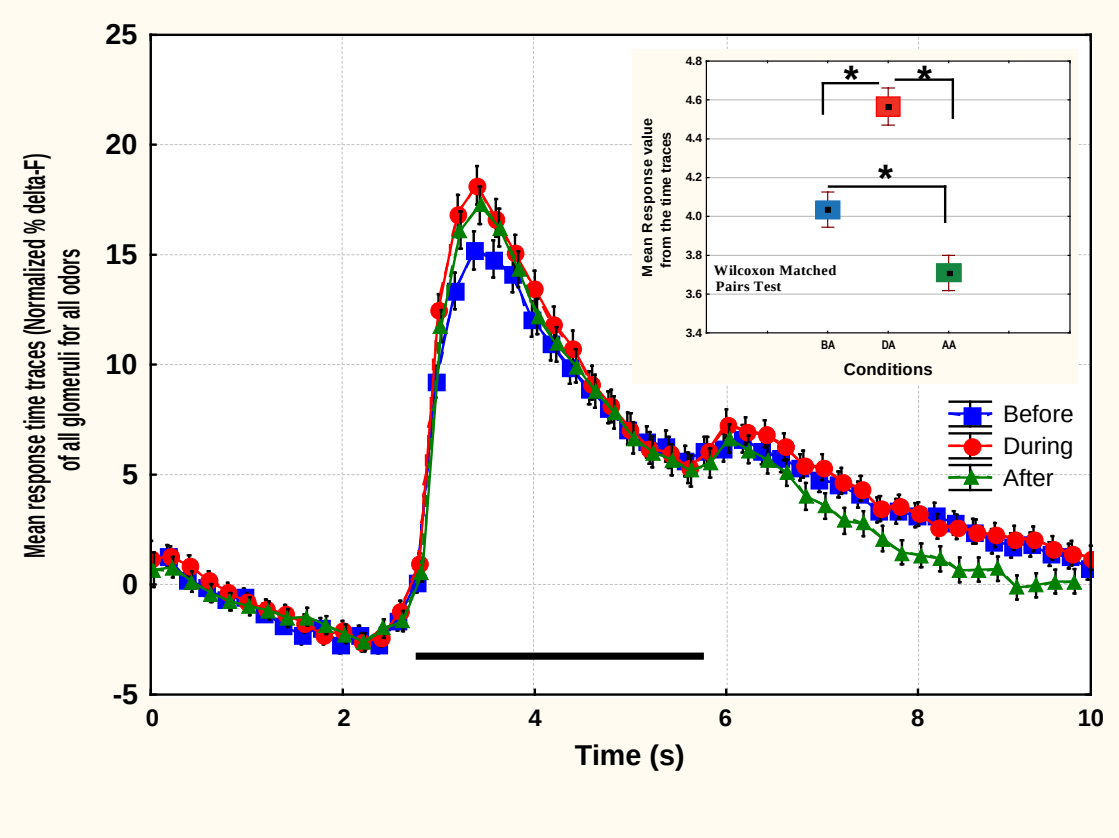


Fig. 3D: Comparisons between the mean response strengths of the AL glomeruli to the test odors of the three conditions; before, during and after adaptation with the mixture of 4 odors: In this plot the mean response time series of the glomerular ensemble (113 glomeruli pooled from the 5 bees) of the three conditions were represented with the three colors; blue, red and green, respectively represented the conditions of 'before', 'during' and after adaptation with the synthetic odor mixture. The x and y axes represented the same variables as mentioned in Fig. 3A. Measurements of odor responses were performed at the same time (the black bar under line graphs) as described in Fig. 3A. Vertical bars in the line graphs represented the 95% confidence intervals of the means. Statistically significant interaction was ($p < 0.05$) found between the glomerular response and the experimental condition using the Friedman ANOVA test. This was followed by the Wilcoxon matched pairs test (with the Bonferroni correction) to compare between the pairs of conditions. Comparisons revealed the significant increase in response strength during the adaptation compared to the other two conditions as well as the significant decrease after the adaptation compared to 'before'. Results of the Wilcoxon matched pairs test were shown in inset at the top right corner of the figure. Mean response strengths were calculated from the same three line graphs and represented with the box and whisker plots (shown on the y axis; mean ± standard error) and with the same color code (denoting the 3 experimental conditions). Significant differences in the inset plot were denoted by asterics. The x-axis of the inset-plot showed the abbreviations of the three experimental conditions; BA stood for before adaptation, DA for during adaptation and AA for after adaptation.

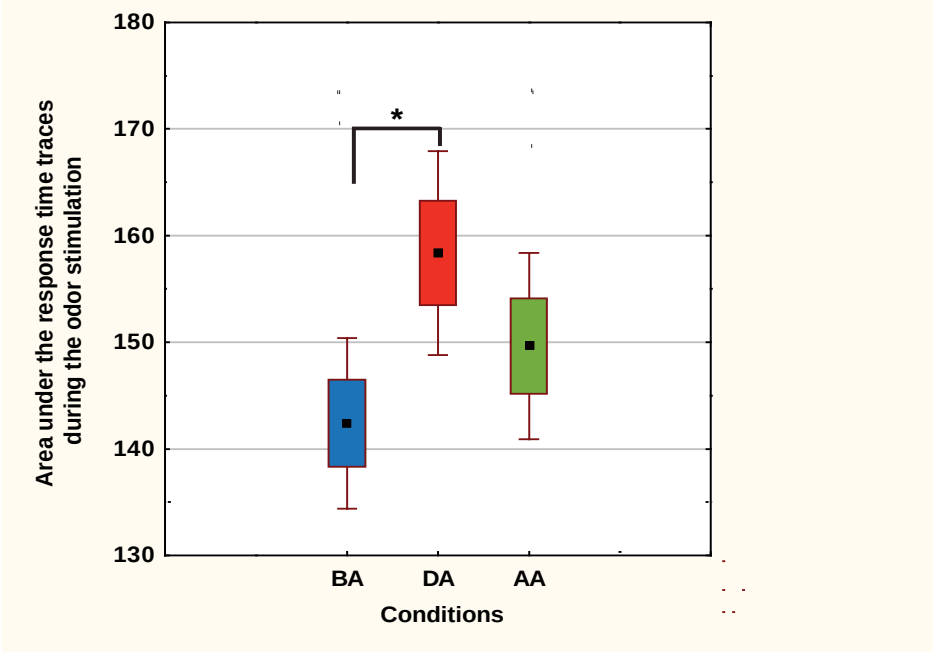


Fig. 3E: Comparisons between the mean integrated response intensities during the odor stimulation of the three experimental conditions (adaptation with the synthetic mixture): Adaptation with the synthetic odor mixture changed the mean strength of glomerular responses (between the 3 conditions) during the time of odor stimulation (3 sec) as measured by the integrated intensity or area under the response time traces (represented on the y-axis). The mean values were represented with the box and whisker plot (mean ± standard error) and coded with

the same three colors as in Fig. 3A to represent the three experimental conditions. The x-axis showed the abbreviations of the three conditions viz. BA, DA and AA, which were same as described in Fig. 3A. Significant increase in the mean integrated intensity was found during the adaptation compared to before. However, differences between 'after' and before the adaptation or 'during' and after the adaptation were not found significant. Significant differences were denoted by asterics.

The results of the gross analysis showed that unlike the colony odor experiment, *olfactory adaptation with the background of synthetic odor mixture enhanced the strength of the odor evoked calcium responses of AL glomeruli compared to the un-adapted condition*. However, *removal of the adaptation stimulus unlike the colony odor experiment recovered the odor response strength of the glomeruli back to the un-adapted levels* (Fig. 3E). Glomerular responses after the odor offset did not decrease during the adaptation but only after the adaptation compared to the other two conditions (Fig. 3F). The overall response strength of the AL glomeruli although was decreased after the adaptation compared to before (Fig. 3D) however; this was not a strong effect (WMP test only showed significant difference). Hence, the stoppage of the adaptation stimulus only

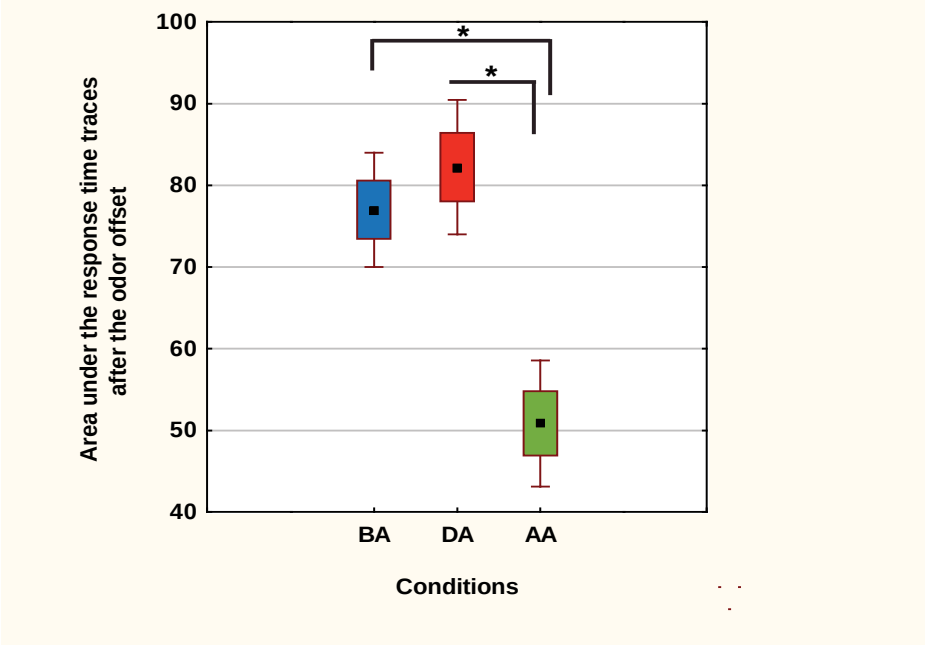


Fig. 3F: Comparisons between the mean integrated response intensities after the odor offset of the three experimental conditions (adaptation with the synthetic mixture): Adaptation with the background of synthetic odor mixture changed the mean strength of glomerular

responses (between the 3 conditions) after the odor offset as measured by the integrated intensity or area under the response time traces (represented on the y-axis). Mean values (mean ± standard error) both 'before' and during the adaptation were found significantly higher compared to after-adaptation (significant differences were denoted by asterics). However, the difference between 'before' and during the adaptation was not found significant.

affected the post offset responses of the AL glomeruli unlike the results of the previous experiment (Fig. 3B and 3C). Additionally, more number of common significant effects (significant differences) were found between the two sets of statistical tests when the mixture of four odors was used for the adaptation compared to the colony odor. However, both adaptation stimuli showed the common increase in odor evoked response strength of the AL glomeruli during the adaptation compared to the un-adapted condition as well as showed the lack of adaptation recovery (either during odor stimulation or in the post-offset responses) in glomerular responses.

4.5.3 Individual glomeruli showed the similar or dissimilar types of change in odor responses due to adaptation

After the gross analysis performed on the glomerular ensemble here we investigated the possible changes in odor response strength of the different individual glomeruli due to the olfactory adaptation. A total of 14 glomeruli were selected based on the criterion that these glomeruli were identified in at least 80% of the honeybees in the data sets of the two adaptation experiments. Thirteen of these glomeruli were innervated by the l-ACT and one (T3-45) by the m-ACT tract of the projection neurons. The response strengths of these glomeruli only during the time of odor stimulation were compared between the conditions of before, during and after adaptation. Changes found in the overall response strength were categorized into six different types (described below) when the colony odor was used for adaptation.

***Type-1*:** The mean strength of the odor evoked calcium signals decreased significantly (Fig. 4A) both 'during' and after the adaptation compared to the condition 'before'. However, this decrease in response was not progressive as the difference between 'during' and after adaptation was not found significant. Glomerulus 38 was (results of the statistical tests were given in table-1; see appendix-2, Friedman ANOVA followed by the Wilcoxon matched pairs test) the only member found in this category.

***Type-2*:** The mean response strength of these glomeruli (Fig. 4A) showed significant decrease during the adaptation compared to the other two conditions. Glomeruli 42 (Fig. 4A) and 52 (see Fig. 4B; appendix-2) were found to belong to this type.

***Type-3*:** Mean response strength in type-3 glomeruli increased significantly (Fig. 4A) during the adaptation compared to the conditions of 'before' and after adaptation. However, no significant difference was found between 'before' and after adaptation. Two members were found in this category viz. glomerulus 28 (Fig. 4A), 29 (Fig. 4B; appendix-2).

***Type-4*:** Significant increase in the mean strength of the odor evoked responses was found in these glomeruli (Fig. 4A) after the removal of background adaptation compared to both 'before' and during adaptation. Three glomeruli were clustered in this category viz. 47 (Fig. 4A), 49 and 33 (Fig. 4B; appendix-2).

***Type-5*:** Type-5 glomeruli showed significant increase (Fig. 4A) in the mean response strength both 'during' and after the adaptation compared to 'before' with no significant difference in response strength found between the conditions of 'during' and after adaptation. The m-ACT glomerulus T3-45 was an exception as this one also showed the significant increase after the adaptation compared to 'during' (Fig. 4B; appendix-2). This category had the maximum number of four glomeruli viz. glomerulus 35 (Fig. 4A), 36, 60 and T3-45 (Fig. 4B; appendix-2).

***Type-6*:** No change in the response strength (Fig. 4A) was found between the three conditions in this type. Glomerulus 48 (Fig. 4A) and 17 (Fig. 4B; appendix-2) were found in this category.

Repeated measurement ANOVA found the significant response × condition effect (data not shown) for 13 out of the 14 glomeruli (except for glomerulus 29) however; for only glomerulus 28, 42 and T3-45 (3 glomeruli out of 14; data not shown for the other 11 glomeruli) significant condition effect was found (Glomerulus 28: $F(2,285) = 7.14$, $p = 0.0009$; Glomerulus 42: $F(2,285) = 3.45$, $p = 0.03$; Glomerulus T3-45: $F(2,237) = 3.35$, $p = 0.03$). These results showed that odor evoked response strength of the AL glomeruli although changed significantly between the different conditions but only for three glomeruli the differences in mean response strength were found significant between the

three experimental conditions. Results from the two sets of statistical test confirmed the fact that at least glomerulus T3-45 (type-5) did not show the adaptation recovery in odor

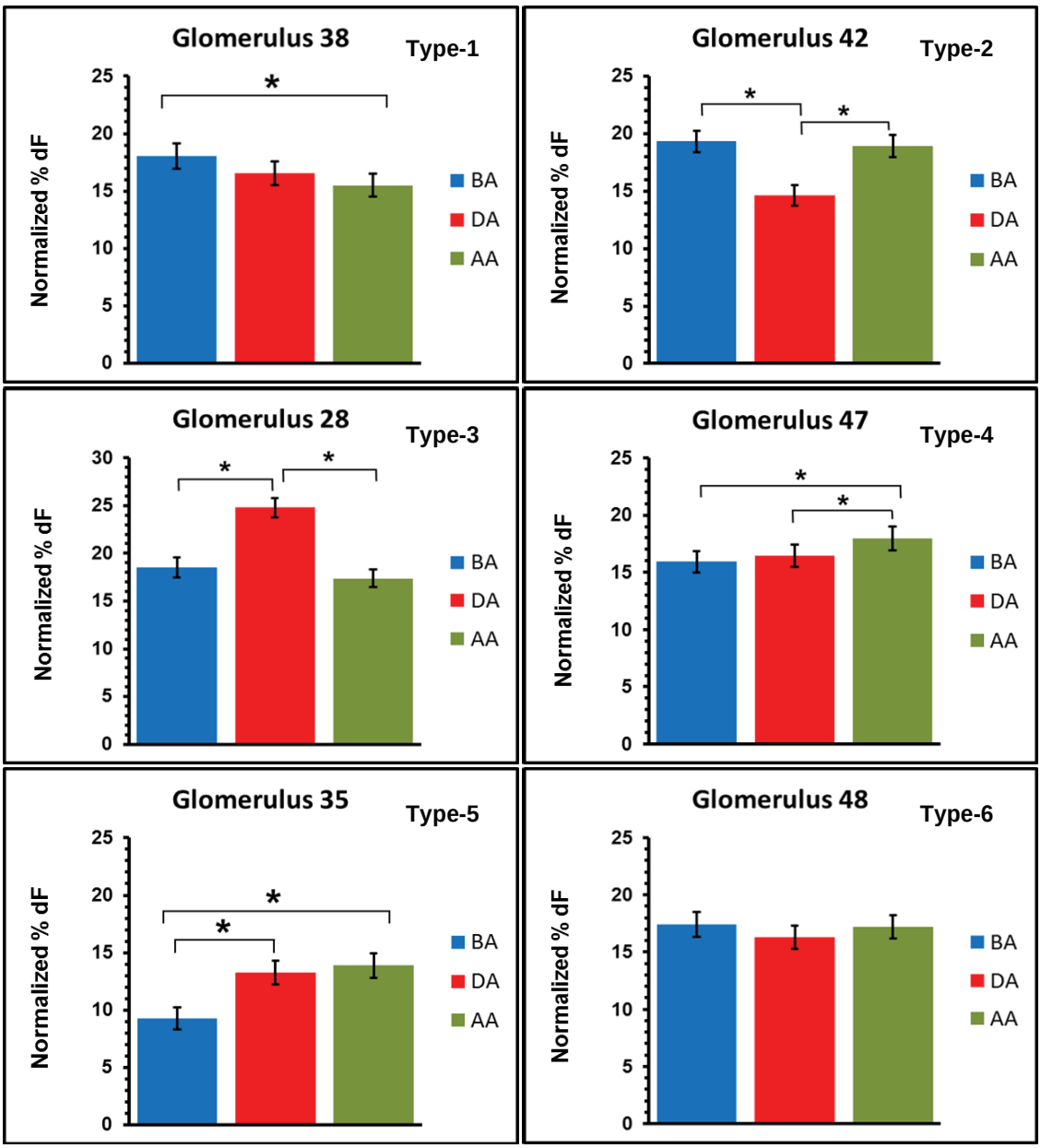


Fig. 4A: Glomerular types differed with respect to their patterns of change in odor response strength (adaptation with the colony odor): Six different glomerular types (mentioned at the top right corner of each sub-plot) were found in the adaptation experiment with the colony odor. These categories were represented in this figure with the six sub-plots. These categories incorporated the 14 glomeruli which were innervated by both the l-ACT (13 of them) and m-ACT (1 glomerulus) tracts of the projection neuron. Only the response patterns of the six glomeruli

were shown here as the representatives of each type (rests were shown in Fig. 4B; appendix-2). The y-axis showed the mean response strength (normalized % delta-F) of the single glomeruli to all test odors only during the time of odor stimulation and the colored bars represented the mean ± standard error. The three experimental conditions were represented with the three colors (blue, red and green were respectively denoted the conditions of before, during and after adaptation; the abbreviations BA, DA and AA were same as described before in Fig. 3A). Friedman ANOVA and the Bonferroni corrected Wilcoxon matched pairs test were performed to test the differences in mean response strength between the three conditions. The results of the statistical tests were shown in table-1 (see appendix-2); the significant differences were denoted in the figure by the asterics.

responses (appendix-2: significantly higher mean value of response strength 'after' compared to before adaptation; Bonferroni post hoc test $p = 0.03$). While considering the results of the Wilcoxon matched pairs test, type-4 glomeruli along with the type-5 were also found to show the similar enhancement in odor responses after the adaptation compared to 'before'. These results supported the previous result where the post-adaptation enhancement in odor response strength of the PN glomerular ensemble was found (Fig. 3B). On the contrary, glomerulus 38 (type-1) showed the significant decrease in response strength after the adaptation compared to the condition 'before'. Additionally, the odor evoked responses of the type-2 and type-6 (Fig. 4A) glomeruli were found to show the adaptation recovery after the withdrawal of the adaptation background. These results showed that olfactory adaptation with the colony odor changed the odor response strength of the individual glomeruli. These changes were not monotonic as we found both enhancement and decrease in glomerular response strength. Individual glomeruli not only showed the dissimilar pattern of change in their odor response strength but also showed the different temporal dynamics of adaptation recover in odor responses. The reasons for the differences in adaptive responses of the glomeruli were not understood from these results however; such differences possibly enhance the antennal lobe processing of the new odor information (identity and intensity) in the pre-existing olfactory background.

In the other adaptation experiment with the synthetic odor mixture, the same set of 14 glomeruli showed six different types of change in their odor response strength between the three experimental conditions which were described below.

***Type-1*:** Glomerulus 17 found in this category showed the progressive decrease in odor response strength (Fig. 4C) along the three experimental conditions (results of the statistical tests were given in table-2; appendix-2, Friedman ANOVA followed by the

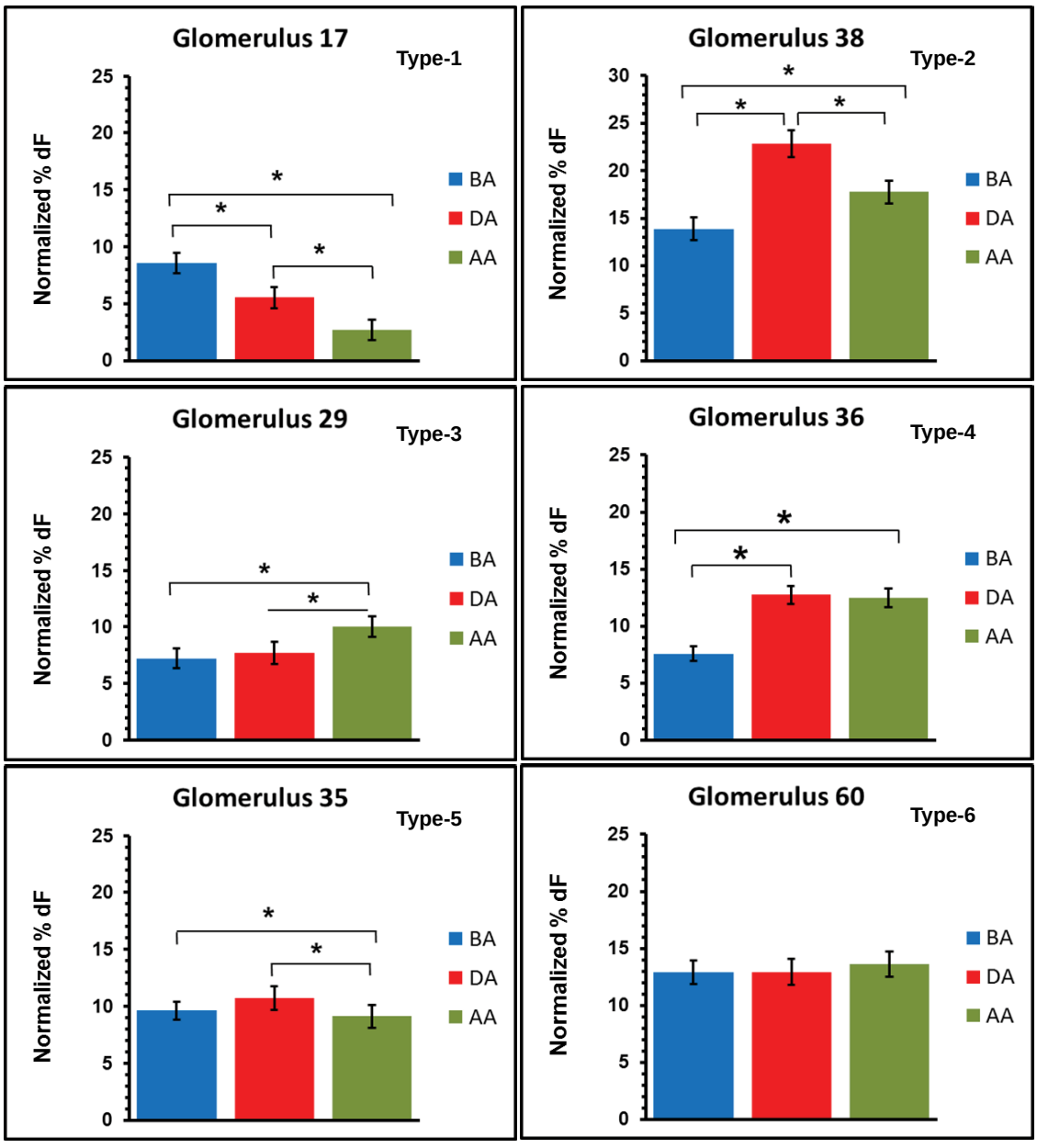


Fig. 4C: Glomerular types differed with respect to their patterns of change in odor response strength (adaptation with the synthetic odor mixture): Six different glomerular types (mentioned at the top right corner of each sub-plot) were found in the adaptation experiment with the synthetic odor mixture. These categories were represented in this figure with the six sub-plots. These categories incorporated the 14 glomeruli which were innervated by both the l-ACT (13 of

them) and m-ACT (1 glomerulus) tracts of the projection neuron. Only the response patterns of the six glomeruli were shown here as the representatives of each type (rests were shown in Fig. 4D; appendix-2). The y-axis showed the mean response strength (normalized % delta-F) of the single glomeruli to all test odors only during the time of odor stimulation and the colored bars represented the mean ± standard error. The three experimental conditions were represented with the three colors (blue, red and green were respectively denoted the conditions of before, during and after adaptation; the abbreviations BA, DA and AA were same as described before in Fig. 3A). Friedman ANOVA and the Bonferroni corrected Wilcoxon matched pairs test were performed to test the differences in mean response strength between the three conditions. The results of the statistical tests were shown in table-2 (see appendix-2); the significant differences were denoted in the figure by the asterics.

Wilcoxon matched pairs test). This type was similar with the type-1 in colony odor experiment, except for the feature of progressive decrease in response strength.

***Type*-2:** This category was found to incorporate the maximum number (5) of glomeruli viz. glomerulus 38 (Fig. 4C), 42, 47, 48 and T3-45 (Fig. 4D; appendix-2). Type-2 glomeruli showed the significant increase in mean response strength during the adaptation compared to both 'before' and after adaptation (results of the statistical tests were given in table-2; appendix-2). They also showed significantly higher responses after the adaptation (except T3-45; contrasting result with the previous experiment) compared to before adaptation. This particular type resembled the type-3 glomeruli in the colony odor adaptation experiment; however, no significant differences in response strength were found between the conditions of 'before' and after adaptation.

***Type-3*:** This type resembled the type-4 glomeruli of the colony odor experiment. Type-3 glomeruli showed significant increase in mean response strength (Fig. 5B) after the adaptation compared to both 'before' and during adaptation. Glomerulus 29 was found in this category (Fig. 4C).

***Type-4*:** Four glomeruli viz. 36 (Fig. 4C), 28, 49 and 52 (appendix-2; Fig. 4D) were found in this category which showed increased responses to the odors both 'during' and after the adaptation compared to 'before'. Similar glomerular type was found (type-5) in the colony odor experiment (Fig. 4A), which also showed the high number of representatives.

***Type-5*:** The mean response intensity of these glomeruli decreased after the adaptation compared to both 'before' and 'during'; glomeruli 35 (Fig. 4C) and 33 (appendix-2; Fig. 4D) were found in this type.

***Type-6*:** Glomerulus 60 (Fig. 4C) was found in this category with no differences found in the mean strength between the three conditions (Fig. 4C).

RM-ANOVA test performed on the response data of these glomeruli showed the significant response × condition effect (data not shown) for nearly half of the glomeruli (6 out of 14) and significant condition effect for 5 out of these 6 glomeruli (Glomerulus 17: $F(2,237) = 4.73$, $p = 0.009$; Glomerulus 36: $F(2,237) = 9.30$, $p = 0.0001$; Glomerulus 38: $F(2,237) = 5.04$, $p = 0.007$; Glomerulus 42: $F(2,237) = 3.54$, $p = 0.03$; Glomerulus 47: $F(2,237) = 6.10$, $p = 0.002$).

Unlike the colony odor experiment, less number of glomeruli changed their response strengths along the experimental conditions when the synthetic odor mixture was used for adaptation. However, in this case little more number of glomeruli was found to show the significant change in their mean response strength between the conditions of before, during and after adaptation. More number of significant differences found in the WMP test compared to the RM-ANOVA indicated the differences in strength between these two types of tests. Considering the results of these two statistical tests, we found that majority of the AL glomeruli showed the enhancement in odor response strength during the time of adaptation compared to the un-adapted condition (before adaptation). Six glomeruli out of 14 in the colony odor experiment and 9 out of 14 in the synthetic odor mixture experiment showed this effect.

More number of glomeruli in the synthetic odor mixture experiment (13 out of 14) did not show the adaptation recover in their odor responses than in the colony odor experiment (8 out of 14). The same glomerulus also showed different patterns of change in the responses strength between these two adaptation experiments. The differential effects of the two background adaptation stimuli can be explained if indicated that these two odor mixtures possibly activated the *different forms or pathways of olfactory adaptation* in the honeybee antennal lobe which were unknown.

4.5.4 Adaptation induced changes in glomerular responses were found to vary with the odor identity

Adaptation with the colony odor or synthetic odor mixture either enhanced or suppressed the overall response strength of the AL glomeruli. However, the gross changes found in

responses of the individual glomeruli were not truly reflecting their responses to the individual test odors. To understand that here, I compared the responses of the three representative glomeruli found in all bees; 17, 28 and 33 to the individual test odors between the three experimental conditions.

Glomerulus 17

This particular glomerulus did not show any significant change in the mean response strength between the three experimental conditions when the colony odor was used as the adaptation stimulus (Fig. 4B). However, odor-wise analysis showed (Fig. 5A) the response enhancement during adaptation compared to the condition before for some of

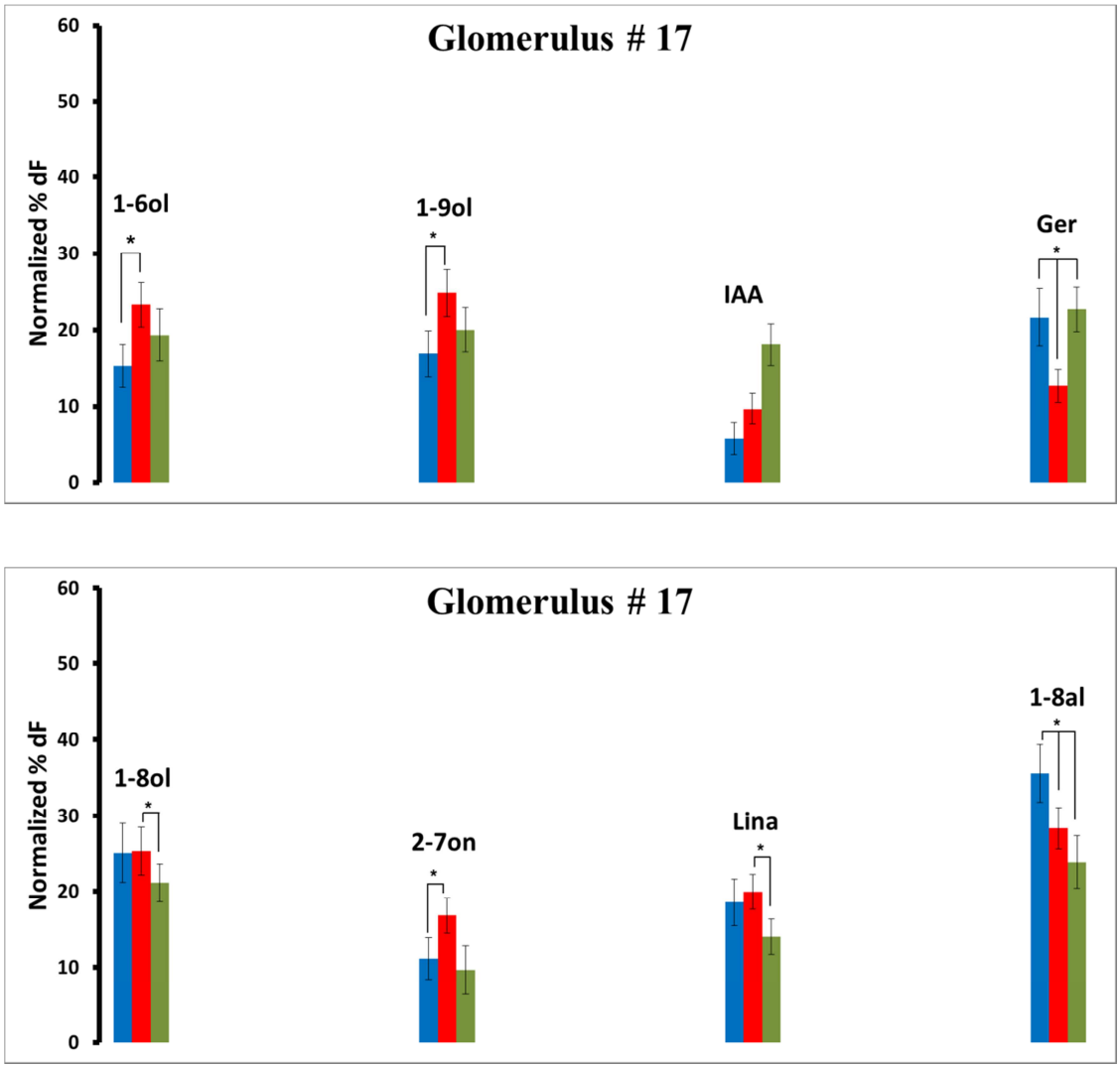


Fig. 5A: Mean response strengths of glomerulus 17 to the test odors (adaptation with the colony odor): The mean response strengths of glomerulus 17 to the individual test odors (1-hexanol (1-6ol), 1-nonanol (1-9ol), isoamyl acetate (IAA), geraniol (Ger), 1-octanol (1-8ol), 2-heptanone (2-7on), linalool (Lina), 1-octanal (1-8al)) during the odor stimulation represented here using the colored bars. Each set of three bars with three different colors represented the responses to the individual odors (abbreviations of the odor names were given on top of the bars) during the three recording conditions (blue, red and green respectively represented before, during and after adaptation; as in Fig. 4A). The y-axis represented the mean response strength (mean ± standard error). The significant differences were denoted with the asterics (results of the statistical tests were given in table-3; appendix-2).

the odors namely, 1-hexanol (1-6ol), 1-nonanol (1-9ol) and 2-heptanone (2-7on). Mean responses to the Nasonov pheromone component, geraniol showed an adaptation induced decrease in the mean response strength compared to the other two conditions and 1-octanol (1-8ol) and linalool showed the significant decrease 'after' compared to during adaptation. A trend of progressive decrease (Fig. 5A) in responses was found for 1-octanal(1-8al) form the un-adapted state until the post-adaptation condition although, the difference between 'during' and after adaptation was not found significant (results of the

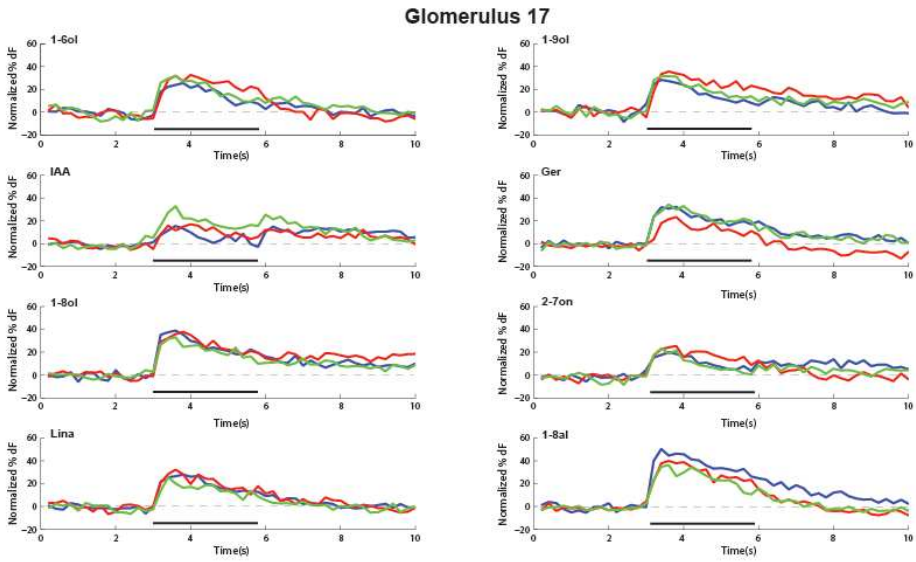
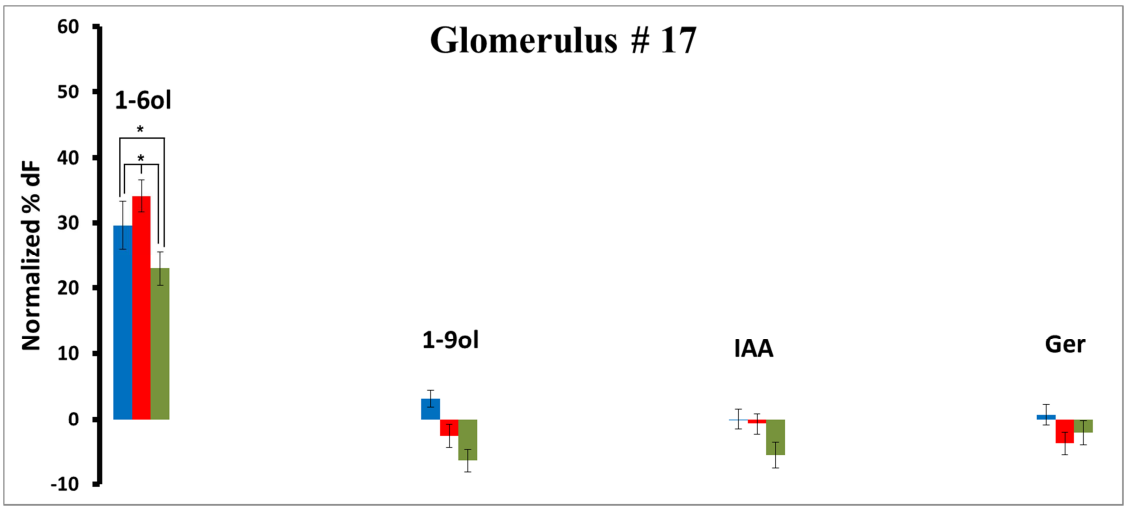


Fig. 5B: Mean response time traces of glomerulus 17 to the test odors (colony odor adaptation): Mean response time traces of glomerulus 17 to the test odors were represented here with the eight sub-plots (abbreviations of the odor names were mentioned on the top left corner of the sub-plots). Each sub-plots showed the three time traces coded with the same three colors

(blue, red and green) to represent the three recording conditions as described in Fig. 5A. The x and y axes were respectively represented the recording time (10 sec) and the odor response strength (in percent normalized delta-F) in each sub-plot. Odor stimulation was denoted with the black bar below the response traces and the dotted line represented the '0' of the y axis.

statistical analysis were shown in table-3; appendix-2, Friedman ANOVA and Wilcoxon matched pairs test). These effects were visible in the odor stimulation windows of the mean response time traces of this glomerulus to the individual test odors (black color bars under the traces represented the odor stimulation window; Fig. 5B).

RM-ANOVA found the significant response × condition effect ($F(28,462) = 1.92$, $p = 0.003$) only for the odor 1-nonanol (data not shown for other odors) however, no significant difference was found between the mean responses of the three conditions (data not shown). RM-ANOVA performed on this glomerulus previously showed the similar (although the data not shown) results as found here and also supported the results of the gross analysis (Fig. 4B; see appendix-2) that glomerulus 17 did not show any change in response strength for the test odors between the three experimental conditions.



Adaptation of glomerulus 17 with the mixture of four odors (Fig. 4C) reduced the mean response strength progressively along the three conditions, which was found to be true for most of the individual test odors such as 1-nonanol, IAA, geraniol, 1-octanol, 2-heptanone and linalool (Fig. 5C). However, these responses were very weak and (nearly

at '0' value of the % delta-F/F as shown in the mean-response time traces; Fig. 5D), hence, no conclusions were drawn for these odors. However, for the floral odor 1-6ol an

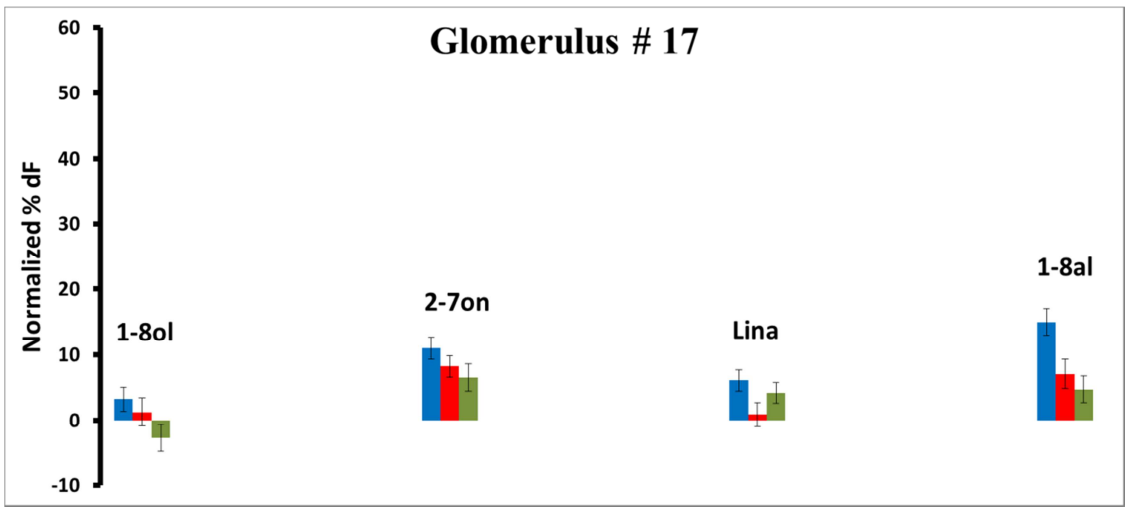


Fig. 5C: Mean responses of glomerulus 17 to the test odors (adaptation with the mixture of four odors): The mean response strengths of glomerulus 17 to the individual test odors during the odor stimulation were represented in this figure using the colored bars. Each set of three bars with three different colors represented the responses to the individual odors during the three recording conditions as mentioned in Fig. 5A. The x and y-axes represented the same variables as described in Fig. 5A. The significant differences were denoted with the asterics (results of the statistical tests were given in table-4; appendix-2).

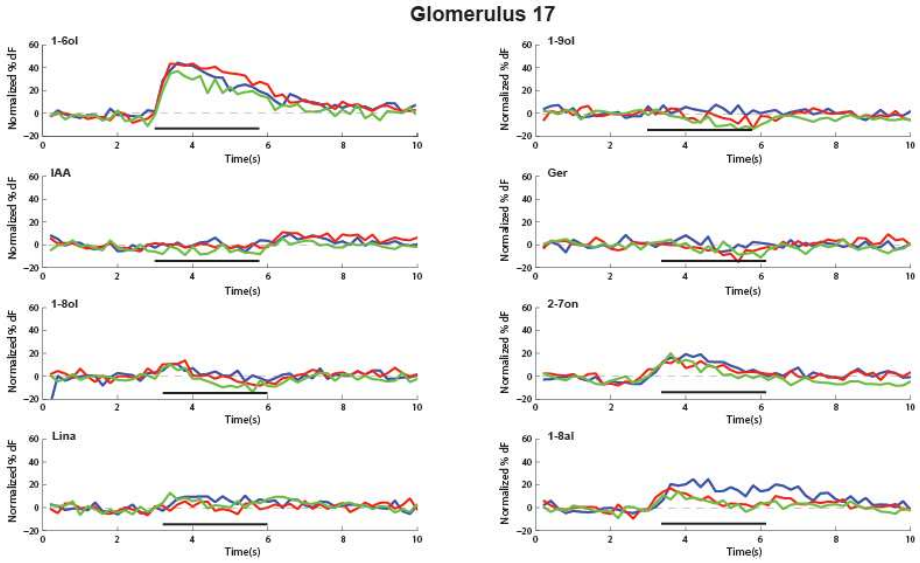
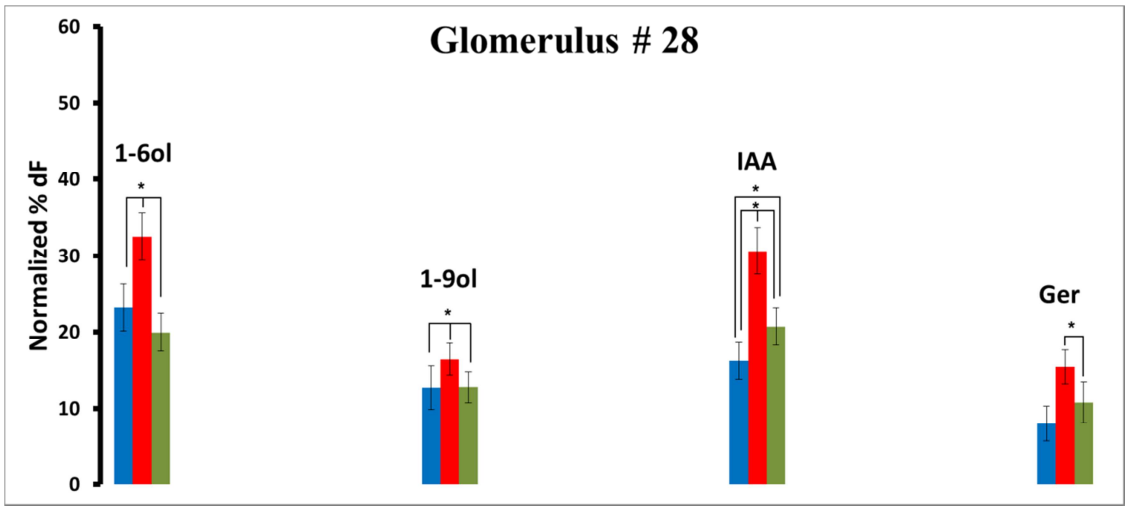


Fig. 5D: Mean response time traces of glomerulus 17 to the test odors (adaptation with the mixture of four odors): Mean response time traces of glomerulus 17 to the test odors were represented here with eight the sub-plots (abbreviations of the odor names were mentioned on the top left corner of the sub-plots). Each of the sub-plots showed the three time traces coded with the same three colors (blue, red and green) to represent the three recording conditions (as in Fig. 5B). The x and y axes represented the same variable as mentioned in Fig. 5B.

enhancement in responses (found also in the time traces; Fig. 5D) was found during the adaptation compared to the other two conditions (results of the statistics shown in table-4; appendix-2). In addition, the mean response strength for 1-6ol was found to decrease after the adaptation compared to 'before' (Fig. 5C). RM-ANOVA showed the non-significant response × condition effect ($F(28, 378) = 1.4$, $p = 0.056$) as well as the non-significant change in mean response strength between the experimental conditions ($F(2, 27) = 1.8$, $p = 0.17$) for 1-6ol (data not shown for other odors).

Glomerulus 28

The mean response strength of glomerulus 28 was found to increase significantly during the colony odor adaptation compared to both 'before' and after adaptation (Fig. 4A). This hold true in the odor-wise analysis as majority of the odors (results of the statistical tests are shown in table-5; appendix-2) showed the response enhancement during adaptation



compared to the other two conditions (Fig. 5E). Responses to 1-octanal decreased both 'during' and after adaptation compared to 'before'. These differences were also visible in

the odor stimulation windows of the mean response time traces of the individual test odors (Fig. 5F).

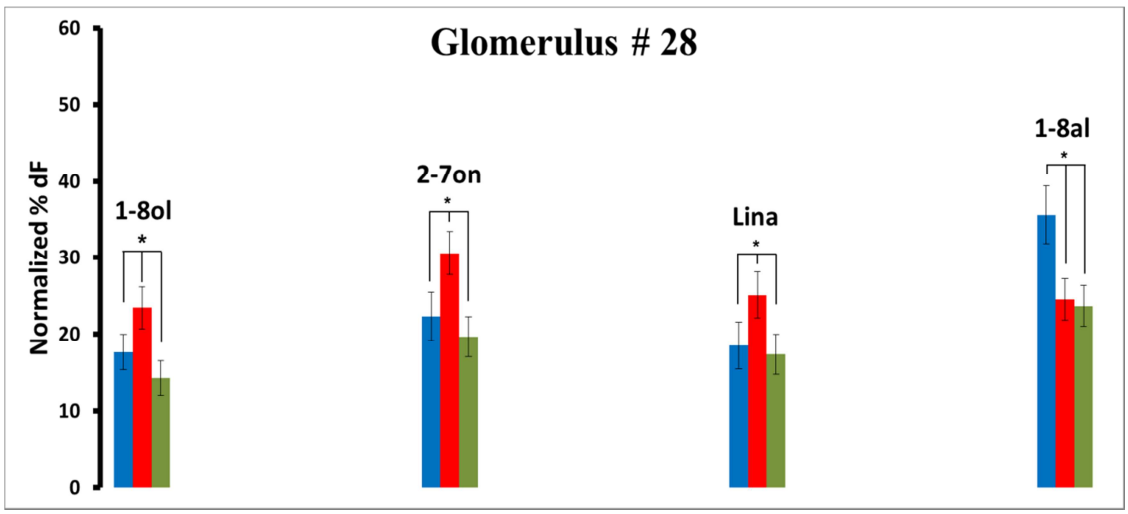


Fig. 5E: Mean responses of glomerulus 28 to the test odors (adaptation with the colony odor): The mean response strengths of glomerulus 28 to the individual test odors (1-hexanol (1-6ol), 1-nonanol (1-9ol), isoamyl acetate (IAA), geraniol (Ger), 1-octanol (1-8ol), 2-heptanone (2-7on), linalool (Lina), 1-octanal (1-8al)) during the odor stimulation were shown here using the colored bars. Each set of three bars with three different colors represented the responses to the individual odors for the three recording conditions (blue, red and green were denoted before, during and after adaptation). The y-axis showed the mean response strengths of glomerulus 28 (mean ± standard error). The significant differences were denoted with the asterics (results of the statistical tests were given in table-5; appendix-2).

Repeated measurement ANOVA found the significant response × condition effect for 5 out of the 8 test odors (1-6ol: $F(28, 462) = 1.84$, $p = 0.005$; IAA: $F(28, 462) = 2.44$, $p = 0.00007$; 2-7on: $F(28, 462) = 1.56$, $p = 0.03$; 1-8ol: $F(28, 462) = 1.54$, $p = 0.03$; Ger: $F(28, 462) = 2.55$, $p = 0.0003$) which indicated the change in response strengths along the three conditions (data not shown for other odors), however, only for isoamyl acetate (IAA) significant difference in mean response strength between the experimental conditions was found ($F(2, 33) = 3.73$, $p = 0.03$). These results were similar with the previous results of the RM-ANOVA for this glomerulus (see page 33).

Adaptation with the synthetic odor mixture elevated the mean response strength of glomerulus 28 both ‘during’ and after the adaptation compared to ‘before’ (results of WMP test; Fig. 4D in appendix-2). Odor-wise analysis, however, found that responses to

majority of the odors were very weak (Fig. 5G) as found for glomerulus 17 (Fig. 5C), hence, no statistical analysis was performed on these data (see the mean response time traces; Fig. 5H). Only 1-hexanol showed the significant response enhancement 'during' compared to after adaptation (statistical analysis in table-6; appendix-2). RM-ANOVA found no (data not shown for individual odors) significant difference between the mean response strength of the pairs of experimental conditions for the test odors (including for 1-6ol: between during and after adaptation p = 0.07).

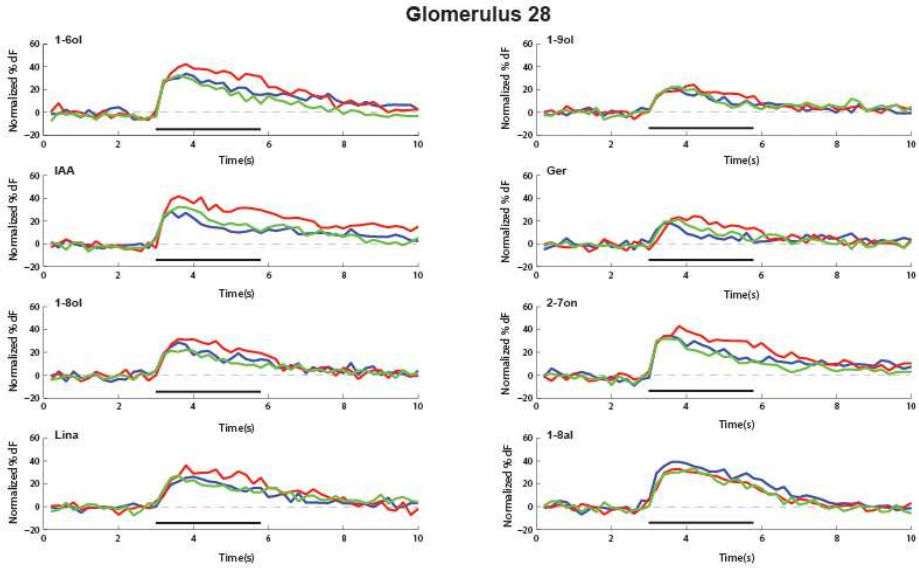


Fig. 5F: Mean response time traces of glomerulus 28 to the test odors (adaptation with the colony odor): Mean response time traces of glomerulus 28 to the test odors were shown in this figure with the eight sub-plots. The time traces with three different colors and the two axes in each sub-plots represented the same recording conditions and variables as mentioned in Fig. 5B.

Glomerulus 33

Glomerulus 33 showed the after-adaptation increase in the response strength during the colony odor experiment compared to the conditions of 'before' and during adaptation (results of WMP test; Fig. 4B, appendix-2). Responses to 1-nonaol and 1-octanol showed the adaptation induced decrease (Fig. 5I) as well as the post-adaptation recovery (results of the statistical tests were given in table-7; appendix-2) whereas the floral odor1-hexanol showed the after-adaptation enhancement in responses compared to 'during' (Fig. 5I).

Glomerulus 33 showed no change in responses for 1-octanal; however, IAA, geraniol and linalool showed very weak responses during all conditions (no statistics done for these

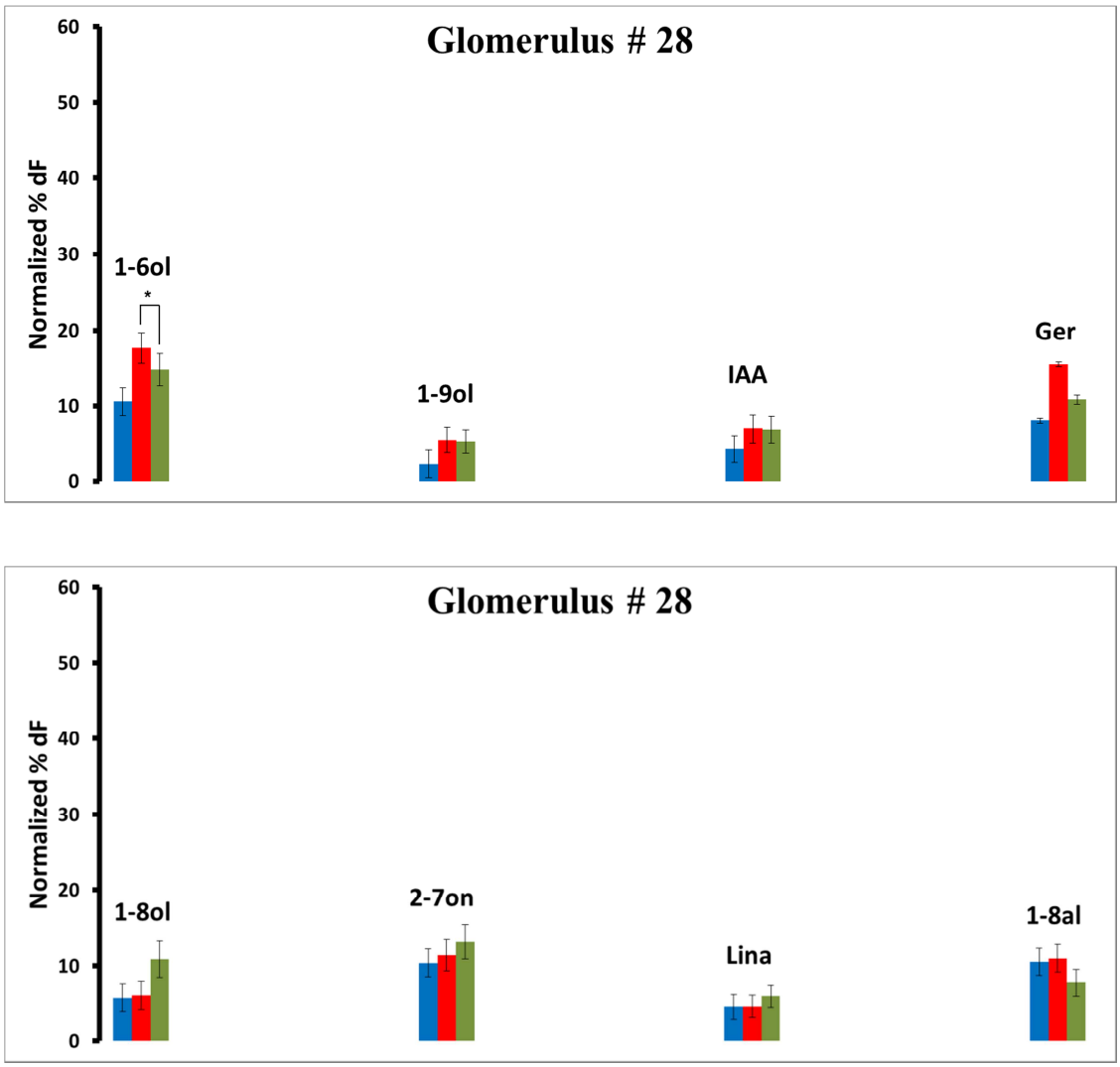


Fig. 5G: Mean responses of glomerulus 28 to the test odors (adaptation with the synthetic odor mixture): The mean response strengths of glomerulus 28 to the individual test odors during the odor stimulation were shown here using the colored bars. Each set of three bars with three different colors represented the responses to the individual odors for the three recording conditions as described in Fig. 5A. The x and y axes represented the same variables as mentioned in Fig. 5B. The significant differences were denoted with the asterics (results of the statistical tests were given in table-6; appendix-2).

odors). These effects were visible in the odor stimulation windows of the mean response time traces of this glomerulus (Fig. 5J). Repeated measurement ANOVA found no

significant difference in mean response strength between the three experimental conditions for 1-hexanol, 1-nonaol and 1-octanol (results not shown).

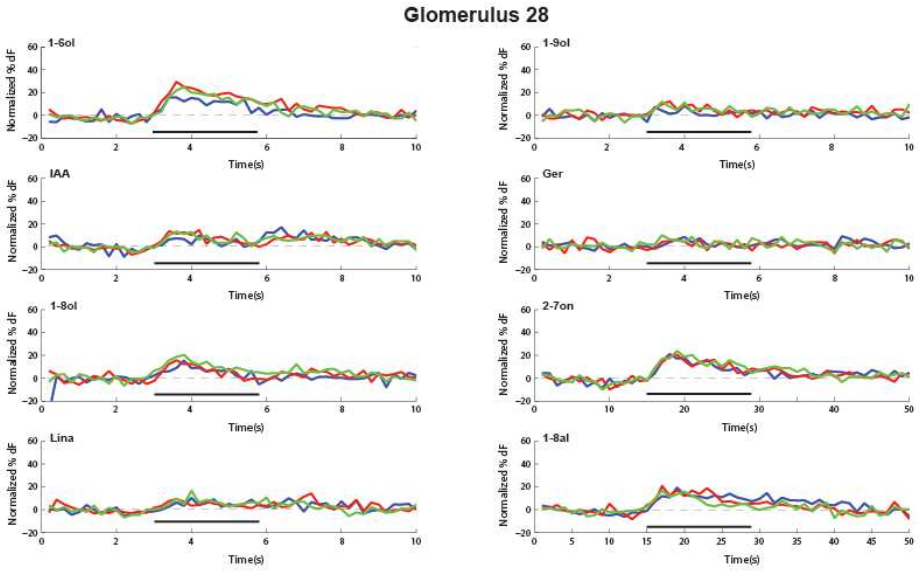


Fig. 5H: Mean response time traces of glomerulus 28 to the test odors (adaptation with the synthetic odor mixture): Mean response time traces of glomerulus 28 to the test odors were shown here with the eight sub-plots (odor names were mentioned on the top left corner of the sub-plots). Each of the sub-plots showed the three traces coded with the same three colors (blue, red and green) to represent the three recording conditions (same as in Fig. 5B). The x and y axes represented the same variables as described in Fig. 5B.

Adaptation with the synthetic odor mixture reduced (Fig. 4D) the mean odor response strength of glomerulus 33 after the removal of adaptation compared to 'during'. The same effect was found for 1-6ol as the mean response strength after the adaptation decreased compared to 'during' (Fig. 5K). However, the mean responses during the adaptation were found to decrease for 2-heaptanone compared to both 'before' and after adaptation (Fig. 5K). 1-octanal showed the gradual decrease (see table-8 for statistical analysis; appendix-2) in response strength along the three experimental conditions (confirmed also by the mean response time traces; Fig. 5L) as found before for glomerulus 17 (Fig. 5C). RM-ANOVA like the colony odor experiment also found no significant difference between the mean odor response strengths of the three conditions for this glomerulus (results not shown).

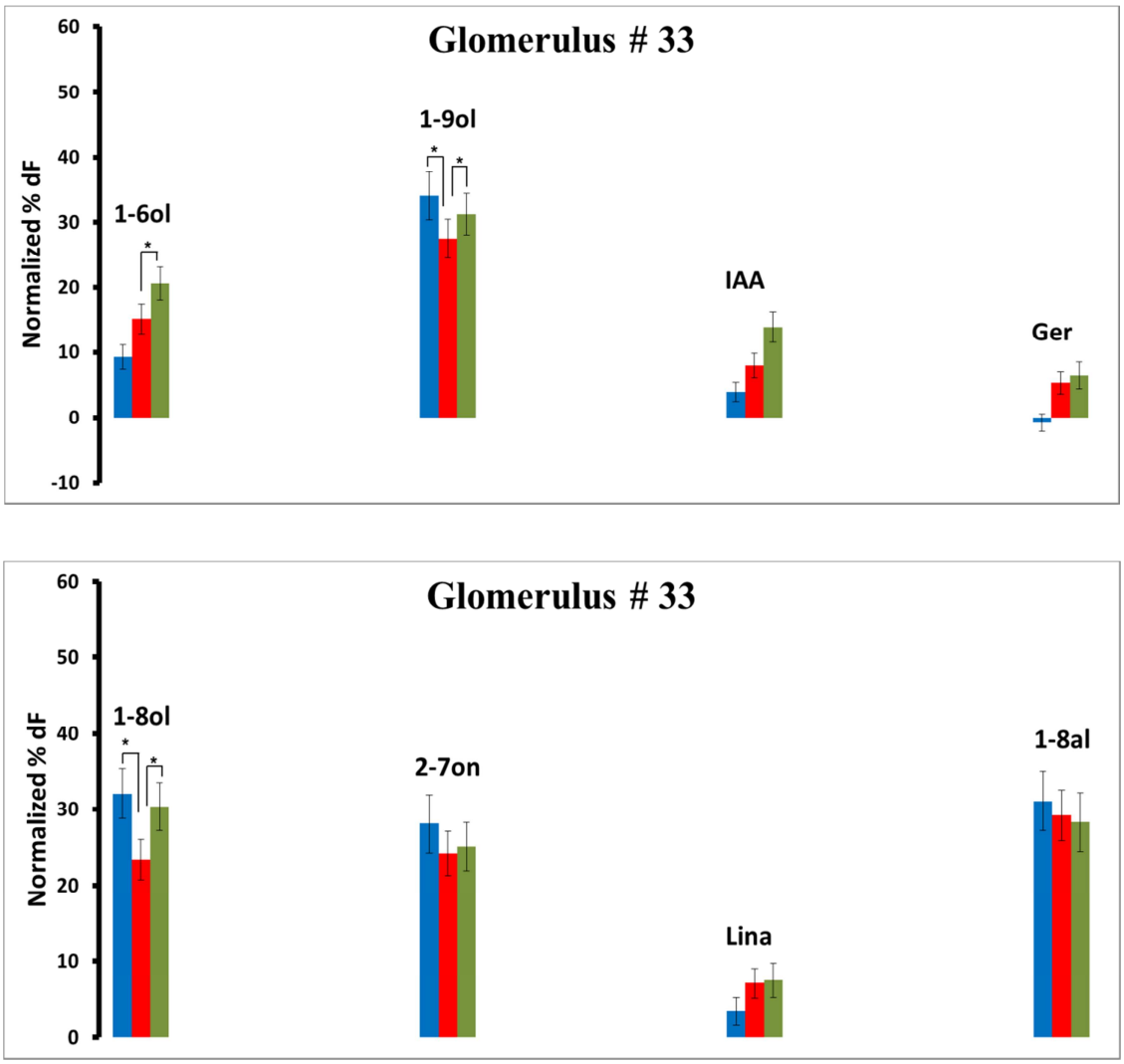


Fig. 5I: Mean responses of glomerulus 33 to the test odors (adaptation with the colony odor): The mean response strengths of glomerulus 33 to the individual test odors (1-hexanol (1-6ol), 1-nonanol (1-9ol), isoamyl acetate (IAA), geraniol (Ger), 1-octanol (1-8ol), 2-heptanone (2-7on), linalool (Lina), 1-octanal (1-8al)) during the odor stimulation were represented in this figure. Each set of three bars with three different colors represented the responses to the individual test odors during the three recording conditions (same color code as described in Fig. 5A). The y-axis represented the mean response strength of glomerulus 33 (mean ± standard error). The significant differences were denoted with the asterics (results of the statistical tests were given in table-7; appendix-2).

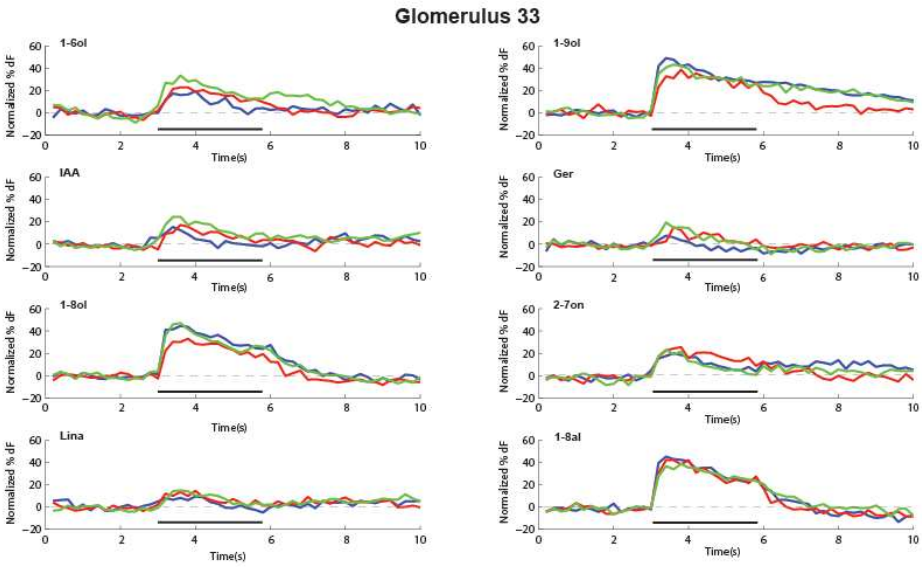


Fig. 5J: Mean response time traces of glomerulus 33 to the test odors (adaptation with the colony odor): Mean response time traces of glomerulus 33 to the test odors were shown here with eight sub-plots. Each sub-plot showed the three time traces coded with the same three colors (blue, red and green) to represent the three recording conditions as described in Fig. 5B. The x and y axes represented the same variables as mentioned in Fig. 5B.

Apart from the similarities found between the results of the gross and odor-wise analyses, many odor specific changes in glomerular response strength were disclosed in this analysis, which were not visible in the gross-response analysis. It was concluded from these results that olfactory adaptation either with the odor mixture of know (synthetic mixture) or unknown complexity (odor of the honeybee colony) did not increase or decrease the response strength of the antennal lobe glomeruli monotonically to the different odors. The same set of dendritic branches of the PNs innervating a certain glomerulus showed the odor specific change in response (increase or decrease) strength due to the long-term olfactory adaptation.

Weak responses of these three glomeruli to the entire set of test odors found in the synthetic mixture adaptation experiment *did not reflect the response scenario of the other glomeruli such as 36, 38, 42, 47 or 52*. This later group of glomeruli and others showed strong responses to the test odors throughout the different recording conditions when the synthetic odor mixture was used for adaptation.

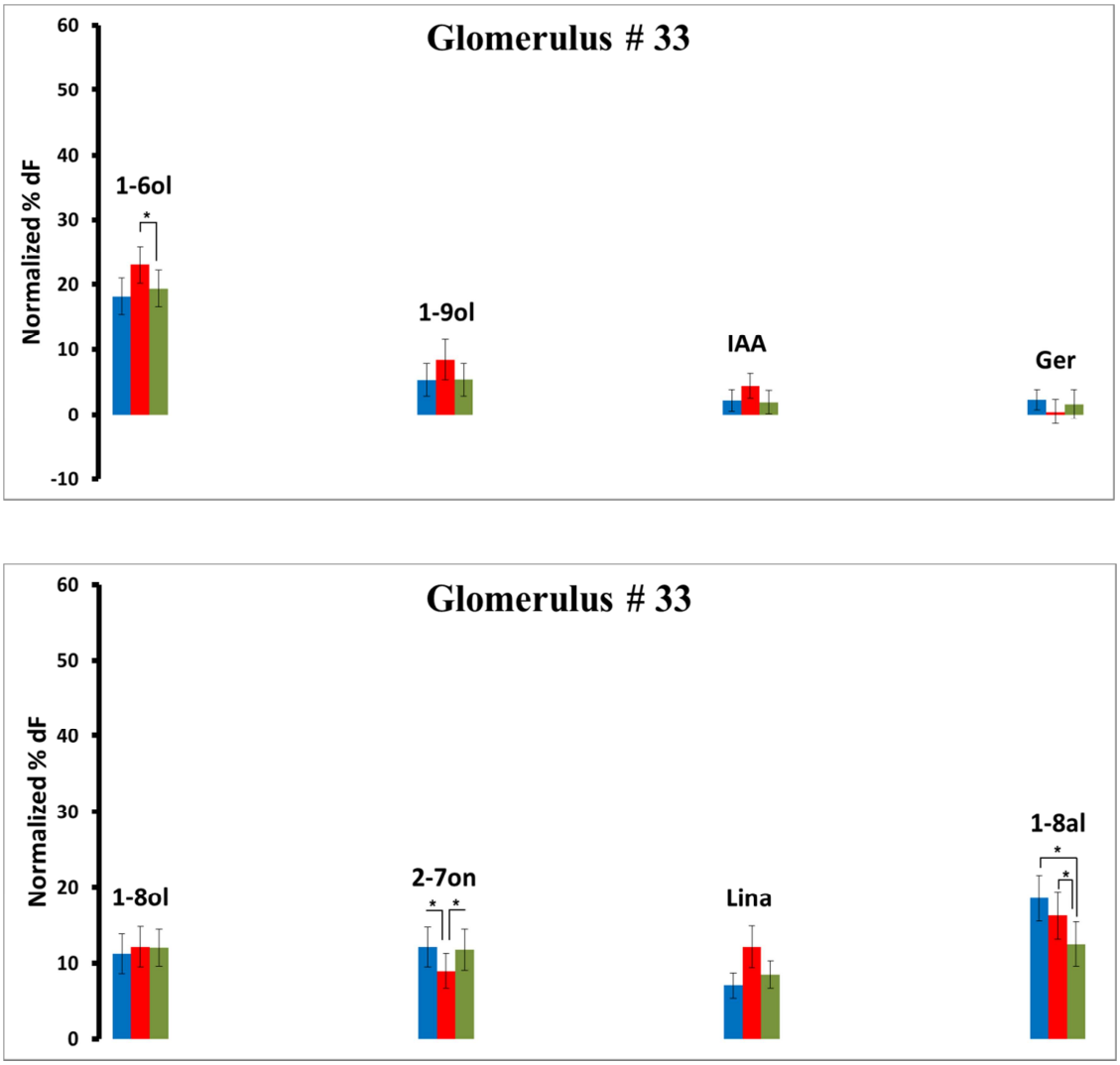


Fig. 5K: Mean responses of glomerulus 33 to the test odors (adaptation with the synthetic odor mixture): The mean response strengths of glomerulus 33 to the individual test odors during the odor stimulation were represented in this figure. Each set of three bars with three different colors represented the responses to the individual test odors during the three recording conditions (same color code as described in Fig. 5A). The y-axis represented the mean response strength of glomerulus 33 (mean ± standard error). The significant differences were denoted with the asterics (results of the statistical tests were given in table-8; appendix-2).

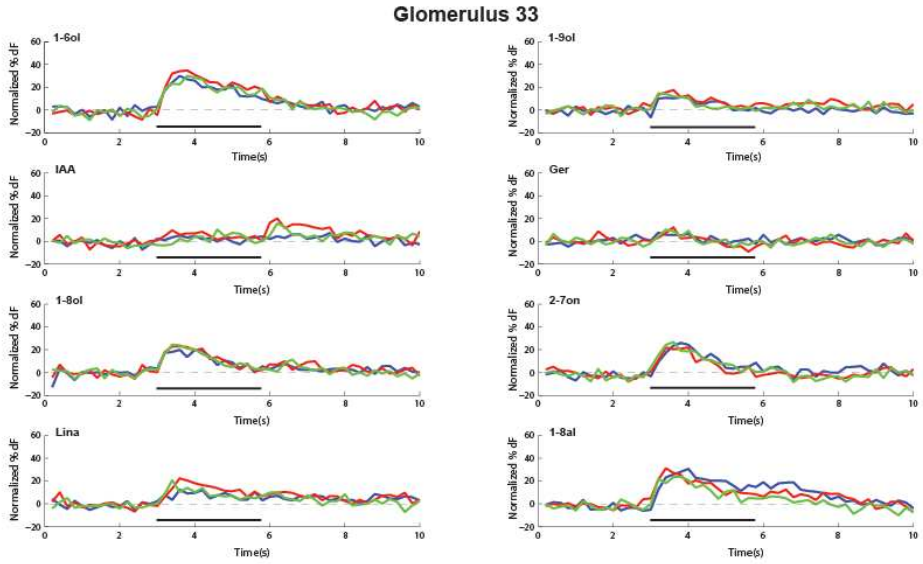


Fig. 5L: Mean response time traces of glomerulus 33 to the test odors (adaptation with the synthetic odor mixture): Mean response time traces of glomerulus 33 to the test odors were shown here with the eight sub-plots. Each sub-plot showed the three time traces coded with the same three colors (blue, red and green) to represent the three recording conditions as described in Fig. 5B. The x and y axes represented the same variables as mentioned in Fig. 5B.

4.5.5 Categories of test odors

We found that response strength of the individual glomeruli varied for the individual test odors between two adaptation experiments. However, response strength of the glomeruli in most of the cases was found to increase and decrease respectively for 1-hexanol and 1-octanal during adaptation with the two background odor stimuli compared to the un-adapted conditions. Here, it was analyzed whether the AL glomeruli in the pooled data (separately for the two adaptation experiments) showed any average pattern in responses to the individual test odors for the three experimental conditions or there were no patterns. We quantified the integrated response strengths (or areas under the response time traces during the odor stimulation) of the glomerular ensemble to the individual odors for the conditions of before, during and after adaptation and compared these values using the Wilcoxon matched pairs test. The results were also analyzed with the repeated measurement ANOVA using the intensity values (directly from the time traces) during the time of odor stimulation in place of the integrated intensities. The set of test odors

showed three types of change (described below) in glomerular response strength when the colony odor was used as the adaptation stimulus.

***Type-1*:** The antennal lobe glomeruli showed (Fig. 6A) two similar types of change for the type-1 odors; either a progressive increase in the integrated intensity of the calcium signals along the three conditions or an increase after the adaptation compared to both 'before' and 'during'. The floral odor, 1-hexanol and the sting alarm pheromone (SAP) odor isoamyl acetate showed the first and the nasonov pheromone component, geraniol showed the second type of change in glomerular responses. Glomerular ensemble also showed the increase in response strength for the other odor 1-nonanol but only the difference between 'after' and before adaptation was found significant (results of the Friedman ANOVA and the Wilcoxon matched pairs tests were given in table-9; appendix-2). Hence, the post-adaptation responses elicited by the type-1 odors never showed the adaptation recovery.

***Type-2*:** Only 1-octanal was found in this category (Fig. 6A) with the adaptation recovery found in the response strength of the glomerular ensemble. However, the mean value of the integrated intensity decreased significantly during adaptation compared to the other two conditions.

***Type-3*:** The other two sting alarm pheromone odors viz. 2-heptanone and 1-octanol along with the floral odor linalool were incorporated in this category. For the type-3 odors no significant change in glomerular response strength was found between the three conditions (Fig. 6A). However, common with the other floral odors (1-6ol and 1-9ol), linalool also showed the increase in glomerular response intensity both 'during' and after the adaptation compared to before, but these differences were not significant.

RM-ANOVA (performed with the normalized % delta-F values in place of the integrated intensities) found the significant response × condition effect for all of the test odors (data not shown), although the AL glomeruli showed the significantly different mean responses only for 1-hexanol ($F(2, 795) = 3.4$, $p = 0.03$) and isoamyl acetate ($F(2, 795) = 7.32$, $p = 0.0007$). Bonferroni post hoc test confirmed the significant increase in mean responses(for both odors) after the adaptation compared to 'before' (1-hexanol: $p = 0.02$, Isoamyl acetate: $p = 0.0004$), without any significant difference found between the 'during' and before adaptation. Current results of the WMP test (Fig. 6A) were in line

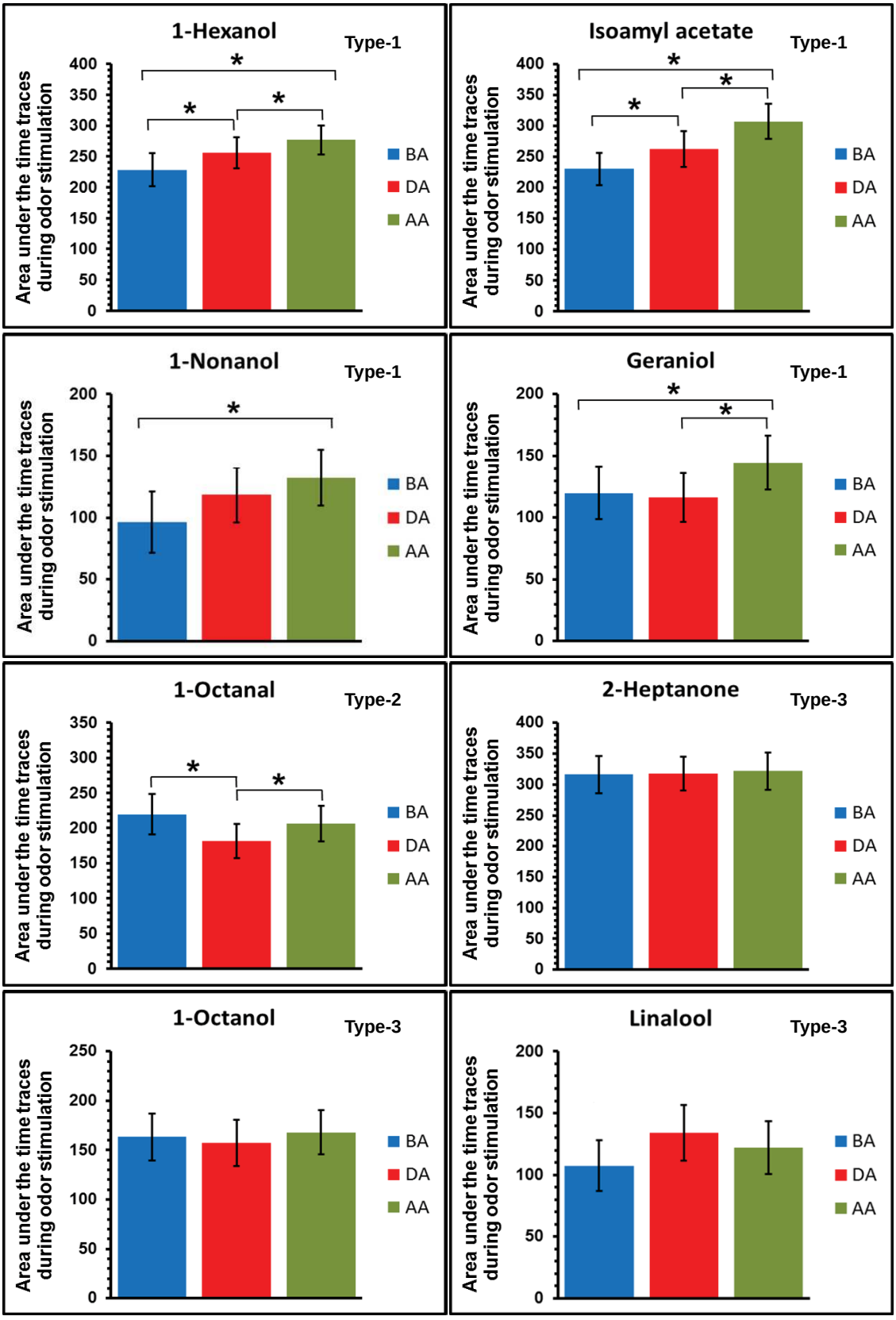

1-Hexanol
Type-1
Isoamyl acetate
Type-1
1-Nonanol
Type-1
Geraniol
Type-1
1-Octanal
Type-2
2-Heptanone
Type-3
1-Octanol
Type-3
Linalool
Type-3
Area under the time traces during odor stimulation
BA
DA
AA
*

Fig. 6A: Odor categories in the adaptation experiment with the colony odor: Three different odor types (mentioned at the top right corner of each sub-plot) emerged when the glomeruli were adapted with the colony odor. The categorization was based on the type of changes found in the glomerular response strength to the test odors during the three recording conditions. Integrated response intensities or areas under the time traces of the glomerular responses to the odors were calculated and represented on the y-axis (mean ± standard error). Each of the eight sub-plots showed the three colored bars (blue, red and green) and abbreviations (BA, DA and AA) which respectively reprsented the same color codes and abbreviations of three experimental conditions as described in Fig. 4A. Type-1 had the maximum number of members (4) with the gradual enhancement found in the glomerular response strength along the different conditions. Type-3 odors with 3 members showed no significant change in response strength between the conditions. Friedman ANOVA and Wilcoxon matched pairs test (with Bonferroni correction) were performed to compare between the mean response strength of the three conditions. The results of the statistical tests were given in table-9 (appendix-2); the significant differences were only denoted in the sub-plots by the asterics.

with the previous results (Fig. 5A – 5L) for 1-hexanol as the antennal lobe glomeruli showed increase in response strength for this odor during the adaptation with colony odor compared to the condition 'before'. Although, 1-octanal unlike before (Fig. 5A – 5L) did not show the progressive decrease (Fig. 6A) in integrated response strength of the glomeruli, however showed the significant decrease during the adaptation compared to the other two conditions. Differences in results between the two sets of statistical tests were found again like before, with the higher number of significant differences showed by the WMP test compared to the RM-ANOVA.

When the mixture of four pure odors or synthetic odor mixture was used for the background adaptation five different odor types emerged, which were described below.

***Type-1*:** Floral odor 1-hexanol and sting alarm pheromone (SAP) odor isoamyl acetate (IAA) were found (Fig. 6B) in this category. The description of this type was similar with the type-1 odor of the colony odor experiment (Fig. 6A); significant increase in the integrated response intensity of the glomeruli both 'during' and after the adaptation compared to the condition 'before'. Both 1-hexanol and isoamyl acetate (IAA) again (statistics shown in table-10; appendix-2) were found to enhance the glomerular response strength like before (Fig. 6A) however; unlike the colony odor experiment the post adaptation responses of IAA were recovered back to the un-adapted levels (no significant difference found between 'before' and after adaptation).

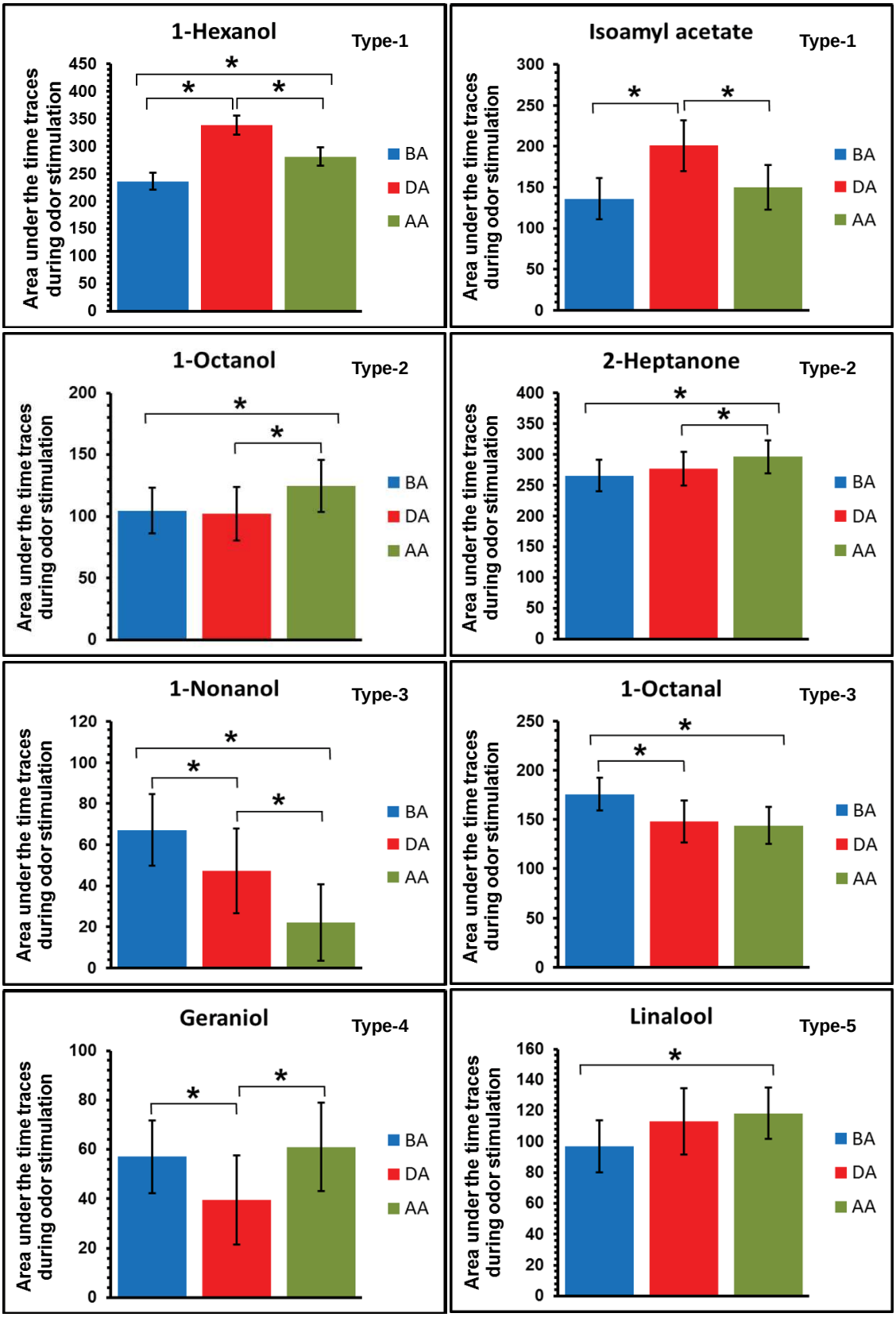

1-Hexanol
Type-1
Isoamyl acetate
Type-1
1-Octanol
Type-2
2-Heptanone
Type-2
1-Nonanol
Type-3
1-Octanal
Type-3
Geraniol
Type-4
Linalool
Type-5
Area under the time traces during odor stimulation
BA
DA
AA
*

Fig. 6B: Test odor categories during the adaptation with synthetic odor mixture: Five different odor types (mentioned at the top right corner of each sub-plot) emerged when the glomeruli were adapted with the mixture of four pure odors. More number of odor categories was found in the odor mixture experiment compared to the colony odor experiment. Odors like 1-hexanol and isoamyl acetate showed similar changes in glomerular responses for both adaptation stimuli but 1-octanol and 2-heptanone which did not show any change in the previous experiment showed the response enhancement after the adaptation in this experiment. Friedman ANOVA and the Bonferroni corrected Wilcoxon matched pairs test were performed to compare between the mean response strengths of the three conditions. The results of the statistical tests were given in table-10 (appendix-2); the significant differences were only denoted in the figure by the asterics.

***Type-2*:** For the type-2 odors significant increase (Fig. 6B) in the integrated response intensity of the glomerular ensemble was found after the adaptation compared to the other conditions. The two other sting alarm pheromone compound, 2-heptanone and 1-octanol were found in this category. These odors however, did not show any change in responses during the colony odor experiment (Fig. 6A).

***Type-3*:** Progressive decrease in the integrated response intensity was found from 'before' until the post adaptation conditions (Fig. 6B) in this category. 1-Octanal like in the previous experiment (Fig. 6A) was found to show the response decrease both during and after the adaptation compared to before. 1-nonanol, unlike the colony odor experiment (Fig. 6A) also showed the progressive decrease in glomerular response strength along the three conditions.

***Type-4*:** Type-4 odorant, geraniol showed the adaptation recovery in glomerular responses (Fig. 6B) along with the decrease in the mean response intensity during the adaptation compared to the other two conditions.

***Type-5*:** The type-5 odor (Fig. 6B), linalool like the colony odor experiment (Fig. 6A) did not show any change in the response intensities between the conditions of 'before' and during adaptation however, the after-adaptation responses did not recovered back to the to the un-adapted levels ('after' was significantly higher than 'before').

Repeated measurement ANOVA (performed with the normalized % delta-F values in place of the integrated intensities) like previously found the significant response × condition effect for all odors (data not shown), however, showed the significant condition effect (significant differences in mean response strength between the three conditions) only for 1-hexanol ($F(2, 675) = 16.02$, $p = 0.000000$; Bonferroni post hoc test supported the results of the WMP test: between 'before' and 'during' $p = 0.000000$, 'before' vs.

'after' p = 0.04 and 'during' vs. 'after' p =0.004), 1-nonanol (F (2, 675) = 5.48, p = 0.004; Bonferroni post hoc test showed significant difference between 'before' and 'after' p = 0.003 but no significant difference found between 'before' and 'during') and isoamyl acetate (F (2, 675) = 5.69, p = 0.003; Bonferroni post hoc test found significant difference between 'before' and 'during' p = 0.004 and between 'during' and 'after' p = 0.03).

The categorization of the test odors in these two adaptation experiments showed the differential effects of the two adaptation stimuli on the odor evoked responses of the glomeruli. These effects potentially indicated the activation of the different pathways of adaptation machineries in the honeybee AL which was unknown. However, the potentially different effects of the two adaptation stimuli showed some similar outcomes; similarities in the glomerular response patterns found for the odors 1-hexanol, isoamyl acetate (increase in response strength during adaptation than before adaptation) and 1-octanal (decrease in response strength during adaptation than before adaptation).

4.5.6 Adaptation changed the odor representation pattern in glomeruli

Euclidean distance measurement has been popularly used in the behavioral and physiological experiments to calculate the similarity between the response patterns of the odors both in the insect (Ditzen *et al.,* 2003; Rath *et al.,* 2011) and vertebrate (Olsson 1994; Bathellier *et al.,* 2008) models. Experiments reported here already showed that responses strengths of the AL glomeruli to the odors were changed during the adaptation and even persisted after the removal of the adaptation stimuli. Hence, Euclidean distances between the different conditions (before, during and after adaptation) were calcultaed for the inidividual odors and compared to investigate whether olfactory adaptation influenced the odor representation patterns in the odor coding space of the AL glomeruli.

1-Hexanol

Floral odor 1-hexanol showed (Friedman ANOVA and Wilcoxon matched pairs test: results of the statistical tests were given in table-11; appendix-2) significant differences in the Euclidean distances (ED) calculated for the pairs of experimnetal conditions (thee sets of ED values were calculated between the conditions: between before-during, during-after and before-after adaptation). Introduction of adaptation stmulus (condition), the

colony odor induced more dissimilarity in the overall glomerular response pattern of 1-hexanol (significantly higher mean ED value of the before-during comparison than the during-after) than the removal of the adaptation stimulus (condition). However, removal of the background adaptation stimulus was found to enhance this dissimilarity further compared to the initial condition of no-adaptation (significantly higher mean ED value of the after-before comparison compared to the before-during). In fact, the highest mean value of ED was found in the comparison between 'before' and 'after' adaptation (Fig. 7A). Repeated measurement ANOVA supported these results as the mean ED values quantified with the condition pairs were found to show significant differences (significant condition effect: $F(2,42) = 5.03$, $p = 0.01$). Bonferroni post hoc test showed the significant difference ($p = 0.01$) between the maximum (comparison between 'after' and 'before') and minimum (comparison between during-after) mean ED values of the condition pairs. These results confirmed the long lasting effects of the colony odor adaptation since the glomerular respresentation patterns of 1-hexanol showed significant difference between the post-adaptation and the un-adapted conditions (before adaptation) after the interval of 5 min for adaptation recovery. When the synthetic odor mixture was used for adaptation, significant differences were found (results of the statistical tests were given in table-12; appendix-2) between the three sets of ED values quantified from the pairs of recording conditions (Fig. 7A). Like before (colony odor experiment) minimum mean ED value was found when the response patterns were compared between during and after adaptation. However, (unlike the previous result) adaptation with the synthetic odor mixture led to the highest separation in glomerular reresesntation pattern (highest mean ED value) and the removal of adaptation stimulus decreased the Euclidean distance significantly comapred to the introduction of the adaptation condition (significantly lower mean ED value of the after-before pair compared to the before-during pair). RM-ANOVA showed the significant condition effect ($F(2,42) = 8.58$, $p = 0.0007$) as well as the significantly different mean ED values for the before-during and during-after ($p = 0.0004$) comparisons.

1-Nonanol

1-nonanol, like 1-hexanol showed significant differences in Euclidean distances (table-11; appendix-2) calculated for the pairs of experimental conditions (ED values were calculated between the three pairs of condition: before-during, during-after and before-after adaptation with the colony odor). Adaptation with the colony odor led to the highest separation in gloemrular response pattern (highest mean ED value) of 1-nonanol. Minimim mean value of ED was found in the comparison between 'during' and after adaptation (Fig. 7B). This showed that adaptation-induced changes in the glomerular representation pattern of 1-nonanol did not show the recovery after the removal of adaptation stimulus. However, unlike 1-hexanol removal of adaptation reduced the ED values significantly compared to the introduction of the adaptation stimulus (significantly lower mean ED value of the after-before comparison compared to the before-during comparison). Repeated measurement ANOVA showed the significant condition effect ($F(2,42) = 5.40$, $p = 0.008$), along with the significantly different mean ED values between the before-during and during-after comparisons (Bonferroni post hoc test: $p = 0.008$).

When the synthetic odor mixture (Fig. 7B) was used as adaptation stimulus Friedman ANOVA and Wilcoxon matched pairs test (table-12; appendix-2) found significant differences between the three sets of ED values. Adaptation condition induced more dissimilarity in the glomerular respresentation pattern of 1-nonanol compared to the removal of the adaptation stimulus. This was visible in the significantly higher mean ED value for the before-during comparison then the during-after pair. This again showed that

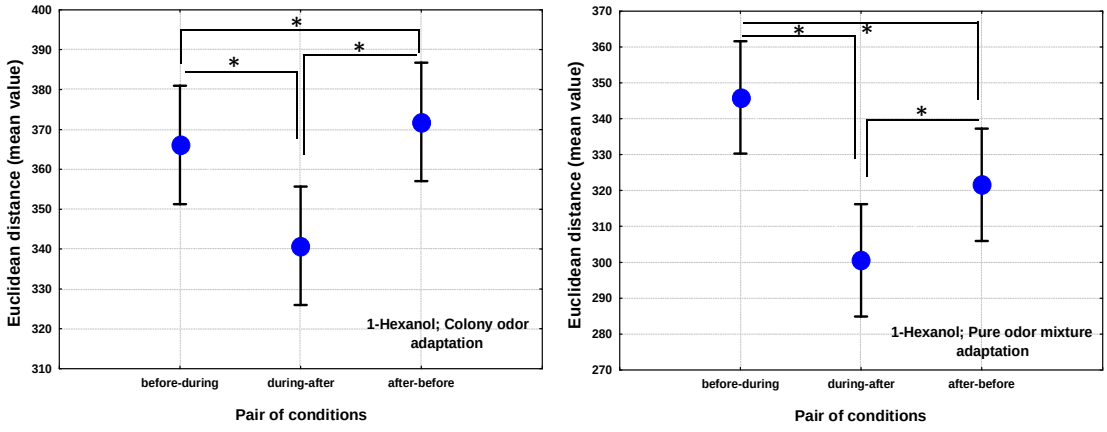


Fig. 7A: Comparison between the Euclidean distances calculated for 1-hexanol between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot represented the mean (blue-filled circles) Euclidean distances (EDs) quantified for 1-hexanol between the three pairs of conditions (between before-during, during-after and between after-before adptation) in the adaptation experiment with the colony odor. Introduction of adaptation stimulus induced more separation in glomerular response pattern of 1-hexanol compared to the removal of adaptation stimulus. Removal of the colony odor stimulus led to further increase in the glomerular representation patters compared to the un-adapted condition (significantly higher mean value of the after-before pair compared to the before-during comparison). Significant differences between the mean ED values were denoted with the asterics.
The second sub-plot showed the mean (blue-filled circles) Euclidean distances (EDs) quantified for 1-hexanol between the pairs of conditions (between before-during, during-after and between after-before adptation) in the adaptation experiment with the synthetic odor mixture. Introduction of adaptation stimulus led to the highest seperation in glomerular representation pattern (GRP) of 1-hexanol however, unlike the previous experiment removal of the adaptation stimulus did not show any further increase in the GRP compared to the un-adapted condition, rather significant decrease in mean ED value was found for the after-before compared to the before-during comparison. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

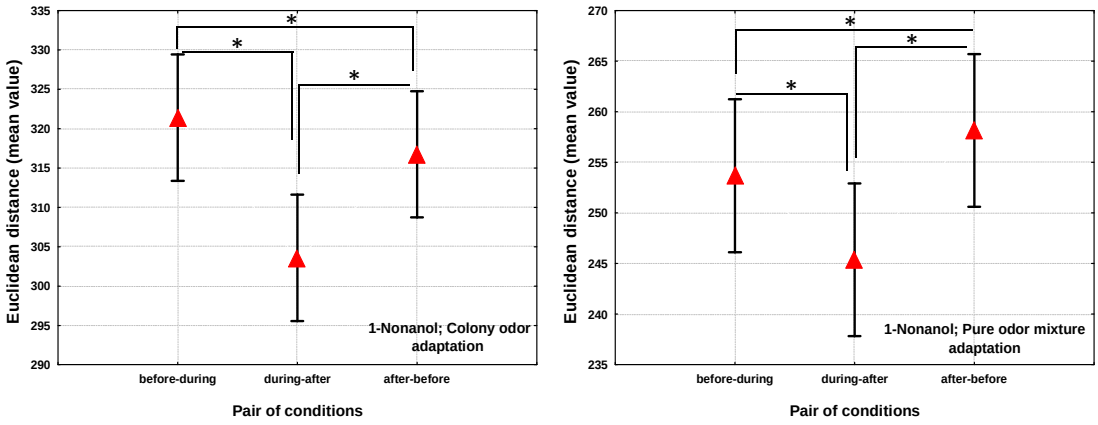


Fig. 7B: Comparison between the Euclidean distances calculated for 1-nonanol between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot represented the mean (red-filled triangle) Euclidean distances (EDs) quantified for 1-nonanol between the pairs of conditions (between before-during, during-after and between after-before adptation) in the adaptation experiment with the colony odor. Introduction of adaptation stimulus led to the highest seperation in glomerular representation pattern (GRP) of 1-nonanol however, removal of the adaptation stimulus showed the significant decrease in mean ED value (mean ED value in after-before comparison was less than the before-during comparison). Significant differences between the mean ED values were denoted with the asterics.
The second sub-plot represented the mean (red-filled triangle) Euclidean distances (EDs) quantified for 1-nonanol between the pairs of conditions (between before-during, during-after and between after-before adptation) in the adaptation experiment with the synthetic odor mixture. Introduction of adaptation stimulus induced more separation in glomerular response pattern of 1-nonanol compared to the removal of adaptation stimulus. Removal of the colony odor stimulus

led to further increase in the glomerular representation patters compared to the un-adapted condition (significantly higher mean value of the after-before pair compared to the before-during comparison). Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

adaptation induced changes in glommerular representation pattern (GRP) of 1-nonanol did not show the recovery when the adaptation condition was withdrawned, rather more separation in response pattern was found after the removal of the adaptation stimulus (significantly higher mean value of the after-before pair compared to the before-during comparison). However, RM-ANOVA unlike before showed no significant difference (meaning no change in GRP of 1-nonanol between the three conditions) between the sets of ED values (condition effect: $F(2,42) = 3$, $p = 0.06$). This discrepancy in results between the two sets of statistical tests indicated that adaptation effect of the syntheric odor mixture was weaker than the colony odor.

Isoamyl acetate

The sting pheromone component isoamyl acetate (IAA) showed significant differences in the Euclidean distances between the three recording conditions (table-11; appendix-2) (with the highest mean ED value found between the before-after comparison and the lowest between the during-after comparison) when the glomeruli were adapted with the colony odor (Fig. 7C). Adaptation condition significantly increased the distance in the glomerular response pattern of IAA compared to the removal of adaptation condition (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular respresentation pattern of IAA remained dissimilar after the adaptation (without further change in the ED; non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition due to the long lasting effects of adaptation. RM-ANOVA showed the non-significant condition effect ($F(2,42) = 2.13$, $p = 0.13$), meaning that there was no significant difference found between the mean ED values of the different pairs of comparisons. Contradictions bewteen these two sets of statistical tests probably indicated the fact that RM- ANOVA only highlighted the stronger differences (or effects) compared to the results of the Friedman ANOVA or WMP test which also picked up the weaker effects while

comapring the columns of values of the variables. Synthetic odor-mixture adaptation showed the identical (Fig. 7C) change in mean ED values (Friedman ANOVA and the WMP test) of the three pairs of comparisons (see table-12; appendix-2). Contrary to the results (significant differences found between the mean ED values) of the WMP test, RM-ANOVA again showed no significant change (Condition effect: F (2,42) = 0.88, p = 0.4) between the three sets of ED values.

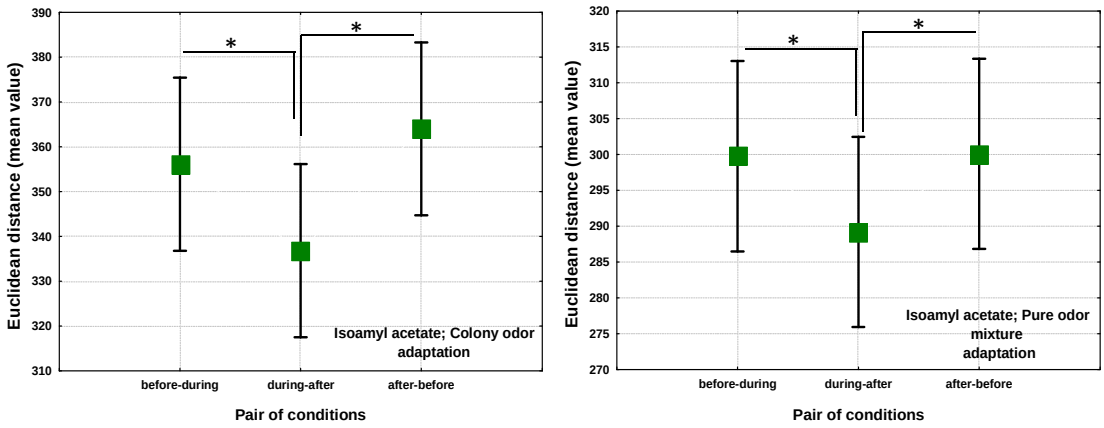


Fig. 7C: Comparison between the Euclidean distances calculated for isoamyl acetate between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot represented the mean (squares filled with green color) Euclidean distances calculated for isoamyl acetate (IAA) between the pairs of conditions in the adaptation experiment with the colony odor. Adaptation induced significantly more separation in glomerular response pattern of IAA compared to the removal of the adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular response patter of IAA after the adaptation remained different (without further change in the EDs; non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition due to the long lasting effects of adaptation.
The second sub-plot showed the mean (squares filled with green color) Euclidean distances calculated for IAA during the adaptation experiment with the synthetic odor mixture. Identical changes in Euclidean distances along the experimental conditions were found with the colony odor adaptation experiment. The x and y axes reprsented the same parameters as mentioned in Fig. 7A. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

Geraniol

The floral as well as the nasonov pheromone compound, geraniol showed significant differences in the ED values calculated between the pairs of experimental conditions

(results shown in table-11; appendix-2) with the maximum mean ED value found in the comparison of response patterns between 'before' and during adaptation. Adaptation with the colony odor (Fig. 7D) significantly increased the dissimilarity in the GRP of geraniol compared to the removal of adaptation stimulus (significantly higher mean ED value of before-during comparison compared to both during-after and after-before comparisons). However, unlike other odors, mean Euclidean distance between 'after' and before adaptation also decreased (not significantly) compared to the mean ED value found between 'before' and during adaptation. RM-ANOVA however, did not find any significant change in ($F(2,42) = 0.83$, $p = 0.4$) the glomerular response patterns along the different conditions. However, during the synthetic odor mixture adaptation experiment (Fig. 7D) the mean ED values of the different conditions were found to differ significantly in the WMP test (see table-12; appendix-2). The process of adaptation led to the highest separation in gloemrular response pattern (highest mean ED value) of geraniol and minimim ED value was found in the comparison between 'during' and after adaptation. This showed that adaptation-induced changes in the glomerular representation pattern of geraniol did not show the post-adaptation recovery back to the un-adapted state when the adaptation stimulus was removed (significantly lower mean ED value of the after-before comparison compared to the before-during comparison). RM-ANOVA also showed the significant condition effect: $F(2,42) = 5.12$, $p = 0.01$) along with the significant difference between the mean ED values of the before-during and during-after pairs (Bonferroni post hoc test: $p = 0.008$).

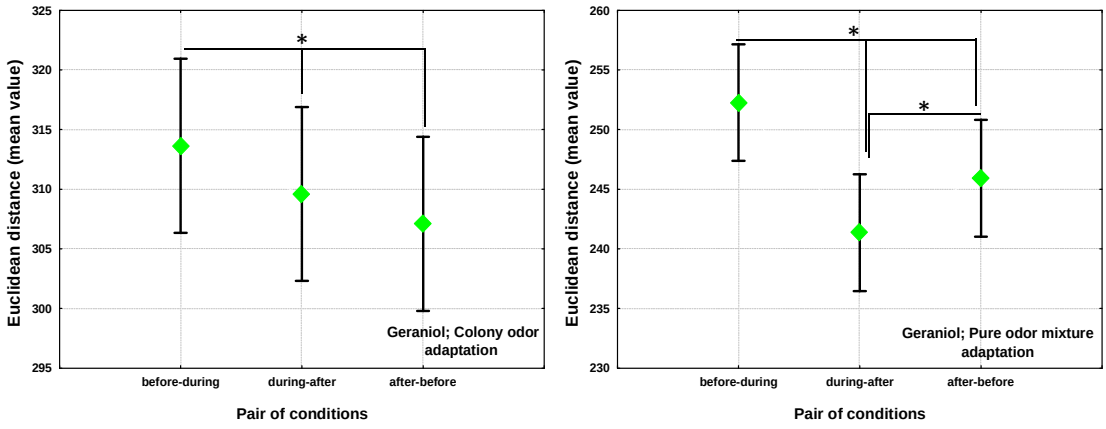


Fig. 7D: Comparison between the Euclidean distances calculated for geraniol between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot represented the mean (diamonds filled with green color) Euclidean distances calculated for geraniol in the adaptation experiment with the colony odor. Adaptation induced significantly more separation in the glomerular response pattern compared to the removal of the background adaptation (significantly higher mean ED value of the before-during pair compared to both during-after and after-before comparisons). However, unlike other odors, mean ED of the after-before comparison decreased (not significantly) compared to the comparison between during and after.
The second sub-plot represented the mean (diamonds filled with green color) Euclidean distances (EDs) quantified for geraniol between the pairs of conditions in the adaptation experiment with the synthetic odor mixture. Introduction of adaptation stimulus led to the highest seperation in glomerular representation pattern (GRP) of geraniol however, removal of the adaptation stimulus showed the significant decrease in mean ED value (mean ED value in after-before comparison was less than the before-during comparison). Meaning that adaptation-induced changes in GRP did not recover back to the un-adapted state when the background adaptation stimulus was removed. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

1-Octanol

1-octanol showed minimum number of significant differences (table-11; appendix-2) in the pair-wise comparisons (WMP test) of ED values in the colony odor experiment (Fig. 7E). No significant difference was found between the before-during and during-after pairs however, removal of the adaptation stimulus decreased the mean ED value as found in the significantly lower mean value of the after-before comparison compared to the mean value of the before-during comparison. This showed that post-adaptation glomerular response pattern of 1-octanol although showed the adaptation recovery but it was not fully recovered. RM-ANOVA also found no significant difference in the mean ED values (non-significant condition effect: $F(2,42) = 0.35$, $p = 0.7$). Hence, the effect of colony odor adaptation was not found strong like the other odors for 1-octanol. Adaptation condition with the synthetic odor mixture (Fig. 7E) induced significantly more separation in the response pattern of 1-octanol compared to the removal of adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair; table-12 in the appendix-2). Glomerular representation patter of 1-octanol after the adaptation remained dissimilar (without further change in the ED; non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition due to the long lasting effects of

adaptation. RM-ANOVA although did not find any significant difference between the mean ED values of the three pairs of comparisons (F (2,42) = 1.8, p = 0.16).

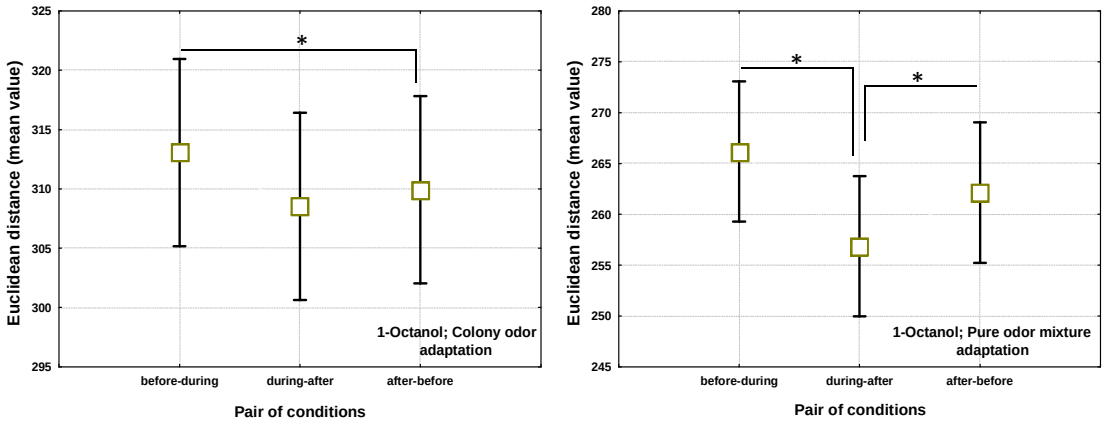


Fig. 7E: Comparison between the Euclidean distances calculated for 1-octanol between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot showed the mean (squares with green boarder) Euclidean distances calculated for 1-octanol between the pairs of conditions in the adaptation experiment with the colony odor. No significant difference was found between the before-during and during-after pairs however, removal of adaptation stimulus decreased the EDs as found in the reduced mean ED value of the after-before comparison compared to the mean value of the before-during pair. This meant that unlike other odors (1-6ol, 1-9ol, IAA, geraniol) glomerular response pattern of 1-octanol although did show more adaptation recovery however, did not recover back fully to the un-adapted pattern. Significant difference between the two mean ED values was denoted with the asterics.
The second sub-plot represented the mean (squares with green boarder) Euclidean distances calculated for 1-octanol in the adaptation experiment with the synthetic odor mixture. Adaptation induced significantly more separation in the glomerular response pattern compared to the removal of the adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular response patter of 1-octanol after the adaptation remained different (non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition due to the prolong after-effects of adaptation. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

2-Heptanone

This sting alarm pheromone odor 2-heptanone showed significant differences in mean ED values quantified for the three pairs of experimental conditions in the adaptation (Fig. 7F) experiment with the colony odor (table-11; appendix-2). Adaptation condition induced significantly more separation in the glomerular response pattern of 2-heptanone compared to the removal of adaptation stimulus (significantly lower mean ED value

found for the during-after pair compared to the before-during pair). Glomerular response patter of 2-heptanone after the adaptation remained dissimilar (non-significant difference between the after-before and the before-during pairs) compared to the un-adapted condition due to the prolong post-effects of adaptation. RM-ANOVA however, showed no significant difference between the mean ED values of the three experimental conditions (non-significant condition effect: F (2,42) = 0.41, p = 0.6). Adaptation experiment with the synthetic odor mixture (Fig. 7F) showed more number of significant differences (table-12; appendix-2). The minimum mean ED value was found in the comparison between 'during' and after adaptation. Introduction of adaptation stimulus or condition induced more dissimialrities or separation in the glomerular response pattern of 2-heptanone compared to the removal of the adaptation stimulus (condition). This was visible in the significantly higher mean ED value found between 'before' and during adaptation compred to the during-after comparison. This showed that adaptation induced changes in the glomerular representation pattern of the sting pheromone odor 2-heptanone did not show the adaptation recovery when the adaptation stimulus was removed, rather more dissimilarity in response pattern was found after the removal of adaptation stimulus compared to the un-adapted condition (significantly higher mean value of the after-before pair compared to the before-during pair). The difference between during-after with the after-before was also found significant. RM-ANOVA like the colony odor experiment showed no significant difference (F (2, 42) = 1.9, p = 0.16) in the ED values calculated between the pairs of experimental conditions.

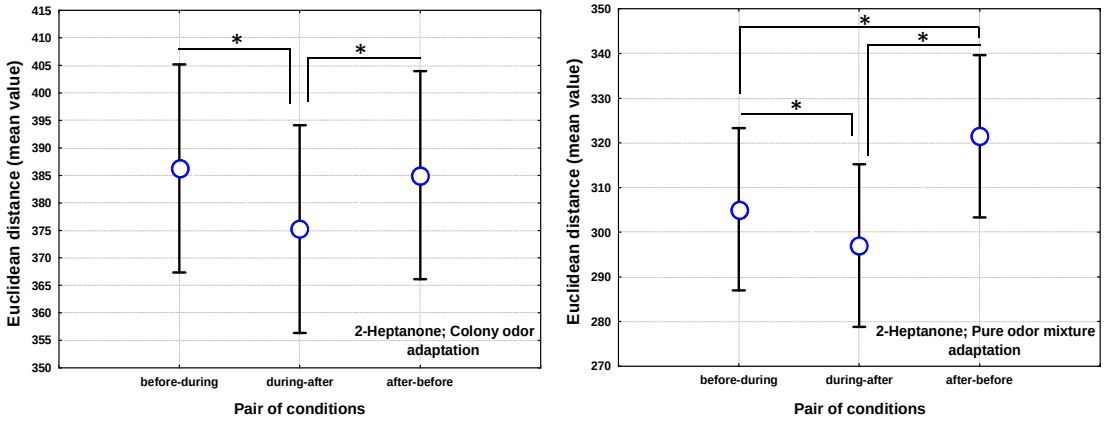


Fig. 7F: Comparison between the Euclidean distances calculated for 2-heptanone between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot represented the mean (circles with blue boarder) Euclidean distances calculated for 2-heptanone between the pairs of conditions in the adaptation experiment with the colony odor. Introduction of the adaptation stimulus significantly increased the EDs compared to the removal of the adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular response patter of 2-heptanone after the adaptation remained different (non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition.
The second sub-plot represented the mean (circles with blue boarder) Euclidean distances calculated for 2-heptanone during the adaptation experiment with the synthetic odor mixture. Adaptation condition induced more separation in glomerular response pattern compared to the removal of the adaptation condition. This was visible in the significantly higher mean ED value of the before-during compred to the during-after comparison. This showed that adaptation induced changes in glomerular representation of 2-heptanone did not recover back to the un-adapted pattern when the adaptation stimulus was withdrawned, rather more separation in response pattern was found after the adaptation compared to the un-adapted condition (significantly higher mean value of the after-before pair compared to the before-during pair). The difference between during-after with the after-before was also found significant. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

Linalool

Linalool showed no significant difference (Fig. 7G) in Euclidean distances in the Friedman ANOVA (table-11) as well as in the RM-ANOVA tests ($F(2,42) = 0.03$, $p = 0.9$) in the colony odor adaptation experiment. However, adaptation with the synthetic odor mixture (Fig. 7G) induced significantly more separation (Fig. 7G) in the glomerular response pattern (Table-12; appendix-2) of linalool compared to the removal of the adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular response pattern to linalool after the adaptation remained dissimilar (non-significant difference found between the after-before and the before-during pairs) compared to the un-adapted condition due to the long lasting after ffects of adaptation. RM-ANOVA contradicted this result with the non-significant condition effect ($F(2,42) = 2.28$, $p = 0.1$).

1-Octanal

1-Octanal showed significant differences in ED values quantified between the different experimental conditions (colony odor adaptation; Fig. 7H) both in the Friedman ANOVA (table-11) and in the RM-ANOVA tests ($F(2,42) = 5.5$, $p = 0.007$). Like other odors

minimum change in response patterns was found between 'during' and after adaptation and the maximum change was manifested between 'before' and after adaptation (Bonferroni post hoc test showed the significant difference between the two mean ED values; p = 0.005). Hence, the post-adaptation glomerular response patterns of 1-octanal did not recover back to the un-adapted levels. When the mixture of synthetic pure odorants was used for the adaptation, significant difference between the Euclidean distances were found in both (Table-12; appendix-2) statistical tests (RM-ANOVA showed the significant condition effect: F (2,42) = 6.2, p = 0.004). Significant difference between the maximum (calculated between 'before' and during adaptation) and the minimum (calculated between 'during' and after adaptation) mean ED values was found in the Bonferroni post hoc test (p = 0.003).

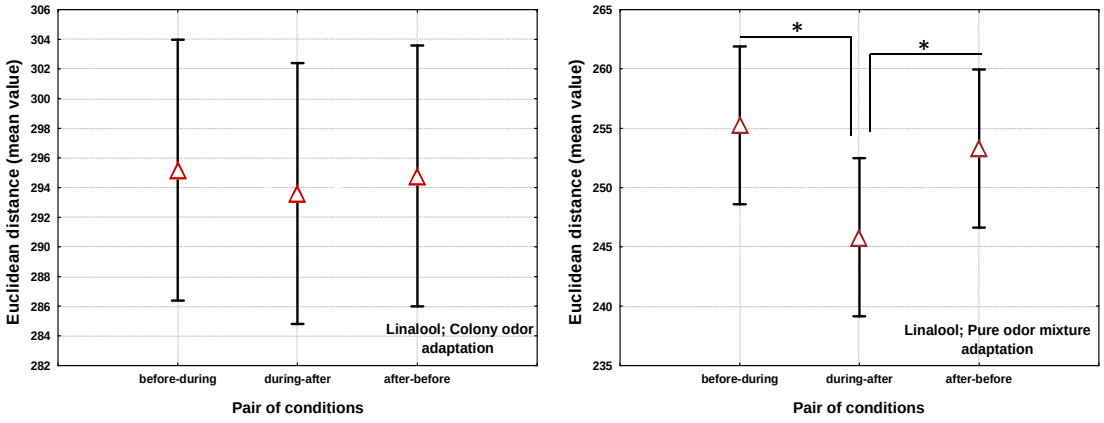


Fig. 7G: Comparison between the Euclidean distances calculated for linalool between the pairs of conditions in the two adaptation experiments: The first sub-plot represented the mean (triangles with red boarder) Euclidean distances (EDs) calculated for linalool between the three pairs of conditions in the adaptation experiment with the colony odor. No significant difference in the mean ED values was found in this case. However, adaptation with the synthetic odor mixture (the second sub-plot) significantly increased the distances in the glomerular response patterns of linalool compared to the removal of the adaptation stimulus (significantly lower mean ED value found for the during-after pair compared to the before-during pair). Glomerular response patter to linalool after the adaptation remained different (non-significant difference between after-before and the before-during pairs) compared to the un-adapted condition due to the prolong after-effects of adaptation. Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

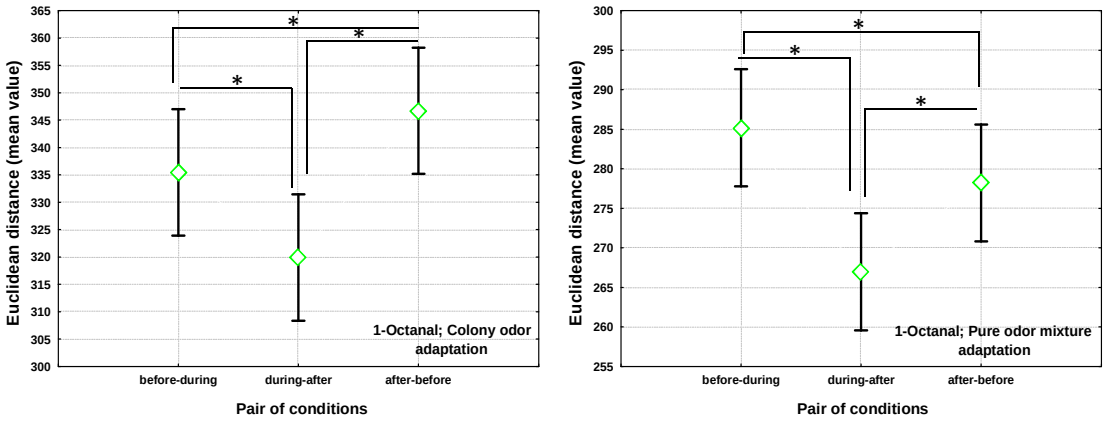


Fig. 7H: Comparison between the Euclidean distances calculated for 1-octanal between the pairs of experimental conditions in the two adaptation experiments: The first sub-plot showed the mean (diamonds with green boarder) Euclidean distances calculated for 1-octanal between the three pairs of conditions in the adaptation experiment with the colony odor. Adaptation condition induced more separation in glomerular response pattern compared to the removal of the adaptation background. Additionally, the adaptation induced changes in glomerular representation of 1-octanal did not recover back to the un-adapted state when the adaptation condition was withdrawned, rather more separation in response pattern was found after removal of adaptation stimulus compared to the un-adapted condition (significantly higher mean value of the after-before pair compared to the before-during pair).
The second sub-plot represented the mean (squares with green boarder) Euclidean distances calculated between the same three pairs of conditions in the adaptation experiment with the synthetic odor mixture. The process of adaptation led to the highest separation in gloemrular response pattern (highest mean ED value) however, removal of the adaptation stimulus signifcantly reduced the mean value of Euclidean distance (ED value of the after-before comparison was less than the value found between the before-during pair). No further change in ED value was found after the removal of adaptation stimulus (non-significant difference between the mean values of after-before and before-during comparisons). Significant differences between the mean ED values were denoted with the asterics. The x and y axes in both sub-plots respectively reprsented the pair of conditions and the mean ED values (mean ± 95% confidence interval).

4.6 Discussion

4.6.1 Methodological considerations

Honeybees, in our experiments were exposed to the constant flow of background odor stimulus for ~ 20 min for the physiological adaptation of the antennal lobe glomeruli. For both background odor stimuli viz. the colony odor and the mixture of four pure odors or the synthetic mixture no significant change in the intracellular calcium concentration of projection neurons was detected during this 20 min time. According to our definition of

adaptation (given previously) this did not confirm the adaptation of the glomeruli. Single odor stimuli used in these experiments (test odors) faithfully elicited calcium responses throughout the 3 sec time of odor delivery in the glomeruli. During the process of adaptation we recorded the glomerular calcium signals for 100 sec (10 times longer than odor measurements). It was rather surprising that such long recordings did not detect any calcium signals evoked by the background adaptation stimuli both during the onset of the stimuli as well as during the later time points. Behavioral adaptation in the adult fruit fly, *Drosophila melanogaster* (Störtkuhl *et al.,* 1999) was reported to arise within 15 sec of the odor exposure and from 30 min onwards in the *Caenorhabditis elegans* (Colbertand Bargmann 1995). Fast synaptic depression mechanism operating between the mitral/tufted cells of the lateral olfactory tract and the pyramidal cells (PRCs) of the piriform cortex was known to adapt the olfactory responses of the PRCs within 50 sec of odor exposure (Best and Wilson 2004; Best *et al.,* 2005). If similar type of fast synaptic depression mechanism (within the time scale of seconds) operates at the receptor neuron's input to the projection neuron in the honeybee antennal lobe in response to the continuous background of adapting stimuli, then we would be able to record the calcium signals after the stimulus onset. It was not understood why the signal acquisition protocol did not detect any responses however, electrophysiological recordings (with higher temporal resolution) in these experiments probably would have disclosed the response adaptation process of the antennal lobe neurons to the background odor stimuli.

4.6.2 Colony odor and synthetic odor mixture differentially influenced the odor response strength of the glomeruli

Background adaptation with the odor extracted from the honeybee colony reduced the mean response strength of the glomerular ensemble as found in the gross analysis. This overall inhibitory effect of the adaptation stimulus however, did not affect the odor evoked responses of the glomeruli as they showed the significant enhancement in responses during the time of odor stimulation. Rather the responses after the odor offset were found to contribute more to the overall inhibitory effect of the colony odor adaptation. Contrasting result was found when honeybees were adapted with the synthetic odor mixture. Gross analysis of the ensemble response strength as well as during the time

of odor stimulation revealed the significant increase in glomerular response strength during the adaptation compared to the initial state of no-adaptation. Common increase in glomerular response strength during the odor stimulation (3 sec) was found both when the odor mixture of unknown (colony odor) and known (synthetic mixture) complexities were used for background adaptation. However, the overall opposite effects of these two stimuli on the glomerular response strength indicated that these two background odor stimuli probably activated the different forms or pathways of adaptation in the network of the antennal lobe. In honeybee, physiological characterization of the adaptation mechanisms in the first or in the second order olfactory neurons was not reported. However, results found in our study showed that adaptation did not monotonically decreased the odor responses of the second order neurons (projection neurons), rather exhibited the dual effects of increase and decrease in the gross response strength of the AL glomeruli, depending on the identity of the adaptation stimulus. Similar adaptation induced enhancement or suppression in odor responses were found in the olfactory bulb neurons of rat (Mair 1982). In addition, mathematical modeling supported the possibility of enhancement in the sensitivity of mitral cells to the newly emerging odor in the pre-existing background of another odor stimulus (Li 1990).

4.6.3 Odor responses of the Glomeruli did not show the adaptation recovery

Adaptation-recovery of odor responses was expected in these experiments when honeybees were kept undisturbed for 5 min after the removal of the background odor stimuli. However, results of the Wilcoxon matched pairs test (WMP test) and the repeated measurement ANOVA revealed the further increase in glomerular response strength (on top of the adaptation induced increase) during the odor stimulation after the removal of the colony odor background. Results of these statistical tests however, showed the significant decrease in the post-odor offset responses after the removal of adaptation compared to the other two conditions when the background of synthetic odor mixture was used. In addition to these gross response analyses many individual glomeruli in both adaptation experiments did not show the odor response recovery after the stoppage of the adaptation stimuli. In the colony odor experiment, reduction of glomerular responses in addition to the increase was also found when the data was analyzed with the WMP test.

This was the other '*methodological consideration*' that our protocol did not show the adaptation-rescue of odor responses of the glomeruli back to the un-adapted levels. In *Drosophila* behavioral recovery from the state of olfactory adaptation was reported to take place within 1.5 min (Störtkuhl *et al.,* 1999) and in the receptor neurons of salamander, the process of olfactory de-adaptation was reported to occur within 6.5 min (Zufall *et al.,* 2000). These were the important information based on which the 5 min time of the adaptation recovery was selected in our protocol, which clearly found inadequate to rescue the odor responses of glomeruli after the constant exposure of the antennal lobe neuropil for ~ 40 min to the background adaptation stimuli (20 min + recording time for odor responses). However, the phenomena of prolong adaptation recovery was reported in other model systems such as in the *Caenorhabditis elegans* (over 3 hours of adaptation recovery in behavioral assay: Colbert and Bargmann 1995) and in the silkworm moth *Antheraea polyfemus* (receptor neurons were reported to take over 1 hour to recover; Kaissling *et al.,* 1987). In the housefly *Musca domestica*, effects of background adaptation with higher concentrations of pure odorant on the receptor's responses to the test odors did not recover fully within the 15 min time, used for the adaptation recovery (Kelling *et al.,* 2002). Mitral cells of the rat olfactory bulb were also reported to consume 30 – 50 min time for the response recovery when they were adapted for 1 hour with the constant background odor (Chaput and Panhuber 1982). It is possible that like the mitral cells of rat or the receptor neurons of *Musca domestica* or *Antheraea polyfemus*, honeybee projection neurons innervating the AL glomeruli also require longer period of time than 5 min for the adaptation recovery, which explains the limitation in our protocol. Additionally, we could not discard the possibility that prolong exposure to the background odor stimuli imparted some form of non-specific sensitization in the odor responses of glomeruli.

4.6.4 Glomeruli showed similar or dissimilar types of change in odor response strength

AL glomeruli were categorized into different types according to their types of change in the odor response strength along the conditions of before, during and after adaptation. Results of the WMP test showed that apart from the differential response patterns of the

same glomerulus with the two background adaptation stimuli, majority of the glomeruli showed the enhancement in odor response strength during adaptation compared to 'before'. Results of the RM-ANOVA test although limited the number of significant differences in both adaptation experiments, however, preserved the significant differences for the different patterns of change in response strength (e.g. gradual decrease in responses from the un-adapted through adapted into the post-adaptation conditions or adaptation induced increase in responses which persisted even after the removal of background adaptation stimuli). In both rat and mouse models cortical olfactory adaptation of the pyramidal cells (PRCs) due to the synaptic depression in input signals of the mitral/tufted cells not only ceased the responses of the PRCs to the unchanging background odor but also preserved its responses to the incoming new odor stimuli (Wilson 1998; Kadohisa and Wilson 2006). Mitral cells on the other were found to respond continuously to the trivial, static background both in absence and presence of the new odor stimulus. This specific pattern of cellular responses was considered as the possible mechanism contributes to the behavioral task of odor-background segmentation (Linster *et al.,* 2007). In our adaptation experiments projection neurons of the honeybee antennal lobe behaved like the rodent cortical pyramidal cells rather than the mitral cells (its vertebrate analogue). This although sounds anomalous; however, similar type of mechanism operating between the olfactory receptor neurons (ORNs) and projection neurons (PNs) might account for the response adaptation of the PNs to the constant background odor stimuli along with the enhancement in response strength of the PNs to the newly arriving test odors. This type of mechanism can preserve the selectivity in responses of the AL glomeruli to the odors under condition of olfactory adaptation. In addition, this also indicated that ORNs in honeybee might act like the receptor neurons in vertebrate and responded continuously to the background odor without any adaptation induced exhaustion (Lancet 1986; Pryor *et al.,* 1970). Alternately, prolong adaptation (~ 20 min) in these experiments probably reduced (inhibited) the strength of some of the ORN-LN (local inhibitory interneurons) connections, which led to the reduced inhibition of the sub-set of PNs and further increase in their odor responses compared to the background-less un-adapted condition. This particular trend in the PN's or their innervated glomeruli was also associated with the glomeruli which showed the adaptation

induced decrease in their odor response strength. The same model might explain this behavior by considering that some of the ORN-LN connections were activated (increase in strength) during the time of adaptation which eventually reduced the PN's responses to the test odors due to elevation in the LN mediated inhibition. This proposed mechanism was based on the idea that olfactory receptor neurons had strong influences on the adaptive odor response strengths of the antennal lobe glomeruli however; this model needs to be tested.

Heterogeneity in the functional classes of receptor neurons was possibly also reflected in the results when we analyzed the individual glomerular responses to the individual test odors. The three glomeruli selected in this analysis (glomerulus 17, 28 and 33) showed the excitation as well as inhibition to the different test odors with both adaptation stimuli. One possible explanation of this type of dual behavior might be that different sub-set of synapses between the ORNs and PNs (with receptors for the different odors) within the same glomerulus behaved differentially due to the differential effects of adaptation on the receptor neuron's activity. This possibly contributed to the plasticity mechanism of the single glomeruli responded either in an excitatory or in an inhibitory manner to the different test odors and along the three conditions of background adaptation.

Reports on the number of neurons showed the estimated innervation of about 5 projection neurons (PNs) inside a single honeybee glomerulus (Galizia CG 2008). Hence, it also possible that those projection neurons of the same glomerulus are functionally different which finally leads to the simultaneous enhancement or decrease in the glomerular odor response strength. Although, this possibility has no supporting evidence in honeybee until now; however, in rat Padmanabhan and Urban found the intrinsic heterogeneity in the spiking rate of the mitral cells innervating the same glomerulus of the olfactory bulb (Padmanabhan and Urban 2010). Additionally, the possible involvements of the feed-back signals from the higher brain centers to regulate the adaptive odor responses of the AL glomeruli also need to be investigated.

4.6.5 Odor categories

Floral odor 1-hexanol (1-6ol) and the sting alarm pheromone odor isoamyl acetate (IAA) showed the common increase in glomerular response strength during the adaptation

compared to 'before' with both adaptation stimuli. On the other hand for 1-octanal, AL glomeruli showed the significant decrease in response strength during the adaptation with both background odor stimuli compared to the un-adapted condition. In addition to the similar types of change found between the two adaptation experiments, glomerular response strength showed opposite changes for 1-nonanol. Adaptation induced changes in the glomerular response strength to the different test odors were similar or dissimilar from each other like we found for the individual glomeruli. However, the reason for the specific increase in response strength to the floral and sting pheromone odor was not understood and requires further investigation. One interesting aspect in the data was that AL glomeruli showed the adaptation induced enhancement in response strength to 1-hexanol which persisted even after the withdrawal of the adaptation stimulus, whereas the glomerular responses were found to decrease progressively from the un-adapted state until the post-adaptation condition for 1-9ol. These two odors were not only used as test odors in these experiments but they were also part of the synthetic odor mixture that was used for adaptation. Hence, PNs probably did not recognize these odor components of the synthetic odor mixture individually as they did not show any self-adaptation type effect in responses. The word 'self-adaptation effect' specifically means the phenomena of decrease in neuronal responses to an odor when the same odor is used in the background for olfactory adaptation. In our result PNs seemed to treat the mixture of four pure odors as separate odor than its components and increased the response strength for 1-hexanol. The reduction in response strength found for 1-nonanol although might argue against the idea that PNs did not show any self-adaptation type effect however; it was known that PNs in the honeybee AL can represent (or process) the information of the quaternary odor mixture differently (like a separate odor) than the individual components (Deisig *et al*., 2006).

4.6.6 Dissimilarities in the odor representation patterns due to adaptation

Prolong adaptation with the constant odor background not only changed the strength of odor evoked responses of the AL glomeruli but also changed the glomerular representation patterns of the test odors. Distances or dissimilarities in odor representation patterns in the glomerular coding space between the conditions of before,

during and after adaptation were measured by the linear Euclidean distances. With both adaptation stimuli, it was found for nearly all of the test odors that background adaptation brought about significant separation in representation patterns in comparison with the pattern of the un-adapted state. In comparison, removal of the adaptation stimuli changed the odor representation patterns much less. These effects resulted in the significantly higher Euclidean distances between the glomerular odor representation patterns of 'before' and 'during' as well as between 'before' and after adaptation compared to the comparison between 'during' and after adaptation. This again showed the long lasting effects of olfactory adaptation on the odor representation patterns of the antennal lobe glomeruli. Significant changes in Euclidean distances might be associated with many factors such as the changes in response strength or the number of activated glomeruli or their response latencies which are complicated to illustrate. However, significant increase in the measured Euclidean distances due to olfactory adaptation and its persistence even after the removal of the adaptation stimuli clearly showed that prolong exposure of the AL glomeruli to the habitat odor of honeybee colony or the mixture of pure odorants enhanced the specific and stable forms of odor discrimination which probably signified the more reliable or elaborated representation of the different molecular features of odor moieties in the glomerular coding space.

4.7 Comments and outlook

Olfactory adaptation enhanced as well as inhibited the response intensities of the projection neurons innervating the antennal lobe glomeruli of the honeybee *Apis mellifera*. *This particular result in association with the significant changes found in the odor representation patterns confirmed that olfactory adaptation in this protocol changed the odor coding scheme of the antennal lobe glomeruli*. In vertebrate models (rat, mouse) the process of olfactory adaptation not only was found to preserve the neuronal responses to the novel odor but also enhanced the odor-background discrimination task behaviorally. Adaptation may well be the physiological mechanism through which animals reduce the overall responsiveness to the static and often meaningless odor background and preserve the selectivity and sensitivity of responses to the new odor stimuli. In other words olfactory adaptation probably helps to filter and

enhance the responses to the dynamic environment from the unchanging background content of information. We did not detect the calcium signals during the 20 min expose of honeybees with the background odor stimuli. One can expose bees to single or mixture of odors for progressively higher period of time and record the calcium signals from the AL glomeruli. This will be an easier way to estimate the adaptation time of the AL glomeruli with the criterion of showing no calcium responses to the further exposure of odors.

Extension of our adaptation experiments can incorporate the signal recording from the antennal lobe with higher temporal resolution to understand the dynamics of the neuronal processes when achieving the state of adaptation. Apart from the projection neurons, recordings from the AL interneurons and the antennal receptor neurons can reveal their responsibilities in adaptive changes of odor responses. Neuronal populations of the higher brain areas such as the Kenyon cells of the mushroom body can also be targeted for calcium imaging studies to understand their roles in the regulation of odor responses during the process of olfactory adaptation. Behavioral mechanisms and physiological correlates of olfactory learning under conditions of adaptation with background odor stimuli are least known and is undoubtedly an exciting avenue of future research to understand the computation of the olfactory system driving the discrimination between the target and the background in different contexts.

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Appendix-2

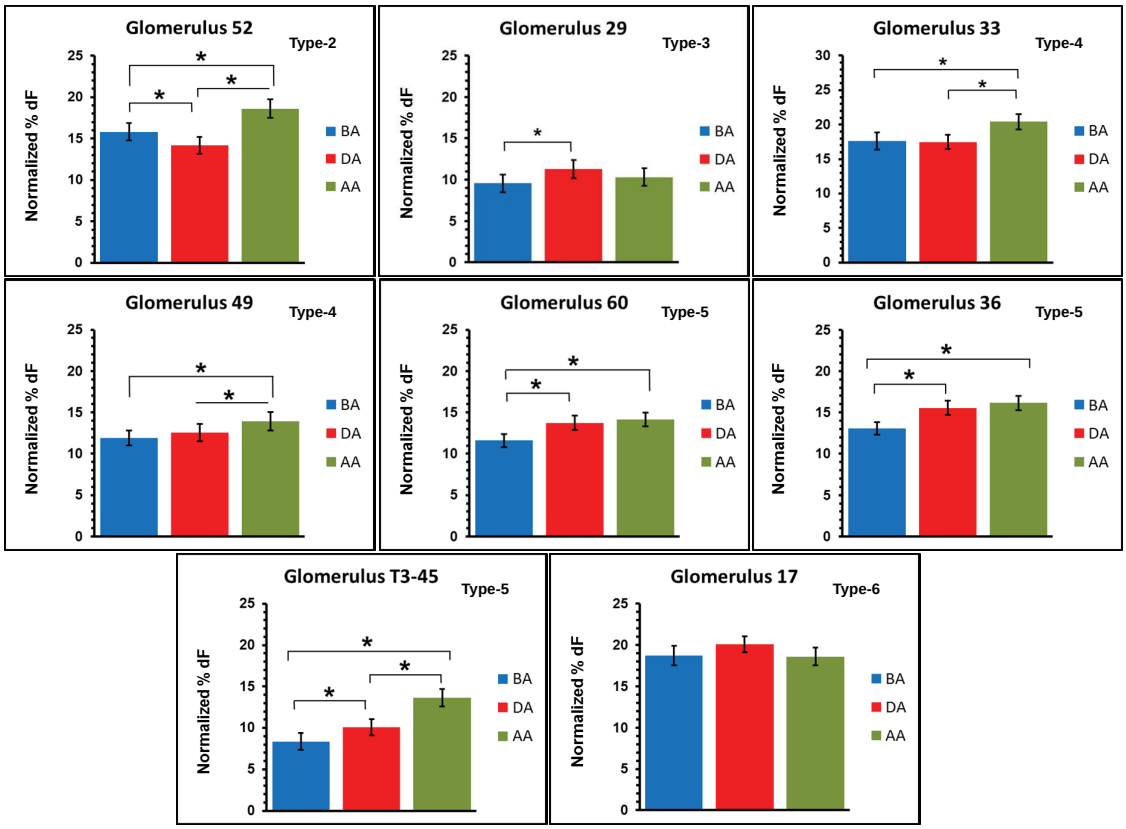

Fig. 4B: Glomerular types differed with respect to their patterns of change in response strength to the test odors between the conditions of before, during and after adaptation with the colony odor: The 6 different glomerular types (mentioned at the top right corner of each sub-plots) found in the adaptation experiment with the colony odor were already described in chapter-3 (fig. 4A). The 6 representative glomeruli were shown in Fig. 4A; the response patterns of the rests were shown here. Each sub-plot contained 3 bars with different colors (colors represented the same three conditions as explained in Fig. 4A) which showed the mean response strengths of the individual glomeruli to the test odors during the three recording conditions (the abbreviations for conditions were also same as described in Fig. 4A). The y-axis was showed the mean values of responses (normalized percent change in delta-F: mean ± standard error). Results of the statistical tests were given in table-1 (Friedman ANOVA followed by the Bonferroni corrected Wilcoxon matched pairs test); the significant differences between means were denoted with the asterics in the figure.

Table-1: Results of the statistical tests for the 14 selected gloemruli in the adaptation experiment with colony odor; refernce to Fig. 4A (Chapter-3) and 4B (appendix-2): The first three columns respectively represented the glomerular type and name, χ^2 values and degrees of freedom and the probability values (results from Friedman ANOVA test). The second three columns showed the probability (p) values found in the Wilcoxon matched pairs test (WMP test with Bonferroni correction) when the odor response strenghths between the pairs of conditions were compared (between before and during; 1st coulum, between during and after; 2nd coulum and between before and after; 3rd coulum). Type-6 glomerulus 17 showed non-significant p value in the Friedman ANOVA, hence, no WMP test was performed.

Glomerular type & number	Friedman ANOVA χ^2 and degree of freedom (df)	p value	Wilcoxon matched pairs test with Bonferroni correction p value (before vs. during)	(during vs. after)	(before vs. after)
(type-1) 38	$\chi^2 = 8.51$ df = 2	0.014	0.02	0.053	0. 000083
(type-2) 42	$\chi^2 = 91.73$ df = 2	0.00000	0.000000	0.000000	0.48
(type-2) 52	$\chi^2 = 81.85$ df = 2	0.00000	0.000004	0.000000	0.000000
(type-3) 28	$\chi^2 = 161.41$ df = 2	0.00000	0.000000	0.00	0.16
(type-3) 29	$\chi^2 = 7.38$ df = 2	0.00000	0.010	0.14	0.049
(type-4) 47	$\chi^2 = 30.08$ df = 2	0.00000	0.15	0.00038	0.000002
(type-4) 33	$\chi^2 = 65.51$ df = 2	0.00000	0.059	0.000000	0.000000
(type-4) 49	$\chi^2 = 28.87$ df = 2	0.00000	0.20	0.0019	0.000001
(type-5) 35	$\chi^2 = 66.08$ df = 2	0.00000	0.000000	0.05	0.000000
(type-5) 60	$\chi^2 = 39.69$ df = 2	0.00000	0.000000	0.62	0.000000
(type-5) 36	$\chi^2 = 34.07$ df = 2	0.00000	0.000000	0.16	0.000000
(ty-5) T3-45	$\chi^2 = 34.07$ df = 2	0.00000	0.000000	0.16	0.000000
(type-6) 48	$\chi^2 = 11.22$ df = 2	0.0036	0.78	0.054	0.17
(type-6) 17	$\chi^2 = 4.17$ df = 2	0.124	-	-	-

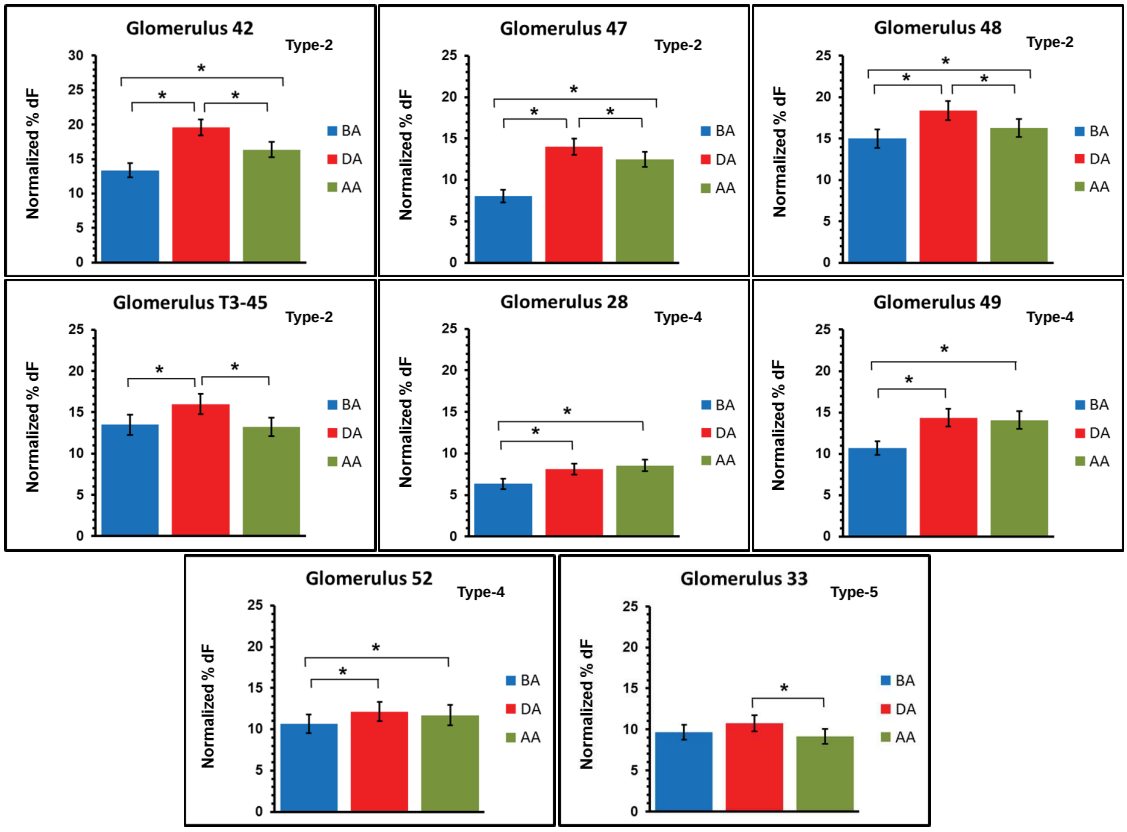

Fig. 4D: Glomerular types differed with respect to the patterns of change in response strengths to the test odors between the conditions of before, during and after adaptation with the synthetic odor mixture: The 6 different glomerular types (mentioned at the top right corner of each sub-plots) found in the adaptation experiment with the synthetic odor mixture were already described in chapter-3 (fig. 4C). The 6 representative glomeruli were shown in Fig. 4C; the response patterns of the rests were shown here. Each sub-plot contained 3 bars with different colors (colors represented the same three conditions as explained in Fig. 4A) which showed the mean response strengths of the individual glomeruli to the test odors during the three recording conditions (the abbreviations for conditions were also same as described in Fig. 4A). The y-axis was showed the mean values of responses (normalized percent change in delta-F: mean ± standard error). Results of the statistical tests were given in table-2 (Friedman ANOVA followed by the Bonferroni corrected Wilcoxon matched pairs test); the significant differences between means were denoted with the asterics in the figure.

Table-2: Results of the statistical tests for the 14 selected gloemruli in the adaptation experiment with synthetic odor mixture; refernce to Fig. 4C (Chapter-3) and 4D (appendix-2): The first three columns respectively represented the glomerular type and name, χ^2 values and degrees of freedom and the probability values (results from Friedman ANOVA test). The second three columns showed the probability (p) values found in the Wilcoxon matched pairs test (WMP test with Bonferroni correction) when the odor response strenghths between the pairs of conditions were compared (between before and during; 1st coulum, between during and after; 2nd coulum and between before and after; 3rd coulum).

Glomerular type & number	Friedman ANOVA χ^2 and degree of freedom (df)	p value	Wilcoxon matched pairs test with Bonferroni correction p value (before vs. during)	(during vs. after)	(before vs. after)
(type-1) 17	χ^2 = 182.07 df = 2	0.00000	0.000000	0.000000	0.00
(type-2) 38	χ^2 = 118.27 df = 2	0.00000	0.000	0.000000	0.000000
(type-2) 42	χ^2 = 146.30 df = 2	0.00000	0.000	0.000000	0.000000
(type-2) 47	χ^2 = 162.97 df = 2	0.00000	0.000	0.037	0.000000
(type-2) 48	χ^2 = 53.37 df = 2	0.00000	0.000000	0.000038	0.011
(ty-2) T3-45	χ^2 = 29.64 df = 2	0.00000	0.000002	0.000000	0.57
(type-3) 29	χ^2 = 44.83 df = 2	0.00001	0.10	0.000000	0.000000
(type-4) 36	χ^2 = 126.63 df = 2	0.00000	0.000000	0.48	0.000000
(type-4) 28	χ^2 = 23.18 df = 2	0.00001	0.000009	0.414	0.000000
(type-4) 49	χ^2 = 50.54 df = 2	0.00000	0.000000	0.78	0.000000
(type-4) 52	χ^2 = 15.32 df = 2	0.00047	0.00014	0.88	0.018
(type-5) 35	χ^2 = 18.52 df = 2	0.00010	0.312	0.00043	0.00010
(type-5) 33	χ^2 = 7.34 df = 2	0.025	0.30	0.0015	0.24
(type-6) 60	χ^2 = 12.48 df = 2	0.0019	0.41	0.030	0.063

Table-3: Results of the statistical tests for gloemrulus 17 (response strengths to the test odors in the adaptation experiment with colony odor; reference to Fig. 5A Chapter-3): The first three columns respectively represented the glomerular type and name, χ^2 values and degrees of freedom and the probability values (results from Friedman ANOVA test). The second three columns showed the probability (p) values found in the Wilcoxon matched pairs test (WMP test with Bonferroni correction) when the odor response strenghths between the pairs of conditions were compared (between before and during; 1st coulum, between during and after; 2nd coulum and between before and after; 3rd coulum). No statistical analysis was performed on responses to isoamyl acetate both before and during adaptation as well as with responses to 2-heptanone after the adaptation (due to weak responses).

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 12.77$ df = 2	0.0016	0.000000	0. 055	0.03
1-Nonanol	$\chi^2 = 15.411$ df = 2	0.0004	0.000002	0. 032	0.20
Geraniol	$\chi^2 = 19.033$ df = 2	0.00007	0.00030	0.000000	0.62
1-Octanol	$\chi^2 = 9.78$ df = 2	0.0075	0.99	0.0116	0.046
2-Heptanone	$\chi^2 = 8.22$ df = 2	0.016	0.0029	-	-
Linalool	$\chi^2 = 11.33$ df = 2	0.0034	0.27	0.000001	0.028
1-Octanal	$\chi^2 = 14.74$ df = 2	0.00063	0.000051	0.049	0.000050

Table-4: Results of the statistical tests for gloemrulus 17 (responses to the test odors in the adaptation experiment with synthetic odor mixture; reference to Fig. 5C Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests. Analysis was only conducted for 1-hexanol since glomerulus 17 responded weaky to the other odors.

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 59.05$ df = 2	0.00000	0.0013	0.000000	0.00021

Table-5: Results of the statistical tests on the response strengths of gloemrulus 28 to the test odors in the colony odor adaptation experiment; reference to Fig. 5E (Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests. No statistical analysis was performed with responses to geraniol before the adaptation.

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 48.21$ df = 2	0.00000	0.000000	0.000000	0.04
1-Nonanol	$\chi^2 = 11.07$ df = 2	0.0039	0.0086	0.004	0.50
Isoamyl acetate	$\chi^2 = 68.76$ df=2	0.00000	0.000000	0.000015	0.000000
Geraniol	$\chi^2 = 14.07$ df = 2	0.00088	-	0.0024	-
1-Octanol	$\chi^2 = 24.348$ df = 2	0.0001	0.000082	0.000000	0.026
2-Heptanone	$\chi^2 = 51.95$ df = 2	0.0034	0.0029	0.0004	0.16
Linalool	$\chi^2 = 22.67$ df = 2	0.00001	0.27	0.000001	0.028
1-Octanal	$\chi^2 = 50.80$ df = 2	0.00000	0.000000	0.589	0.000000

Table-6: Results of the statistical tests on the response strengths of gloemrulus 28 to the individual test odors in the synthetic odor mixture adaptation experiment; reference to Fig. 5G (Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests. Statistical analysis was performed only with the response data of 1-hexanol (only between during and after adaptation since responses before adaptation was weak)

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 29.64$ df = 2	0.00000	-	0.033	-

Table-7: Results of the statistical tests on the response strengths of gloemrulus 33 to the individual test odors in the colony odor adaptation experiment; reference to Fig. 5I (Chapter-3): Responses to 5 odors were shown here (the rest 3 odors showed weak responses), but 1-hexanol showed weak responses before the adaptation (no statistics performed on that data).

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 53.34$ df = 2	0.00000	-	0.000011	-
1-Nonanol	$\chi^2 = 9.54$ df = 2	0.0084	0.00016	0.0034	0.398
1-Octanol	$\chi^2 = 23.41$ df = 2	0.0001	0.000000	0.000001	0.16
2-Heptanone	$\chi^2 = 0.73$ df = 2	0.692	0.21	0.86	0.29
1-Octanal	$\chi^2 = 0.87$ df = 2	0.64	0.44	0.62	0.67

Table-8: Results of the statistical tests on the response strengths of gloemrulus 33 to the test odors in the adaptation experiment with synthetic odor mixture; reference to Fig. 5K (Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests. Responses to 4 out of 8 odors were found stronger and analyzed with the statistical tests.

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 5.75$ df = 2	0.056	0.017	0.0094	0.24
1-Octanol	$\chi^2 = 1.24$, df = 2	0.537	0.73	0.54	0.76
2-Heptanone	$\chi^2 = 7.09$, df = 2	0.02	0.004	0.0082	0.92
1-Octanal	$\chi^2 = 15.57$ df = 2	0.00042	0.12	0.007	0.00005

Table-9: Results of the statistical tests for the odor-categorization (colony odor adaptation); reference to Fig. 6A (Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests. AL glomeruli did not show any significant change in response strength to 2-heptanone between the three conditions of adaptation with the colony odor.

Odor type & name	Friedman ANOVA χ^2 and degree of freedom (df)	p value	Wilcoxon matched pairs test with Bonferroni correction p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol (type-1)	$\chi^2 = 9.75$ df = 2	0.0076	0.0038	0. 0160	0.00049
1-Nonanol (type-1)	$\chi^2 = 11.10$ df = 2	0.0038	0.030	0. 11	0.00045
Isoamyl acetate (type-1)	$\chi^2 = 36.81$ df=2	0.00000	0.0032	0.000003	0.000000
Geraniol (type-1)	$\chi^2 = 15.97$ df = 2	0.00034	0.97	0. 002	0.0048
1-Octanol (type-3)	$\chi^2 = 4.26$ df = 2	0.11	0.29	0.13	0.87
2-Heptanone (type-3)	$\chi^2 = 1.50$ df = 2	0.47	-	-	-
Linalool (type-3)	$\chi^2 = 5.96$ df = 2	0.050	0.025	0.033	0. 80
1-Octanal (type-2)	$\chi^2 = 15.34$ df = 2	0.00047	0.0095	0. 55	0.0064

Table-10: Results of the statistical tests for the odor-categorization (odor mixture adaptation experiment); reference to Fig. 6B (Chapter-3): The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests.

Odor type & name	Friedman ANOVA χ^2 and degree of freedom (df)	p value	Wilcoxon matched pairs test with Bonferroni correction p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol (type-1)	$\chi^2 = 55.93$ df = 2	0.00000	0.000000	0. 000000	0.00047
1-Nonanol (type-3)	$\chi^2 = 18.48$ df = 2	0.00010	0.0090	0.0010	0.000002
Isoamyl acetate (type-1)	$\chi^2 = 21.74$ df=2	0.00002	0.000000	0.000018	0.35
Geraniol (type-4)	$\chi^2 = 11.30$ df = 2	0.0035	0.0050	0. 00053	0.73
1-Octanol (type-2)	$\chi^2 = 8.39$ df = 2	0.015	0.0040	0.13	0.011
2-Heptanone (type-2)	$\chi^2 = 10.33$ df = 2	0.0057	0.14	0. 010	0.002
Linalool (type-5)	$\chi^2 = 4.70$ df = 2	0.09	0.13	0.22	0. 014
1-Octanal (type-3)	$\chi^2 = 11.53$ df = 2	0.0031	0.0090	0.96	0.005

Table-11: Results of the statistical tests (for the Euclidean distance measurements in the colony odor adaptation experiment): Results of the individual odors were shown here for the colony odor adaptation experiment; refenrence to the Fig. 7A, B, C, D, E, F, G and H (chapter-3). The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests.

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 109.98$ df = 2	0.00000	0.000000	0.000000	0.000037
1-Nonanol	$\chi^2 = 85.60$ df = 2	0.00000	0.000000	0.000000	0.000041
IAA	$\chi^2 = 76.38$ df = 2	0.00000	0.000000	0.000000	0.28
Geraniol	$\chi^2 = 18.96$ df = 2	0.00008	0.001	0.054	0.000016
1-Octanol	$\chi^2 = 6.78$ df = 2	0.033	0.02	0.7	0.007
2-Heptanone	$\chi^2 = 20.16$ df = 2	0.00004	0.000013	0.000000	0.87
Linalool	$\chi^2 = 3.2$ df = 2	0.21	-	-	-
1-Octanal	$\chi^2 = 125.9$ df = 2	0.00000	0.000000	0.00	0.000000

Table-12: Results of the statistical tests (for the Euclidean distance measurements in the synthetic odor mixture): Results of the individual odors were shown here for the synthetic odor mixture adaptation experiment; refenrence to the Fig. 7A, B, C, D, E, F, G and H (chapter-3). The orders The columns of this table represented the same parameters as described in Table-1 with the same sequence of statistical tests.

Odor name	Friedman ANOVA		Wilcoxon matched pairs test with Bonferroni correction		
	χ^2 and degree of freedom (df)	p value	p value (before vs. during)	(during vs. after)	(before vs. after)
1-Hexanol	$\chi^2 = 75.66$ df = 2	0.00000	0.000000	0.000000	0.000000
1-Nonanol	$\chi^2 = 44.91$ df = 2	0.00000	0.000001	0.000000	0.001
IAA	$\chi^2 = 23.70$ df = 2	0.00001	0.01	0.000027	0.5
Geraniol	$\chi^2 = 46.74$ df = 2	0.00000	0.000000	0.0002	0.000000
1-Octanol	$\chi^2 = 29.98$ df = 2	0.00000	0.000001	0.000018	0.02
2-Heptanone	$\chi^2 = 202.51$ df = 2	0.00000	0.000000	0.000000	0.000000
Linalool	$\chi^2 = 43.63$ df = 2	0.00000	0.000000	0.000000	0.051
1-Octanal	$\chi^2 = 71.26$ df = 2	0.00000	0.000000	0.000000	0.000000

Chapter -5

Discussion

In the 'Introduction' (chapter-1) of this dissertation I tried to explain the connections between the three chapters with respect to their research goals or interests and methods. One of the important motivations of these chapters in this thesis was to understand the olfactory perception and learning in honeybee in presence of the complex odor environment.

Discussion of results; chapter-2

In chapter-2 we investigated whether the hygienic bees were better in learning the odors associated with the *Varroa* infection than the non-hygienic bees. In the first experiment honeybees from both genetic lines were found to fail to learn the discrimination between the odors of the healthy and infected wax caps. Both types of wax caps used in this experiment emanated the mixture of odors which bees learned without learning the difference between the two types of mixtures. Presence of the common wax compounds in higher concentrations over the lower concentrations of the disease associated chemicals in the volatile odor profiles of the two types of wax pieces might be one of the possible reasons for the complete generalization (or no discrimination) in responses between these wax stimuli. Alternately, the isolation procedure possibly affected the chemical composition of the wax pieces. However, we did not check the alternative possibility as whether bees can recognize and discriminate between these two types of wax stimuli inside the colony or in other words when adapted with the background of colony odor. This can be an interesting experiment to perform in future to answer the original question in the context of honeybee colony. Results of the following experiment showed that only the hygienic but not the non-hygienic bees were able to differentiate between the volatile odor-bouquets of the healthy and *Varroa* parasitized pupae. This result was important as better discriminability of the hygienic bees over the non-

hygienics between the volatile odors of the healthy and infected pupae indicated the possible *important roles of the olfactory processes to the efficient recognition of the Varroa infected brood by the hygienic bees* which possibly contribute to their higher resistance against this ectoparasitic mite. Similar to the previous experiment with wax odors, conditioning with pupal odors was also not repeated with the background adapting stimulus of the colony odor. Hence, possible effects of olfactory adaptation with the background of colony odor on the discrimination between the volatile odors of the pupae or the wax caps in these two genetic lines were untested in this study. Interestingly, hygienic bees showed the higher discriminability or lower generalization between the pupal odors only when the *Varroa* parasitized pupae but not the un-parasitized pupae were used as the rewarded stimulus or CS+. In the opposite combination of the two CS stimuli both hygienic and non-hygienic bees were found to fail to learn the task of discrimination. This type of asymmetric learning was also found previously in the study conducted by Masterman and colleagues. They found that bees form their hygienic line learned the olfactory discrimination task (in PER paradigm) better when the chalkbrood infected pupae were used as the CS+ and the healthy pupae as the CS- but not *vice versa* (Masterman *et al.,* 2000). The possible reason of the asymmetric salience between the volatile odors of the healthy and the diseased brood was unclear but found commonly between their and our studies independent of the specificity of infection.

The better olfactory discriminability of the hygienics than the non-hygienic bees towards the odor stimuli represented the health status of the brood did not confirm whether these two lines differed generally in their olfactory learning abilities. To test that, bees were conditioned with the sting alarm pheromone odor, isoamyl acetate (IAA) but were tested during the memory retention tests with the novel or untrained odor (a new CS), 1-hexanal along with the CS+ odor IAA. This protocol tested the effect of olfactory generalization between the trained and the novel odors. Bees from both genetic lines were found to learn the CS+ (IAA) similarly during the absolute conditioning but non-hygienic bees compared to the hygienics showed significantly higher odor generalization during both the short-term and the long-term memory retention tests. Additionally, the non-hygienic bees showed consistently strong responses to the filter paper during the two retention tests. Honeybees from both lines showed higher odor generalization when conditioned

with the same odor IAA in presence of the colony odor background used for adaptation. However, this effect was related with the lower learning during the conditioning compared to the previous experiment when bees did not receive any background adaptation. This reduction in olfactory learning was found stronger in non-hygienics than in the hygienic bees. When bees from these two lines were conditioned differentially with the high concentrations of the floral odors such as geraniol and 1-hexanol, non-hygienic bees were again failed to learn the discrimination both during the conditioning as well as the retention tests. Hygienic bees learned the discrimination task between these two odors, like the bees in our institute's garden as reported previously (Malun *et al.*, 2002). The overall *higher odor generalization* showed by the non-hygienic bees between the geraniol and 1-hexanol, between the trained odor IAA and the novel odor 1-hexanal along with the unusually high response levels to the stimulus like filter paper demonstrated that either this honeybee line had general deficit in learning the odor stimuli or some form of specific deficit to learn the odors in the PER conditioning paradigm. Alternately, the non-hygienic bees possibly suffered some form of long-term arousal or sensitization effect which suppressed the effects of olfactory learning and elevated the average responses to all kinds of CS stimuli (including the filter paper) for the entire experimental season. Irrespective of these possibilities it was concluded that hygienic bees performed significantly better than the non-hygienic bees in the entire set of conditioning experiments conducted during the summer and autumn (2009). It was also concluded that olfactory adaptation with the colony odor background reduced the learning of isoamyl acetate significantly during the conditioning in both genetic lines but for the non-hygienic bees the effects were stronger. It was unclear from these results whether this was a specific effect of the colony odor or specific reduction found in the conditioned responses to IAA. These possibilities are of general interest to be investigated in future to understand the behavioral learning mechanisms in honeybees under conditions of olfactory adaptation. However, the better performance of the hygienic bees in our study closely resembled the learning behavior of honeybees in general, so the differences between these the hygienic and non-hygienic lines most likely were raised due to the consistent poor performance of the non-hygienic bees rather due to the true superior performance of the hygienic line. In absence of any third group of

honeybees conditioned and tested in parallel with these two lines, the possible superiority of the hygienic bees in their olfactory learning abilities over the non-hygienic bees could not be confirmed. However, successful discrimination between the volatile odor profiles of the *Varroa* parasitized and the un-parasitized brood showed by the hygienic bees strongly indicated the possibility of the *olfactory recognition of diseased brood as the mechanism of behavioral resistance of the hygienic lines against the Varroa mite.*

Future perspective

Uncapping experiments performed previously towards the *Varroa* parasitized brood (Bienefeld & Zautke 2006) as well as the recent report by Schöning and colleagues confirmed that brood-removal or hygienic behavior is not triggered by the identity of pathogen but by the extent of damage inflicted on the brood in course of the infection (Schöning *et al.,* 2011). This idea was supported by the previous report that honeybees use the same defense mechanism, the hygienic behavior to resist both the chalkbrood and the American foulbrood diseases (Spivak and Gilliam 1998a, 1998b). Colonies breed for the resistance against the chalkbrood and the American foulbrood also showed resistance against the *Varroa* mite (Ibrahim and Spivak 2006). In comparison to the chalkbrood or foulbrood infection, *Varroa* causes relatively subtle damage on the brood since many infected broods were found to develop normally (Schöning *et al.,* 2011). Hence, understanding the mechanisms of hygienic behavior against the *Varroa destructor* is important to develop the honeybee lines with biological resistance against the *Varroa* pathogenesis and other brood diseases (chalkbrood, American and European forms of foulbrood). In addition, it will also stop the use of toxic chemicals in the bee-keeping industry for controlling infestations. The results in chapter-2 although showed the significantly better olfactory learning abilities of the hygienic bees than the non-hygienics, however, did not confirm the true superiority of the hygienic bees due to the consistently poor learning and memory performances of the non-hygienic bees. However, if one can confirm the fact that hygienic bees have higher discriminability and sensitivity to the brood odors (healthy and *Varroa* parasitized) than the non-hygienic bees, then the next step will be to investigate the possible differences in expression patterns of genes associated with the olfactory pathways in the brain neuropiles of the hygienic and non-

hygienic bees. Knowledge of the differential expression patterns or levels of genes (using the microarray analysis) in various tissues of the nervous system (antennae, antennal lobe and mushroom body) of the hygienic *vs.* non-hygienic bees can be used to understand the molecular basis of the disease resistance. This set of genomic information can also be used to develop the diagnostic tools such as the genetic kits with the markers of disease-resistance which will enable us to recognize the non-resistant honeybee lines for efficient breeding for the higher resistance against the *Varroa* pathogenesis.

Common aspect; chapter-2 and chapter-3

Results in chapter-2 showed that only hygienic bees were able to discriminate between the volatile odor profiles of the healthy and the *Varroa* infested pupae. These odor profiles represented the mixture of odors emanated from the body of the two types of pupae. However, in place of the odor mixture, bees of the same hygienic lines in chapter-3 were conditioned differentially (in the cumulative conditioning assay) with the pure odors namely, ocimen and phenethyl acetate respectively represented the odors of the healthy and the chalkbrood infected larvae. The overall conditioned responses (CRs) and discriminability of the hygienic bees to the pupal volatiles although were low (relatively complex task) but conditioning with pure odors increased the CRs and the odor discriminability during the cumulative conditioning assay (relatively simpler task). However, these two conditioning experiments commonly mimicked the task of odor driven differentiation between the healthy and the diseased brood inside the colony.

Discussion of results; chapter-3

The motivation of chapter-3 was to characterize the different learning and memory performance classes of the honeybee population with the approach of analyzing the performance of individual bees. We found high variability in the performance scores of the individuals (within the population) in the different learning related features e.g. the rate of CS+ learning for which high heterogeneity in population was also found previously (Pamir *et al.,* 2011). This high variability most likely reflected the variability in olfactory learning performance of the forager bees present in the natural population.

This variability probably indicates the differences in genetic background and the history of odor learning of the forager bees. Under this condition the best and the worst cumulative performers were selected with the arbitrary criteria (not based on any statistical criteria) of the higher and lower ranges of cumulative scores. Although arbitrary, but the selection procedure had the support from the other data that showed the presence of the extreme scorer categories in the score histograms of the individual quantified features related with olfactory learning and memory. The best cumulative performers showed good performances throughout the assay. They learned the CS+ stimuli fast and reliably during the differential conditionings, discriminated strongly between the CS+ and CS- odors both during the conditioning trials and the memory retention tests as well as showed high sensitivity in responses to the different dilutions of the CS stimuli during the tests. The high scores in the different learning and memory related features of the best cumulative scorers led to the high correlations between these features. The only exception was the feature which quantified the responses to the filter paper and paraffin oil. This group of high scorers actually showed strong responses to the filter paper and paraffin oil initially during the 1st test. However, the responses were reduced during the successive 2nd test in each of the two phases of the cumulative assay which eventually lowered the correlation of this feature with the others. The poor cumulative performers on the other hand performed consistently bad throughout the different phases of the cumulative assay. Consistently weak CS+ learning, odor discriminability during the conditionings and the retention tests with the overall weak responses to any type of CS stimuli of these bees was associated with the low correlations between the scores of the same set of learning and memory related features. However, sucrose responses of the low scorers did not show any compromise during the entire assay. Hence, it was concluded that deficit in olfactory learning and memory processes rather not the compromised PER responses resulted in the overall poor performance of the low cumulative scorers throughout the assay. We found that bees used in the cumulative assay did not show any significant variation in olfactory learning and memory performances along the different time points of the season (summer and autumn 2010), although the seasonal effects on olfactory learning were reported previously for honeybees (Blažytė-Čereškienė and Skirkevičius 2006; Hadar and Menzel 2010). The

possible reasons for the differences in results between these two studies with the cumulative conditioning assay were unclear but differences in genotypes of the honeybees (backcrossed hygienic bees *vs.* colonies breed for no selection trait), olfactory learning protocols (cumulative conditioning *vs.* absolute and reversal conditioning) as well as the experimental time points during the season (Čereškienė and colleagues conducted their experiments in Lithuania where spring extends from March to July and autumn from September until November, unlike in Berlin where July is the middle of summer and November is the beginning of winter) together probably contributed to this differences. Honeybees does not have any genetic component to perform good or bad in the olfactory PER conditioning paradigm since it is an artificial method (laboratory assay). However, this paradigm can test the effectiveness of the natural genetic components of odor learning. This particular information along with the contributions of the individual backcrossed colonies to the populations of both the best and the poor cumulative scorers, and the absence of seasonal effect on the learning related performances of bees suggested that cumulative conditioning assay and the selection criteria most likely sorted out the truly superior and inferior olfactory performers of the natural population.

Although these two types of cumulative scorers showed substantial variability in their learning and memory performances, but they had the common high correlation in scores between the CS+ learning speed (Acq1) and the odor discriminability (Disc1) during the 1st differential conditioning. In fact, the high correlation between these two features was found in the other types of performer classes selected with the different criteria and for the entire experimental population of honeybees. Performance scores of Acq1 contributed directly to the scores of Disc1 due to the way they were quantified but the scoring procedure did not dictate the high correlation between the Acq1 and Disc1 as the same two features (Acq2 and Disc2) during the 2nd phase of the assay showed the lower correlation values. This inconsistency was explained by the fact that honeybees learned and discriminated better between the odors during the 1st phase compared to the 2nd phase of the assay. Higher generalization between the CS+ and CS- stimuli during the 2nd phase of the assay reduced the scores and correlations between the features such as Acq2 and Disc2. During the 3rd memory retention test of the cumulative assay (2nd phase) bees were

able to discriminate well between the training concentrations of the CS+ and CS- odors but showed more generalization in responses when the dilutions of the CS stimuli were presented. Hence, the high number of incorrect responses to the dilutions of the CSs did not reflect the possible deficit in learning during the 2nd phase, rather most likely indicated the hunger responses in bees due to the prolong odor training and test protocol (for 3 hrs) of the 1st phase of the assay. The possible effects of hunger responses might have contributed to the higher number of incorrect responses when the dilutions of the CS stimuli were presented however; exposure to the highest concentrations of the CSs restored the specific higher responses of bees to the CS+ compared to the CS- stimuli (due to concentration effect).

We also found that scores of Disc1 (odor discrimination during the 1st differential conditioning) among the other features was singly able to predict the final performance scores of the best and the poor cumulative scorer classes with highest probabilities. In other words, ability to learn concomitantly the meanings of the rewarded (CS+) and the unrewarded (CS-) odor stimuli was the most important feature found in this assay which strongly influenced the final performance levels of the two types of extreme cumulative scorers. However, these two classes of cumulative scorers were selected by the scores of the Disc1 only with the probability values nearly 0.7, rather not high as 0.9 or more to be adequately sure about the usefulness of the feature Disc1 for the selection of the cumulative performers. This clearly indicated the *variability in the learning and memory performances of these bees during the cumulative conditioning assay* such that the lower performance score in one or more features was compensated by the higher performance score in one or others. This eventually resulted in the failure of selecting these bees with high probabilities using the scores of the single features. This was rather obvious, since the overall high variability of the whole experimental population (as discussed before) was also found in the learning and memory performances of these two types of scorers

Acq 1 and Disc1 showed high linear correlation between their scores, but the higher scores of Acq1 were only able to select the 54% bees which were high Disc1 scorers, whereas, all high Disc1 scorers were found to score high in Acq1. The 2nd finding was trivial but the 1st was not, as it demonstrated that fast and consistent responses (learning) to the rewarded odors (CS+) were insufficient for the fast and consistent learning of the

CS- odors during the differential conditioning. In other words, *learning speed of the CS- stimuli was found independent of learning speed of the CS+ stimuli*.

Learning dynamics of the CS+ and the CS- odors were found to vary in different performer classes. The best cumulative scorers and the high scorers of Acq1 showed the concomitant learning of the CS+ and CS- odor stimuli which was not found in the poor cumulative or the low Acq1 scorers. The feature Acq1 represented both the speed and consistency of CS+ learning in this assay. Analysis separated these two aspects of Acq showed that the early but not the delayed learners and / or responders to the CS+ stimuli during the 1st DC exhibited the 'together-learning' of the CS+ and CS- odor stimuli. This revealed the fact that learning of the rewarded and the unrewarded odor stimuli *do not follow any common or general dynamics in bees rather the learning dynamics of these two stimuli depend on the type of learning performers*. However, the high cumulative scorers and the fast and consistent CS+ learners showed the stronger effects of odor generalization (between the CS+ and CS- odors) and the sucrose mediated arousal compared the low Acq1 and cumulative scorers during the 1st differential conditioning. These effects, found in the high scorers were suppressed by the fast learning of the CS- stimuli which led to the concomitant learning of the CS+ and CS- stimuli. The underlying reasons for the initial higher number of responses to the CS- stimuli (due to high generalization and effects of sucrose arousal) in the high cumulative and Acq 1 scorers were unknown; however, possible higher sensitivity of the olfactory and/or gustatory receptors of these bees might have controlled the early higher responsiveness to both types of odor stimuli which was suppressed soon by the strong effects of learning. Overall we found that bees learning speed of the CS+ stimuli and odor discriminability were the two most important features that dominate the overall or cumulative performance levels of bees in the cumulative assay. However, it is possible that other olfactory learning assays in honeybee may pick between these two features or any other behavioral feature to be the strongest predictor of the individual's overall performance.

Future perspective

Fast learners of the CS+ odors and bees with strong ability to discriminate between odors performed consistently well in the cumulative conditioning assay. *In vivo* calcium imaging or electrophysiological recordings from the specific neuronal population (e.g., Kenyon cells in the mushroom body) of these bees can be highly interesting to understand the neurophysiological basis of fast and concomitant learning of multiple odor information. Consecutive olfactory and visual learning assays in honeybee can be developed to investigate the possible correlations between the learning performances in these two sensory modalities. This approach can be useful to understand the nature of the common behavioral machinery regulating the learning of these two important sensory stimuli. Although the results of the gene expression study were not reported in this dissertation, however, any robust and conserved genetic signature for the different types of olfactory learning and memory performers can be used to understand the underlying genetic / molecular networks regulating the olfactory learning-related performances in honeybee.

Contradictory aspect; chapter-2 and chapter-4

In chapter-2 honeybees of the hygienic and non-hygienic lines were trained with the sting alarm pheromone compound, isoamyl acetate (IAA) both in presence and absence of the background odor of honeybee colony. The constant background odor was used to adapt the bees behaviorally while they were conditioned with the IAA and latter tested with the novel odor, 1-hexanal apart from the conditioned odor, IAA to test the effects of odor generalization. Bees of the hygienic and non-hygienic lines were found to learn the conditioned stimulus isoamyl acetate significantly less in the adapted state compared to the un-adapted state (when conditioned in the background of laboratory). They also showed higher odor generalization when the colony odor was applied for olfactory adaptation. Hence, we concluded that background adaptation with the odor of honeybee colony inhibited the olfactory learning as well as elevated the effects of odor generalization in bees. In chapter-4 we performed *in vivo* calcium imaging in the honeybee antennal lobe (AL) to understand the potential effects of olfactory adaptation

on the glomerular odor coding. Similar to chapter-2, constant background of colony odor was also used in chapter-4 for adaptation of the AL glomeruli which was followed the recording of glomerular calcium responses to the different test odors under the adapted condition. Isoamyl acetate was used as one of the test odors and it was found that adaptation with the colony odor increased the glomerular response strength significantly to this odor compared to the un-adapted condition. In addition, adaptation with the colony odor was also found to increase the linear distance between the glomerular representation patterns (Euclidean distances) of IAA compared to the other two conditions (details given in 'Discussion; chapter-4').

Significant increase in the strength of glomerular calcium signals along with the change in the representation patter apparently did not explain the result of chapter-2, that adaptation with the colony odor declined the olfactory learning in bees for isoamyl acetate. However, olfactory learning experiments performed in chapter-2 were different than the odor coding (perception) experiments performed in chapter-4. The latter set of experiments did not incorporate any sucrose or other US-component. Hence, classically no learning component was involved in our calcium imaging study. However, adaptive response of glomeruli to the isoamyl acetate mimicked the increased calcium signals of the AL glomeruli for the learned rewarded odor (although IAA was not used as conditioned odor) after the olfactory PER conditioning (Faber *et al.,* 1999, Rath *et al.,* 2011). The sugar US of these studies seemed to be replaced by the adapting background odor of honeybee colony in our imaging study as they both enhanced the odor response strength of the AL glomeruli. But, on the other hand colony odor adaptation decreased the olfactory learning in both hygienic and non-hygienic bees (chapter-2). The contradiction between the behavioral and physiological data was not understood, but probably indicated the fact that behavioral outcomes in animals in kind type of experiment may not be correlated with the physiological outcomes of the few neurons in another type of experiment.

Discussion of results; chapter-4

Honeybees were exposed for 20 min either with the background odor of unknown complexity such as the odor of the honeybee colony (the habitat odor) or with the odor

mixture of known complexity (equal v/v mixture of four odors) for adaptation of the antennal lobe glomeruli. Under the adaptation condition both background stimuli were found to increase the average response strength of the glomeruli only during the time of odor stimulation to the set of test odors compared to the initial un-adapted condition. However, when glomerular responses before, during and after the odor stimulation were considered these two adaptation stimuli showed opposite type of change in the average odor responses strength of the glomeruli. Adaptation with the colony odor reduced and with the synthetic odor mixture increased the average glomerular response strength to the odors. The overall inhibitory effect of the colony odor adaptation however did not affect the odor evoked responses of the glomeruli as they showed the adaptation induced enhancement in responses during the time of odor stimulation (mentioned before). The dissimilar effects of the two background adaptation stimuli on the overall response strength of the glomeruli probably indicated the different effects of these odor mixtures on the glomerular network of the AL in terms of activating the different forms or pathways of olfactory adaptation. However, neither of these possibilities was investigated further in this study. Further research in this direction can reveal the possible physiological mechanisms of the glomerular sub-units in the honeybee antennal lobe. Glomerular responses to the test odors, after the removal of the background adaptation conditions, did not recover back to the initial un-adapted levels. Removal of the colony odor led to the further increase in odor responses on top of the adaptation induced increase. For the synthetic odor mixture, post-odor offset responses of the AL glomeruli were decreased after the removal of the background adaptation condition. Adaptation-recovery of odor responses was expected in these experiments as bees were kept undisturbed for 5 min after the stoppage of the background odor stimuli. However, 5 min time period used in the protocol proved to be insufficient for the responses recovery of the projection neurons innervating the honeybee AL glomeruli. Mitral cells in the rat olfactory bulb were reported to consume 30 – 50 min for the response recovery after being adapted for 1 hour with the constant background odor (Chaput and Panhuber 1982) stimulus. Olfactory receptor neurons in the silkworm moth, *Antheraea polyfemus* were reported to take more than 1 hour for the complete adaptation recovery (Kaissling *et al.,* 1987). In the housefly *Musca domestica,* it was also reported that odor responses of the

olfactory receptor neurons (ORN) did not recover from the effects of background adaptation with the higher concentrations of the pure odorants within 15 min of time (Kelling *et al.,* 2002). It is possible that like the rat mitral cells or the receptor neurons of the housefly and the silkworm moth, honeybee projection neurons (PNs) also require time longer than 5 min for adaptation recovery. The limitation of our experimental protocol that we did not find the adaptation recovery however was interesting in itself to indicate the possible slow adaptation recovery of the projection neurons in the honeybee AL.

Apart from the gross or average analyses of the glomerular response intensities, individual glomeruli were also analyzed for the possible adaptation induced changes in their odor response strengths. In this analysis we found that majority of glomeruli with both adaptation stimuli showed the enhancement in their response strength to the test odors during the time of adaptation compared to before. However, the same glomerulus showed different types of changes during the adaptation with the two background odor stimuli. This again demonstrated the differential effects of these two adapting stimuli (colony odor and synthetic odor mixture) on the AL network to induce either the different kinds or activate the different pathways of adaptation. The reasons for the increase in odor response strength of the glomeruli during the adaptation with background odor stimuli in these experiments were not further investigated. However, one possible explanation of this behavior might be that prolong exposure (for 20 min) of the antennal lobe neuropil to the background odor stimuli reduced (inhibited) the strength of the ORN-LN (local inhibitory interneurons) connections, which led to reduced inhibition at the PN-LN connections and subsequent increase in the odor response strength of the PNs compared to the background-less un-adapted condition. Opposite type of changes in the strength of these synaptic connections induced by the background adaptation probably resulted in the increased inhibition in some of the PN-LN connections and subsequent decrease in the odor response strength of some of the glomeruli innervated by these specific sub-sets of PNs (e.g. Glomerulus 17 and 42 respectively for the synthetic and the colony odor adaptation experiments). This model was based on the basic assumption that olfactory receptor neurons have strong influences on the adaptive odor responses of the

antennal lobe neuropil which needs to be tested. In addition, individualistic nature of the glomeruli in terms of processing odor information also contributed to this model.

Prolong adaptation with the constant odor background not only changed the strength of odor evoked responses of the AL glomeruli but also changed the glomerular representation patterns of the test odors. Distances or dissimilarities in odor representation patterns in the glomerular coding space between the conditions of before, during and after adaptation were measured by the linear Euclidean distances. With both adaptation stimuli, it was found for nearly all of the test odors that background adaptation brought about significant separation in representation patterns compared to the pattern of the un-adapted state. In comparison, removal of the adaptation stimuli changed the odor representation patterns much less. These effects resulted in the significantly higher Euclidean distances between the glomerular odor representation patterns of 'before' and 'during' as well as between 'before' and after adaptation compared to the comparison between 'during' and after adaptation. This again showed the long lasting effects of olfactory adaptation on the odor representation patterns of the antennal lobe glomeruli. Significant changes in Euclidean distances might be associated with many factors such as the changes in response strength or the number of activated glomeruli or their response latencies which are complicated to illustrate. However, significant increase in the measured Euclidean distances due to olfactory adaptation and its persistence even after the removal of the adaptation stimuli clearly showed that prolong exposure of the AL glomeruli to the habitat odor of honeybee colony or the mixture of pure odorants enhanced the specific and stable forms of odor discrimination which probably signified the more reliable or elaborated representation of the different molecular features of odor moieties in the glomerular coding space.

This study contributed to the understanding of the adaptive transformations of odor representations in the primary olfactory neuropil of the honeybee central nervous system; the strategy that bees probably employ in the natural conditions when they perceive odors in the background of other odor stimuli.

Future perspective

In vivo calcium imaging assays merging the phenomena of olfactory adaptation and learning in honeybees can be used to understand the adaptive processing of the learned odor information in the different brain neuropiles both during and after learning. Not only the antennal lobe neurons but also cells of the higher processing station such as the Kenyon cells are of great interest in this regard to investigate the roles of the mushroom body neuropil in the processing of the learned odor information under adapted condition.

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