

RESEARCH ARTICLE

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

Mira C. Becker, Wolfgang Rössler and Martin Fritz Strube-Bloss*

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 that were or were not 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.

KEY WORDS: PER conditioning, Positive and negative patterning, Multimodal integration, Olfaction, Vision, Associative learning and memory

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., 2011a,b). 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. (2011a,b) 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 represent 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 have previously been termed configural or elemental processing roles, respectively. 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 from the relevance of other colours perceived by bees (Papiorek et al., 2016). 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 electroretinogram recordings for all visual stimuli. This ensured that all wavelengths evoked distinct receptor neuron activity (Fig. S1). Furthermore, tests showed that all wavelengths used 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

Behavioral Physiology & Sociobiology (Zoology II), Biozentrum, University of Würzburg, Am Hubland, 97074 Würzburg, Germany.

*Author for correspondence (martin.strube-bloss@uni-wuerzburg.de)

 M.F.S., 0000-0001-8264-7534

Received 8 February 2019; Accepted 2 May 2019

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 1 h, 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.

MATERIALS AND METHODS

Experimental animals

Foragers of *Apis mellifera carnica* Pollman 1879 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 (w/w) *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 (w/w) and adapted to the light conditions in the laboratory (dimmed room with red light, average temperature: 24°C). A recent study showed 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 min⁻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 to determine 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 (v/v). Six µl of the odour solution was pipetted on filter paper strips (1×8 cm) in three drops of 2 µl in a row and placed into the stimulation pipette.

Light stimulation

We used three different LED light sources: 375 nm (UV, intensity: 7.5×10¹³ photons cm⁻² s⁻¹, TRU Components, Conrad, Hirschhaid, Germany), 465 nm (blue, intensity: 6.5×10¹³ photons cm⁻² s⁻¹, Avago Technologies, Broadcom Inc., San José, CA, USA) and 525 nm (green, intensity: 3.93×10¹³ photons cm⁻² s⁻¹, Avago Technologies, Broadcom Inc., San José, CA, USA) (Fig. 1B).

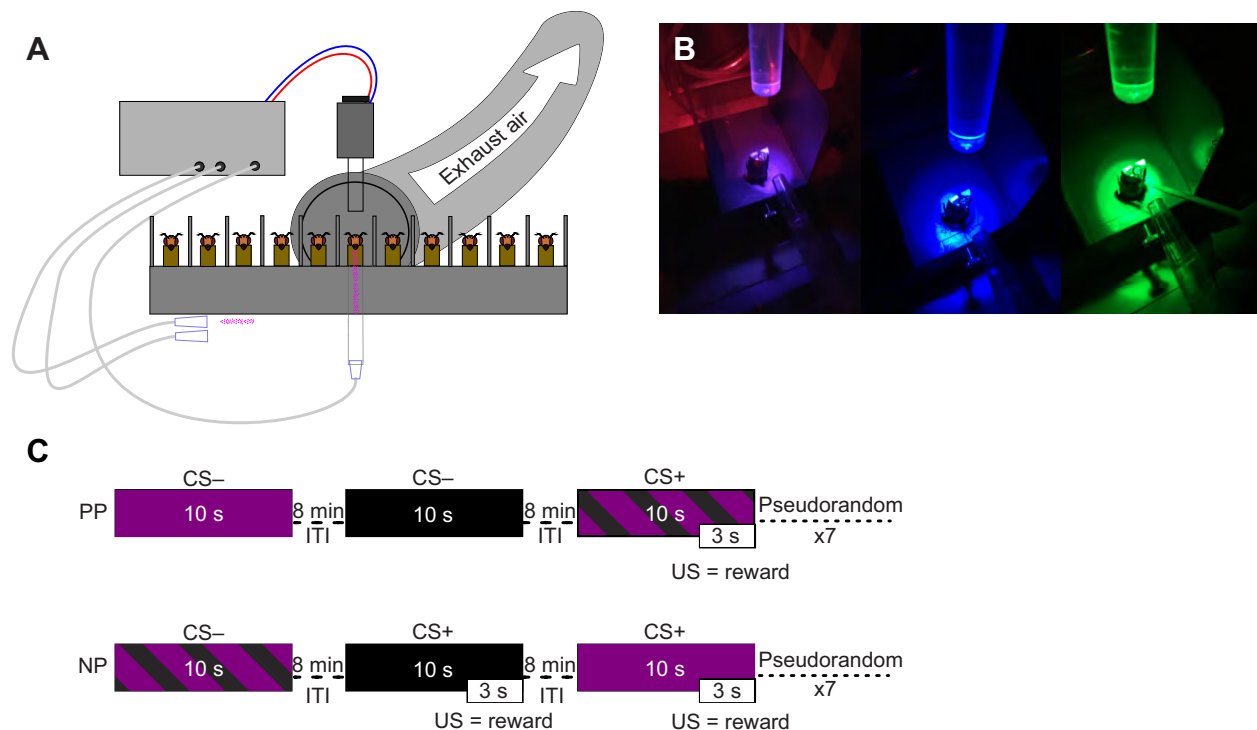


Fig. 1. Conditioning of a restrained bee to olfactory–visual stimuli. (A) A cold light-guide system made of an acrylic glass cylinder was positioned 3 cm above the tested subject and orthogonal to the odour presentation device. Odours were presented using multiple pipettes with filter paper strips. Subjects 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 (375 nm, UV; 465 nm, blue; 525 nm, green). (C) Schematic of positive (PP) and negative (NP) patterning experiments using UV element as example. During PP, UV (purple) and an odour (black) are presented unrewarded (CS-), whereas their compound is rewarded (CS+). Stimulation lasted 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. CS, conditioned stimulus; ITI, inter-trial interval; US, unconditioned stimulus.

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 3 cm 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 stimulation. 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 3 s 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 8 min 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 such a way that each stimulus occurred only twice in a row, but randomly. Furthermore, we made sure to always start with a CS-. All experiments were performed 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 nine 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 1 h 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 analysis of variance (ANOVA) for repeated measurements for within-group and between-group comparisons on the most suitable model. Even though ANOVA is usually 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-blue ($n=66$, Fig. 2A) or geraniol-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 had never been 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 was used as visual element during PP. In total, we trained 121 bees to discriminate an olfactory-visual compound of geraniol-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

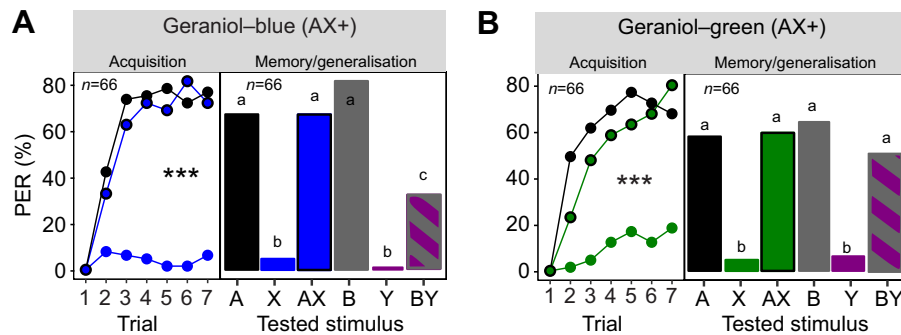


Fig. 2. Acquisition trials and memory/generalisation tests in positive patterning experiments including blue and green as element of the trained compound. (A,B) During acquisition, the compound geraniol–blue (black–blue in A, $n=66$) and geraniol–green (black–green in B, $n=66$) were rewarded, whereas 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 there were significant differences between proboscis extension response (PER) rates during the memory test (for blue: Cochran's Q test= 156.4035 , d.f.=5, $P<0.0001$; for green: Cochran's Q test= 116.2846 , d.f.=5, $P<0.0001$). Generalisation to a novel odour (B, citronellol; grey) and compound (BY, grey–purple) was high, whereas generalisation to novel UV (Y, purple) was low in both groups (pairwise Wilcoxon sign test: different letters indicate significant differences; Bonferroni-corrected threshold for multiple comparisons: $\alpha'=0.003$).

rewarded, the PER performance to the odour alone was significantly increased compared with that of the compound, whereas UV 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).

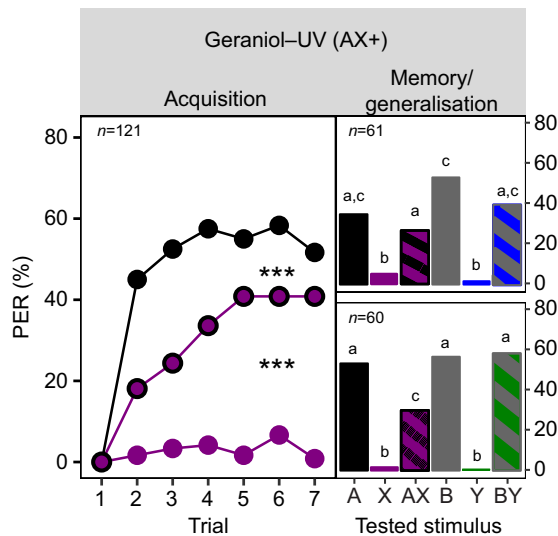


Fig. 3. Acquisition trials and memory/generalisation tests in positive patterning experiments including UV as element of the trained compound. Bees ($n=121$) learned to discriminate all three stimuli (rewarded: geraniol–UV, black–purple; unrewarded: geraniol, black; UV, purple; ANOVA, $***P<0.0001$). Although not rewarded, the associative strength to geraniol seemed to be highest and was also memorised 1 h later. To test for generalisation, we introduced the single odour citronellol (B, grey) and blue light (Y, blue) and its compound (BY, grey–blue) in half of the bees ($n=61$; upper panel, Cochran's Q test= 81.789 , d.f.=5, $P<0.0001$) and in the other half ($n=60$; lower panel, Cochran's Q test= 114.655 , d.f.=5, $P<0.0001$) green light (Y, green) and its compound (BY, grey–green). 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 compound UV stimulus (pairwise Wilcoxon sign test: different letters indicate significant differences; Bonferroni-corrected threshold for multiple comparisons: $\alpha'=0.003$).

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, blue), were rewarded (CS+), but their compound (geraniol–blue) 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 both light-only and odour-only stimuli, reaching equally high learning rates ($\sim 60\%$). 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, UV) as well as their compound (citronellol–UV) 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 with an olfactory–visual compound including the same odour. However, this difference in generalisation predominantly occurred when UV was introduced as an element of a novel olfactory–visual compound (Figs 2 and 4).

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

In negative patterning experiments including UV, 121 bees were conditioned in total. The bees had to learn that the single elements, geraniol or UV, were rewarded (CS+), but their olfactory–visual compound (CS–) was not. The PER rates for the latter were significantly lower compared with the single elements (Fig. 5). Thus, if UV 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).

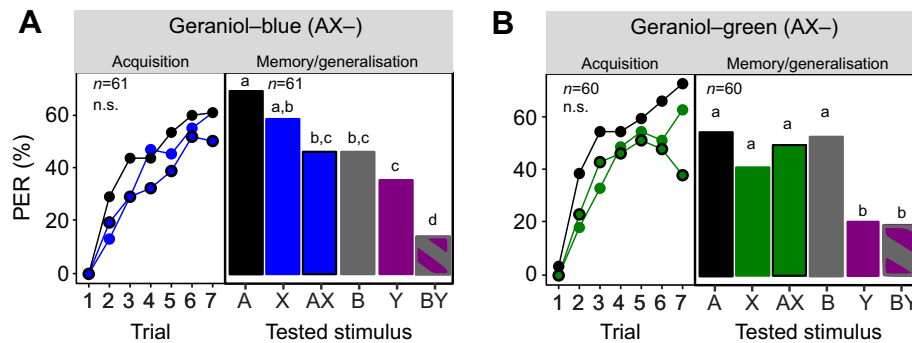


Fig. 4. Acquisition trials and memory/generalisation tests in negative patterning experiments including blue and green light as trained elements. (A,B) During acquisition, single elements geraniol (black), blue (A, $n=61$) and green (B, $n=60$) were rewarded, whereas the compounds geraniol–blue (black–blue 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.). During the memory test in A, bees differentiate the single odour element from the compound and did not generalise that information to a novel odour (B, citronellol; grey) (Cochran's Q test=62.536, d.f.=5, $P<0.0001$). During the memory test in B, bees did not differentiate the single elements from the compound and generalised the novel odour element (B, citronellol; grey) (Cochran's Q test=50.213, d.f.=5, $P<0.0001$). However, in both groups (A and B) neither the novel UV (Y, purple) nor the novel odour–UV compound (BY, purple–grey) was generalised to the conditioned single elements (pairwise Wilcoxon sign test: different letters indicate significant differences; Bonferroni-corrected threshold for multiple comparisons: $\alpha'=0.003$).

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 was the only difference, we

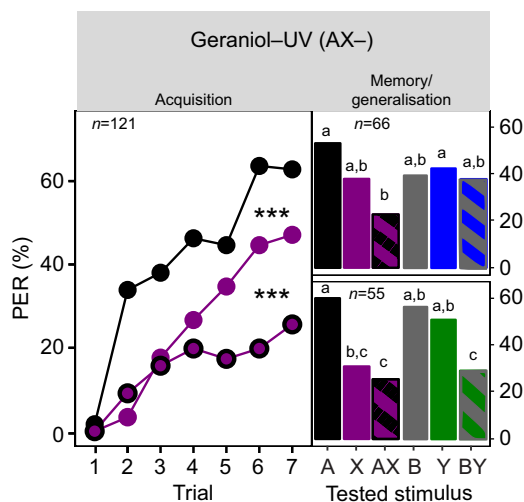


Fig. 5. Acquisition trials and memory/generalisation tests in negative patterning experiments including UV as trained element. The bees ($n=121$) could differentiate between single elements (geraniol, black; UV, purple; CS+) and their compound (geraniol–UV, black–purple; CS–). The odour element evoked 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 formed. One group received blue light, citronellol and their compound ($n=66$, grey–blue, upper panel, Cochran's Q test=23.037, d.f.=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, d.f.=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 also showed a significant differentiation to the novel compound (Cochran's Q test=39.2804, d.f.=5, $P<0.0001$) (pairwise Wilcoxon sign test: different letters indicate significant differences; Bonferroni-corrected threshold for multiple comparisons: $\alpha'=0.003$).

had to keep the odour information constant. In total, we trained four groups of bees to separate blue and UV light, as well as green and UV. The odour–UV compound was either unrewarded (Fig. 6A and Fig. 7A) or rewarded (Fig. 6B and Fig. 7B). For the pure-light discrimination, this kind of inverted meaning did not have any influence on wavelengths separation (Figs 6 and 7, right subpanels). 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 Fig. 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 Fig. 7B). This suggests that modulation of light perception by the odour element of an olfactory–visual compound occurs and thus 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 (Figs 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 1 h 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

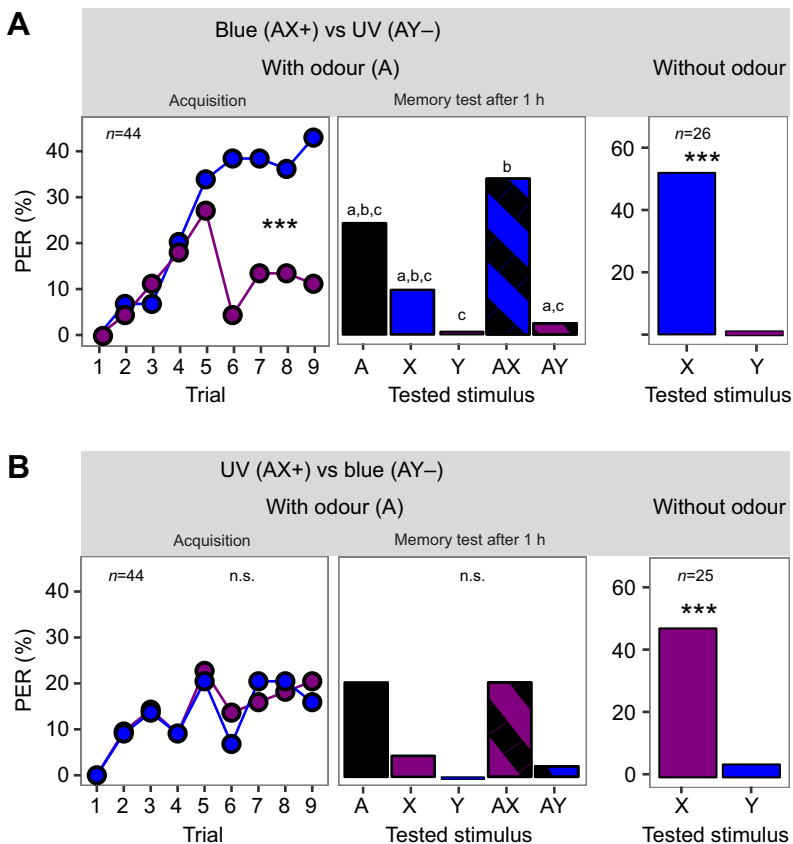


Fig. 6. Acquisition trials and memory test in differential olfactory-visual compound conditioning with blue and UV light. (A) An odour–blue compound was presented rewarded (AX+; blue–black) and an odour–UV compound was presented unrewarded (AY–; purple–black). Note, the odour element was the same. During the memory test (middle panel), both compounds and its single elements were presented. Right panel shows pure light separation (different experiment). (B) Rewarded and unrewarded compounds presented in 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 significant 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 independently if rewarded or unrewarded (right panels, χ^2 test, $***P<0.001$).

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 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 results and our own.

Different learning capabilities for UV compared with 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 one theory or the other, depending on the wavelength. Bees could not solve the PP and NP task if blue or green light was used as the visual element (Figs 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). In contrast, the patterning experiments including

UV as an element could be solved (Figs 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 might be processed differentially, resulting in a different associative strength during our cross-modal conditioning experiments compared with green and blue light.

UV, but not blue or green light, 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 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 (Figs 6 and 7, upper panels), but not if UV was an element of the rewarded compound (Figs 6 and 7, lower panels), even though the involved single light elements could be differentiated in either case (Figs 6 and 7, right hand panels). Similarly, studies with Africanised *A. mellifera* on absolute and discriminant learning tasks with visual

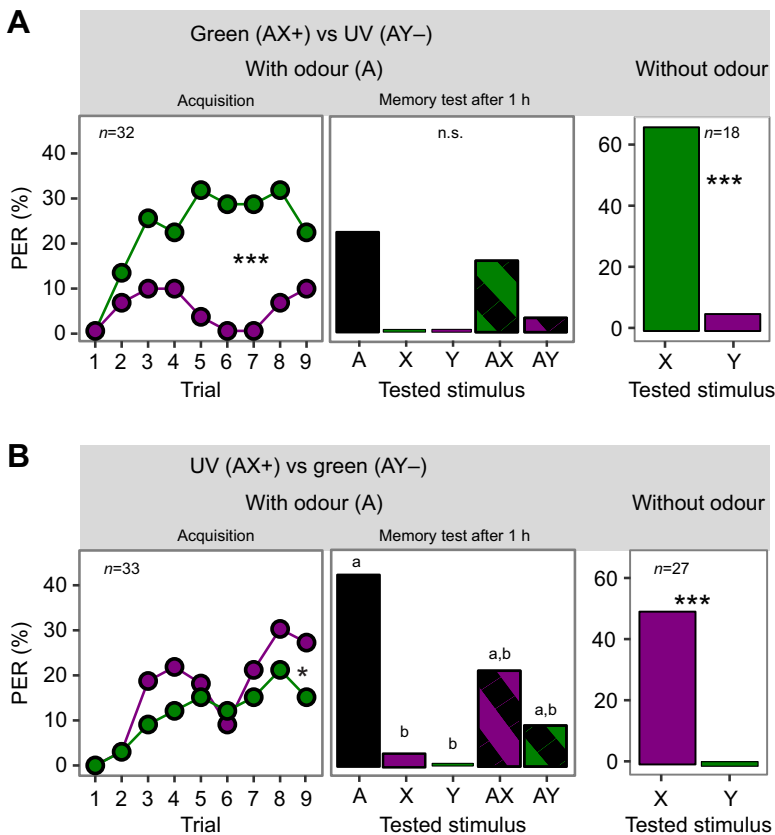


Fig. 7. Acquisition trials and memory test in differential olfactory-visual compound conditioning with green and UV light. (A) During acquisition, bees discriminated between a rewarded odour–green compound (AX+) and an unrewarded odour–UV compound (AY–) ($n=32$, Wilcoxon signed rank test, $***P<0.001$). (B) When rewarded and unrewarded stimuli were reversed, this discrimination was much lower, but still present ($n=33$, Wilcoxon signed rank test, $*P<0.05$). However, in both cases, bees did not memorise 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, χ^2 test, $***P<0.001$).

stimuli could show only that learning performance depended on the quality of the colour of the light stimulus, with lower learning performances for violet light compared with 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 that to the single odour element (Fig. 5). Moreover, the odour element did not dominate in the associative strength of the compound as it did in NP experiments including green and blue light (Fig. 4). Thus, in this experimental context, the odour–UV compound is perceived as a unique cue and not as the sum of the associative strength of the single elements. If UV is presented without any odour context, it can be learned and differentiated from other light stimuli as well (Figs 6 and 7, right panels). Thus, in general, bees had no difficulty associating UV with reward. This is why we think that the interaction (integration) of the odour–light pathway might be different if UV is included compared with green or blue, and that this might be based on neurobiological differences. Although we cannot completely exclude experience-driven responses to UV, 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 in our studies was not polarised as it would be 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 a lack

of information. These findings suggest that the differential effects with UV may be due to differences in internal processing of UV and light polarisation information, compared with blue or green light. Indeed, studies in various insect species show that information about polarised UV 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 showed 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 (KCs), 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 that did not respond to any of the presented stimuli (iv) may become recruited after associative conditioning, as we showed 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 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 that depend on the presented wavelength of light stimuli, supporting a distinct processing pathway for UV compared with other wavelengths.

Acknowledgements

The authors thank Dirk Ahrens for beekeeping and Leonie Lichtenstein for her advice in PER conditioning with monochromatic light. Furthermore, we thank Leonie and Matthias Lichtenstein for providing the program 'TimingProtocol'.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.C.B., M.F.S.-B.; Validation: W.R., M.F.S.-B.; Formal analysis: M.C.B., M.F.S.-B.; Investigation: M.C.B., M.F.S.-B.; Resources: W.R., M.F.S.-B.; Writing - original draft: M.C.B.; Writing - review & editing: M.C.B., W.R., M.F.S.-B.; Visualization: M.C.B., M.F.S.-B.; Supervision: M.F.S.-B.; Funding acquisition: M.F.S.-B.

Funding

This work was funded by the Deutsche Forschungsgemeinschaft (STR 1334/3-1) to M.F.S.-B. Further support was provided by the Faculty of Biology at the University of Würzburg.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.201483.supplemental>

References

- Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107–119. doi:10.1037/0735-7036.97.2.107
- Deisig, N., Lachnit, H., Giurfa, M. and Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learn. Mem.* **8**, 70–78. doi:10.1101/lm.8.2.70
- Deisig, N., Lachnit, H., Sandoz, J.-C., Lober, K. and Giurfa, M. (2003). A modified version of the unique cue theory accounts for olfactory compound processing in honeybees. *Learn. Mem.* **10**, 199–208. doi:10.1101/lm.55803
- Deisig, N., Sandoz, J.-C., Giurfa, M. and Lachnit, H. (2007). The trial-spacing effect in olfactory patterning discriminations in honeybees. *Behav. Brain Res.* **176**, 314–322. doi:10.1016/j.bbr.2006.10.019
- Dobrin, S. E. and Fahrbach, S. E. (2012). Visual associative learning in restrained honey bees with intact antennae. *PLoS ONE* **7**, e37666. doi:10.1371/journal.pone.0037666
- Dyer, A. and Garcia, J. (2014). Color difference and memory recall in free-flying honeybees: forget the hard problem. *Insects* **5**, 629. doi:10.3390/insects5030629
- Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G. P., Simonov, V. and Wong, B. B. M. (2012). Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. *Proc. R. Soc. B* **279**, 3606–3615. doi:10.1098/rspb.2012.0827
- Ehmer, B. and Gronenberg, W. (2002). Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). *J. Comp. Neurol.* **451**, 362–373. doi:10.1002/cne.10355
- Gerber, B. and Smith, B. H. (1998). Visual modulation of olfactory learning in honeybees. *J. Exp. Biol.* **201**, 2213–2217.
- Giurfa, M. (2003). Cognitive neuroethology: dissecting non-elemental learning in a honeybee brain. *Curr. Opin. Neurobiol.* **13**, 726–735. doi:10.1016/j.conb.2003.10.015
- Giurfa, M. (2004). Conditioning procedure and color discrimination in the honeybee *Apis mellifera*. *Naturwissenschaften* **91**, 228–231. doi:10.1007/s00114-004-0530-z
- Grob, R., Fleischmann, P. N., Grübel, K., Wehner, R. and Rössler, W. (2017). The role of celestial compass information in *Cataglyphis* ants during learning walks and for neuroplasticity in the central complex and mushroom bodies. *Front. Behav. Neurosci.* **11**, 226. doi:10.3389/fnbeh.2017.00226
- Held, M., Berz, A., Hensgen, R., Muenz, T. S., Scholl, C., Rössler, W., Homberg, U. and Pfeiffer, K. (2016). Microglomerular synaptic complexes in the sky-compass network of the honeybee connect parallel pathways from the anterior optic tubercle to the central complex. *Front. Behav. Neurosci.* **10**, 186. doi:10.3389/fnbeh.2016.00186
- Homberg, U., Heinze, S., Pfeiffer, K., Kinoshita, M. and el Jundi, B. (2011). Central neural coding of sky polarization in insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **366**, 680–687. doi:10.1098/rstb.2010.0199
- Hori, S., Takeuchi, H., Arikawa, K., Kinoshita, M., Ichikawa, N., Sasaki, M. and Kubo, T. (2006). Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera* L. *J. Comp. Physiol. A* **192**, 691–700. doi:10.1007/s00359-005-0091-4
- Jernigan, C. M., Roubik, D. W., Wcislo, W. T. and Riveros, A. J. (2014). Color-dependent learning in restrained africanized honey bees. *J. Exp. Biol.* **217**, 337–343. doi:10.1242/jeb.091355
- Kevan, P. G. and Lane, M. A. (1985). Flower petal microtexture is a tactile cue for bees. *Proc. Natl. Acad. Sci. USA* **82**, 4750–4752. doi:10.1073/pnas.82.14.4750
- Kirkerud, N. H., Schlegel, U. and Galizia, C. G. (2017). Aversive learning of colored lights in walking honeybees. *Front. Behav. Neurosci.* **11**, 94. doi:10.3389/fnbeh.2017.00094
- Lichtenstein, L., Sommerlandt, F. M. J. and Spaethe, J. (2015). Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning. *PLoS ONE* **10**, e0134248. doi:10.1371/journal.pone.0134248
- Lichtenstein, L., Lichtenstein, M. and Spaethe, J. (2018). Length of stimulus presentation and visual angle are critical for efficient visual PER conditioning in the restrained honey bee, *Apis mellifera*. *J. Exp. Biol.* **221**, jeb179622. doi:10.1242/jeb.179622
- Lunney, G. H. (1970). Using analysis of variance with a dichotomous dependent variable: an empirical study. *J. Educ. Meas.* **7**, 263–269. doi:10.1111/j.1745-3984.1970.tb00727.x
- Mansur, B. E., Rodrigues, J. R.V. and Mota, T. (2018). Bimodal patterning discrimination in harnessed honey bees. *Front. Psychol.* **9**, 1529. doi:10.3389/fpsyg.2018.01529
- Matsumoto, Y., Menzel, R., Sandoz, J.-C. and Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. *J. Neurosci. Methods* **211**, 159–167. doi:10.1016/j.jneumeth.2012.08.018
- Menzel, R. (1981). Achromatic vision in the honeybee at low light intensities. *J. Comp. Physiol.* **141**, 389–393. doi:10.1007/BF00609941
- Menzel, R. and Blakers, M. (1976). Colour receptors in the bee eye - Morphology and spectral sensitivity. *J. Comp. Physiol.* **108**, 11–13. doi:10.1007/BF00625437
- Menzel, R. and Greggers, U. (1985). Natural phototaxis and its relationship to colour vision in honeybees. *J. Comp. Physiol. A* **157**, 311–321. doi:10.1007/BF00618121
- Mobbs, P. (1982). The brain of the honeybee *Apis mellifera*. I. The connections and spatial organization of the mushroom bodies. *Phil. Trans. R. Soc. Lond. B* **298**, 309–354. doi:10.1098/rstb.1982.0086
- Mota, T., Giurfa, M. and Sandoz, J.-C. (2011a). Color modulates olfactory learning in honeybees by an occasion-setting mechanism. *Learn. Mem.* **18**, 144–155. doi:10.1101/lm.2073511

- Mota, T., Roussel, E., Sandoz, J.-C. and Giurfa, M.** (2011b). Visual conditioning of the sting extension reflex in harnessed honeybees. *J. Exp. Biol.* **214**, 3577-3587. doi:10.1242/jeb.062026
- Myers, K. M., Vogel, E. H., Shin, J. and Wagner, A. R.** (2001). A comparison of the Rescorla-Wagner and Pearce models in a negative patterning and a summation problem. *Anim. Learn. Behav.* **29**, 36-45. doi:10.3758/BF03192814
- Niggebrügge, C., Leboulle, G., Menzel, R., Komischke, B. and de Ibarra, N. H.** (2009). Fast learning but coarse discrimination of colours in restrained honeybees. *J. Exp. Biol.* **212**, 1344-1350. doi:10.1242/jeb.021881
- Papiorek, S., Junker, R. R., Alves-dos-Santos, I., Melo, G. A. R., Amaral-Neto, L. P., Sazima, M., Wolowski, M., Freitas, L., Lunau, K. and Dafni, A.** (2016). Bees, birds and yellow flowers: pollinator-dependent convergent evolution of UV patterns. *Plant Biol.* **18**, 46-55. doi:10.1111/plb.12322
- Plath, J. A., Entler, B. V., Kirkerud, N. H., Schlegel, U., Galizia, C. G. and Barron, A. B.** (2017). Different roles for honey bee mushroom bodies and central complex in visual learning of colored lights in an aversive conditioning assay. *Front. Behav. Neurosci.* **11**, 98. doi:10.3389/fnbeh.2017.00098
- Raguso, R. A.** (2004). Why are some floral nectars scented? *Ecology* **85**, 1486-1494. <https://esajournals.onlinelibrary.wiley.com/doi/abs/10.1890/03-0410>
- Riveros, A. J. and Gronenberg, W.** (2009). Learning from learning and memory in bumblebees. *Commun. Integr. Biol.* **2**, 437-440. doi:10.4161/cib.2.5.9240
- Riveros, A. J. and Gronenberg, W.** (2012). Decision-making and associative color learning in harnessed bumblebees (*Bombus impatiens*). *Anim. Cogn.* **15**, 1183-1193. doi:10.1007/s10071-012-0542-6
- Rossel, S. and Wehner, R.** (1984). How bees analyse the polarization patterns in the sky. *J. Comp. Physiol. A* **154**, 607-615. doi:10.1007/BF01350213
- Rybak, J. and Menzel, R.** (1993). Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. *J. Comp. Neurol.* **334**, 444-465. doi:10.1002/cne.903340309
- Scheiner, R., Page, R. E. and Erber, J.** (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie* **35**, 133-142. doi:10.1051/apido:2004001
- Schiestl, F. P. and Schlüter, P. M.** (2009). Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annu. Rev. Entomol.* **54**, 425-446. doi:10.1146/annurev.ento.54.110807.090603
- Schildberger, K.** (1983). Local interneurons associated with the mushroom bodies and the central body in the brain of *Acheta domesticus*. *Cell Tissue Res.* **230**, 573-586. doi:10.1007/BF00216202
- Schmitt, F., Stieb, S. M., Wehner, R. and Rössler, W.** (2016). Experience-related reorganization of giant synapses in the lateral complex: Potential role in plasticity of the sky-compass pathway in the desert ant *Cataglyphis fortis*. *Dev. Neurobiol. Wiley Online Libr.* **76**, 390-404. doi:10.1002/dneu.22322
- Schürmann, F. W.** (1987). The architecture of the mushroom bodies and related neuropils in the insect brain. In *Arthropod Brain: Its Evolution, Development, Structure, and Functions* (ed. A.G. Gupta), pp. 231-275. New York: John Wiley & Sons.
- Stone, T., Webb, B., Adden, A., Weddig, N. B., Honkanen, A., Templin, R., Wcislo, W., Scimeca, L., Warrant, E. and Heinze, S.** (2017). An Anatomically Constrained Model for Path Integration in the Bee Brain. *Curr. Biol.* **27**, 3069-3085.e11. doi:10.1016/j.cub.2017.08.052
- Strausfeld, N. J.** (2002). Organization of the honey bee mushroom body: Representation of the calyx within the vertical and gamma lobes. *J. Comp. Neurol.* **450**, 4-33. doi:10.1002/cne.10285
- Strube-Bloss, M. F., Nawrot, M. P. and Menzel, R.** (2011). Mushroom body output neurons encode odor-reward associations. *J. Neurosci.* **31**, 3129-3140. doi:10.1523/JNEUROSCI.2583-10.2011
- Strube-Bloss, M. F., Nawrot, M. P. and Menzel, R.** (2016). Neural correlates of side-specific odour memory in mushroom body output neurons. *Proc. R. Soc. B* **283**, 20161270.
- Strube-Bloss, M. F. and Rössler, W.** (2018). Multimodal integration and stimulus categorization in putative mushroom body output neurons of the honeybee. *R. Soc. Open Sci.* **5**, 171785. doi:10.1098/rsos.171785
- Vogt, K., Yarali, A. and Tanimoto, H.** (2015). Reversing stimulus timing in visual conditioning leads to memories with opposite valence in drosophila. *PLoS One* **10**, e0139797. doi:10.1371/journal.pone.0139797
- Wagner, A. R.** (1971). Elementary associations. In *Essays in neobehaviorism: A Memorial Volume to Kenneth W. Spence* (ed. H. H. Kandler and J. T. Spence), pp. 187-213. New York: Appleton-Century-Crofts.
- Williams, D. A. and Braker, D. S.** (1999). Influence of past experience on the coding of compound stimuli. *J. Exp. Psychol. Anim. Behav. Process.* **25**, 461. doi:10.1037/0097-7403.25.4.461
- Yilmaz, A., Grübel, K., Spaethe, J. and Rössler, W.** (2019). Distributed plasticity in ant visual pathways following colour learning. *Proc. Biol. Sci.* **286**, 20182813. doi:10.1098/rspb.2018.2813