# Principles of olfactory-visual integration to form a common percept in honeybees

## Prinzipien der olfaktorisch-visuellen Integration des Lernverhaltens der Honigbiene

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## Abstract

The honeybee is a well studied and important organism in neuroethology. The possibility to train them with a classical conditioning paradigm and their miniature brain provide a perfect requisite to investigate the neuronal principles of learning and memory. Honeybees use visual and olfactory cues to detect flowers during their foraging trips. Hence, the reward association of a nectar source is a multi-modal construct, which has at least two major components - olfactory and visual cues.

It is still an open question, how both sensory components are converged in the mushroom body, which represent the multi-modal integration centre of the honeybee brain. The main goal of this study, is to investigate the processing of multiple modalities and how a reward association is formed. This includes, how and wether both sensory modalities interfere during learning. Thus, in this study stimulation with UV, blue and green light was used to evoke distinct photoreceptor activities in the compound eye. Furthermore, three different odours (Geraniol, Citronellol and Farnesol) were used. These stimuli were tested in three different experimental series.

The first experiment involved classical differential conditioning of the single modalities - odour and colour. Honeybees showed high learning performances in differentiating olfactory stimuli and also reliable responses for visual conditioning. Furthermore, a temporal discrepancy in the stimulus length for best learning in the olfatcoty and visual cues was found.

In the second series, it was tested how multi-modal compounds are perceived. This includes, unique cues (configural processing) or the sum of the single components of a compound (elemental processing). This was tested by combining single odour components with monochromatic light in a positive (PP) and negative patterning (NP) experiment. During PP, the olfactory-visual compound was rewarded, whereas the single components were unrewarded. In contrast, during NP the single components were reinforced, but the compound was not. In addition, the ability to distinguish between two different light stimuli presented as a part of an olfactory-visual compound with the same odour component during acquisition was tested. In a memory test, the light stimuli were presented again as a compound and in addition as the single components. The results revealed that bees used elemental processing with compounds containing green and blue light. In contrast, when UV light was presented the bees used configural processing.

Finally, a third experiment was conducted at the neuronal level. Multi-unit recordings were established to provide a suitable method to analyse extrinsic neurons at the mushroom body output region, the so called ventral lobe of the pedunculus. Here, three different odours (Geraniol, Farnesol and Citronellol), two colours (green and blue) and two combined stimuli (colour + odour) were chosen as stimuli, to search for possible variations in processing stimuli with different modalities. Two units could be detected that responded mainly to visual stimuli.

## Zusammenfassung

Die Honigbiene ist ein gut untersuchter und wichtiger Organismus für die neuroethologische Forschung. Die Möglichkeit sie auf klassische Weise zu Konditionieren und ihr relativ kleines Gehirn macht sie zum idealen Untersuchungs-Gegenstand um die neuronalen Prinzipien des Lernens und der Gedächtnisbildung zu erforschen. Während des Furagierens nutzen Honigbienen beides: visuelle und olfaktorische Merkmale der Futterplanzen. Daher ist die Belohnungs-Assoziation mit der Nektar-Belohnung ein multi-modales Konstrukt, welches aus mindestens zwei Hauptkomponenten, den olfaktorischen und den visuellen Reizen, besteht.

In dieser Arbeit soll untersucht werden, wie olfaktorische und visuelle Reize verarbeitet werden und wie sie im Pilzkörper, dem multi-modalen Integrationszentrum des Bienengehirnes, konvergieren. Wie beide sensorischen Modalitäten integriert werden um eine gemeingültige Belohnungs-Assoziation zu bilden, ist immer noch eine offene Frage. Weiterhin ist unklar ob und wie sie miteinander interferieren. Die hier dargestellten Studien nutzen Stimulationen mit UV, blauem und grünem Licht um unterschiedliche Photorezeptor Aktivitäten im Komplexauge auszulösen. Des Weiteren wurden drei verschiedene Duftkomponenten (Geraniol, Citronellol und Farnesol) verwendet. Diese Stimuli wurden in drei verschiedenen Experiment-Reihen gestestet. Das erste Experiment umfasste die klassische differentielle Konditionierung der Einzelmodalitäten (Duft und Farbe). Honigbienen zeigten eine hohe Lernfähigkeit bei der Unterscheidung zweier olfaktorischer Reize sowie eine solide Lern-Leistung während der Konditionierung mit Licht.

Im zweiten Experiment wurde getestet, ob ein zusammengesetzter Reiz aus beiden Modalitäten als Summe der einzelnen Elemente (elementare Verarbeitung) oder als unikaler Reiz (konfigurale Verarbeitung) wahrgenommen wird. Hierbei wurde monochromatisches Licht und einzelne Duftkomponenten in *positive patterning*- (PP) und *negative patterning*-Experimenten (NP) getestet. Beim PP, wurde der zusammengesetzte Reiz belohnt, wohingegen die Einzelkomponenten unbelohnt blieben. Dagegen wurden beim NP nur die Einzelkomponenten belohnt, aber nicht ihre Kombination. Außerdem wurde der Frage nachgegangen, ob die Fähigkeit zur Differenzierung unterschiedlich ist, wenn zwei verschiedene Lichtreize teil einer olfaktorisch-visuellen Kombination sind, oder nicht. Interessanterweise zeigten die Verhaltensleistungen einen prominenten Fall von konfiguraler Verarbeitung, allerdings nur wenn UV-Licht ein Element der olfaktorisch-visuellen Zusammensetzung war. Die Ergebnisse der Experimente mit blauem oder grünem Licht hingegen, unterstützen die Theorie einer elementaren Verarbeitung. Abschließend wurde mittels elektrophysiologischer *multi-unit*-Aufnahmen eine passende Methode etabliert, um die extrinsischen Neurone des Pilzkörpersausganges zu analysieren. Hierbei wurden drei verschiedene Düfte und zwei Farben sowie zwei Kombinationen aus Farbe und Duft getestet, um mögliche Variationen der multimodalen Reiz-Verarbeitung zu untersuchen. Zwei neuronale Einheiten (*units*) wurden gefunden, welche hauptsächlich auf Lichtreize antworteten.

# Contents

# Part I

# Main introduction

## Our environment is a multi-modal construct

We usually perceive our environment with various senses, like the smelling or tasting of food (Calvert et al., 2004). Not only the distinct smell or sweet taste of an apple has a certain association with the fruit, but also the predominantly red colour of the ripe fruit could tell us if it is delicious or if it is not eatable. Early in our childhood we learn to associate the bitter and sour taste of an unripened fruit with its colour, while trying to eat for instance a green apple or green berries. Consequently, we avoid fruits that have these visual signals. Comprising, a certain combinations of taste, smell and colour represents a multi-modal cue if something is providing a good food source or not. Several studies reveal, that it is sufficient to present only one stimuli of such a compound to elicit the same responses in human brains as it would the whole compound. The mechanism is the reactivation of crossmodal semantic associations by the human hippocampus (Gottfried & Dolan, 2003).

Not only humans perceive their environment as a multi-modal construct, as it was shown for flies (*Drosophila melanogaster*) and honeybees (*Apis mellifera*; Small, 2004; Leonard & Masek, 2014). Since the first behavioural observations of Karl von Frisch (Von Frisch, 1950), the honeybee's ability to perceive environmental properties with different modalities was object of many studies. During their foraging trips, they have to manage a multi-facetted optical flow of colour patches and detect the visual patterning that represents a batch of flowers, which represent their food resource. Those have different sensory cues with a multi-modal characteristic. Not only the scent of the flower bouquet, but also the destinct visual properties of a blossom could predict, if nectar and pollen is of good qualitiy (Leonard & Masek, 2014; Junker & Parachnowitsch, 2015). During the approach to a flower, bees would first receive the visual cue and then the odour cue of the plant. Thus, the colour is predicting the context of the odour cue (Chakroborty et al., 2016; Filla & Menzel, 2015; Gerber & Smith, 1998). Visual patternings on the blossom can serve as a cue to find the nectaries (Eugene Jones & Buchmann, 1974; Papiorek et al., 2015). Other modalities like gustatory or tactile information play an additional role during the perception of the food resource (Ruedenauer et al., 2015, 2018; Scheiner et al., 2001).

Despite the broad investigation of uni-modal properties of a flower stimulus, e.g. olfactory and visual cues (Spaethe et al., 2001; Lichtenstein et al., 2015, 2018; Takeda, 1961), their multi-modal compound represents still an under representated scientific field (Leonard & Masek, 2014; Mansur et al., 2018). Visual and olfactory cues are first processed at a peripheral level, the optic and olfactory lobes, and then projected via destinct pathways to higher order brain neuropils, the mushroom body (MB) calyces (Maronde, 1991; Paulk et al., 2014; Galizia & Kimmerle, 2004). Thereby, the mushroom bodies represent the multi-modal integration centre of the honeybee brain.

## Learning paradigms

Honeybees show their amazing learning abilities not only in experiments with free-flying conditions. They can be observed under controlled laboratory conditions too (Bitterman et al., 1983; Matsumoto et al., 2012; Brandes et al., 1988). Classical conditioning experiments (Pavlov, 1927) with honeybees based on the natural behavioural response to gustatory stimuli, the extension of the proboscis to uptake nectar (proboscis extension response, PER). First experiments using a PER protocol included olfactory stimuli with absolute and differential conditioning paradigms (Takeda, 1961). The learning mechanism in these paradigms underly appetitive processes (Menzel et al., 1993). Commonly, a sugar reward is presented to the bees by touching the antennae with a soaked tooth pick and then the bees are allowed to take up the reward for few seconds (Bitterman et al., 1983). This situation represents the unconditioned stimulus (US) of the conditioning paradigm. As the conditioned stimulus (CS) different stimulus types with various modalities, for example odour, colours or patterns could be tested. The CS is presented paired or unpaired with the US, resulting in different learning properties (Hammer & Menzel, 1995). In differential conditioning, two different types are presented, one is rewarded (CS+) and the other one remains unrewarded (CS-; Hammer, 1997). A special case of this differential learning task, is the so termed positive and negative patterning. The positive patterning includes the reward of the compound, whereas the single components would be unrewarded. In the negative patterning the reward situation would include the single components, but the compound is left non-reinforced. First experiments included odour compositions, in which bees had to learn that only a destinct combination of different odours represents the rewarded CS (Deisig et al., 2001, 2003, 2007). There are two learning strategies bees could use, while solving these task. One is the elemental processing theory (Spence et al., 1971) the other the configural processing theory (Williams & Braker, 1999; Myers et al., 2001). During elemental processing, the compound would represent the addition of the single components, and therefore the response to the compound would be higher than to the single components. Another strategy could be, that the tested individuals concentrate on one component to solve the positive patterning. The negative patterning can only be solved by using the configural processing, where the compound represents a unique cue (Deisig et al., 2001, 2003). While each single component has its own association with the reward, the unrewarded compound would elicit a novel response in the brain. Therefore, the response to it would by lower as to the single rewarded components. Hence, the positive and negative patterning experiments represent a promising learning paradigm for investigating cross-modal interactions during memory acquisition.

## Hypotheses and study aims

The aim of this thesis is to investigate the perception of olfactory and visual stimuli, and also their multi-modal compound in an established study organism, the honeybee. The processing of these stimuli is not only restricted to peripheral sensory neuropils, but also relies on the properties of the higher order neuropil, the mushroom body region in the honeybee brain. Therefore different methods will be used. The first two chapters will focus on the behavioral level. A classical differential conditioning paradigm, tested the learning abilities of honeybees during presentation of pure visual and olfactory stimuli and how the temporal discrepancy of the stimulus length for the best learning results for both modalities could be overcome. The obtained results should build a prerequisite for the published manuscript in the second chapter, exposing a more sophisticated task, the learning of olfactory-visual compounds and their single components in positive and negative patterning experiments. Furthermore, olfactory-visual compounds are presented with a consistent odour component. The third chapter will focus on the neuronal level using state-of-the-art techniques to investigate in the role of the multi-modal integration centre in the honeybee brain, the mushroom body. The multi-unit recording method provides insights in the neuronal activity of the mushroom body output region. In addition, backfill stainings with a fluorescent dye allow visualisation of optical and olfactory tracts and the verification of the recording electrode position.

# Part II

# Experiments

# 1 Classical conditioning of single modalities

## 1.1 Introduction

One reason why honeybees are well studied, is that individual worker bees can be trained to a classical conditioning paradigm, the Pavlovian conditioning protocol (Pavlov, 1927). To address questions regarding learning and memory, the so called proboscis extension response (PER) protocoll was developed in 1961 by Kimihisa Takeda (Takeda, 1961). First experiments included olfactory conditioning, where bees were harnessed in small tubes, able to move their proboscis, mandibles and their antennae (Bitterman et al., 1983; Takeda, 1961). During learning trials, the bees were presented different stimuli. In a Pavlovian conditioning paradigm (Pavlov, 1927) the stimulus that represents the reward is called the unconditioned stimulus (US). In the case of honeybees, this is normally a sucrose solution (represents the nectar of a flower) and can elicit an innate response (PER). The stimulus that should be learned is defined as conditioned stimulus (CS) and usually represents a neutral stimulus (no response). Through forward pairing the CS with the US, individuals learn to associate that a CS forecasts a delivery of the US (Pavlov, 1927).

### 1.1.1 Olfactory learning

The beginning of investigating in olfactory learning with a Pavlovian learning paradigm (Pavlov, 1927) and the PER was made by Takeda (Takeda, 1961) and later revised by Bitterman (Bitterman et al., 1983). There are two types of conditioning protcols which can be used for addressing olfactory learning abilities in harnessed honeybees. One is the simpler absolute conditioning, where individuals have to associate only one odour (CS) with the forward paired reward (US; Takeda, 1961). Despite mandatory control groups with backward pairing for this design (Hellstern et al., 1998) it is suitable for investigating simple questions of olfactory learning. A more complex design is the differential conditioning (Bitterman et al., 1983). Two odours are presented to the experimental animals during the acquisition trials. One odour is paired with the reward (CS+) and the other conditioned stimulus is presented unrewarded (CS−). This paradigm also serves as a within-group control for associative abilities of the bees. This design allowed researchers to study a various field of questions regarding olfactory learning. For example the generalisation of different odours (Guerrieri et al., 2005; Laska et al., 1999) and input sides during conditioning (Sandoz et al., 2001, 2003, 2002).

In experiments with olfactory compounds, honeybees showed an impressing ability to solve positive and negative patterning learning paradigms, suggesting that they use the so called unique cue theory rather than elemental processing (Deisig et al., 2001, 2003, 2007). During elemental processing, they would associate each component of the compound as a single element and summarise them (Spence et al., 1971). Whereas in configural learning strategies, the compound has its own novel association during classical conditioning, making it unique (Williams & Braker, 1999; Myers et al., 2001).

The role of a well timed stimulus length is crucial for honeybees to learn a given stimulus. In most experiments, a stimulus length of six seconds is used, with a forward paired three seconds presentation of the US at the end of the six seconds CS (Bitterman et al., 1983; Matsumoto et al., 2012). Also the intertrial interval (ITI) is an important aspect during learning trials, since memory consolidation needs a certain amount of time (Menzel et al., 2001).

### 1.1.2 Visual learning

Visual conditioning was more sophisticated, yielding in contradictory statements about the feasibility of such studies. The first successful experiments with visual learning tasks with harnessed honeybees required ablation of the antennae (Niggebrügge et al., 2009). Yet, advancing the experimental setup and learning conditions led to more reliable performances of bees during visual conditioning. In experiments with a paired odour-colour stimuli, the visual stimuli could predict a rewarding situation without blocking the learned olfactory stimuli (Gerber & Smith, 1998). Recent studies could show, that honeybees were able to solve a differential conditioning paradigm, with an CS presentation of only five seconds (Dobrin & Fahrbach, 2012). After these first successful studies, further investigations on the visual learning performances were done by testing different stimulus lengths and light transmissions and visual angles (Lichtenstein et al., 2018, 2015). Thereby it was shown, that honeybees performed best (PER rates up to 70 % with a CS length beginning with >10 seconds and a visual angle of 70° what is also corresponding to other studies (Jernigan et al., 2014; Riveros & Gronenberg, 2012). It is suggested, that visual conditioning takes longer, since the neuronal processing of a visual stimulus is more complex than an olfatcory stimulus.

### 1.1.3 Study aims

The aim of the experiments presented here, is to develop the basement for a multi-modal conditioning paradigm and therefore to investigate the temporal discrepancy between the best olfactory and the best visual learning during classical PER conditioning in honeybees. The hypotheses are, that honeybees show more learning capacities in differential olfactory learning, with a fast learning performance, regarding the stimulus length and the number of trials in contrast to visual learning. Furthermore, all tested odours should be learned equally well. During visual conditioning bees should be able to learn visual stimuli as well and will be able to discriminate two different colours, dependent on the distance between tested wavelengths. The results should represent the prerequisite for creating a learning paradigm with olfactory-visual compounds.

## 1.2 Material and Methods

### 1.2.1 Experimental subjects

For all experiments individuals of the european honeybee *Apis mellifera carnica* were used. Foragers were caught in the morning at the entrance of the hive with single glass vials and cooled on ice until they stopped moving. For further handling they were harnessed in small metal tubes and fixed with tape, so that the proboscis, mandibles and the antennae could move freely (Bitterman et al., 1983). The wings were protected by a small piece of paper, to avoid sticking to the tape. The bees were fed with 50 % sucrose solution (weight/weight) *ad libidum* and let them acclimatise to room temperature in a small dark box with high humidity. After one hour individuals were chosen for experiments by testing their PER. A toothpick soaked with 50 % sugar solution was held to the antennae and if the subjects moved their proboscis above a horizontal line between the mandibles, the bee was used for further experiments. Individuals were then placed in a plastic sleigh with single compartments, divided by plastic walls.

### 1.2.2 Stimuli

#### Odours

Three different odors were used for olfactory conditioning Geraniol, Citronellol and Farnesol (Sigma-Aldrich, Germany). Each odorant was diluted in Paraffin oil 1:100 (vol/vol) and stored in a small glass vials in the refrigerator until usage. Geraniol is an acyclic monoterpene allylalcohol (Fig 1.1A) which is a component of odour compounds in laurel, rose and coriander (Hagvall et al., 2007). Furthermore, it is a component of the pheromone released by the nasonov gland of honeybee, which is used for marking suitable food sources (Free, 1968). Citronellol (Fig 1.1B) is also an acyclic monoterpene allylalcohol with a rosier scent and is a component of citronell oil. Farnesol (Fig 1.1C) is a primary alcohol of the sesquiterpenes and has a floral scent. It is also a component of the pheromone released by the nasonov gland of honeybees (Pickett et al., 1980).

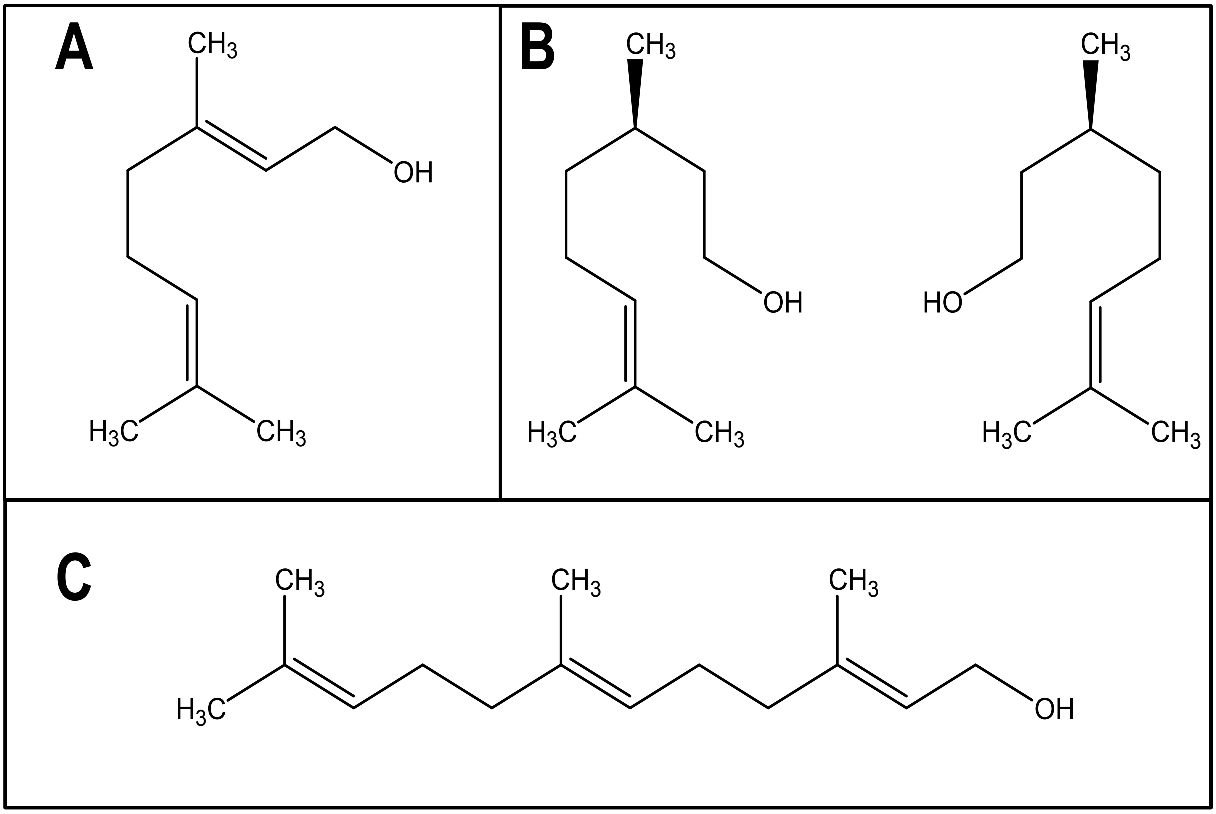

**Figure 1.1: Structural formulas of applied olfactory stimuli:** **A**) Geraniol, **B**) (R)-Citronellol (left) and (S)-Citronellol (right) and **C**) Farnesol.

### LED lights

Three different LED lights were chosen for experiments : UV (375 nm, intensity: 7.5 x 1013 photons/cm$^2$/s, TRU Components, Conrad, Hirschhaid, Germany), blue (465 nm, intensity: 6.5 x 1013 photons/cm$^2$/s, Avago Technologies, Broadcom Inc., San José, CA, USA) and green (525 nm, intensity: 3.93 x 1013 photons/cm$^2$/s, Avago Technologies, Broadcom Inc., San José, CA, USA; Fig 1.2A). All three wavelengths are in range of the receptor sensitivity (Fig 1.2B) and can be separated on the receptor level of the honeybee eye (see supplemental material in Becker et al., 2019).

## 1.2.3 Training apparatus

The olfactory stimuli device was built out of glass pipettes and teflon tubes. The consistent air flow (1 l/min) was led through a main pipe, to which two small pipes were attached. One of the small pipes contained a filter paper stripe (1 x 8 cm) with 6 µl of the stimuli odour. The other contained a blank paper stripe as the continuous airflow. The air flow was generated via a Synchtec CS-55 (Ockenfels Syntech GmbH, Kirchzarten, Germany) and for the stimulus/continuous flow adjusted to 0.5 l/min. An air exhauster was installed behind the training apparatus, to avoid accumulation of the odorants during the training procedure. The main pipe was positioned two centimetres in front of the tested animal. The visual stimuli device was

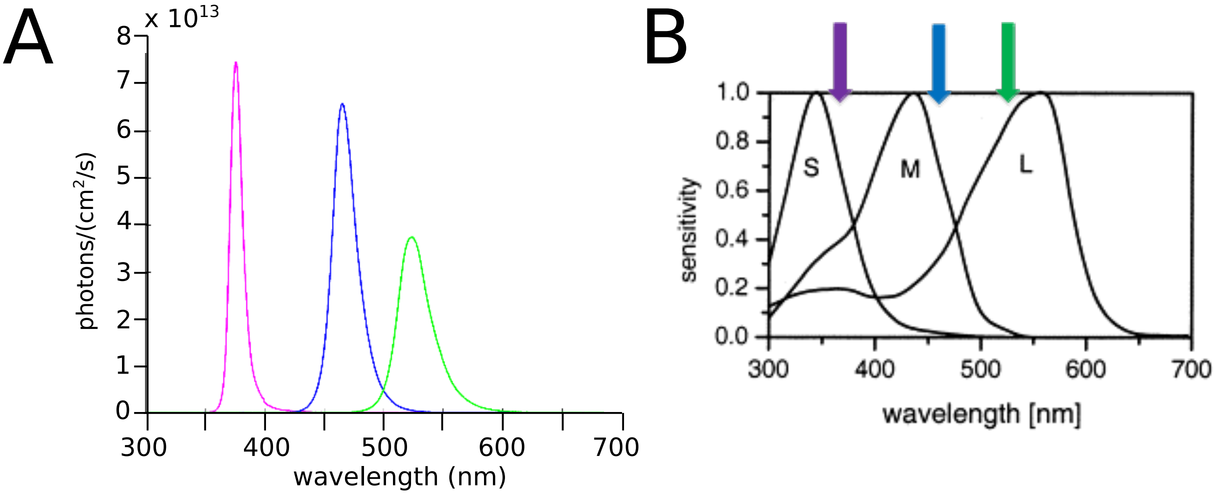

**Figure 1.2: Wavelengths of applied visual stimuli: A)** Intensity spectras of UV, blue and green, **B)** Receptor sensitivity of the thee receptor types of the honeybee eye (modified from Vorobyev et al., 2001). Coloured arrows (purple = 375 nm, blue = 465 nm, green = 525 nm) indicate the wavelength of the used LED lights.

built out of a cold light guide system of acrylic glass and three different LED lights (UV, green, blue). The acrylic glass rod was hanging three centimetres above the trained animal and was positioned orthogonal to the olfactory stimuli device. Both devices were controlled via the foot pedal of the Synchtec CS-55. The TTL pulses of the Synchtec CS-55 enabled synchronising the visual stimulation with the olfactory stimulation. The computer program "TimingProtocol" (Lichtenstein et al., 2018) was used to give acoustical and visual cues to the experimenter.

### 1.2.4 Training procedure

A differential conditioning paradigm was used, consisting of nine non-reinforced stimulus (CS-) presentations and nine reinforced stimulus (CS+) presentations. Each trial was initiated by sliding the sleigh side-wards, bringing the tested animal in a frontal position to the stimuli device. A resting phase of 15 s was obligatory before and after giving the CS. Olfactory stimuli had a stimulus length of six seconds, whereas visual stimuli lasted for 10 seconds. For reinforced trials, subjects were rewarded with an unconditioned stimulus (US) consisting of 30 % sugar solution during the last three seconds of the CS+ presentation. During unrewarded trials, a dry toothpick was presented in the same manner to avoid conditioning to the hand's movement of the experimenter. In total, 18 trials were performed in a pseudo random order. For visual conditioning, a memory test was performed one hour after acquisition, where both CS+ and CS- were presented once. The inter trial interval (ITI) for each animal was set to eight minutes for all trials. In a second series of olfactory conditioning with only seven trials, a stimulus length of 10 seconds was tested, to ensure that the odour was still present in the airflow during prolonged experiments.

### 1.2.5 Response measurements and statistical analyses

PER measurement was done by using a binary code. If the bee extended its proboscis over a virtual horizontal line between the mandibles before US presentation a PER (1) was counted. Only bees that survived the whole experiment and showed more than three times a PER to the US (0 as binary code) were taken for statistical analyses. All analyses were done with R Studio (Version 1.0.143, RStudio, Inc). For the acquisition trials a Wilcoxon signed rank test for in-group comparisons was used. For the memory test a $\chi^2$-test was used. Descriptive analyses were done by using the package ”ggplot2” (ggplot2 version 3.0.0, Wickham, 2016) and the percentage of the binary PER recordings during the acquisition trials (learning curves) and for the one trial memory test (bar plots) during visual conditioning. The alpha level was set to 0.05 for all statistical analyses.

## 1.3 Results

### 1.3.1 Olfactory conditioning

In total 183 individuals were trained to three different odour pairings with a stimulus length of 6 s. One group was trained to Citronellol (CS+) and Farnesol (CS−; n=27, Fig 1.3, left upper panel) and another was trained vice versa (n=27, Fig 1.3, left lower panel). Both groups were able to discriminate reliably between the given stimuli beginning with the third trial, resulting in high PER rates (about 80 %) for the CS+. For the groups that were trained to Citronellol (CS+) and Geraniol (CS−; n=35, Fig 1.3, middle upper panel) and vice versa (n=41, Fig 1.3, middle lower panel), also both groups were able to discriminate both odours starting with the thrird trial. But PER rates were higher in the group that received Geraniol as the reinforced stimulus (Fig 1.3, middle lower panel). Similar results were found in the odour pairing Farnesol and Geraniol (Fig 1.3, right panels). The group that received Geraniol as the rewarded odour (Fig 1.3, right lower panel) showed higher PER rates compared to the group that received Farnesol as the CS+ (Fig 1.3, right upper panel), where PER rates reached only about 60 % in the last two conditioning trials.

In a second series of experiments, 20 animals were tested with a stimulus length of 10 s during a differential conditioning with Geraniol (CS+) and Citronellol (CS−), to test whether the prolonged stimulus length had an impact on the learning performance. The animals showed a significant ability in discriminating both odours, beginning already with the seconds trial (n=20, Fig 1.4), for all 7 trails.

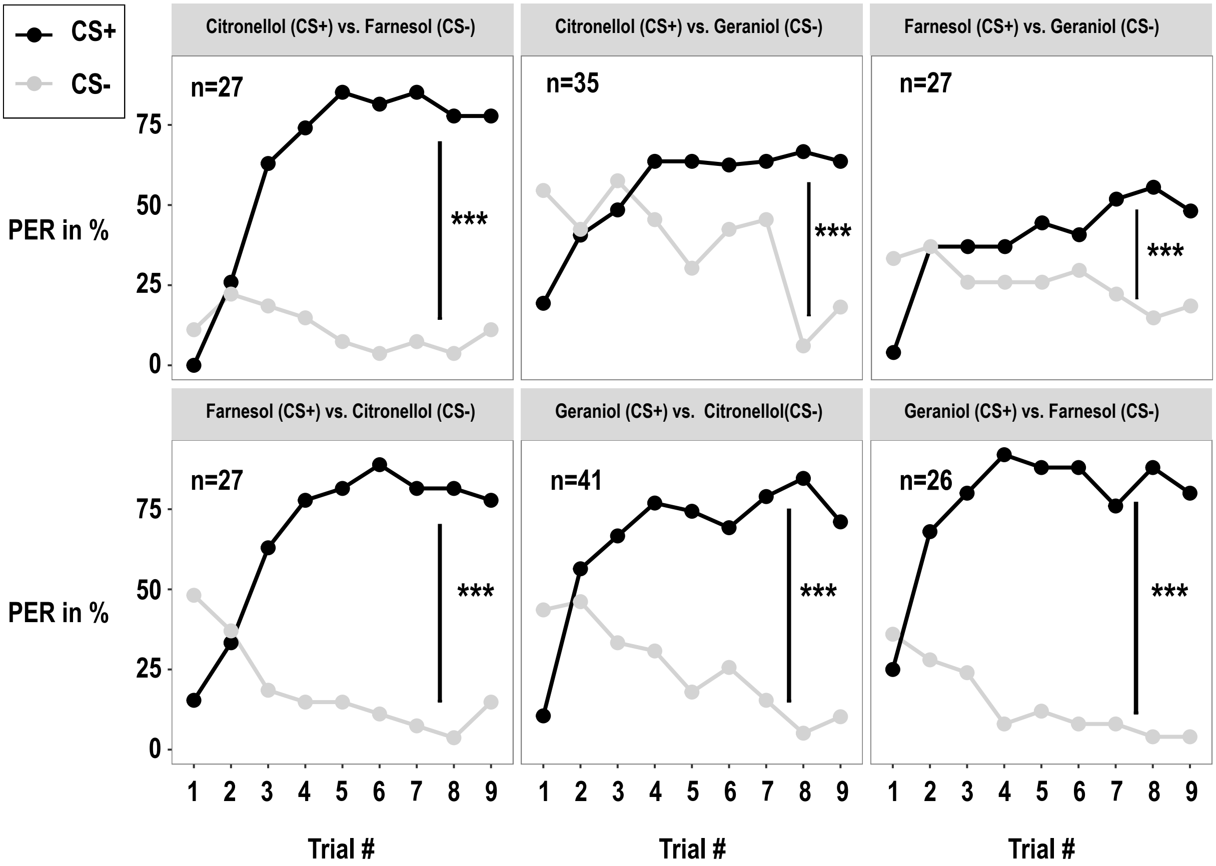

**Figure 1.3: Acquisition curves for differential conditioning with three different odour pairings:** At the left upper panel the acquisition trails for Citronellol vs. Farnesol, in the middle upper panel for Citronellol vs. Geraniol and at the right upper panel for Farnesol vs. Geraniol (and vice versa in the lower parts) are shown. Honeybees learned to discriminate between all odour pairings (Wilcoxon signed rank test, *** $p < 0.001$).

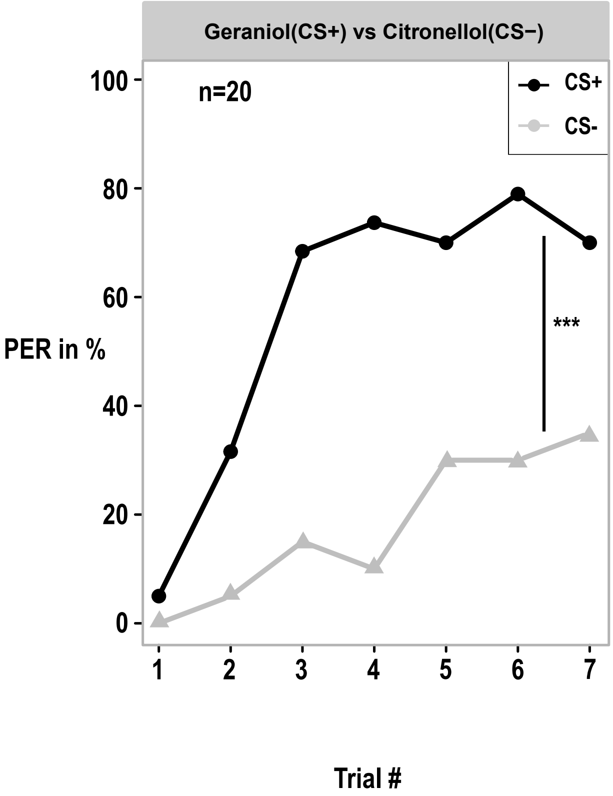

**Figure 1.4: Acquisition curves for differential conditioning with 10 s stimulus length:** 20 individuals were tested with Geraniol and Citronellol during seven trials. Bees were able to discriminate between the odours (Wilcoxon signed rank test, *** $p < 0.001$).

### 1.3.2 Visual conditioning

In total 225 animals were conditioned to a visual discriminating task with three colour pairings. Only 154 of them received a memory test after 1 h due to a changing experimental design. The test was added some time after the establishment of the experiment, resulting in more animals that received only acquisition trials, but did not perform a memory test (compare Fig 1.5C, E and F). Two groups were tested with UV as the reinforced stimulus and received blue (n= 25, Fig 1.5A) or green (n= 27, Fig 1.5D) as the unrewarded colour stimulus. In both groups the individuals were able to discriminate the stimuli on a reliable basis after the third conditioning trial and could conserve the learned information during the memory test. Highest PER rates were in all cases slightly above 40 %. The next two groups were trained to blue as the CS+ and UV (n=26, Fig 1.5B) or green (n=56 for acquisition, n=29 for memory test, Fig 1.5E) as the CS−. In both groups the bees were able to discriminate both colours during acquisition and the memory test, but only the blue (CS+) versus UV (CS−) pairing reached about 40 % PER rates. The combination blue (CS+) versus green (CS−) elicited only about 30 % PER rates. Similar results were found in the last two groups, with green as the reinforced colour stimuli. The group tested with UV as the CS− (n=27 for acquisition, n=18 for memory test Fig 1.5C) showed higher PER rates than the group tested with blue as the non-reinforced

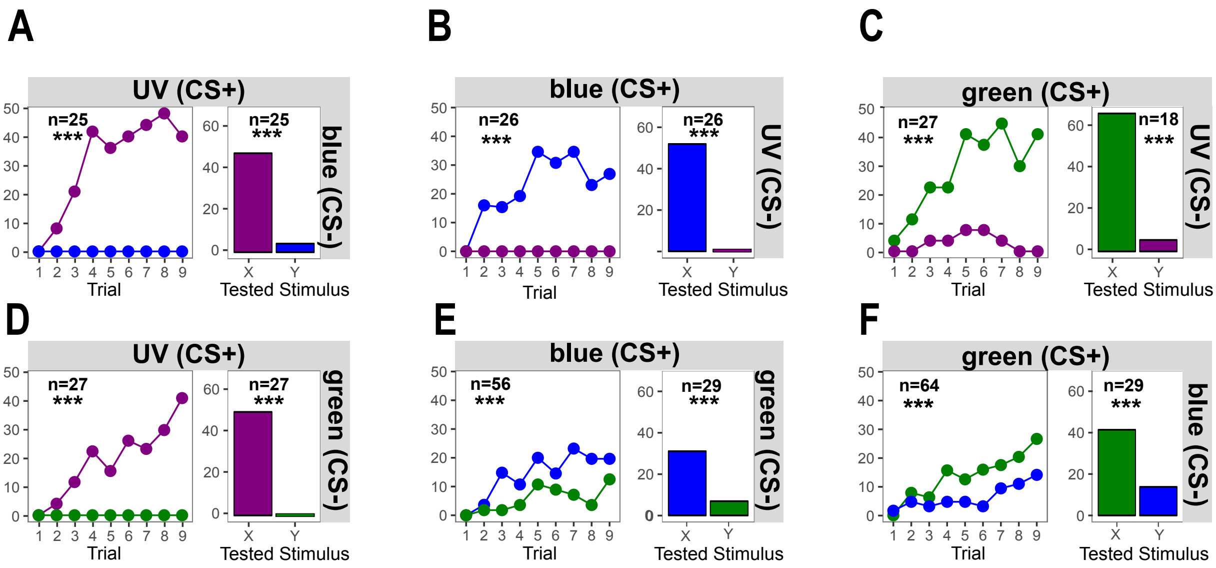

**Figure 1.5: Acquisition and memory performance of honeybees during differential conditioning with three colour pairings: A)** Honeybees were able to discriminate between UV and blue during acquisition and in the memory test. **B)** Also tested vice versa, bees showed a high learning performance. **C)** The pairing green vs. UV was also significantly discriminated by the tested animals and in **D)** vice versa. **E)** During the acquisition with blue and green and during the memory test bees showed the ability to discriminate the colour pairings. This was also the case if tested vice versa in **F)** (Acquisition: Wilcoxon signed rank test, *** $p < 0.001$; Memory test: $\chi^2$-test, *** $p < 0.001$).

stimuli (n=64 for acquisition, n=29 for memory test, Fig 1.5F). Nevertheless both groups were able to significantly discriminate both stimuli after the third conditioning trial.

## 1.4 Discussion

Olfactory and visual stimuli were tested in a classical differential conditioning task with honeybees. Individuals showed significant differences in their responses between the CS+ and CS− for both odour and colour stimuli.

For olfactory conditioning, these results were not surprising. Several studies could show that the ability of honeybees to discriminate between two olfactory stimuli is reliable (Bitterman et al., 1983; Takeda, 1961), even for complex odour compounds (Laska et al., 1999; Guerrieri et al., 2005; Deisig et al., 2003). The stimulus length seems not to affect the learning performance, since a stimulus length of three seconds was still sufficient to elicit a PER (for a detailed summary see Matsumoto et al., 2012). The prolonged stimulus length of 10 s had also no effect in this study, since the airflow was adjusted in a manner that the odour was not volatilised too early during the stimulus. That implies that the stimulus/continuous airflow was slower than the consistent airflow (see Chapter 1.2.3), resulting in a slower emptying of the odour molecules in the odour container. Interestingly, PER rates for the CS+ in groups with Geraniol as the non-reinforced stimulus (Fig 1.3 upper middle and upper right panel) were lower than in the other tested groups (Fig 1.3). Geraniol is a component of the recruiting pheromone of the nasonov gland in honeybees (Free, 1968). That implies, that Geraniol may have an innate preference in this context, since the tested indivdiuals were not naïve and had most likely extensive foraging experiences. Therefore, they might have an existent positive association to this odour, resulting in higher responses for the unrewarded stimulus than for the other tested odours. For further experiments, it is also mentionable, that trials in olfactory conditioning started randomised with the CS+ or the CS−, resulting in responses to the unrewarded stimulus in trials, where the CS+ was presented first. Thus, all following experiments were started with the CS− as the first trial, to avoid this phenomenon.

In experiments with visual stimuli, honeybees showed also the ability to learn the discrimination task. All tested pairings could be significantly differentiated during acquisition and the memory test (Fig 1.5). The stimulus length of 10 s was shown to be sufficient already in recent studies (Lichtenstein et al., 2018, 2015). As already described by Menzel (Menzel & Snyder, 1974), the distance between the tested wavelength is a criterion how reliable honeybees discriminate and learn the visual stimuli, with best learning performances for the UV and blue range (Lichtenstein et al., 2015; Menzel & Blakers, 1976). In the study presented here, honeybees showed higher PER rates for colour pairings that include UV as a conditioned stimuli (Fig 1.5A, B, C, D). The PER rates for the blue versus green pairing were not as high as the latter mentioned, and the relative amount of animals responding to both stimuli was higher (Fig 1.5E, F). These findings were also conserved during the memory test. Interestingly the UV stimuli could be learned as good as the other two wavelengths, despite recent studies suggest a poorer learning performance for UV light in africanised honeybees (Jernigan et al., 2014).

The PER rates for olfactory conditioning were higher than for visual conditioning, even though PER rates in recent studies (Lichtenstein et al., 2018) were nearly as high as those for learning trials with odours (for a detailed summary see Matsumoto et al., 2012). The harnessed situation is a very artificial situation to the honeybees. Their learning performances for colourful objects, such as artificial flowers or even patternings and symbols is much better in a more natural experimental setup, such as free-flying learning paradigms or in y-maze arenas (Giurfa, 2004; Schultheiss et al., 2017). The importance of differentiating certain wavelengths or colour patterns seems to have more impact on the orientation during the foraging flight and the approach to the blossom. It could be shown that certain spots or colour patterns on the flower attract the pollinators, when they are in a close range and act as a signal for a promising food source (Papiorek et al., 2015; Dyer et al., 2012). The associative strength of olfactory stimuli seems to be higher than those for visual stimuli alone under harnessed conditions (Gerber & Smith, 1998, compare Fig 1.3, Fig 1.4 and Fig 1.5). Basically, the reward association is built faster during olfactory conditioning, since there is a direct connection to the VUMx1 neuron in the olfactory pathway (Hammer, 1993). Whereas the association with a visual rewarded stimuli takes longer

(Lichtenstein et al., 2018), resulting in a temporal discrepancy of the stimulus length for best learning performances for olfactory and visual conditioning. This factor should be considered regarding the conditiong of olfactory-visual compounds, consequentially presenting the compound with a sufficient stimulus length for both components.

### 1.4.1 Conclusions

For testing olfactory-visual compounds in further experiments, these results are a premise for developing a multi-modal conditioning paradigm that is suitable for the major challenge to combine olfaction and vision to a compound in a straightforward fashion. The stimulus length of 10 s and the reliable ability to learn and discriminate visual stimuli against UV is a mandatory element for further investigations. The detailed description of this study is the main topic of the next chapter.

# 2 UV-light in olfactory-visual compounds

This chapter adresses the question how UV light perception is modulated in an olfactory-visual compound during classical conditioning. These results are subject in the following manuscript: **Mira C. Becker, Wolfgang Rössler, Martin Fritz Strube-Bloss** (2019) UV-light perception is modulated by the odour element of an olfactory-visual compound in restrained honeybees. **Journal of Experimental Biology** *accepted manuscript.*

**Figure 2.1: Honeybee foraging on an apple blossom**: Flowers represent a multi-modal construct, consisting of olfactory, visual, gustatory and tactile cues to determine the quality of a food resource.

# UV-light perception is modulated by the odour element of an olfactory-visual compound in restrained honeybees

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## Abstract

Honeybees use visual and olfactory cues to detect flowers during foraging trips. Hence, the reward association of a nectar source is a multimodal construct which has at least two major components – olfactory and visual cues. How both sensory modalities are integrated to form a common reward association and whether and how they may interfere, is an open question. The present study used stimulation with UV, blue and green light to evoke distinct photoreceptor activities in the compound eye and two odour components (Geraniol, Citronellol). To test if a compound of both modalities is perceived as the sum of its elements (elemental processing) or as a unique cue (configural processing) we combined monochromatic light with single odour components in positive (PP) and negative patterning (NP) experiments. During PP, the compound of two modalities was rewarded, whereas the single elements were not. For NP, stimuli comprising a single modality were rewarded, whereas the olfactory-visual compound was not. Furthermore, we compared the differentiation abilities between two light stimuli with and without being part of an olfactory-visual compound. Interestingly, the behavioural performances revealed a prominent case of configural processing, but only in those cases when UV light was an element of an olfactory-visual compound. Instead, learning with green- and blue-containing compounds rather supports elemental processing theory.

# INTRODUCTION

Angiosperms attract suitable pollinators using sophisticated olfactory and visual cues culminating in impressive cases of pollinator manipulations for maximizing pollen transfer (Schiestl and Schlüter 2009). Honeybees are well known pollinators that associate the multifaceted features of a flower with its rewarding components – pollen and nectar (Dyer et al. 2012; Dyer and Garcia 2014; Giurfa 2004; Raguso 2004). It remained unclear, however, how combinations of different sensory modalities are learned and shape behavioural decisions.

In a controlled laboratory environment, honeybees can be classically conditioned using the well-established proboscis extension response (PER) paradigm. This assay was used, for example, to ask whether honeybees can discriminate between olfactory stimuli (for a detailed summary see for example: Matsumoto et al. 2012) or between visual stimuli, for example when monochromatic lights were associated with a reward (Dobrin and Fahrbach 2012; Hori et al. 2006; Lichtenstein et al. 2018) or punishment (Mota et al.2011). In an aversive operant conditioning paradigm using a walking arena, bees could learn that a certain wavelength is signalling safety (Kirkerud et al. 2017), a behaviour that was also observed in *Drosophila* (Vogt et al. 2015). Original colour learning experiments with harnessed honeybees were only successful after cutting off both antennae (Niggebrügge et al. 2009). However, recent modifications of the PER protocol proved that bees reliably learn visual stimuli with both antennae intact (Dobrin and Fahrbach 2012; Lichtenstein et al. 2018; Lichtenstein et al. 2015). This opened up the prerequisite for our study addressing the question how honeybees combine olfactory and visual cues to form a common percept of a visited flower. Previous studies provided arguments for the existence of such interactions between the two modalities. Experiments by Mota et al. (2011) suggest that the visual component is less important in an olfactory context and more difficult to learn but can act as a cue during olfactory conditioning. An earlier study by Gerber and Smith (1998) shows that visual pre-training modulates learning of an odour stimulus. However, these studies used both modalities temporally separated in different experimental phases and designs, whereas the natural situation during a flower visit provides visual and olfactory cues simultaneously with the reward. Hence, the flower-reward association might represents a combination of olfactory and visual stimuli, with a complex ranking and interplay during a close-up situation (Kevan and Lane 1985; Menzel and Greggers 1985).

To address this question, we adapted a positive patterning (PP) and negative patterning (NP) paradigm that had previously been used to investigate whether an odour mixture of two single odour components are perceived as a unique cue or as the sum of its single elements (Deisig et al. 2001; Deisig et al. 2003; Deisig et al. 2007). The two options previously had been termed configural or elemental processing roles, respectively. Most recent findings (Mansur et al. 2018) suggest that bees may even use a pronounced form of a configural learning strategy (Williams and Braker 1999) to combine both modalities. However, this might depend on the visual stimulus used during the experiments, since not all wavelengths may have the same impact. For example, the natural context of UV light is different

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from the relevance of other colours perceived by bees (Papiorek et al. 2015). We therefore included the three monochromatic wavelengths forming the basis for the trichromatic visual system of the bee: UV, blue and green (Hori et al. 2006; Menzel 1981; Menzel and Blakers 1976) in our experimental paradigm. To test our visual stimulation device, we performed electroretinogramme recordings for all visual stimuli. This ensured that all wavelengths evoked distinct receptor neuron activity (cp. supplemental material, Fig. S1). Furthermore, tests showed that all used wavelengths were discriminated by the bees in a classical differential conditioning experiment. To find out which learning strategy might be used during olfactory-visual integration, we presented the olfactory-visual compound as the reinforced stimulus (PP) or as the non-reinforced stimulus (NP), with the single elements being unrewarded (PP) or rewarded (NP), respectively. In addition to a memory test after one hour, we included generalisation tests for a novel odour and light element as well as their odour-light compound stimulus. This allowed us to test if bees judge novel stimuli in a similar way compared to the modality combinations they were confronted with during training. In a second series of experiments, we tested if two olfactory-visual compound stimuli can be discriminated in a classical differential learning experiment and if odour presence has an influence on the separation between two visual stimuli.

## MATERIAL AND METHODS

### *Experimental animals*

Foragers of *Apis mellifera carnica* were caught individually in small glass vials at the entrance of the hive in the morning at our departmental bee station (University of Würzburg) before each experiment during the summer season (May-September) 2017. During the winter season (October 2017 - February 2018) bees were maintained in a heated glasshouse with an artificial light source and fed with pollen and 50% sugar solution (weight/weight) ad libitum. Only pollen foragers were collected for the experiments, since their sucrose responsiveness is high for the used reward sucrose concentration of 30%, whereas water and nectar foragers show a rather variable gustatory response score (Scheiner et al. 2004) which might influence the learning performance. Bees were immobilised on ice and harnessed in small metal tubes that allow the proboscis and the antennae to move (Bitterman et al. 1983). About 1.5 h before the experiment started, bees were fed ad libitum using a 30% sugar solution (weight/weight) and adapted to the light conditions in the laboratory (dimmed room with red light, average temperature: 24°C). A recent study could show, that there is no difference between bees tested with dimmed light or under moderate ambient illumination similar to natural conditions (Lichtenstein et al. 2018). Ten minutes before the first conditioning trial bees were tested for intact proboscis extension response (PER) by touching the antennae with a toothpick soaked with 30% sugar solution (w/w) without subsequent feeding. Only individuals showing a PER were chosen for experiments.

### *Odour stimulation*

We used a Syntech CS-55 (Ockenfels Syntech GmbH, Kirchzarten, Germany) generating a continuous air flow of 1.0 l $m^{-1}$ and added a stimulus flow of 0.5 l $min^{-1}$ which was shifted between a blank and a stimulus pipette to prevent mechanical stimulation (Fig. 1A). We tested all single odour components which could be differentiated in classical conditioning experiments (data not shown). The odours (Geraniol and Citronellol; Sigma-Aldrich, Germany) were diluted in Paraffin oil 1:100 (vol/vol). Six µl of the used odour-solution were pipetted on filter paper stripes (1 x 8 cm) in three drops of two µl in a row and placed into the stimulation pipette.

### *Light stimulation*

We used three different LED light sources : 375 nm (UV, intensity: 7.5 x $10^{13}$ photons/cm²/s, TRU Components, Conrad, Hirschhaid, Germany), 465 nm (blue, intensity: 6.5 x $10^{13}$ photons/cm²/s, Avago Technologies, Broadcom Inc., San José, CA, USA), and 525 nm (green, intensity: 3.93 x $10^{13}$ photons/cm²/s, Avago Technologies, Broadcom Inc., San José, CA, USA) (Fig. 1B). To control and synchronise the light stimulation with the odour presentation, we used the TTL output of the Syntech CS-55. Our custom build light device was positioned three centimetres above the tested bee and orthogonal to the odour stimulation device (Fig. 1).

### *Training procedure*

The restrained experimental bees were placed on a plastic sleigh with multiple bees and walls between individual bees to avoid cross stimulations. The sleigh was moved along a horizontal line in front of the stimulation devices. An air extractor behind this setup ensured that the tested odours were not accumulating around the bee (Fig. 1A, B). The timing of the training procedure was adapted from previous studies (Lichtenstein et al. 2018; Lichtenstein et al. 2015; Riveros and Gronenberg 2009; Riveros and Gronenberg 2012). Before and after each stimulation bees had a 15 s resting phase in front of the airstream to accustom to the training situation. Durations of the conditioned stimuli (CS- and CS+) were set to 10 s. The unconditioned stimulus (US, 30% sugar solution (w/w)) during reinforced trials (CS+) was presented overlapping for the last three seconds of the CS. The US was presented on a toothpick. A dry toothpick was presented in the same way during CS- trials, to avoid conditioning to the toothpick presentation itself. We used an eight-minute inter trial interval (ITI). ITI timing was controlled via a custom-made software “TimingProtocol” (freely available on request, Lichtenstein et al. 2018) which provided the experimenter with acoustic cues for experimental control.

## *Experimental design*

### Positive and negative patterning

*Acquisition trials*

During positive patterning (PP), the olfactory-visual compound was presented rewarded (**AX+[CS+]**) and the single elements, “odour” (**A-[CS-]**) and “light” (**X-[CS-]**) were non-reinforced (Fig. 1C, first row). During negative patterning (NP), the compound was non-reinforced (**AX-**), but the single elements were rewarded (**A+, X+**) (Fig. 1C, second row). Each stimulus was repeated seven times, resulting in a total of 21 trials. Hence, conditioned bees received seven reinforced trials during PP and 14 reinforced trials during NP. CS+ and CS- stimuli were presented pseudorandomised in a way that each stimulus occurred only two times in a row, but randomly. Furthermore, we made sure to always start with a CS-. All experiments were obtained during the summer season.

*Memory and Generalisation test*

One hour after the last acquisition trial a memory and generalisation test was conducted. The learned single elements (**A, X**) and the compound (**AX**), as well as two novel elements (**B, Y**) and a novel compound (**BY**) were presented randomised one time each.

### Differential conditioning of two compounds with same odour component

*Acquisition trials*

To test if bees learn to discriminate two different olfactory-visual compound stimuli we made sure that the bees did not use the odour information alone by using the same odour in both compounds. Each stimulus was presented 9 times, resulting in a total of 18 trials. For each pair of stimuli, we tested two groups of animals in which we exchanged the CS+ and CS-. Furthermore, we combined this olfactory visual compound training with the results we obtained from purely visual conditioning of the light components to ask if the odour component has a reinforcing influence on visual learning. All experiments were obtained during the winter season.

*Memory test*

The memory test was performed one hour after the last acquisition trial and contained presentations of the conditioned compounds (**AX** and **BX**) and of each single component (**A, B, X**), to test if the latter can substitute for the conditioned compound after acquisition. In total, the memory test comprised five trials in random order.

### *Response measurement and statistical analyses*

A PER was counted if the bees extended their proboscis over a virtual line between the mandibles. A binary response (1) during acquisition was counted in cases when the response to the CS stimulation occurred before the US had been presented. Only bees that survived the entire experimental procedure were taken for statistical analysis.

All analyses were done with R Studio (Version 1.0.143, RStudio, Inc.). For descriptive analyses we used the package “ggplot” and plotted the percentage of the binary PER recorded during the acquisition trials (learning curves) and for the one trial generalisation/memory tests (bar plots). For positive and negative patterning experiments, we computed different generalised linear models (GLMs) and used an analyses of variance (ANOVAs) for repeated measurements for within-group and between-group comparisons on the most suiting model. Even though ANOVA usually is not allowed for dichotomous data such as those of the PER experiments, Monte Carlo studies have shown that ANOVA can be used under certain conditions (Lunney 1970), which are met by our experiments: equal cell frequencies and at least 40 degrees of freedom in the error term. For post hoc comparisons, we used Tukey HSD tests. For statistical analyses of differential conditioning of two compounds, we used Wilcoxon signed rank tests for within-group comparisons. For the memory tests, we used Cochran’s Q test for within-group comparisons. For significant differences, pairwise comparisons using the Wilcoxon sign test (with Bonferroni correction) were performed. The alpha level was set to 0.05 for all statistical analyses.

## Results

### *Patterning experiments*

#### Odour dominates olfactory-visual compound learning

During positive patterning (PP) experiments with Geraniol and blue (n=66, Fig. 2A) or Geraniol and green (n=66, Fig. 2B) as the reinforced compound, the animals associated the compound with the reward. However, the learning performance to the olfactory element alone reached the same response rates although it never had been presented rewarded (Fig. 2A, B, left panels). Only the light element was discriminated from the pure odour and the olfactory-visual compound. The same ranking appeared in the memory test (Fig. 2, right panels) reflecting a predominantly odour driven reward association. This was confirmed by the generalisation tests. Here, both the novel odour and the novel olfactory-visual compound were generalised to the initially trained olfactory-visual compound (Fig.2A, B). The only exception occurred in the group that had been trained to a compound containing blue light. Here the novel UV-odour compound was not generalised to the initially trained olfactory-visual compound (Fig. 2A).

### UV component interferes with the olfactory-visual compound

A different picture emerged when UV light was used as visual element during PP. In total, we trained 121 bees to discriminate an olfactory-visual compound of Geraniol and UV (CS+) from its single elements (CS). As in the previous experiments, bees learned to associate the compound with the reward (Fig. 3). However, although not rewarded, the PER performance to the odour alone was significantly increased compared to that of the compound, whereas UV light evoked almost no response and was significantly different from both the compound and the odour stimulus (Fig. 3). Moreover, this effect was still present during the memory test and not generalised to the novel compound stimulus including green light (Fig. 3, right panels).

### Bees learn light and odour elements equally well, but do not differentiate their compound

In negative patterning experiments bees had to learn that the single elements, Geraniol and blue, were rewarded (CS+), but their compound was not (CS-) (n=61, Fig. 4A). In another group we tested green light instead (n=60, Fig. 4B). In both cases, the bees failed to discriminate. However, in our experimental setting bees showed the same learning performance and established a reward association with light only and odour only stimuli as well, reaching equally high learning rates (about 60%). However, there was a tendency that during the memory test the single reward-associated elements can be discriminated from the non-rewarded olfactory visual compound, which was significant for the odour, but not for the blue light (Fig. 4A). For the generalisation test, the single elements (Citronellol and UV) as well as their compound were introduced as novel stimuli. The trained bees did not generalise the single-element reward associations to the novel light only and the novel odour-light compound, but generalised the single odour element (Fig. 4A, B). This illustrates, that after our training procedure a novel odour element was perceived differently compared to an olfactory-visual compound including the same odour. However, this difference in generalisation predominantly occurred when UV-light was introduced as an element of a novel olfactory-visual compound (Fig. 2 and 4).

### Bees solve negative patterning when UV is an element of the olfactory-visual compound

In negative patterning experiments including UV light, 121 bees were conditioned in total. The bees had to learn that the single elements, Geraniol and UV, were rewarded (CS+), but their olfactory-visual compound (CS-) was not. The PER rates for the latter were significantly lower compared to the single elements (Fig. 5). Thus, if UV light is part of the olfactory visual compound stimulus the compound can be differentiated from its single elements. However, this was not the case for other tested wavelengths (Fig.4). During the memory test the single reward associated odour element was differentiated significantly from the olfactory visual compound, whereas the single reward associated light was not. However, the trained bees generalised to the novel compounds and their single elements when blue light was introduced (Fig. 5, upper panel), but only to the single elements when green light was the novel stimulus component (Fig. 5, lower panel).

### *UV-light perception is modulated in an olfactory-visual compound*

To understand how an odour element can modulate light perception, we trained bees to discriminate two olfactory-visual compound stimuli. To make sure that light identity is the only difference, we had to keep the odour information constant. In total, we trained 4 groups of bees to separate blue and UV light as well as green and UV light. The odour-UV compound was either unrewarded (Fig. 6A and 7A) or rewarded (Fig. 6B and 7B). For the pure-light discrimination this kind of inverted meaning did not have any influence on wavelengths separation (cp. right subpanels Fig. 6 and 7). However, a different picture emerged when the light information was part of an olfactory-visual compound. Bees could differentiate better between the lights if UV was part of the unrewarded compound (Fig. 6A and 7A). Instead, in cases when UV was part of the rewarded compound, bees were only able to discriminate the most different wavelengths (Fig. 6B and 7B). This suggests modulation of light perception by the odour element of an olfactory-visual compound and represents a case of sophisticated cross modal stimulus interactions.

## Discussion

### Odour dominates olfactory-visual compound learning in PP

The ability to discriminate the individual modalities odour and light and their olfactory-visual compound was investigated using positive (PP) and negative patterning (NP) experiments. During PP, when only the olfactory-visual compound was rewarded, honeybees showed, in addition to the reward associated compound, a high response to single olfactory stimuli (Fig. 2 and 3), even though they had never been rewarded in this experimental context. This mostly olfactory driven reward association could be confirmed by a memory and a generalisation test one hour after the last conditioning trial. We therefore conclude that bees cannot solve cross-modal PP discrimination of olfactory and visual information. In contrast, a recent study showed that bees can solve this problem starting with the 6th conditioning trial (Mansur et al. 2018). This is equivalent to the number of learning trials we performed in our study. Thus, the number of conditioning trials alone cannot explain this discrepancy. Unfortunately, Mansur et al. (2018) did not test if the established olfactory-visual compound reward association was also generalised to a novel olfactory-visual compound and its single elements to strengthen their findings.

### Bees learned light and odour elements equally well in NP

In the NP experiments, honeybees showed equally high learning performances to both, the odour element and the light element which they could not differentiate from the unrewarded compound if blue and green light was an element (Fig. 4). A trend in separating the olfactory-visual compound from the pure odour might be established during the memory test (Fig. 4A, B, right panels). However, if UV-light was one of the elements bees solved the NP task and memorised that information which they partially generalised to novel stimuli (Fig. 5). Our results, therefore, are partially in line with the observations by Mansur et al. (2018) who also reported the capability of solving cross modal NP. In contrast to their experiments, where the reward association of the visual element occurred delayed and stayed at a rather low level, bees in our NP paradigm learned the individual visual and olfactory elements equally well, from the beginning of the acquisition phase (Fig. 4). One explanation for this might be that bees in the study by Mansur et al. (2018) had a different stimulus situation, which might have caused discrepancy between their and our results.

### Different learning capabilities for UV compared to green or blue light

There are different strategies for solving complex learning tasks such as positive and negative patterning experiments. The elemental learning strategy describes learning of a compound by summing up its single elements, whereas the configural learning strategy describes the compound as a unique cue during conditioning (Giurfa 2003; Deisig et al. 2001; Deisig et al. 2003). Interestingly, we found arguments supporting the one or the other theory depending on the wavelength. Bees could not solve the PP and NP task if blue or green light were used as the visual elements (Fig. 2 and 4) and showed similar high PER rates for the olfactory-visual compound (CS-). This suggests the summation of the single rewarded elements and supports the elemental processing theory (Wagner 1971). Contrary, the patterning experiments including UV light as an element could be solved (Fig. 3 and 5) supporting a configural character of olfactory-visual compound processing. Most interestingly, during PP trials, UV as a non-rewarded light element lowered the response to the rewarded UV-odour compound, whereas the single odour element, which was also not rewarded, evoked the highest PER rate (Fig. 3). This could mean that the negative reward association of the single light element lowered the perception of the odour light compound which is mainly driven by the odour perception. During NP trials the bees were able to solve the patterning task, which is only possible using a configural learning strategy (Myers et al 2001) (Fig. 5). Hence, this suggests that UV-light might be processed differentially resulting in a different associative strength during our cross-modal conditioning experiments compared to green and blue light.

### UV-light, but not blue or green, interferes with the olfactory-visual compound

When we trained honeybees to discriminate two olfactory-visual compound stimuli, we had to keep the odour information constant to ensure that the bees did not use olfactory information to solve the discrimination task. Hence, we varied the visual element of the two compounds and compared it to the discrimination of the light elements when presented without odour. Interestingly, the ability to discriminate two olfactory-visual compounds depended on the visual element (wavelength) we included. Two olfactory-visual compounds could be significantly differentiated if UV was an element of the unrewarded olfactory-visual compound (Fig. 6 and 7, upper panels), but not if UV was an element of the rewarded compound (Fig. 6 and 7, lower panels), even though the involved single light elements could be differentiated in either case (Fig. 6 and 7, right hand panels). In a similar line, studies with Africanised *A.mellifera* on absolute and discriminant learning tasks with visual stimuli only could show that learning performance depended on the quality of the colour of the light stimulus, showing lower learning performances for violet light compared to blue or green light (Jernigan et al. 2014).

Furthermore, our results show that the PER rates in response to the generalisation test after PP were significantly lower if the novel odour-light compound contained UV as an element (Fig. 2). In cases when UV was an element of the reinforced compound during acquisition, bees showed a high generalisation to novel odour and light elements (Fig. 3). During NP including UV, the associative strength of UV is significantly lower than the one to the single odour element (Fig. 5). Moreover, the odour element is not dominating the associative strength of the compound like in NP-experiments including green and blue (Fig. 4), because the compound stays significantly lower. Thus in this experimental context the odour-UV-light compound is perceived as a unique cue and not as the sum of the associative strength of the single elements. If UV light is presented without any odour context it can be learned and differentiated from other light stimuli as well (Fig. 6 and 7 right panels). Thus, in general bees had no difficulty associating UV with reward. This is the reason, why we think that the interaction (integration) of the odour-light pathway might be different if UV light is included compared to green and blue and that this might be based on neurobiological differences. Although we cannot completely exclude experience driven responses to UV light, we assume different processing pathways for the tested wavelengths. Recent studies in the honeybee show that visual learning involves the central complex and the mushroom bodies, with the vertical lobes of the mushroom bodies being involved in differential learning of visual stimuli (Plath et al. 2017). Since the UV light in our studies was not polarised like in a natural foraging context (Rossel and Wehner 1984), it is possible, that the bees could not associate the UV stimuli in a natural context due to lacking information. These findings suggest that the differential effects with UV light may be due to differences in internal processing of UV and light polarisation information, compared to blue or green light. Indeed, studies in various insect species show that information about polarised UV light received by photoreceptors in the dorsal rim area of the compound eye is bundled via the anterior optic tract to the anterior optic tubercle, lateral complex and central complex (anterior sky-compass pathway) (e.g. Homberg et al. 2011; Held et al. 2016; Schmitt et al. 2016; Grob et al. 2017; Stone et al. 2017). Furthermore, studies in ants could show that colour learning and long-term memory formation elicited plastic changes in the optic lobes, central complex and the anterior optic tubercle, suggesting that multiple brain levels are involved in visual learning (Yilmaz et al. 2019).

### The neural level of olfactory-visual integration

Multimodal sensory integration involves convergence of different sensory pathways at a higher brain level. The honeybee's mushroom body (MB) represents such a high-order sensory integration centre. The MB intrinsic neurons number up to ~170.000 Kenyon Cells (KC) with dendritic arborisations organised in concentric layers within the input region, the MB calyx (Mobbs 1982; Strausfeld 2002). Each layer within the MB calyx is preferentially innervated by one modality: For instance, the outer lip region receives olfactory information from projection neurons of the antennal lobe, whereas visual projection neurons of the optic lobes innervate the collar, and the basal ring is innervated by both modalities (e.g. Mobbs 1982; Schildberger 1983; Schürmann 1987; Ehmer and Gronenberg 2002; Strausfeld 2002). Hence, KCs receiving input from the different compartments of the MB calyx provide a computational space for simultaneous processing of activity triggered by visual and olfactory input.

The MB output is conveyed to ~400 MB output neurons (MBON; Rybak and Menzel 1993). Hence, the relatively large coding space of activity in a large number of KCs converges to a few hundred MBONs that potentially combine input from different modalities represented in groups of KCs. Recently, we exposed honeybees to olfactory, visual and olfactory-visual compound stimuli and recorded MBON activity (Strube-Bloss and Rössler 2018). Interestingly, we found four types of response behaviours in MBONs. MBONs sensitive to light only (i), to odours only (ii), to light and odours (iii), and MBONs that did not respond to any of the presented stimuli (iv). This suggests that the modality-specific layered input of the MB is conserved in subpopulations of MBONs (i, ii), but a substantial proportion of MBONs integrate olfactory and visual information across MB input layers (iii). The subpopulation of MBONs which did not respond to any of the presented stimuli (iv) may become recruited after associative conditioning, as we could show earlier (Strube-Bloss et al. 2011). Moreover, MBONs hold the capacity to combine complex stimulus features like odour and its spatial occurrence (Strube-Bloss et al. 2016). We therefore propose that reward associations to an olfactory-visual compound stimulus may recruit initially non-responsive MBONs which will encode the multimodal reward association during memory retention, a hypothesis we are currently testing.

## Conclusion

Overall, the patterning experiments suggest that an olfactory-visual compound stimulus is perceived as the sum of its single elements and, therefore, follows elemental processing. However, UV light seems to have a special effect since olfactory-visual compounds containing UV-light were discriminated from its single elements during NP experiments. This supports configural processing of the single elements. Furthermore, the discrimination between UV versus blue and UV versus green is affected when the visual stimuli were part of an olfactory-visual compound. Thus, olfactory-visual integration follows sophisticated cross modal stimulus interactions which depend on the presented wavelength of light stimuli supporting a distinct processing pathway for UV light compared to other wavelength.

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## Competing interests

The authors declare no competing or financial interests.

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## Author Contributions

MSB conceived the study. MSB and MCB designed the experiments. MCB collected and analysed PER data and MSB collected and analysed ERG data. MCB, WR and MSB discussed and interpreted the results. MCB drafted the manuscript. All authors wrote the paper and approved the final version of the manuscript.

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## Figures

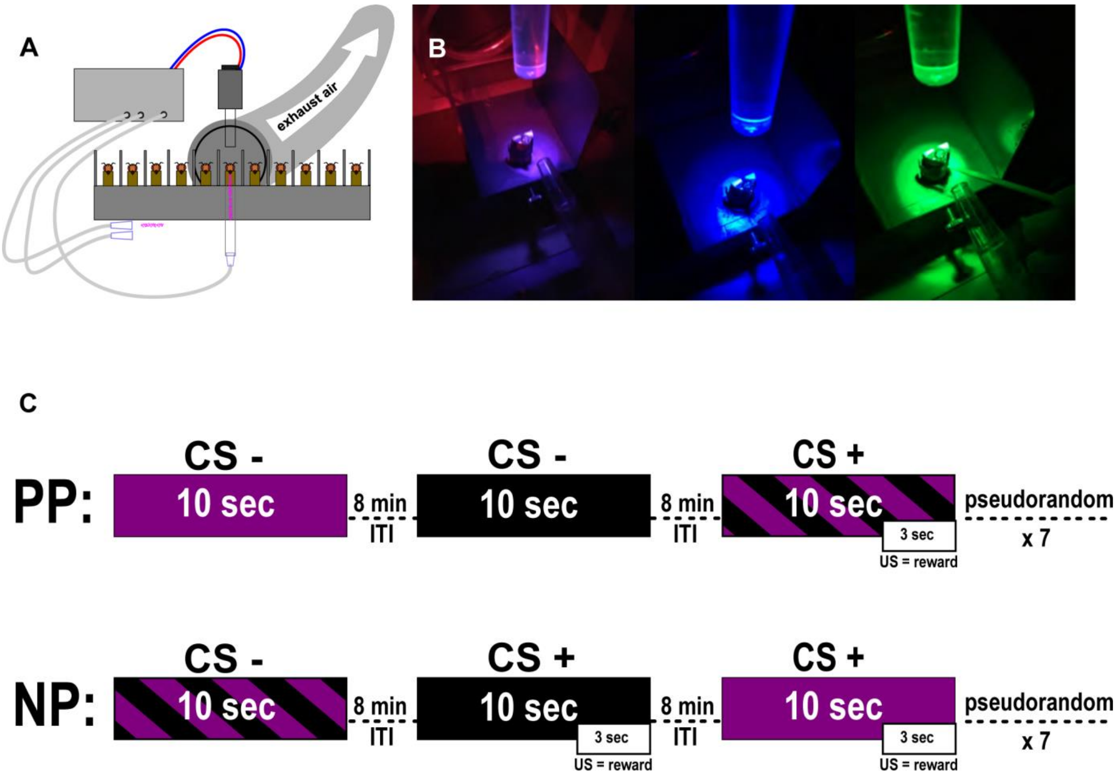

**Figure 1: Conditioning of a restrained bee to olfactory-visual stimuli. A:** A Cold-light-guide system made of an acrylic glass cylinder was positioned three centimeters above the tested subject and orthogonal to the odour presentation device. Odours were presented using multiple pipettes with filter paper strips. Subjects were placed in a plastic sleigh with single compartments. To avoid odour-accumulation, an air extractor was located behind the sleigh. **B:** We used three different LED lights (375nm - UV, 465nm - blue, 525nm - green). **C**: Schematic of positive (PP) and negative (NP) patterning experiments using UV light element as example. During PP UV (purple) and an odour (black) is presented unrewarded (CS-), whereas their compound is rewarded (CS+). Stimulation lasted always 10 s. During CS+ stimulation the US was presented with a 3 s overlap at the end of the stimulus. During NP the single elements were rewarded, but the compound was not.

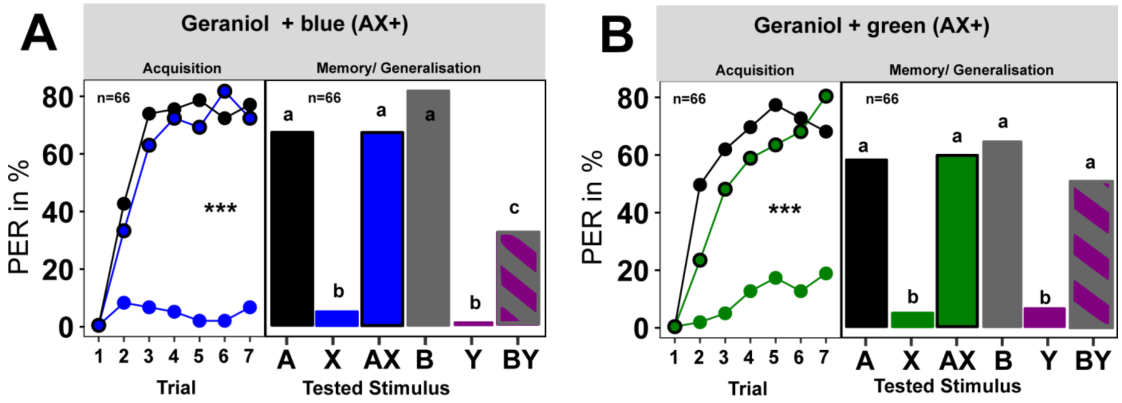

**Figure 2: Acquisition trials and memory/generalisation tests in positive patterning experiments including blue and green as element of the trained compound.** During acquisition the compound Geraniol + blue (black-blue in **A**, n=66) and Geraniol + green (black- green in **B**, n=66) were rewarded whereas single elements Geraniol (black), blue (blue) and green (green) were not rewarded. Bees learned to discriminate between visual element and compound (ANOVA, for both groups: p < 0.00001), but not between odour element and compound (ANOVA, for blue: Tukey HSD = -4.046875, p = 0.6; for green: Tukey HSD = -0.307692, p = 0.185). In both groups were significant differences between PER rates during the memory test (for blue: Cochran’s Q Test = 156.4035, df = 5, p < 0.0001; for green: Cochran’s Q Test =116.2846, df = 5, p < 0.0001). Generalisation to a novel odour (Citronellol; grey) and compound (grey-purple) was high, whereas generalisation to novel UV-light (purple) was low in both groups (Pairwise Wilcoxon sign test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: α’ = 0.003).

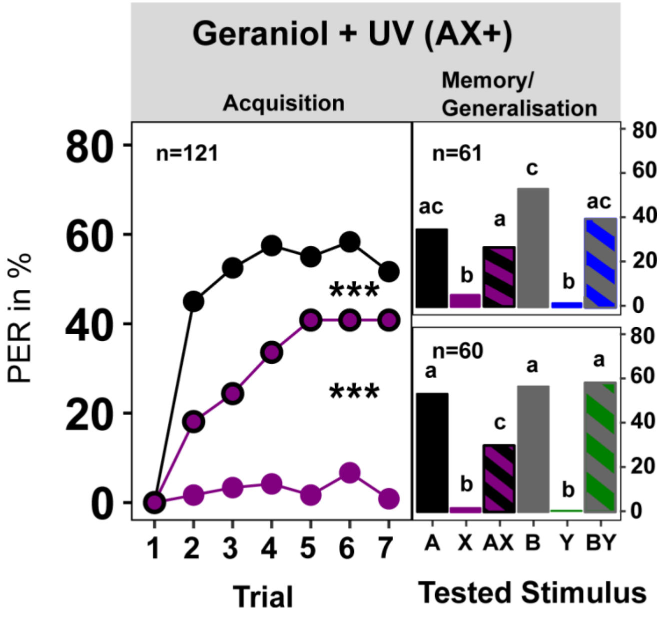

**Figure 3: Acquisition trials and memory/generalisation tests in positive patterning experiments including UV light as element of the trained compound**. Bees (n=121) learned to discriminate all three stimuli (rewarded: Geraniol + UV = black-purple; unrewarded: Geraniol = black, UV = purple; ANOVA: *** $p < 0.0001$). Although not rewarded, the associative strength to Geraniol seemed to be highest and was also memorised one hour later. To test for generalisation, we introduced the single odour Citronellol (grey) and blue light (blue) and its compound (grey-blue) in half of the bees (n=61; upper panel, Cochran's Q Test = 81.789, df = 5, $p < 0.0001$) and in the other half (n=60; lower panel, Cochran's Q Test = 114.655, df = 5, $p < 0.0001$) green light (green) and its compound (grey-blue). In both groups bees generalised the odour and the novel odour-light compounds. However, there is a trend (upper panel) and a significant difference (lower panel) between the novel compound and the memorised UV-compound stimulus (Pairwise Wilcoxon sign test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: α' = 0.003).

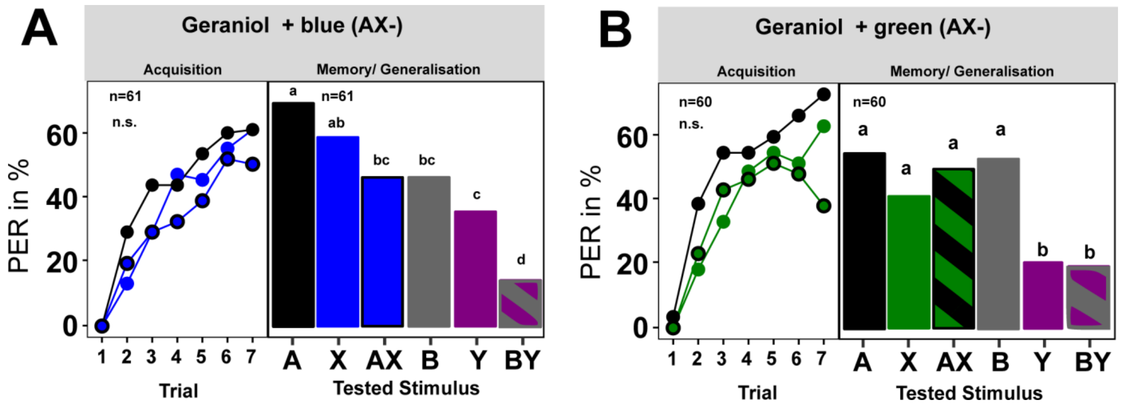

**Figure 4: Acquisition trials and memory/generalisation tests in negative patterning experiments including blue and green light as trained elements.** During acquisition single elements (Geraniol = black, blue (in **A**, n=61) and green (in **B**, n=60)) were rewarded, whereas the compound (Geraniol + blue = black-blue (in **A**) and Geraniol + green = black-green (in **B**) were not rewarded. Bees learned light and odour equally well, which they could not differentiate from their compound (ANOVA, blue: p = 0.239; green: p = 0.023, Tukey HSD = n.s.). **A:** During the memory test bees differentiate the single odour element from the compound and did not generalise that information to a novel odour (Citronellol; grey) (Cochran's Q Test = 62.536, df = 5, p < 0.0001). **B:** During the memory test bees did not differentiate the single elements from the compound and generalised the novel odour element (Citronellol; grey)(Cochran's Q Test = 50.213, df = 5, p < 0.0001). However, in both groups (**A** and **B**) neither the novel UV-light nor the novel odour-UV compound was generalised to the conditioned single elements (Pairwise Wilcoxon sign test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: α' = 0.003).

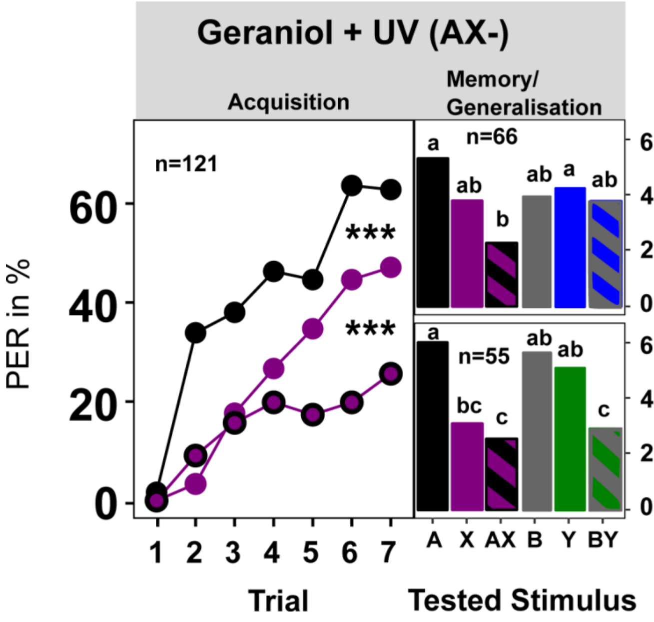

**Figure 5: Acquisition trials and memory/generalisation tests in negative patterning experiments including UV-light as trained element.** The bees (n=121) could differentiate between single elements (Geraniol = black, UV = purple; CS+) and their compound (Geraniol + UV = black-purple, CS-). The odour element evoked a significantly higher PER performance than the light element (odour: ANOVA, Tukey HSD= -26.074, P <0.001; light: ANOVA, Tukey HSD= -9.364, P=0.023). For the memory and generalisation test two groups were divided. One group received blue light, Citronellol and their compound (n=66, grey-blue, upper panel, Cochran’s Q Test = 23.037, df = 5, p = 0.0003), the other group green light, Citronellol and their compound (n=55, grey-green, lower panel, Cochran’s Q Test = 39.2804, df = 5, p < 0.0001) as novel stimuli. Both groups memorised the trained odour element and significantly differentiated it from the compound. Group one (upper panel) generalised the novel odour and light elements as well as their compound to the trained elements. The other group (lower panel) generalised the novel elements to the learned elements, but showed also a significant differentiation to the novel compound (Cochran’s Q Test = 39.2804, df = 5, p < 0.0001) (Pairwise Wilcoxon sign test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: α’ = 0.003).

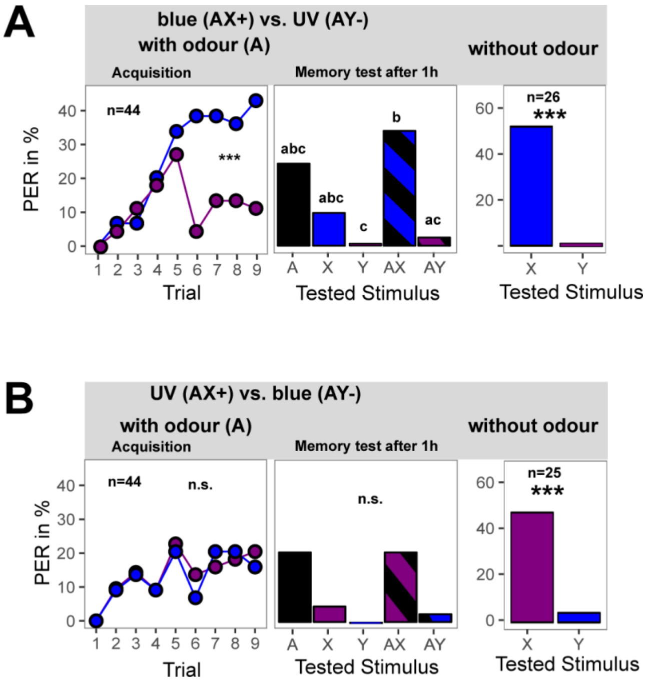

**Figure 6: Acquisition trials and memory test in differential olfactory-visual compound conditioning. A**: An odour-blue compound was presented rewarded (AX+; blue-black) and an odour-UV compound was presented unrewarded (AY-; purple-black). Note, the odour element was the same. During the memory test (middle panel), both compounds and its single elements were presented. Most right panels show pure light separation (different experiment). **B**: Same as in **A**, but rewarded and unrewarded compounds were presented reverse. During Acquisition bees discriminate the olfactory-visual compounds only if the UV-odour compound was unrewarded (n=44, Wilcoxon Signed Rank test, *** $p < 0.001$, in **A**), but not if it was the rewarded compound (n=44, Wilcoxon Signed Rank test, n.s. $p > 0.05$, in **B**). The separation between compounds was significand during the memory test in **A** (but not for **B**. The reward associated compound was generalised to its single odour element and stays separated from the unrewarded compound although odour identity is the same (middle panel in **A**; Cochran's Q test with pairwise Wilcoxon sign test for memory test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: $\alpha' = 0.005$). Note, the pure light stimuli can be separated independent if rewarded or unrewarded (most right panels, $\chi^2$ - Test, *** $p < 0.001$).

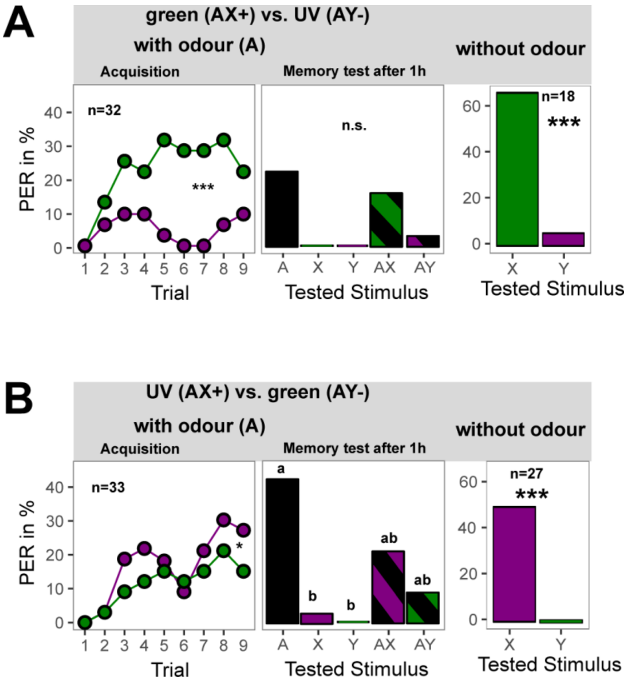

**Figure 7: Acquisition trials and memory test in differential olfactory-visual compound conditioning.** Same as in Figure 6 but the blue light was replaced by green. **A**: During acquisition bees discriminated between a rewarded odour-green compound (AX+) and an unrewarded odor-UV compound (AY-) (n=32, Wilcoxon Signed Rank test, *** $p < 0.001$). **B**: When we reversed rewarded and unrewarded stimulus this discrimination was much lower, but still present (n=33, Wilcoxon Signed Rank test, * $p < 0.05$). However, in both cases bees did not memorised that information and partially generalised between single elements and compound (middle panels; Cochran’s Q test with pairwise Wilcoxon sign test for memory test: Different letters indicate significant differences; Bonferroni corrected threshold for multiple comparisons: $\alpha' = 0.005$). Note, the pure light stimuli can be separated independent if rewarded or unrewarded (right panels, $\chi^2$ - Test, *** $p < 0.001$).

*Journal of Experimental Biology: doi:10.1242/jeb.201483: Supplementary information*

## Supplementary Information: separation of UV, blue and green light at the level of the activity in the honeybee's photoreceptor neurons of the compound eye

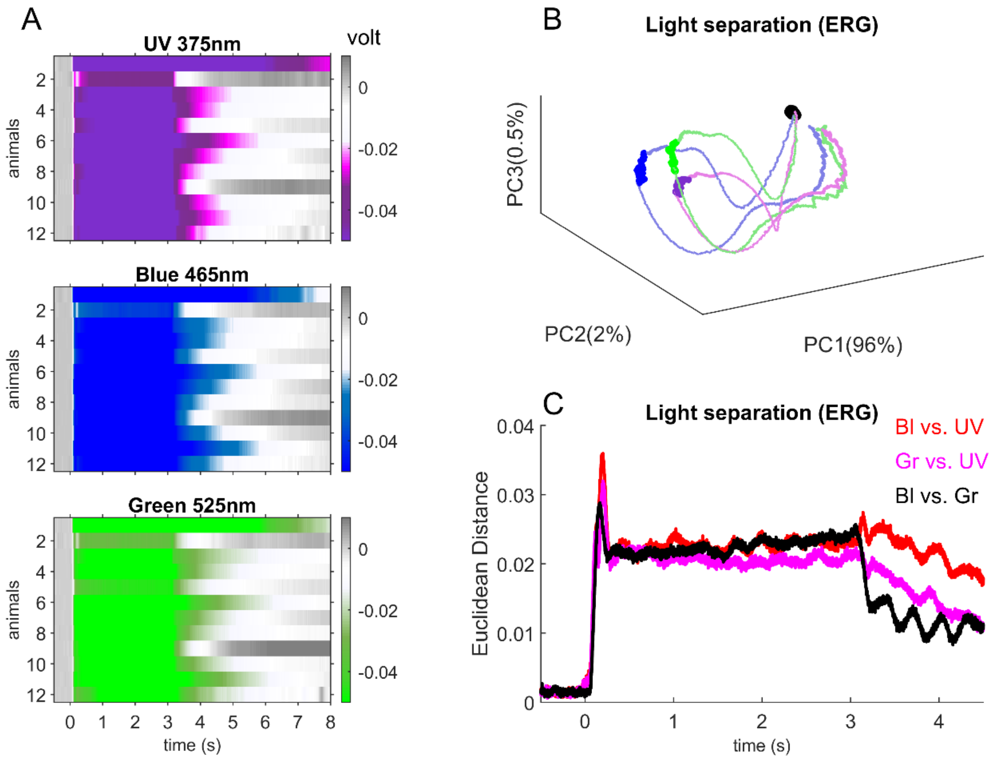

**Figure S1: Stimulus separation by photoreceptor neurons in the bee's compound eye.** Standard electro-retino-grams (ERG) were measured. Each stimulus was repeated 3 times in every animal**.** **A)** Averaged ERG activity of 12 bees in response to UV-light (upper panel), blue light (middle panel) and green light (lowest panel). Stimulation starts at time zero lasting for 3 seconds. **B)** Principal component analysis of the population vectors shown in **A** revealed a distinct representation of the different light stimuli. 500ms of baseline activity before stimulus onset are marked in black. The last two seconds of light stimulation is marked in thick lines using the same colour code as in A. **C)** Pairwise calculation of the Euclidean distances between the light induced population vectors shown in **A** revealed a pronounced differentiation by the recorded activity between all three light stimuli outlasting the entire stimulation time.

# 3 Neuroanatomy and electrophysiology

## 3.1 Introduction

As already mentioned, the honeybee is a well studied organism concerning behavioral biology and ecology. Additionally, these bees represent a suitable study organism for brain physiology. Although they have a “mini-brain”, many neurological processes can be investigated using a combination of diverse state-of-the-art methods. This includes for instance different staining methods, confocal microscopy, and electrophysiology (Menzel, 2001; Galizia & Kimmerle, 2004; Brandt et al., 2005).

### 3.1.1 Olfactory and visual pathways

Environmental stimuli (olfactory and visual) are primarily processed to a first order neuropil in the periphery of the brain (Maronde, 1991; Galizia & Kimmerle, 2004; Paulk et al., 2014). Olfactory input is received via the antennae, a sensory organ consisting of multiple sensory sensilla that show sex-specific differences in honeybees (Kropf et al., 2014; Maronde, 1991). The olfactory sensilla contain olfactory receptor neurons (ORNs), which extend their neurons to the glomeruli of the antennal lobes (AL; Anton & Homberg, 1999). This spheroidal structures encode for specific odorant mixtures (Kelber et al., 2010; Meyer & Galizia, 2012; Strube-Bloss et al., 2012; Streinzer et al., 2013b; Carcaud et al., 2015). The AL are the analogous part to the olfactory bulb in mammals in the honeybee brain (Hildebrand & Shepherd, 1997; Wilson & Mainen, 2006). Information is then processed via the dual olfactory pathway to higher order neuropils (Kirschner et al., 2006; Brill et al., 2013, 2014). Visual coding takes place in the peripheral optic neuropiles - the retina, the lamina, the medulla and the lobula (Menzel, 2001; Streinzer et al., 2013a; Paulk et al., 2014), which are connected via visual interneurons (Maronde, 1991). The retina of the honeybee eye consists of about 5,000 ommatidia (Jander & Jander, 2002). These trichromatic ommatidia can differ in their receptor composition (Wakakuwa et al., 2005). The spectral sensitivity is accomplished by three rhodopsin-like pigments with absorbance maxima at 350 nm (UV), 440 nm (blue) and 540 nm (green; Menzel & Blakers, 1976). UV-receptors form long visual fibres, that penetrate the lamina with different patterns of short arborisations and then project to the first or second layer of the medulla (Menzel & Blakers, 1976). Colour sensitive visual neurons project to the anterior central brain, whereas motion sensitive neurons project to the posterior part of the central brain (Paulk et al., 2009). As a consequence thereof a divergence of neuronal responses to colour stimuli is reflected among the neurons of the medulla and their following structures (Dyer et al., 2011). The divergence is conserved in the next downstream preserving and amplifiyng neuropil, the lobula, which is also a layered structures with two main regions (Hertel et al., 1987). The inner layers are colour sensitive and the outer layers contains achromatic and motion-sensitive regions (Paulk et al., 2008; Ribi & Scheel, 1981). The achromatic properties are then processed further to the posterior lateral protocerebrum, whereas chromatic cues are projected preferentially to the anterior lateral protocerebrum including the mushroom bodies (Paulk et al., 2008). The processing pathway for polarised light includes mainly the three ocelli, but also the dorsal rim area of the honeybee eye (Wehner & Bernard, 1993). This information is projected predominantly to the lateral complex, central complex and anterior optic tubercle (AOTu), the so-called sky-compass pathway, which plays an important role in orientation during flight movements (Held et al., 2016; Schmitt et al., 2016; Grob et al., 2017; Stone et al., 2017; Homberg et al., 2011). Moreover, the AOTu, is also involved in chromatic processing (Mota et al., 2013; Dyer et al., 2011).

Visual and odorant information is then processed via projection neurons (PNs) to the MB, the integration centre of the insect brain, where stimuli of different modalities are converged and modulated (Giurfa & Sandoz, 2012; Groh et al., 2012; Brill et al., 2013, 2014; Hussaini & Menzel, 2013). The MB represents an information centre, which is able to store memory and modulate olfactory input from the AL via a combination of oscillation, circuit properties and other selective filters (Perez-Orive et al., 2002). The main sensory input regions of the mushroom body are the calyces, that show an age-related synaptic plasticity and contain the about 170,000 intrinsic neurons, the so called Kenyon cells (KC; Groh et al., 2004, 2012; Menzel & Manz, 2005; Okada et al., 2007). The MB calyces are innervated by one modality, respectively. The outer lip receives input from olfactory projection neurons, whereas the collar is mainly innervated by PN from the optic lobes. However, the basal ring is suggested to comprise both modalities (Mobbs, 1982; Strausfeld, 2002; Ehmer & Gronenberg, 2002; Schürmann, 1987; Schildberger, 1983). Since the MB output region contains only ∼400 output neurons (Rybak & Menzel, 1993), it seems plausible, that this region combines input from different modalities and is coding for a group of KCs. Another important aspect in the modulation and processing of olfactory and visual cues are feedback loops. Here, one example is the dual olfactory pathway, which connects MB, AL and the lateral horn (LH) via the ENs from the mushroom body calcyces (Kirschner et al., 2006; Galizia & Rössler, 2010; Kropf et al., 2014).

### 3.1.2 Principles of multi-unit Recording

Electrophysiology investigates the electrical activities in living organisms or cells. Two common techniques are used to detect electrical impulses of nerve or muscle membranes. One is the intracellular recording technique, that uses different methods, like current clamp, voltage clamp

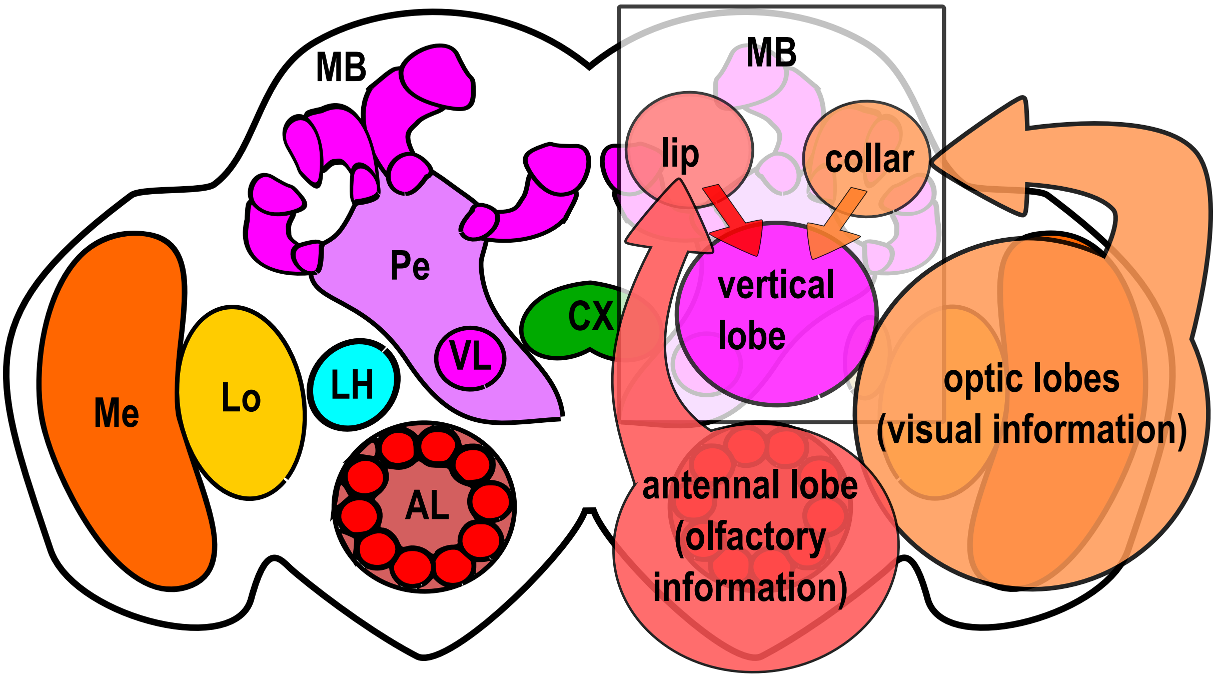

**Figure 3.1: Schematic view of the honeybee brain.** The optic lobes: Medulla (Me, orange) and Lobula (Lo, yellow), antennal lobe (AL, red) with the mushroom body (MB) with pedunculus (Pe, light magenta), olfactory lip (red) and visual collar (orange), the lateral horn (LH, cyan) vertical lobe (VL, magenta), central complex (CX, green)

or the patch clamp technique (Hodgkin & Huxley, 1939). Despite it's accuracy of recording the activity of single cells, this technique has some disadvantages recording activites of living cells. For example, to maintain *in situ* recordings of intact KC or PN, it is neccesary to severe the head from the body of the bee (Kropf & Rössler, 2018). In contrast, the extracellular technique provides the possibility to record neuronal activity in living animals and examine action potentials during behavioural observations. The electrode consist of multiple wires (for example a triode) and is positioned directly in the brain region of interest of the experimental animal (Strube-Bloss et al., 2016, 2012). Since the electrode is not positioned intracellular, it is not possible to assign a certain activity to single cells. The multiple wires allow to built the difference of recorded electrophysiological activities in neuron populations. This also leads to the fact, that multiple units are recorded (since it is not know, which exact neuron is recorded, the term unit is used). The extracellular technique was chosen, not only because it is possible to combine with fluorescence stainings to verify electrode position and reconstruct sensory pathways, but also to add a behavioural observations on living indivdiuals, e.g. classical conditioning using the PER protocol.

### 3.1.3 Study aims

The multi-unit recording technique was used to register neural activity of single mushroom body output neurons (MBON) during repeated stimulation with the single modalities (odour, light) and the olfactory-visual compound. Since the MB receives multi-modal input, it can be expected that the MBONs are multimodal reflecting integrative activity. This activity will be characterised and compared to the neural activity induced by the single modalities, odour and light. To verify the recording at the mushroom body output region, the position of the triode is stained and a backfill staining of the peripheral neuropils, optic lobes or antennal lobes, respectively, was conducted. Furthermore, these techniques represent a prerequesite for experiments combining electrophysiology and classical conditioning of multi-modal compounds.

## 3.2 Material and Methods

### 3.2.1 Multi-unit recordings

#### Preparation of experimental animals

Forager honeybees (*Apis mellifera carnica*) were caught in the morning at the entrance of the hives in small glass vials and cooled immediately on ice until they stopped moving. Individuals were harnessed in small metal tubes, still able to move their antennae, mandibles and proboscis (Bitterman et al., 1983; Haupt, 2004; Frasnelli et al., 2014; Menzel, 1981). They were fed with 30 % sucrose solution (vol/vol) and kept in a dark, warm and humid box until the start of an experiment. The head was fixed to the tube by using wax. Both antennae were glued to the head capsule with low-melting-point wax (eicosane) and an additional droplet fixed the joint of the antennae. After removing the hair with a scalpel, a small window (1.5 x 3 mm) was cutted between the compound eyes, antennal basis and ocelli. Hypopharyngial glands and tracheal sheath were removed as well as the neurolemma above the region of the alpha-lobes.

#### Olfactory and visual stimulation

A constant air stream was delivered by a custom-made olfactometer, adapted from previous experiments (Galizia et al., 1997; Strube-Bloss et al., 2011). It consisted of 8 channels (three odours and control) built with 5 ml syringes and computer controlled magnetic valves connected by Teflon tubes (Ø 6 mm). The air stream was led through the tubes to the syringes, which contain a piece of filter paper (2 $cm^2$) soaked with 10 µl of the tested odour solutions (Substance Geraniol, Citronellol, Farnesol; diluted to a 0.01 volume concentration with paraffin oil). The needles of the syringes were inserted into a main air stream of the olfactometer, built with a 50 ml Falcon tube supplied with compressing air. During stimulation, the air stream is switched to an odourous one and after 3 s of the stimulus switched back to a clean air stream. The airflow during the stimulus was 1 ml/s.

Visual stimuli were presented with two custom-built cold light guides, which illuminate both eyes of the bee with different colours. Two acrylic glass sticks were illuminated by two LEDs, respectively, and placed at 10 mm in lateral position to the bee. Two different wavelengths were chosen, that were discriminable for honeybees (blue: 465 nm, 6.5 x 1013 photons/cm$^2$/s; green: 525 nm, 3.93 x 1013 photons/cm$^2$/s; for more details see Chapter 1).

Both stimuli types were controlled with a TTL signal generated by the Digital Lynx SX Motherboard (Neuralynx, Bozeman, MT, USA) using a self-written stimulation file. In total 8 stimuli were presented. Paraffin oil as the control stimulus, three different odours, two different wavelengths as single modalities and two compound stimuli consisting of a single colour plus a single odour at the same time. Each stimuli was presented 10 times in a random order. Stimulation was initiated three seconds after beginning of a recording and the duration of each stimuli was three seconds. After stimuli offset, the recording continued for another five seconds and the intermission time was six seconds for each stimuli.

### Electrophysiology and data acquisition

For recording brain activity during olfactory and visual stimulation an extracellulary multi-unit recording technique with a 16-channel digital data acquisition system was used (Neuralynx, Bozeman, MT, USA; Strube-Bloss et al., 2012, 2016; Strube-Bloss & Rössler, 2018). A reference electrode (25 µm, Nilaco, Tokyo, Japan) was positioned in the left part of the brain under the left compound eye. Furthermore, a second electrode (25 µm, Nilaco, Tokyo, Japan) was positioned next to the M17 muscle to record stimulation of the proboscis. The recording triode consisted of three micro wires (polyurethane-coated copper wire, 14 µm in diameter, Elektrisola, Escholzmatt, Switzerland) glued together with wax and attached to an electrode interface board (EIB-18, Neuralynx, Bozeman, MT, USA). All wires, including the recording electrode, reference and the M17 electrode were connected to a head stage preamplifier (HS_16, Neuralynx, Bozeman, MT, USA). The recording triode was carefully positioned at the ventral border of one vertical lobe at a depth of 100 to 250 µm (Strube-Bloss et al., 2011; Brill et al., 2014). The brain was then covered by a small droplet of Kwik-Sil (1:1 mixture of the two components of Kwik-Sil, World Precision Instruments, Friedberg, Germany) to prevent the brain from dehydration and to fix the preparation. The neural activity was recorded from all differential combinations of the three wires of the triode with a sampling rate of 30 kHz and a band-pass filter of 400 Hz - 9,000 Hz using the Cheetah data acquisition software (Cheetah 5, Neuralynx, Bozeman, MT, USA).

### Spike-Sorting and Data Analyses

A high-pass filter (> 600 Hz) was used before spike sorting with a semi-automatic technique (template-matching) on the differential recording channels using the software Spike2 (Cambridge

Electronic Design, Cambridge, UK; Strube-Bloss et al., 2011; Brill et al., 2014). The mean activity and SD of the high-pass filtered channels was calculated and the thresholds for detecting an event was set to 3 x SD. Individual spikes were assigned by computing templates of spike waveforms with events that passed the thresholds. The unit separation was then checked with a principal component analysis (PCA) in the first three dimensions for overlapping with the tools that Spike2 provided. If the distributions of sorted units overlapped, the waveforms were assigned to the same unit. Two units were found in one bee which were further analysed. A visualisation of the waveforms and a peristimulus time histogram (PSTH) was done for each stimuli for each unit, respectively.

### 3.2.2 Staining of electrode position

#### Staining method in wholemounts

To verify the recording position, the electrode was dipped in Alexa Fluor 647 hydrazide (Invitrogen, Paisly, UK, Approximate absorption (Abs): 649 nm, fluorescence emission (Em): 666 nm) before recordings and positioned at the ventral border of one vertical lobe at a depth of 100 to 250 µm. Light exposition was avoided. After the recording, the brain was rinsed in bee ringer and all remaining glands and trachea were removed. A broken tip of a glass electrode was dipped in Microruby (Invitrogen, Paisly, UK, tetramethylrhodamine, fluorescence excitation (Ex): 555 nm, Em: 580 nm) and inserted manually in the neuropil of interest (lamina or antennal lobe) and the brain was carefully rinsed with bee ringer afterwards. The brain was covered by the head capsule or parafilm to avoid dehydration. The experimental animals were kept in a moist dark chamber for 3-4 h at room temperature. The brains were dissected in bee ringer and immediately fixed in cold 4% Formaldehyde (FA) in PBS. Fixation was done under dark conditions overnight at 4 °C on a shaker. The tissue was kept dark from now. The brains were washed 3 x 10 min in PBS on a shaker on the next day and dehydrated in an ethanol series (30%, 50%, 70%, 90%, 95%, 100%, 100%, 100%, 100%), 10 min for each step. Finally, the brains were cleared in methyle salicylate (MS) in glass vials for a few days and mounted on metal object slices in MS for further handling.

#### Confocal Laser-Scanning Microscopy

Whole mount preparations were scanned using a confocal laser-scanning microskope (Leica TCS SP2 AOBS, Leica Microsystems, AG, Wetzlar, Germany) at a final image resolution of 1024 x 1024 pixels with two channels (633 nm for Alexa Fluor 647 hydrazisde and 568 nm for Microruby). Image visualisation was done with FIJI (ImageJ 1.52e, National Institutes of Health, USA) by using the Z-Project tool with maximum intensity alignment for the orthoslices of the two channels. 3-D reconstructions were done with AMIRA 6.0.0 (FEI Visualization Sciences Group, Düsseldorf, Germany) for the recording electrode position, AL, the olfactory tracts, LH, MB and an overview of the central brain.

## 3.3 Results

### 3.3.1 Neuronal activities during olfactory-visual stimulation

Multi-unit recordings lasted up to 3 h for one individual. The recordings from one animal were chosen for further analysis. Extracellular recording, showed excitatory responses (3.2 A) during the 3 s of stimulation. After applying a spike sorting and PCA two units were found that were separated in all three dimensions of the PCA (Fig. 3.2 B). They are termed unit1 (in blue) and unit2 (in green) and showed differences also in the overlay of their waveforms (Fig. 3.2 C) will be described in detail in the following. Unit1 showed activity especially after onset and offset of a visual stimuli, as well as for olfactory-visual compounds (Fig. 3.2 D) and the PSTHs revealed that the spike counts were up to 110 spikes/100 ms for 500 ms after stimulus onset (Fig. 3.3 C, D, G, H, lower panels). After stimulus offset there was also a slight increased spike count for single visual stimuli and olfactory-visual compounds. The number of spike events increased and lasted also longer during the stimuli, with increasing number of presented trials (Fig. 3.3 C, D, G, H, upper panels). Unit1 showed no increased activity during stimulation with pure olfactory stimuli (Fig. 3.3 B, E, F, lower panels) compared to the control with paraffin oil (Fig. 3.3 A). Unit2 showed in general less activity than unit1 and had a constant pattern of firing during the recordings (3.2 C). The PSTHs showed, that there were only slight increased counts of spikes during stimulation with light and olfactory-visual compounds (Fig. 3.4 C, D, G, H, lower panels) but not for pure odour stimuli (Fig. 3.4 B, E, F, lower panels) compared to the stimulation with paraffin oil (Fig. 3.4 A lower panels).

### 3.3.2 Staining of electrode position

In total two wholemounts from different individuals were analysed. In the first individual a backfill of the AL was carried out (Fig. 3.5), whereas the OL was stained in the second individual (Fig. 3.6). For both individuals a staining of the electrode position in the ventral lobe of the MB pedunculus was done. The 3D reconstruction of the central brain of the first wholemount revealed, that the tracer backfilled the AL and the olfactory projection neurons (OPNs) of the medial and lateral antennal lobe tracts (m-ALT and l-ALT) up to the region of the MB lip and LH (Fig. 3.5 A, B). The staining of the electrode position visualised the injection site in the MB output region, the vertical lobe (VL), where the tracer was migrating in the surrounding tissue of the pedunculus (Fig. 3.5 C). The visualisation of the backfill in the OL on both sides showed

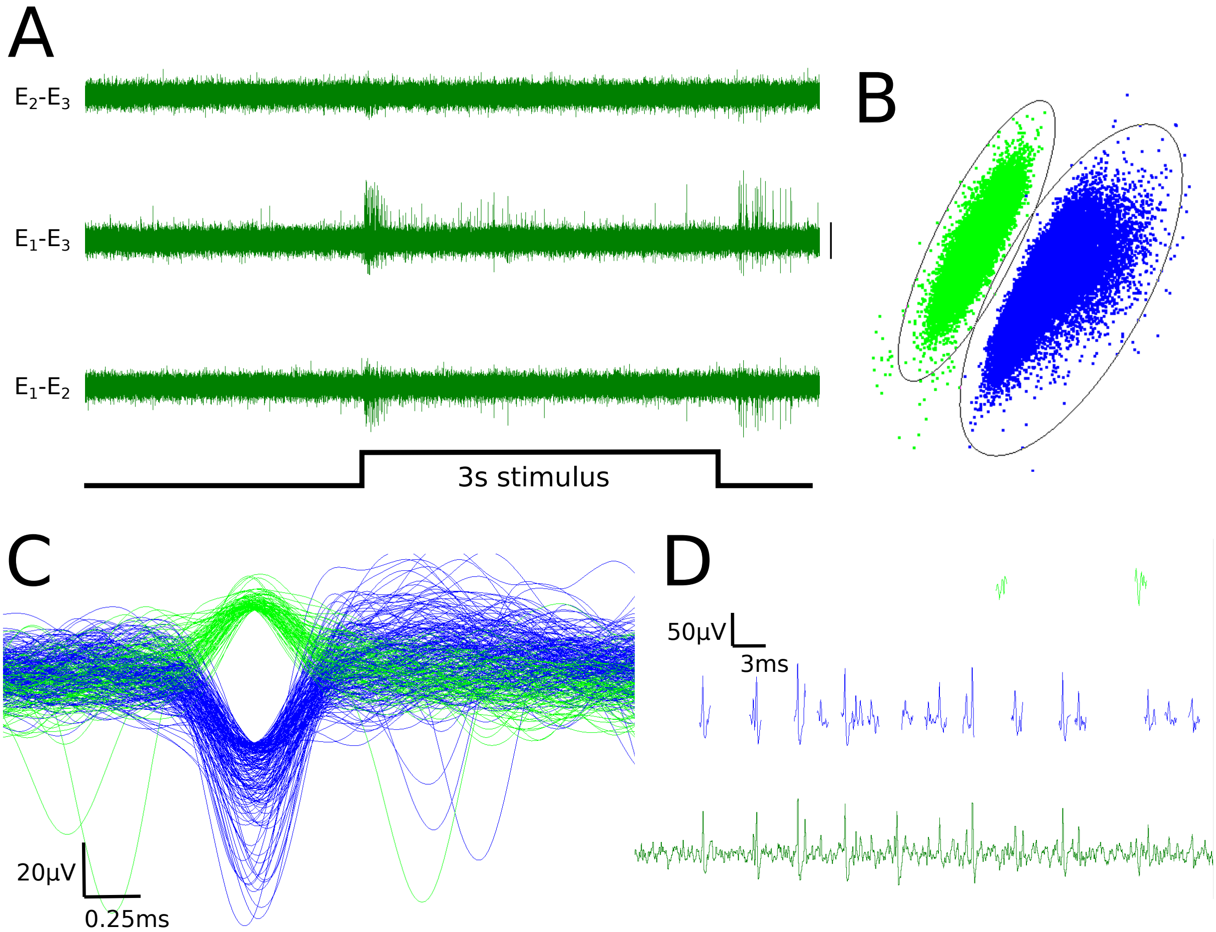

**Figure 3.2: Extracellular recordings at the MB output region and single unit extraction (spike sorting) A**) Extracellular recording showing excitatory responses at the onset and after the offset of a 3 s compound stimuli for each electrode differential. Bar: 50 µV. **B**) View from a 3D clustering of the sorted units with principal component analysis (PCA), indicating the distance of the sorted units to each other (unit1:blue and unit2: light green). **C**) Overlay of the units after spike sorting illustrates the separation of the waveforms. **D**) Colored coded units indicate the action potentials from one channel of the recording in magnification (unit1: blue and unit2: green).

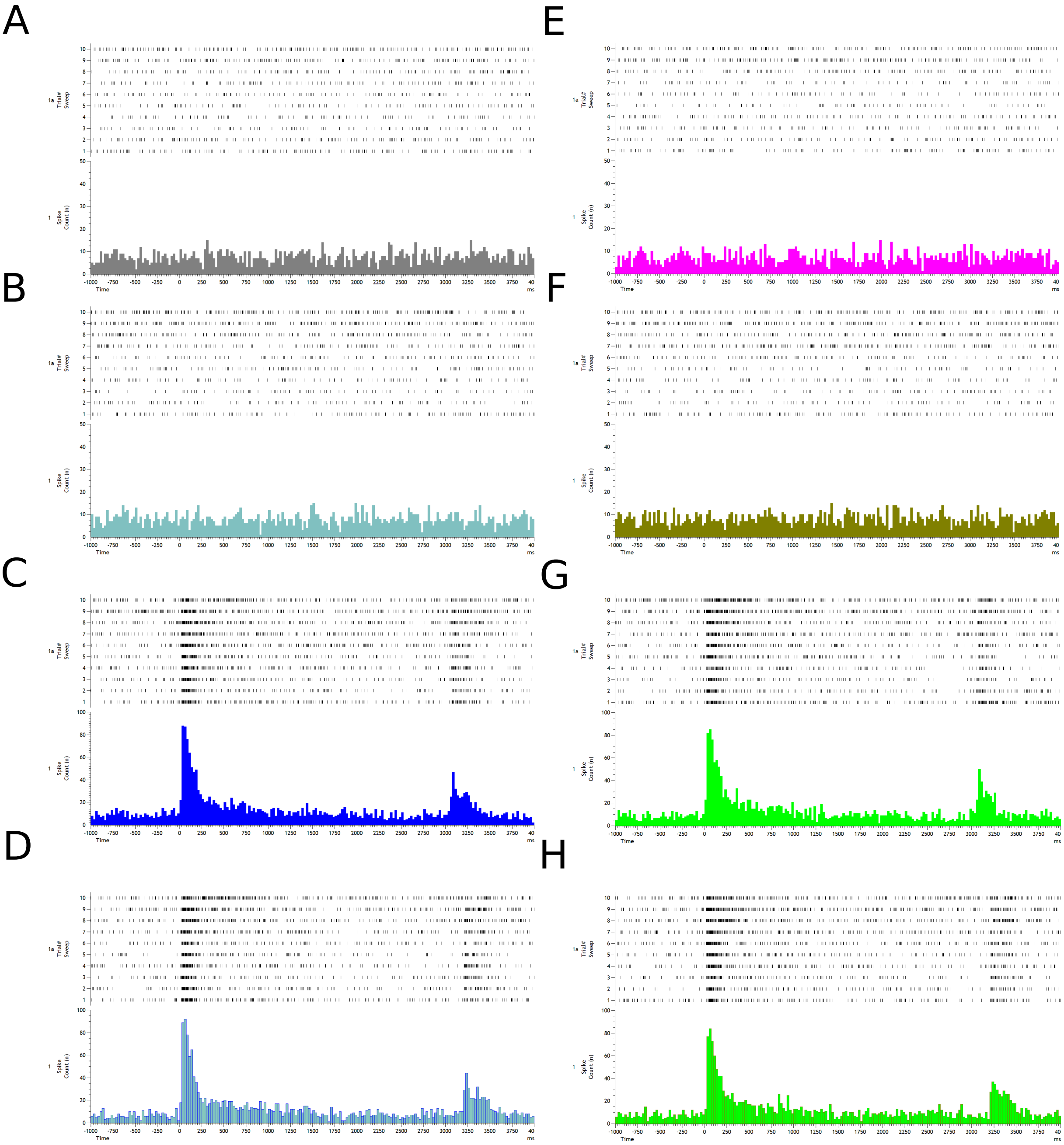

**Figure 3.3: Spike events and peristimulus time histogram (PSTH) of unit1 for stimulus duration of 3000 ms and pre-stimulus events of -1000 ms.** The upper panels show the spike events for the 10 trials, the lower panels show the spike count (n) over time in ms. Note that the stimulus is beginning at 0 ms and ends at 3000 ms **A**) Stimulation trials with paraffin oil. **B**) Stimulation trials with Farnesol. **C**) Stimulation trials with blue light. At the onset and after the offset of the stimulus the spike count is increased. **D**) Stimulation trials with a compound of Farnesol and blue light. At the onset and after the offset of the stimulus the spike count is increased. **E**) Stimulation trials with Geraniol. **F**) Stimulation trials with Citronellol. **G**) Stimulation trials with green light. At the onset and after the offset of the stimulus the spike count is increased. **H**) Stimulation trials with a compound of Citronellol and green light. At the onset and after the offset of the stimulus the spike count is increased.

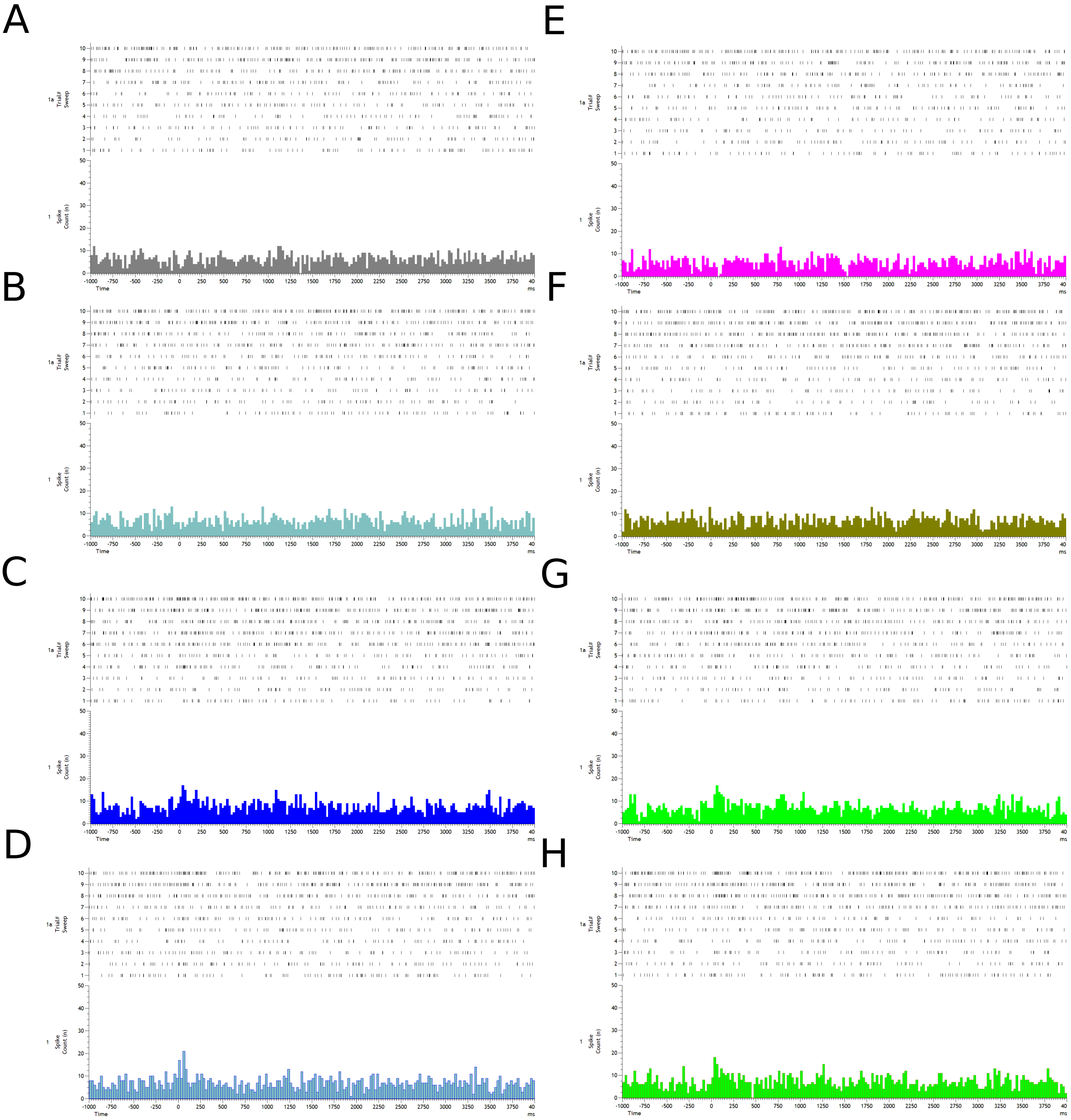

**Figure 3.4: Spike events and peristimulus time histogram (PSTH) of unit2 for stimulus duration of 3000 ms and pre-stimulus events of -1000 ms.** The upper panels show the spike events for the 10 trials, the lower panels show the spike count (n) over time in ms. Note that the stimulus is beginning at 0 ms and ends at 3000 ms **A**) Stimulation trials with paraffin oil. **B**) Stimulation trials with Farnesol. **C**) Stimulation trials with blue light. At the onset of the stimulus the spike count is slightly increased. **D**) Stimulation trials with a compound of Farnesol and blue light. At the onset of the stimulus the spike count is slightly increased. **E**) Stimulation trials with Geraniol. **F**) Stimulation trials with Citronellol. **G**) Stimulation trials with green light. At the onset of the stimulus the spike count is slightly increased. **H**) Stimulation trials with a compound of Citronellol and green light. At the onset of the stimulus the spike count is also slightly increased.

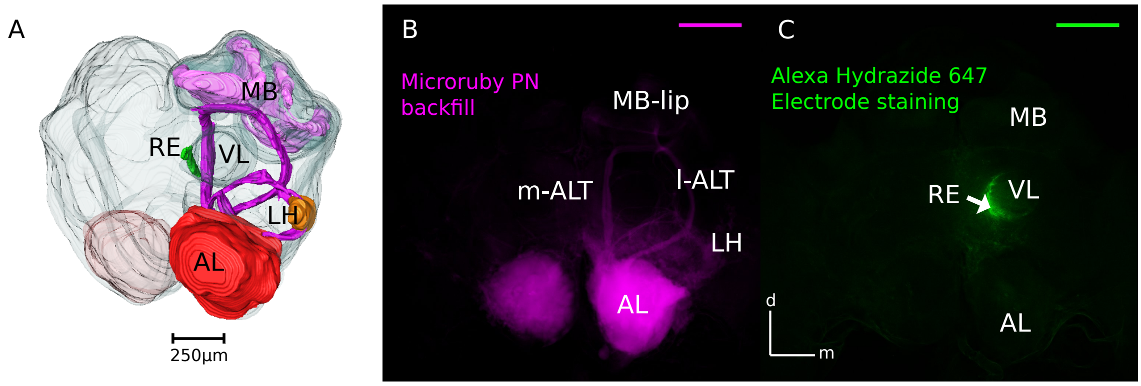

**Figure 3.5: 3D-reconstruction of electrode position and visualisation. A**) 3D reconstructions of the antennal lobe (AL, red) and the olfactory tract (magenta) with the mushroom body (MB) olfactory lip (pink) and the lateral horn (LH, orange) together with the reconstructed recording electrode position (RE, green) at the vertical lobe (VL). **B**) Z-Projection of the ortho-slices with maximum intensity alignment of a backfilling of the uniglomerular neurons projecting from the AL to the MB and LH. The intracellular tracer Microruby (tetramethylrhodamin dextran) was inserted into the AL. **C**) Z-Projection of the ortho-slices with maximum intensity alignment from the staining of the recording electrode position (RE, arrow) with the tracer Alexa hydrazide 647, which migrates to the surrounding tissue in the VL. All scale bars = 250 µm.

the pathway of the visual projection neurons (VPNs, magenta in Fig. 3.6). The staining showed a proximal pathway to the MB calyces and also to the region of the anterior optic tubercle (AOT). The staining of the electrode position (RE, arrow) with Alexa hydrazide 647 is shown in green at the VL of the MB, where the tissue of the pedunculus is also stained with the migrating tracer (Fig. 3.6).

## 3.4 Discussion

### 3.4.1 Multi-modality of the mushroom body output site

The two units found during electrophysiological recordings showed only increased responses to visual stimuli after stimulus onset and after the offset (Fig. 3.2A). The two units did not respond to single odour components, but also their response was not inhibited (see Fig. 3.3 and 3.4). During presentation of olfactory-visual compounds, their activity was similar to those for single visual stimuli, indicating that those units of the MBONs were purely sensitive to light. Furthermore, they showed no differences in their response phase for the two used colours (see Fig. 3.3 and Fig. 3.4), suggesting that a certain coding for different wavelengths is lost after processing in the MB calyces. Their response patterning looks also similar to the MBONs that

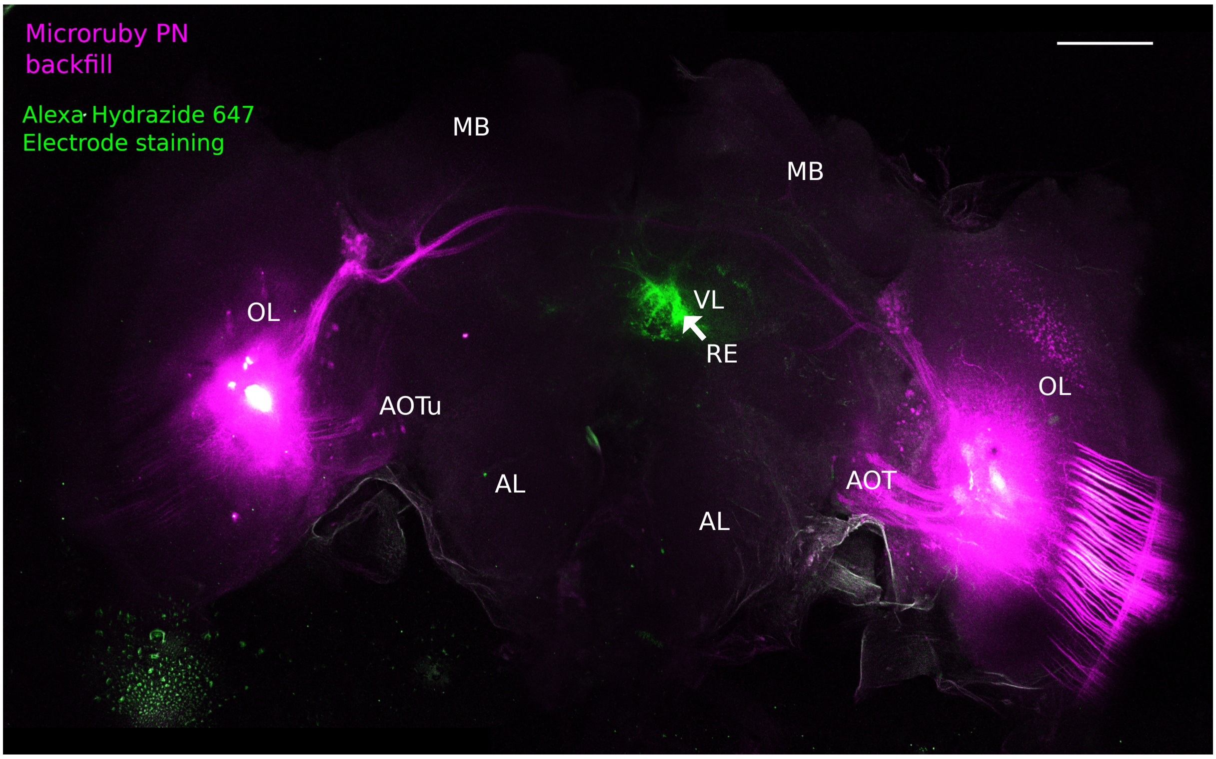

**Figure 3.6: Visualisation of electrode position and optic tracts.** Z-Projection of the ortho-slices with maximum intensity alignment of a backfilling of the projection neurons from the OL to the anterior optic tract (AOT) and anterior optic tubercle (AOTu). The intracellular tracer Microruby (tetramethylrhodamin dextran) was inserted into the OL on both sides.The electrode position (RE, arrow) was stained with the tracer Alexa hydrazide 647, which migrates to the surrounding tissue in the VL. Scale bar = 250 µm.

were sensitive to light stimuli, found in recent studies (Strube-Bloss & Rössler, 2018). Since the recording position was at a depth between 100-250 µm, it seems possible, that those MBONs are in the cluster of the A1, A2, A4, A5 and A7 extrinsic neurons (nomenclature by Rybak & Menzel, 1993). Strube-Bloss & Rössler (2018) could find distinct responses of MBONs for visual and olfactory stimuli, and to their compound. Four different categories could be found, one of them did not respond to any stimuli as already discussed in the manuscript of Chapter 2 (Strube-Bloss & Rössler, 2018; Becker et al., 2019). Therefore, they suggest that those categories are clustered in the same fashion in the output region of the MB, like the organisation of calyx input region, supporting the three-layered organisation of the vertical lobe described by Mobbs (1982). The two units found in this experiment is likely positioned in the cluster region of MBON that respond solely to visual stimuli.

### 3.4.2 Sensory pathways and electrode position

The backfills showed projection neurons to the mushroom bodies for both, olfactory and visual pathways. The OPNs revealed their characteristic distribution from the AL to the MB calyx input region (Mobbs, 1982). As shown in Fig. 3.5B, their pathways proceed in medial (m-ALT) and lateral tracts (l-ALT) through the central brain (CB) to the lip of the MB calyx with projection loop to the LH (Strausfeld, 1976). The typical pathway of the VPNs is displayed in Fig. 3.6. The optic lobes project from the Medulla lateral to the MB calyces. Both pathways are part of the input region the MB calyces (Mobbs, 1982). The olfactory information is mainly processed in the lip region of the calyces, whereas visual information is predominantly processed in the collar region (Ehmer & Gronenberg, 2002; Kirschner et al., 2006; Strausfeld, 2002). In contrast to these unimodal layers the basal ring is suggested to have a multi-modal olfactory-visual input (Gronenberg, 2001). The position of the recording electrode is verified at the MB output region, the vertical lobe (Fig. 3.5C and 3.6). The staining is indicating that responses to light stimuli from the units in the electrophysiological recordings are indeed activities of MBONs and therefore the output from the Kenyon Cells (KC) of the lip region of the MB calyx (Fig. 3.6). The tracer migrated ventral in the lower medial part of the vertical lobe, where the A1 and A2 clusters are located (Rybak & Menzel, 1993). Furthermore, neither in the labeling shown in Fig. 3.5 nor Fig. 3.6 is an evidence that the electrode position meets one of the peripheral pathways, assuming that the recorded signals are not neuron activities from the peripheral optical or olfactory lobes nor the olfactory or visual pathways, respectively.

### 3.4.3 Conclusions

Backfill stainings with Microruby showed the two separated sensory pathways and their input site of the MB calyces. The electrode staining verified the position of the MB output site, the vertical lobe. The electrophysiological recordings revealed neuron populations of MBONs that responded to visual stimuli as single components, but also as apart of an olfactory-visual compound. It is therefore suggested that the MB represents the integration centre for olfactory and visual information, divided in different layers for single modalities, but also for multi-modal compounds. It could be shown in recent studies, that the MBONs respond in four different categories and may be modulated during differential conditioning featuring olfactory-visual compounds and their single modalities (Strube-Bloss & Rössler, 2018). The here presented results suggest, that the used techniques give the opportunity to investigate the processing of multi-modal compounds in extension to the classical conditioning paradigm.

# Part III

# Overall discussion

## Summary of the experiments

In the fundamental experiments including classical differential conditioning of stimuli with single olfactory (Geraniol, Citronellol, Farnesol) and visual (UV, blue, green) components, the honeybees showed a fast and reliable learning performance. Higher PER rates during olfactory conditioning indicate a stronger association of the conditioned stimulus with the reward (Fig. 1.3). Nevertheless, visual conditioning also led to a reliable memory consolidation (Fig. 1.5). There was a temporal discrepancy for the stimulus length, with 10 s for the visual conditioning, whereas olfactory conditioning normally uses a stimulus length of 6 s or less (Matsumoto et al., 2012). It was possible to adapt the olfactory conditioning paradigm to trials with 10 s stimulus length without risking the volatilising of the odour molecules during the stimulus presentation (Fig. 1.4). With these findings it was possible to establish positive and negative patterning experiments with olfactory-visual compounds. The results revealed, that honeybees used different strategies depending on the used wavelength of the multi-modal compound. An elemental processing strategy was used, when blue or green was part of the olfactory-visual compound, whereas UV light needed a configural proccesing (Becker et al., 2019). Additionally, during experiments, where honeybees had to differentiate between compounds with a consistent odour component but different colour components, the UV light element was affecting the compound unlikely blue or green light (Becker et al., 2019). These findings suggest, that UV light is processed via other pathways in the honeybee brain than blue or green light, respectively. There are already several studies on the multi-modal characteristic of the mushroom body (Maronde, 1991; Strube-Bloss & Rössler, 2018; Mobbs, 1982). Thus, this neuropil reflects a multi-modal integration centre. Interestingly, the number of neurons that project to the mushroom body region is much higher than the extrinsic neurons of the output region, the vertical lobe (Groh et al., 2004, 2012; Menzel & Manz, 2005). Therefore, the output region must be the part of the brain where coding and modulation of learnt information of multi-modal cues takes place. Backfill stainings revealed the seperated peripheral pathways for olfactory (Fig. 3.5) and visual information (Fig. 3.6). The different modalities are then processed in different parts of the mushroom body calyces, the olfactory lip and the visual collar (Ehmer & Gronenberg, 2002; Gronenberg, 2001; Maronde, 1991). The verification of the electrode position in Chapter 3, revealed that the recorded neuronal activity are units of the neuron population of the vertical lobe, the mushroom body output region. The two units reported here, responded dominantly to visual information (Fig 3.3 and Fig 3.4), also when a olfactory-visual compound was presented (Fig 3.3 and Fig 3.4). Since it can be excluded through the stainings that signals were recorded from the periphery, the units must be coding after the olfactory-visual integration in the mushroom body had happened.

## Disadvantages of the used methods

Although the used methods are state-of-the-art techniques and PER conditioning represents an expressive paradigm to investigate learning abilities of honeybees, there are still some disadvantages of the methods that are discussed in the following section.

The harnessed condition during PER conditioning is not a natural situation for the honeybee. Despite the possibility to lessen the limmitation of movement of the wings, legs, antennae and mouthparts, the harnessed condition is still not comparable to a foraging situation. Honeybees do not experience an optic flow or the tactile information they receive during normal foraging activities. The temporal spacing of the perception of the multi-modal stimuli is also another important aspect, since the approaching individual would first orientate visually to the food resource and then get the olfactoy input from the odour bouquet of the flower (Gerber & Smith, 1998; Filla & Menzel, 2015). In the next step, again a visual cue will lead the bee to the nectaries, commonly distinct patterns of the blossom that mark this spot. While taking up the nectar reward, both cues are then again simultaneously present for the animal. It is hard to say, when and where exactly the perceptual focus of the bee is and how this foraging approach can be simulated under harnessed conditions. Of course, it would be much easier to study the perception of multi-modal cues during free-flying experiments, but the aim of this work is to establish an experimental setup and learning paradigm, that allows combining classical PER conditioning with electrophysiological recordings. Therefore it was neccesary, to keep the experimental design simple and the external factors downscaled for feasible laboratory conditions. The combining of olfactory and visual cues still remains difficult, since the internal associative strength of both modalities is different (Gerber & Smith, 1998; Kevan & Lane, 1985; Menzel & Greggers, 1985). The olfactory component during a reward association is processed much faster than the visual component, due to the fact that the octopaminergic VUMmx1 neuron is directly linked to the olfactory pathway and also receives subesophageal information (Hammer, 1993). The pathway for visual reward association is not known in complete detail, but it is suggested that it is processed in an indirect way (Lichtenstein et al., 2018).

Furthermore, the visual stimuli is minimised to the wavelength of the colour, shape and patterns of a flower and it's petals are missing. Thus, the cue is not as attractive as it would be in natural conditions or during free-flying experiments with flower dummies. Though it is crucial to use restricted cues to exclude the orientation on patterns or shape, while investigating the influence of certain wavelengths on the learning performance. In addition, it must be verified, that honeybees do not discriminate the colour stimuli by their achromatic cues like light intensities. This was ensured in a previous experimental trial by presenting them the stimuli with transmission filters in seperated acquisition trials and tested with a memory test after 1 h (data not shown).

Besides the used wavelength, also the chosen odour might have disadvantages, thus it can not be excluded that they were not completely novel to the tested individuals. Geraniol is known to play a role in marking suitable food resources, and all tested odours are components of floral odour compounds (Free, 1968; Pickett et al., 1980). Despite it could be shown that honeybees are able to discriminate between the used odours (see Chapter 1.3.1), it would be more accurate to use artificial odour components. It is also not negligible, that odour and colour are presented orthogonally to each other. A more natural situation would be, that both stimuli are presented from the same direction. Due to limitations in the physical layout of electrophiological setup, we desided to adapt this configuration to the PER setup.

Another critical point is, that pollen foragers were used for experiments. They were chosen due to be readily identifiable by their pollen loads. Nectar foragers could be mistaken with water foragers or bees that perform learning flights. Furthermore, nectar foragers show more variability in their gustatory response to different concentrations of sugar solutions (Scheiner et al., 2001) and are thus not readily comparable without testing for their gustatory response scores (GRS). The problem when using pollen foragers is, that they have already an extensive foraging experience, that could effect both colour and odour preferences. One possability to have naïve honeybees that are old enough to show sufficient perceptual skills, is to prepare controlled age cohorts and train them to an artificial pollen feeder. The next encounter is that individual learning differences exist regarding the single individuals (Mansur et al., 2018; Pamir et al., 2011). Mansur et al. could show, that there are at least four types of learners regarding positive and negative patterning experiments with olfactory-visual compounds. One group focussed on the olfactory component of the compound, another only on the visual component. Only one group was able to solve the paradigm in the correct way, and differentiated the compound from it's single components. The last group did not show any ability to solve the paradigm at all. There are several other studies that investigate the individual learning performances and preferences for olfactory or visual stimuli, regarding the the individual differences in learning performances (Pamir et al., 2011; Dyer, 2012).

The disadvantages of the used electrophysiologial recording technique, the multi-unit recording are that it is not possible to exactly distinguish from which neuron the recodings were done. Backfill staining can only verify the recording position after the recordings and often migrates in the surrounding tissue. Therefore it is only an imprecise statement from which neuron population the recodings was maintained. The next handicap is, that only one animal can be recoded at one time and preparation takes a up a lot of time. The used setup also has a disadvantage in presenting olfactory-visual compounds, since it can not start both stimuli types at the exact same time. Hence it was necessary to start the stimulation with 100 ms temporal discrepancy. We chose to start with the olfactory stimulus, because it takes some time for the valves to switch and led the airflow through the pipes. So we would expect the olfactory stimulus to arrive nearly at the same time as the visual stimulus, which is switched on immediatly. This could led to some issues considering the analysis of the compound with the Spike2 software. The marker codes for the compound must be set to the marker of the visual component, to avoid having a 100 ms mismatch in the data. Last but not least, the electrophysiological recordings can be done only under harnessed conditions so far, not representing a natural foraging situation for the honeybee.

## Advantages of the used methods

One advantages of classical PER conditioning is the high sample size that can be obtained within one sampling group. depending on the experimenter's handling skills, up to 11 or 12 animals can be trained with an inter trial interval (ITI) of eight minutes. The interval for one trial is 35 s (15 s resting - 10 s stimulus presentation - 15 s resting) for each animal. Thus there is still a minute left for moving the sleigh in the right position. For experiments with lower trial number, e.g. conditioning of single modalities, it is possible to test two groups on one day, without exhausting the experimenter. Only the positive and negative patterning experiments (21 trials + one hour pausing + 6 trials) is a more time consuming task. Since the bees were rewarded with sugar solutions, they are fed at the same time and survive such harnessed conditions for several days, when kept in a moisture dark box in the interim period. There is also no need for expensive laboratory stuff to obtain an PER conditioning experiment and handling the harnessed honeybees is very easy. Also the data analyses are not very sophisticated, since a binominal data acquisition is used, that can be analysed with all possible spreadsheet programms. To summarise, the PER conditioning paradigm is a convincing sampling method, that is based on precise behavioural observations and can reduce the complexity of a multi-modal compound to a practicable design for controlled laboratory conditions.

The extracelullar long-time multi-unit recodring has it's advantage on the duration an animal can be examined during the recodings. It is possible to record the neuron responses for hours. Not only a living animal can be used, in contrast to intracellular recodings, but it is also possible to study their behavioural responses in the already mentioned classical conditioning paradigms. Next it is also possible to investigate neuron populations and how they may modulate after memory formation (Strube-Bloss et al., 2011). Using several electrodes enables recording from different processing levels, for example the simultaneous recording from the MB output and the antennal lobe (Strube-Bloss et al., 2012) or the dual olfactory pathway (Brill et al., 2013). Together with the backfill staining with fluorescence day, it is achievable to verify the recording electrode position, but also to trace the clusters of the recorded neuron populations and the neuronal pathways of sensory input neuropils. Furthermore, in addition to fluorescence stainings, it is conceivable to implement immunohystochemical stainings to controll for neuropil size or other brain structures and compare animals that showed behavioural learning differences in classical conditioning. Combining all methods, an integrational study on the behavioural, neurophysiological and neuroanatomical level would be imaginable.

## Conclusions and prospects

Each used method has advantages and disadvantages, but overall they are suitable methods to study the integrational characteristic during multi-modal perception in honeybees in a simplified but convincing experiemntal design. Moreover the used methods are combinable to even gain more insight in the integration of olfactory-visual compounds in the honeybee brain. Interestingly, bees used different strategies for solving sophisticated learning task, depending on the used wavelength of olfactory-visual compounds and their single components. How these modalities are processed via different pathways, e.g. UV light and how this affects the multi-modal integration at the MB level is still an interresting topic. The presented methods and their combining might offer the oppurtunity to investiagte these questions.

In future experiments it would be also interresting to investigate other modalities, for example gustatory and tactile information of flowers. There are already studies on tactile learning in honeybees (Scheiner et al., 2001) or the gustatory learning of pollen with different qualities in bumble bees (Ruedenauer et al., 2015). The combining of extracelllular electrophysiological recordings and stainings using fluorescence dyes could be supported by backfill stainings of different MB output layers or intracellular recordings, too. Finally, the long-term goal has to be to adapt the multi-modal flower-reward scenario to a more natural experimental setup. This can include for instance multi-unit recordings combined with a less-harnessed setup as used in experiments with virtual reality (Buatois et al., 2017; Schultheiss et al., 2017).

# Part IV

# Appendix

# Statistics

**Table 1: Single components**: Odour conditioning - Acquisition. Wilcoxon signed-rank test. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| p-value | CS+ | CS- | Significance |
|---|---|---|---|
| 0.000008 | Farnesol | Citronellol | *** |
| 0.000852 | Farnesol | Geraniol | *** |
| 0.0000001 | Geraniol | Citronellol | *** |
| 0.000043 | Citronellol | Geraniol | *** |
| 0.000022 | Geraniol | Farnesol | *** |
| 0.000006 | Citronellol | Farnesol | *** |

**Table 2: Single components**: Colour conditioning - Acquisition. Wilcoxon signed-rank test. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| p-value | CS+ | CS- | Significance |
|---|---|---|---|
| 0.0001979 | UV | blue | *** |
| 0.0001876 | UV | green | *** |
| 0.0000837 | blue | UV | *** |
| 0.0000501 | blue | green | *** |
| 0.0006460 | green | UV | *** |
| 0.0001516 | green | blue | *** |

**Table 3: Single components**: Colour conditioning - Memory Test. Fisher's exact test. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| p-value | CS+ | CS- | Significance |
|---|---|---|---|
| 0.0000000 | UV | blue | *** |
| 0.0000000 | UV | green | *** |
| 0.0000000 | blue | UV | *** |
| 0.0000018 | blue | green | *** |
| 0.0000000 | green | UV | *** |
| 0.0000017 | green | blue | *** |

**Table 4: Acquisition: Positive patterning Geraniol (A) + UV (X)**: ANOVA - summary. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 112253 | 56127 | 77.92 | <2e-16 | *** |
| Residuals | 357 | 257164 | 720 | | | |

**Table 5: Acquisition: Positive patterning Geraniol (A) + UV (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 16.710744 | 8.404364 | 25.017124 | 0.0000094 | *** |
| A-AX | -26.074380 | -34.380760 | -17.768000 | 0.0000000 | *** |
| AX-X | -9.363636 | -17.670016 | -1.057257 | 0.0226275 | *** |

**Table 6: Memory Test: Positive patterning Geraniol (A) + UV (X) with novel compound Citronellol (B) + blue (Y):** Cochran’s Q test. Significant differences are shown in bold font.

| | | | | | |
|---|---|---|---|---|---|
| **Cochran’s Q test** | | | | | |
| Q = 81.7897, df = 5, p-value = 3.542e-16 | | | | | |
| alternative hypothesis: | | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | | |
| **probability in group** | | | | | |
| **X** | **A** | **AX** | **Y** | **B** | **BY** |
| 0.9500000 | 0.6500000 | 0.7333333 | 0.9833333 | 0.4666667 | 0.6000000 |
| **Wilcoxon signed-rank test** | | | | | |
| | **X** | **A** | **AX** | **Y** | **B** |
| **A** | 2.289e-05 | - | - | - | - |
| **AX** | 1.628e-03 | 2.614e-01 | - | - | - |
| **B** | 6.250e-01 | 2.742e-05 | 0.0005150 | - | - |
| **Y** | 2.794e-08 | 1.909e-02 | 0.0003106 | 1.397e-08 | - |
| **BY** | 4.768e-06 | 6.250e-01 | 0.0526012 | 5.811e-06 | 0.09602 |

**Table 7: Memory Test: Positive patterning Geraniol (A) + UV (X) with novel compound Citronellol (B) + green (Y):** Cochran's Q test. Significant differences are shown in bold font.

**Cochran's Q test**
Q = 114.6552, df = 5, p-value < 2.2e-16

alternative hypothesis:
true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **A** | **AX** | **Y** | **B** | **BY** |
|---|---|---|---|---|---|
| 0.9833333 | 0.4666667 | 0.7000000 | 1.0000000 | 0.4333333 | 0.4166667 |

**Wilcoxon signed-rank test**

| | **X** | **A** | **AX** | **Y** | **B** |
|---|---|---|---|---|---|
| **A** | 2.328e-09 | - | - | - | - |
| **AX** | 2.861e-05 | 5.923e-03 | - | - | - |
| **B** | 1.000e+00 | 1.397e-09 | 1.635e-05 | - | - |
| **Y** | 8.731e-10 | 9.121e-01 | 1.366e-03 | 5.821e-10 | - |
| **BY** | 5.821e-10 | 7.863e-01 | 1.366e-03 | 5.821e-10 | 1 |

**Table 8: Acquisition: Positive patterning Geraniol (A) + blue (X)**: ANOVA - summary. Significance codes: '***' p < 0.001, '**' p < 0.01, '*' p < 0.05, 'n.s.' p > 0.005.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 122184 | 61092 | 108.6 | <2e-16 | *** |
| Residuals | 189 | 106284 | 562 | | | |

**Table 9: Acquisition: Positive patterning Geraniol (A) + blue (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: '***' p < 0.001, '**' p < 0.01, '*' p < 0.05, 'n.s.' p > 0.005.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 55.421875 | 45.51885 | 65.32490 | 0.0000000 | *** |
| A-AX | -4.046875 | -13.94990 | 5.85615 | 0.5996501 | n.s. |
| AX-X | 51.375000 | 41.47197 | 61.27803 | 0.0000000 | *** |

**Table 10: Memory Test: Positive patterning Geraniol (A) + blue (X) with novel compound Citronellol (B) + UV (Y):** Cochran's Q test. Significant differences are shown in bold font.

**Cochran's Q test**
Q = 156.4035, df = 5, p-value < 2.2e-16

alternative hypothesis:
true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **A** | **AX** | **Y** | **B** | **BY** |
|---|---|---|---|---|---|
| 0.953125 | 0.343750 | 0.343750 | 1.000000 | 0.203125 | 0.687500 |

**Wilcoxon signed-rank test**

| | **X** | **A** | **AX** | **Y** | **B** |
|---|---|---|---|---|---|
| **A** | 9.095e-12 | - | - | - | - |
| **AX** | 9.095e-12 | 1.000e+00 | - | - | - |
| **B** | 2.679e-01 | 1.705e-12 | 1.705e-12 | - | - |
| **Y** | 5.329e-14 | 4.395e-02 | 5.659e-02 | 1.332e-14 | - |
| **BY** | 3.017e-04 | 9.913e-05 | 1.696e-04 | 3.576e-06 | 2.642e-07 |

**Table 11: Acquisition: Positive patterning Geraniol (A) + green (X)**: ANOVA - summary. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 82539 | 41269 | 57.18 | <2e-16 | *** |
| Residuals | 192 | 138575 | 722 | | | |

**Table 12: Acquisition: Positive patterning Geraniol (A) + green (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 47.200000 | 36.06895 | 58.331051 | 0.0000000 | *** |
| A-AX | -8.307692 | -19.43874 | 2.823358 | 0.1849271 | n.s. |
| AX-X | 38.892308 | 27.76126 | 50.023358 | 0.0000000 | *** |

**Table 13: Memory Test: Positive patterning Geraniol (A) + green (X) with novel compound Citronellol (B) + UV (Y):** Cochran's Q test. Significant differences are shown in bold font.

| **Cochran's Q test** | | | | | |
|---|---|---|---|---|---|
| Q = 116.2846, df = 5, p-value < 2.2e-16 | | | | | |
| alternative hypothesis: | | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | | |
| | | **probability in group** | | | |
| **X** | **A** | **AX** | **Y** | **B** | **BY** |
| 0.9538462 | 0.4307692 | 0.4153846 | 0.9384615 | 0.3692308 | 0.5076923 |
| **Wilcoxon signed-rank test** | | | | | |
| | **X** | **A** | **AX** | **Y** | **B** |
| **A** | 4.366e-10 | - | - | - | - |
| **AX** | 1.659e-09 | 1.000e+00 | - | - | - |
| **B** | 1.000e+00 | 5.239e-09 | 4.366e-10 | - | - |
| **Y** | 1.091e-10 | 5.304e-01 | 7.007e-01 | 1.091e-10 | - |
| **BY** | 7.983e-09 | 5.304e-01 | 4.294e-01 | 1.436e-06 | 0.1552 |

**Table 14: Acquisition: Negative patterning Geraniol (A) + UV (X)**: ANOVA - summary. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 42221 | 21110 | 28.01 | 4.94e-12 | *** |
| Residuals | 360 | 271314 | 754 | | | |

**Table 15: Acquisition: Negative patterning Geraniol (A) + UV (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: ‘***’ $p < 0.001$, ‘**’ $p < 0.01$, ‘*’ $p < 0.05$, ‘n.s.’ $p > 0.005$.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 43.01667 | 34.86183 | 51.171507 | 0.0e+00 | *** |
| A-AX | -17.59167 | -25.74651 | -9.436826 | 1.8e-06 | *** |
| AX-X | 25.42500 | 17.27016 | 33.579840 | 0.0e+00 | *** |

**Table 16: Memory Test: Negative patterning Geraniol (A) + UV (X) with novel compound Citronellol (B) + blue (Y):** Cochran's Q test

| | | | | | |
|---|---|---|---|---|---|
| **Cochran's Q test** | | | | | |
| Q = 23.037, df = 5, p-value = 0.0003321 | | | | | |
| alternative hypothesis: | | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | | |
| | | **probability in group** | | | |
| **X** | **A** | **AX** | **Y** | **B** | **BY** |
| 0.6212121 | 0.4696970 | 0.7727273 | 0.5757576 | 0.6060606 | 0.6212121 |
| **Wilcoxon signed-rank test** | | | | | |
| | **X** | **A** | **AX** | **Y** | **B** |
| **A** | 0.11245 | - | - | - | - |
| **AX** | 0.04852 | 0.0005382 | - | - | - |
| **B** | 0.76172 | 0.27844755 | 0.03319 | - | - |
| **Y** | 1.00000 | 0.1469135 | 0.04852 | 1.0000 | - |
| **BY** | 1.0000 | 0.1124546 | 0.09265 | 0.8281 | 1 |

**Table 17: Memory Test: Negative patterning Geraniol (A) + UV (X) with novel compound Citronellol (B) + green (Y):** Cochran's Q test

| | | | | | |
|---|---|---|---|---|---|
| **Cochran's Q test** | | | | | |
| Q = 39.2804, df = 5, p-value = 2.085e-07 | | | | | |
| alternative hypothesis: | | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | | |
| | | **probability in group** | | | |
| **X** | **A** | **AX** | **Y** | **B** | **BY** |
| 0.6909091 | 0.4000000 | 0.7454545 | 0.4909091 | 0.4363636 | 0.7090909 |
| **Wilcoxon signed-rank test** | | | | | |
| | **X** | **A** | **AX** | **Y** | **B** |
| **A** | 0.00386 | - | - | - | - |
| **AX** | 0.75905 | 0.0003147 | - | - | - |
| **B** | 0.02121 | 0.4526367 | 0.001945 | - | - |
| **Y** | 0.01754 | 0.8469936 | 0.001659 | 0.759048 | - |
| **BY** | 1.00000 | 0.0019455 | 0.846994 | 0.0089590 | 0.00386 |

**Table 18: Acquisition: Negative patterning Geraniol (A) + blue (X)**: ANOVA - summary. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 3054 | 1527 | 1.443 | 0.239 | n.s. |
| Residuals | 180 | 190392 | 1058 | | | |

**Table 19: Acquisition: Negative patterning Geraniol (A) + blue (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 6.000000 | -7.917159 | 19.917159 | 0.5659396 | n.s. |
| A-AX | -9.934426 | -23.851585 | 3.982732 | 0.2129743 | n.s. |
| AX-X | -3.934426 | -17.851585 | 9.982732 | 0.7822947 | n.s. |

**Table 20: Memory Test: Negative patterning Geraniol (A) + blue (X) with novel compound Citronellol (B) + UV (Y):** Cochran's Q test

**Cochran's Q test**
Q = 62.5362, df = 5, p-value = 3.632e-12

alternative hypothesis:
true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **A** | **AX** | **Y** | **B** | **BY** |
|---|---|---|---|---|---|
| 0.4590164 | 0.3606557 | 0.5737705 | 0.6721311 | 0.5737705 | 0.8688525 |

**Wilcoxon signed-rank test**

| | **X** | **A** | **AX** | **Y** | **B** |
|---|---|---|---|---|---|
| **A** | 1.481e-01 | - | - | - | - |
| **AX** | 1.481e-01 | 2.093e-03 | - | - | - |
| **B** | 6.929e-04 | 1.907e-05 | 0.2424387 | - | - |
| **Y** | 1.481e-01 | 3.916e-03 | 1.0000000 | 0.254876 | - |
| **BY** | 3.129e-06 | 1.397e-08 | 0.0001502 | 0.003433 | 0.0006929 |

**Table 21: Acquisition: Negative patterning Geraniol (A) + green (X)**: ANOVA - summary. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** | **Significance** |
|---|---|---|---|---|---|---|
| Stimulus | 2 | 6954 | 3477 | 3.842 | 0.0233 | * |
| Residuals | 177 | 160171 | 905 | | | |

**Table 22: Acquisition: Negative patterning Geraniol (A) + green (X)**: ANOVA - Tukey multiple comparisons of means 95% family-wise confidence level. Significance codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, 'n.s.' $p > 0.005$.

| | **diff** | **lwr** | **upr** | **p adj** | **Significance** |
|---|---|---|---|---|---|
| A-X | 11.766667 | -1.214635 | 24.747969 | 0.0843302 | n.s. |
| A-AX | -14.250000 | -27.231302 | -1.268698 | 0.0275777 | n.s. |
| AX-X | -2.483333 | -15.464635 | 10.497969 | 0.8935108 | n.s. |

**Table 23: Memory Test: Negative patterning Geraniol (A) + green (X) with novel compound Citronellol (B) + UV (Y):** Cochran's Q test

**Cochran's Q test**
Q = 50.2128, df = 5, p-value = 1.254e-09

alternative hypothesis:
true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **A** | **AX** | **Y** | **B** | **BY** |
|---|---|---|---|---|---|
| 0.6000000 | 0.4666667 | 0.5166667 | 0.8000000 | 0.4833333 | 0.8333333 |

**Wilcoxon signed-rank test**

| | **X** | **A** | **AX** | **Y** | **B** |
|---|---|---|---|---|---|
| **A** | 0.192197 | - | - | - | - |
| **AX** | 0.411488 | 7.263e-01 | - | - | - |
| **B** | 0.003433 | 8.225e-05 | 0.0005531 | - | - |
| **Y** | 0.215195 | 1.000e+00 | 0.8727101 | 0.0001981 | - |
| **BY** | 0.002812 | 8.225e-05 | 0.0001049 | 0.8698918 | 0.0001846 |

**Table 24: Different wavelengths with the same odour**: Differential conditioning - Acquisition. Wilcoxon signed-rank test. Significant differences are shown in bold font.

| **p-value** | **CS+** | **CS-** |
|---|---|---|
| 0.926516 | UV + odour | blue + odour |
| 0.033675 | UV + odour | green + odour |
| 0.000013 | blue + odour | UV + odour |
| 0.000196 | green + odour | UV + odour |

**Table 25: Different wavelengths with the same odour**: Differential conditioning with UV (X) versus blue (Y) with odour (A) - Memory test. Cochran's Q test. Significant differences are shown in bold font.

| **Cochran's Q test** | | | | |
|---|---|---|---|---|
| Q = 20.7368, df = 4, p-value = 0.0003571 | | | | |
| alternative hypothesis: | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | |
| **probability in group** | | | | |
| **X** | **Y** | **AX** | **AY** | **A** |
| 0.9545455 | 1.00000 | 0.7954545 | 0.9772727 | 0.7954545 |
| **Wilcoxon signed-rank test** | | | | |
| | **X** | **Y** | **AX** | **AY** |
| **Y** | 1.00000 | - | - | - |
| **AX** | 0.117188 | 0.031250 | - | - |
| **AY** | 0.694444 | 0.312500 | 0.31250 | - |
| **A** | 0.004883 | 0.001221 | 0.03125 | 0.01139 |

**Table 26: Different wavelengths with the same odour**: Differential conditioning with blue (X) versus UV (Y) with odour (A) - Memory test. Cochran's Q test. Significant differences are shown in bold font.

| **Cochran's Q test** | | | | |
|---|---|---|---|---|
| Q = 28.3922, df = 4, p-value = 1.039e-05 | | | | |
| alternative hypothesis: | | | | |
| true difference in probabilities is not equal to 0 sample estimates: | | | | |
| **probability in group** | | | | |
| **X** | **Y** | **AX** | **AY** | **A** |
| 0.9090909 | 1.00000 | 0.6818182 | 0.9772727 | 0.7727273 |
| **Wilcoxon signed-rank test** | | | | |
| | **X** | **Y** | **AX** | **AY** |
| **Y** | 0.20833 | - | - | - |
| **AX** | 0.01587 | 0.001221 | - | - |
| **AY** | 0.0.46875 | 1.00000 | 0.001221 | - |
| **A** | 0.25652 | 0.006510 | 0.534092 | 0.02344 |

**Table 27: Different wavelengths with the same odour**: Differential conditioning with UV (X) versus green (Y) with odour (A) - Memory test. Cochran's Q test. Significant differences are shown in bold font.

**Cochran's Q test**

Q = 33.3333, df = 4, p-value = 1.021e-06

alternative hypothesis:

true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **Y** | **AX** | **AY** | **A** |
|---|---|---|---|---|
| 0.9696970 | 1.00000 | 0.7878788 | 0.9090909 | 0.5757576 |

**Wilcoxon signed-rank test**

| | **X** | **Y** | **AX** | **AY** |
|---|---|---|---|---|
| **Y** | 1.00000 | - | - | - |
| **AX** | 0.117188 | 0.031250 | - | - |
| **AY** | 0.694444 | 0.312500 | 0.31250 | - |
| **A** | 0.004883 | 0.001221 | 0.03125 | 0.01139 |

**Table 28: Different wavelengths with the same odour**: Differential conditioning with green (X) versus UV (Y) with odour (A)- Memory test. Cochran's Q test. Significant differences are shown in bold font.

**Cochran's Q test**

Q = 18.7273, df = 4, p-value = 0.0008891

alternative hypothesis:

true difference in probabilities is not equal to 0 sample estimates:

**probability in group**

| **X** | **Y** | **AX** | **AY** | **A** |
|---|---|---|---|---|
| 1.00000 | 1.00000 | 0.84375 | 0.96875 | 0.78125 |

**Wilcoxon signed-rank test**

| | **X** | **Y** | **AX** | **AY** |
|---|---|---|---|---|
| **Y** | 1.00000 | - | - | - |
| **AX** | 0.12500 | 0.12500 | - | - |
| **AY** | 1.00000 | 1.00000 | 0.2083 | - |
| **A** | 0.07813 | 0.07813 | 1.0000 | 0.1042 |

# List of Abbreviations

AL = antennal lobe

ALT = antennal lobe tract

AOT = anterior optic tract

AOTu = anterior optic tubercle

KC = Kenyon Cells

CB = central brain

CS = conditioned stimulus

EN = extrinsic neurons

FA = Formaldehyde

ITI = intertrial interval

LH = lateral horn

MB = mushroom body

MS = methyle salylicate

MBON = mushroom body output neuron

NP = negative patterning

OL = optic lobe

OPN = olfactory projection neuron

ORN = olfactory receptor neuron

PBS = Phosphate buffered saline

PCA = principal component analysis

PER = proboscis extension response

PN = projection neurons

PP = positive patterning

PSTH = peristimulus time histogram

RE = recording electrode

US = unconditioned stimulus

UV = ultra-violette

VPN = visual projection neuron

VUMmx1 neuron = ventral unpaired median neuron maxillare 1

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# List of Figures

# List of Tables

# Affidavit

I hereby confirm that my thesis entitled “Principles of olfactory-visual integration to form a common percept in honeybees“ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

# Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Prinzipien der olfaktorisch-visuellen Integration des Lernverhaltens der Honigbiene“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

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| **Publication** (complete reference):<br>Becker, M. C., Rössler, W., and Strube-Bloss, M. F. (2019) UV-light perception is modulated by the odour element of an olfactory-visual compound in restrained honeybees, Journal of Experimental Biology, The Company of Biologists Ltd | | | | | |
| **Participated in** | **Author Initials,** Responsibility decreasing from left to right | | | | |
| Study Design<br>Methods Development | MCB<br>MCB | MSB<br>MSB | | | |
| Data Collection | MCB | | | | |
| Data Analysis and Interpretation | MCB | MSB | WR | | |
| Manuscript Writing | | | | | |
| Writing of Introduction | MCB | MSB | WR | | |
| Writing of Materials & | MCB | MSB | | | |
| Methods | MCB | MSB | | | |
| Writing of Discussion | MCB | MSB | WR | | |
| Writing of First Draft | MCB | | | | |

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| **Figure** | **Author Initials,** Responsibility decreasing from left to right | | | | |
| 1 | MCB | | | | |
| 2 | MCB | | | | |
| 3 | MCB | | | | |
| 4 | MCB | | | | |
| 5 | MCB | | | | |
| 6 | MCB | | | | |
| 7 | MCB | | | | |
| S1 | MSB | | | | |

Explanations (if applicable):

I also confirm my primary supervisor's acceptance.

Mira C. Becker

Doctoral Researcher's Name Date Place Signature

The doctoral researcher confirms that she/he has obtained permission from both the publishers and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Mira C. Becker

Doctoral Researcher's Name Date Place Signature

Martin F. Strube-Bloss

Primary Supervisor's Name Date Place Signature

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# Curriculum Vitae