

RESEARCH ARTICLE

Color-dependent learning in restrained Africanized honey bees

C. M. Jernigan^{1,2,*}, D. W. Roubik¹, W. T. Wcislo¹ and A. J. Riveros^{1,3,‡}

ABSTRACT

Associative color learning has been demonstrated to be very poor using restrained European honey bees unless the antennae are amputated. Consequently, our understanding of proximate mechanisms in visual information processing is handicapped. Here we test learning performance of Africanized honey bees under restrained conditions with visual and olfactory stimulation using the proboscis extension response (PER) protocol. Restrained individuals were trained to learn an association between a color stimulus and a sugar–water reward. We evaluated performance for ‘absolute’ learning (learned association between a stimulus and a reward) and ‘discriminant’ learning (discrimination between two stimuli). Restrained Africanized honey bees (AHBs) readily learned the association of color stimulus for both blue and green LED stimuli in absolute and discriminatory learning tasks within seven presentations, but not with violet as the rewarded color. Additionally, 24-h memory improved considerably during the discrimination task, compared with absolute association (15–55%). We found that antennal amputation was unnecessary and reduced performance in AHBs. Thus color learning can now be studied using the PER protocol with intact AHBs. This finding opens the way towards investigating visual and multimodal learning with application of neural techniques commonly used in restrained honey bees.

KEY WORDS: *Apis mellifera*, Classical conditioning, Proboscis extension response

INTRODUCTION

Learning and memory mediate numerous behavioral responses to changing environments (e.g. Thorpe, 1956; Barco et al., 2006; Dukas, 2008). For example, bees optimize foraging efforts by assessing a variable floral market and learning and remembering the stimuli that characterize more profitable flowers, which are preferentially visited (Menzel, 1985). Floral features used by bees include colors (Daumer, 1956; von Frisch, 1967; Menzel, 1985; Galizia et al., 2012), odors (von Frisch, 1967; Galizia et al., 2012; Giurfa and Sandoz, 2012), shapes (Gould, 1985), textures (Kevan and Lane, 1985; Erber et al., 1998), temperature (Whitney et al., 2008), and electrostatic charge (Clarke et al., 2013). Experimentally, perception of floral features can be quantified by conditioning of the proboscis (tongue) extension reflex (PER) of individually restrained bees that are unharmed but prevented from flight. The PER is an unconditioned response (UR) evoked by contact with a sweet substance (the unconditioned stimulus, US) by the antenna, tarsus

or proboscis. Given its reliability – up to 80% of bees learn an association between an odor and a sucrose reward after three training repetitions (Bitterman et al., 1983) – PER conditioning has been widely used to study how European honey bees (*Apis mellifera* Linnaeus) (EHBs) learn and remember olfactory stimuli (acting as conditioned stimuli, CS) (e.g. Takeda, 1961; Daly et al., 2001; Giurfa and Sandoz, 2012).

In contrast to olfactory stimuli, PER conditioning has had limited success in the study of honey bee visual learning (Masuhr and Menzel, 1972; Erber and Schildberger, 1980; Menzel, 1985; Hori et al., 2006; Mota et al., 2011; Dobrin and Fahrbach, 2012). After 10–20 training repetitions, fewer than 40% of EHBs learned a conditioned response to visual stimuli (e.g. Hori et al., 2006; Mota et al., 2011; Sakura et al., 2012; Dobrin and Fahrbach, 2012).

Excluding olfactory input through antennal amputation leads to higher performance during PER visual learning in EHB, with up to 70% color learning in 10 trials (Niggebrügge et al., 2009) (see also Kuwabara, 1957; Hori et al., 2006; Mota et al., 2011). In contrast to EHBs, antennal amputation is not required for PER color conditioning of the bumble bee, *Bombus impatiens* (Riveros and Gronenberg, 2012). Antennal amputation precludes any possibility of simultaneously analyzing how visual and olfactory stimuli interact to influence decisions. Here, we evaluate the potential of the PER protocol to study visual learning in Africanized honey bees (AHB; feral bees near *Apis mellifera scutellata*) (Francoy et al., 2008), which were introduced to the Neotropics over 50 years ago (Smith et al., 1989; Spivak et al., 1991; Schneider et al., 2004; Roubik, 2009). They readily learn olfactory cues following the PER protocol (Abramson et al., 1997; McCabe et al., 2007; Couvillon et al., 2010), yet their performance appears to be poorer (Couvillon et al., 2010) and a previous attempt to condition AHBs to light was unsuccessful (Abramson et al., 1997). We relied on absolute and discriminant learning tasks that were evaluated using colored lights of three different wavelengths. We further compared the performance of AHBs to odor versus color learning, as well as the effect of antennal amputation on acquisition rates.

RESULTS

Experiment 1 (absolute color conditioning)

Bees trained to associate a colored light with a reward versus bees (controls) that received the light stimulus and the sucrose in separate trials differed in their responsiveness to light, depending upon the wavelength used, as shown across trials with color, pairing of stimuli and their interaction as factors (repeated measures analyses: trial: $F_{6,158}=15.27$, $P<0.0001$; pairing: $F_{1,163}=33.99$, $P<0.0001$; color: $F_{2,163}=5.76$, $P=0.004$; color×pairing: $F_{2,163}=10.75$, $P<0.0001$; Fig. 1). Trial-by-trial analyses showed that the effect of pairing was significant after a single rewarding event ($F_{1,163}=0.09$, $P=0.0002$). Differences due to color occurred only after the third rewarding event ($F_{2,163}=0.07$, $P=0.004$). Within colors, bees successfully learned the association (as shown by significant differences between the ‘paired’ and ‘unpaired’ conditions) when blue (B) (repeated measures across trials for the effect of ‘trial’, ‘pairing of stimuli’ and

¹Smithsonian Tropical Research Institute, Balboa, Panama, Republic of Panama.

²Department of Biological Sciences, Butler University, Indianapolis, IN 46208, USA. ³Departamento de Ciencias Fisiológicas, Pontificia Universidad Javeriana, Bogotá, Colombia.

*Present address: School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA.

‡Author for correspondence (andre.riveros@javeriana.edu.co)

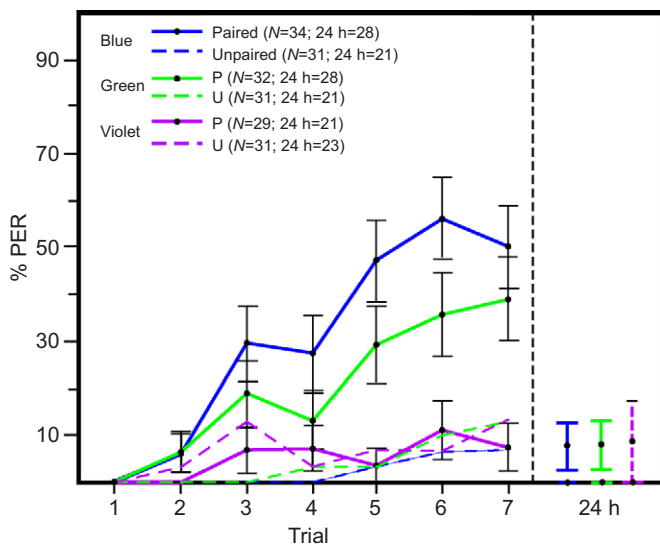


Fig. 1. Learning curves presented as the percent of individual Africanized honey bees responding to trained stimuli for the absolute conditioning protocol over seven learning trials. Conditioned color (blue, green or violet) was paired with a sucrose reward. Controls had color stimulus and sucrose presented in separate trials. The error bars represent standard errors of the mean, and the colored lines represent the stimulus; 24 h memory is shown to the right of the dashed line. PER, proboscis extension response.

the interaction ‘trial×pairing’: trial: $F_{6,52}=9.19$, $P<0.0001$; pairing: $F_{1,57}=36.8$, $P<0.0001$; trial×pairing: $F_{6,52}=6.56$, $P<0.0001$) or green (G) (trial: $F_{6,51}=5.71$, $P=0.0001$; pairing: $F_{1,56}=12.96$, $P=0.0007$; trial×pairing: $F_{6,51}=2.83$, $P=0.019$), but not violet (V) (trial: $F_{6,45}=1.68$, $P=0.15$; pairing: $F_{1,50}=0.025$, $P=0.87$; trial×pairing: $F_{6,45}=0.97$, $P=0.45$) were paired with a sucrose reward. Among colors, the highest percentage of responsiveness was achieved when they were trained to blue (Fig. 1), although this difference is not statistically different from green (color: B vs G: $F_{6,57}=2.469$, $P=0.122$; trial×color: $F_{6,52}=0.559$, $P=0.761$; Fig. 1).

After 24 h following training, bees had almost entirely lost the association to blue (2 out of 28 remembered the association) and green (2 out of 27 remembered).

Experiment 2 (discriminant conditioning)

Bees solved the discrimination task after a few trials when either blue (B+G−: trial: $F_{6,55}=6.23$, $P<0.0001$; reward: $F_{1,60}=13.82$, $P=0.0004$; trial×reward: $F_{6,55}=4.04$, $P=0.002$; B+V−: trial: $F_{6,49}=6.83$, $P<0.0001$; reward: $F_{1,54}=34.31$, $P<0.0001$; trial×reward: $F_{6,49}=6.83$, $P<0.0001$) or green (G+B−: trial: $F_{6,55}=5.79$, $P=0.0001$; reward: $F_{1,60}=22.04$, $P<0.0001$; trial×reward: $F_{6,55}=4.87$, $P=0.0005$; G+V−: trial: $F_{6,62}=6.72$, $P<0.0001$; reward: $F_{1,67}=28.24$, $P<0.0001$; trial×reward: $F_{6,62}=4.97$, $P=0.0003$) was used as the conditioned stimulus (Fig. 2A–D). Although the level of response was similar to that observed during absolute conditioning, we found that a much greater number of bees correctly responded to stimuli in the memory test after 24 h. In particular, the average performance remained the same after 24 h when green was rewarded and violet was not rewarded (Fig. 2D).

In contrast, the bees could not solve the task when violet was presented as the conditioned stimulus (V+B−: trial: $F_{6,52}=2.37$, $P=0.042$, reward: $F_{1,57}=1.31$, $P=0.26$; trial×reward: $F_{6,52}=0.88$, $P=0.51$; V+G−: trial: $F_{6,53}=2.64$, $P=0.026$; reward: $F_{1,58}=0.065$, $P=0.80$; trial×reward: $F_{6,53}=2.17$, $P=0.06$; Fig. 2E,F). Although

there was a significant effect of trial on bee response when violet was rewarded (see above effect of trial), the average response did not significantly differ between the violet and the unrewarded color responses. Nevertheless, bees exhibited a higher percentage of correct responses to violet (absolute: 10%; discrimination: 12–16%) than observed in our absolute conditioning experiment, and we also observed a higher level of memory retention (Fig. 1, Fig. 2E,F).

Experiment 3 (visual versus olfactory learning)

Bees trained to either odor or color significantly differed in response by stimulus, trial and their interaction term (stimulus: $F_{3,141}=27.334$, $P<0.0001$; trial: $F_{9,133}=28.248$, $P<0.0001$; trial×stimulus (Wilks’ lambda): $F_{27,389}=5.743$, $P<0.0001$). Restrained bees correctly responded to odor faster and at a greater percentage, reaching 60–80% by trial 4, whereas bees trained to color only reached ~50% (B+) and 30% (G+) after seven trials (Fig. 3). There was a significant difference between all tested stimuli, with bees learning hexanal best, followed by 2-nonanone, blue and then green. A more detailed analysis of the odors showed that responses to hexanal were significantly greater after the first trial ($F_{1,69}=12.730$, $P=0.0007$). When comparing colors, responses were significantly greater to blue than to green after the sixth trial ($F_{1,72}=9.257$, $P=0.0033$). When comparing hexanal with the two colors, all responses were significantly greater for hexanal than blue or green after the first trial (hexanal–blue: $F_{1,71}=27.058$, $P<0.0001$; hexanal–green: $F_{1,85}=49.447$, $P<0.0001$). For 2-nonanone, responses were significantly greater than green after the first trial ($F_{1,70}=7.130$, $P=0.0094$) and significantly greater than blue after the second trial ($F_{1,56}=5.874$, $P=0.0186$; Fig. 3).

Experiment 4 (effect of antennal amputation on color learning)

There were no significant differences in bees’ responsiveness comparing partially or fully amputated antennae ($F_{1,58}=0.518$, $P=0.474$), thus for the two procedures data were pooled. Overall, bees did not learn the association when any portion of the antennae was removed. Intact bees exhibited performance similar to that observed in our Experiment 1, even though stimulation was directed to the proboscis and not the antennae (Figs 1, 4). Intact bees trained to learn blue and green showed similar performance, while bees trained to violet showed very poor performance. In contrast, bees with amputated antennae rarely learned associations, and never surpassed 5% of PER. Generally, performance between antennal amputation and intact sets of bees differed after the first rewarded trial (Fig. 4) when blue ($F_{1,94}=7.95$, $P=0.006$) was used as the conditioned color, and after the second rewarded event when green was used ($F_{1,58}=10.55$, $P=0.0019$).

DISCUSSION

Overall, AHBs solved the color conditioning learning tasks differentially, according to wavelength used, although memory retention after 24 h was very poor. Performance during color conditioning was significantly lower than olfactory conditioning; performance peaked at seven trials and then decreased in later trials for the odor-trained bees (Fig. 3). We speculate that this may be due to the facts that the study was conducted in very dim light, and the lack of additional light stimuli during olfactory training in particular may have led to decreased responsiveness due to lack of stimulation. Furthermore, excluding olfactory input *via* antennal amputation did not enhance visual learning. It is unclear why AHBs successfully performed tasks that have thus far been quite difficult to achieve in EHBs. Although both subspecies have similar foraging behavior and

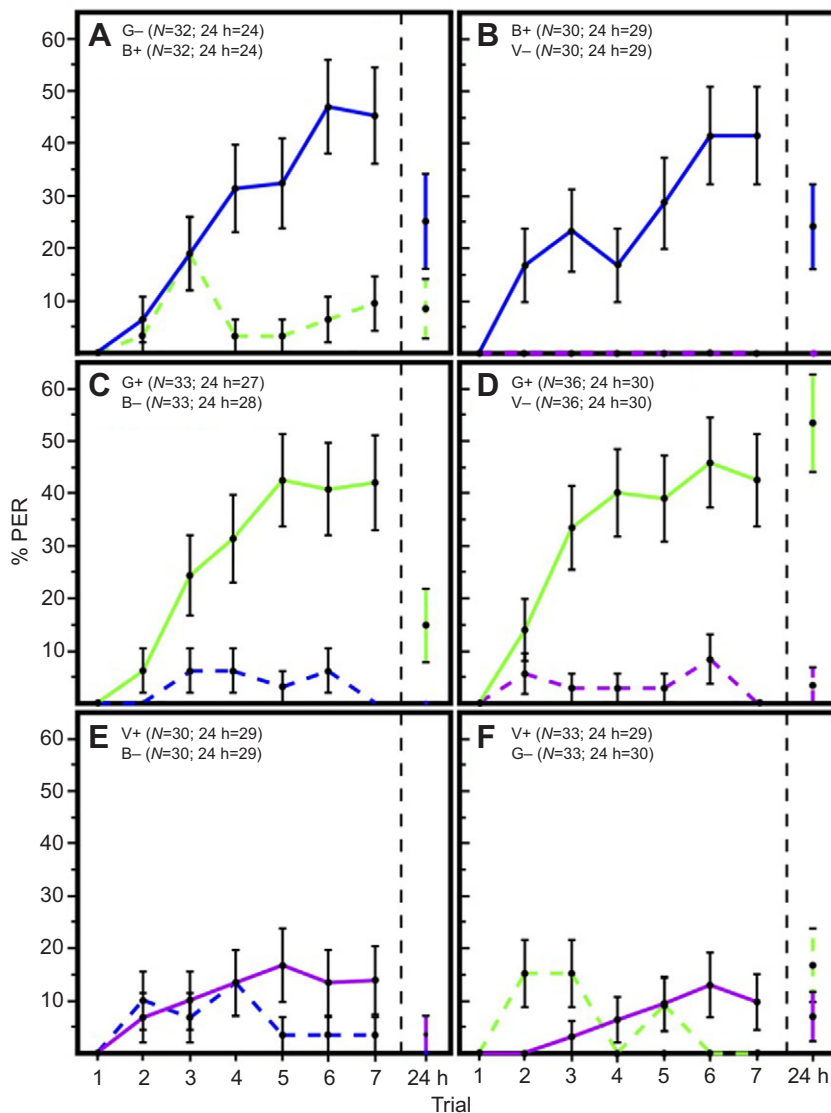


Fig. 2. Learning performance and memory for the discrimination task, shown as percent of the bees that responded to rewarded stimuli, versus the unrewarded stimuli presented in pseudorandom order over 14 stimulus presentations. Here we present all the possible combinations of rewarded and unrewarded colors, for the three colors used: blue (B), green (G) and violet (V). Continuous lines represent the rewarded color; dashed lines represent the unrewarded color. Error bars represent the standard errors of the mean for percentage of individuals responding to the stimulus. Memory after 24 h is shown to the right of the dashed line.

collect resources from diverse flowers, AHBs have lower sucrose response thresholds than EHBs do in PER protocols (Pankiw, 2003), and response sensitivity can be modified by artificial selection (Page, 2013), so they may differ in other sensory modalities as well; this remains to be explored.

We believe that the most likely cause for our success stems from our methodology that harnessed bees with minimal stress. In fact, recently, Dobrin and Fahrback (Dobrin and Fahrback, 2012) trained EHBs to learn visual stimuli in a method using pins around the head to harness bees as in Riveros and Gronenberg (Riveros and Gronenberg, 2012). Direct comparisons of EHB and AHB using the same methods are needed to assess whether they differ in their sensory ecology, or which methods shape the differential outcomes. Bees solved absolute and discriminant conditioning tasks within three training trials, and about 50% exhibited a conditioned response by the end of the session, following seven trials (in absolute learning) or 14 trials (discriminant learning). We found a significant effect of trial on individual bees, which reflects a change in responsiveness during training (i.e. learning). A significant effect of ‘pairing’ reflects between-individual differences in responsiveness due to the association between the colored light and the sucrose reward, consistent with our results indicating that association

depended upon the color used as the conditioned stimulus. These observations were robust, as similar patterns were observed using different colors and manipulations. Color learning took more trials and did not reach the same level of correct response to stimuli, compared with olfactory learning (Fig. 3). We made three major observations when studying the color learning of AHBs: acquisition of the conditioned association was highly context dependent, memory was affected by the conditioning task, and antennal amputation was not necessary for enhanced visual learning.

Context-dependent acquisition

AHBs successfully solved absolute and discrimination learning tasks when blue or green were used as conditioned stimuli, yet failed to solve the same tasks when violet was used. In free-flight experiments, floral color preferred by naïve bees tends to be of shorter wavelength, such as violet and blue (as perceived by humans) (Menzel, 1985; Giurfa et al., 1995). Honey bees are well known to visit the same flower species within a given period of time (‘flower constancy’) (Grant, 1950; Werner et al., 1988). In order to do this visually, the bee must perceive the flowers as the same color through a variety of light conditions throughout the day. According to some color constancy models (e.g. Dyer, 1998; Dyer, 1999),

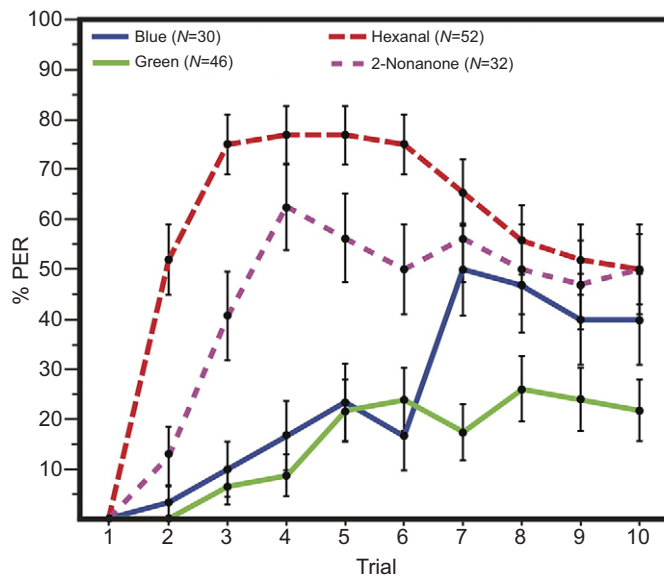


Fig. 3. Comparative olfactory and visual learning performance in restrained PER experiments. Odors are represented as dashed lines, and colors as continuous lines; error bars are standard errors of the mean over 10 learning trials.

however, bees may have difficulty identifying a pure UV stimulus. Moreover, Menzel and Erber (Menzel and Erber, 1978) argued that bees are maximally sensitive to ultraviolet radiation, but that bees do not encounter pure UV radiation at flowers, where it is mixed with long-wavelength light to form ‘bee purple’, which in turn is learned faster.

The violet LED used is capable of stimulating a UV-sensitive photochromic glass lens at the light intensities tested, as well as the short (UV) photoreceptors of the bees. According to a color space model for EHBs it would stimulate the medium (blue) photoreceptor more than the short (UV) and the long (green) photoreceptors (Fig. 5). Thus our violet LED should be a good approximation for the ‘bee purple’ discussed by Menzel and Erber (Menzel and Erber, 1978) (Fig. 5). However, the bees did not learn this stimulus (Figs 1–3).

The bees in our experiments perceived violet as a color and were capable of distinguishing it from blue or green, when blue or green were rewarded (Figs 4, 5). All of the colors were presented at the same light intensity measure in lux. The lux system is based upon the human visual system and thus for the relative sensitivity of EHBs violet should have been the most conspicuous among the three colors presented (Daumer, 1956; Menzel and Blakers, 1976; Menzel and Erber, 1978; Backhaus, 1991). While not directly measured, this is supported by the quantum catch values calculated in our bee color space; all colors including the violet LED appear to be within normal visual ranges for the bees (Fig. 5). Thus the violet wavelength should have been the most conspicuous and the fastest color learned. However, in light of Dyer’s models and our current findings more research is certainly needed. In particular, it would be useful to conduct a study in which bees are tested with a series of wavelengths between blue and UV. Sakura et al. (Sakura et al., 2012) showed a pattern opposite to our findings when polarized light was used as the conditioned stimulus in the PER protocol. They showed that bees solved a discrimination task when polarized ultraviolet light (UV), but not polarized blue or green, was used as the rewarded stimulus. Thus polarization may

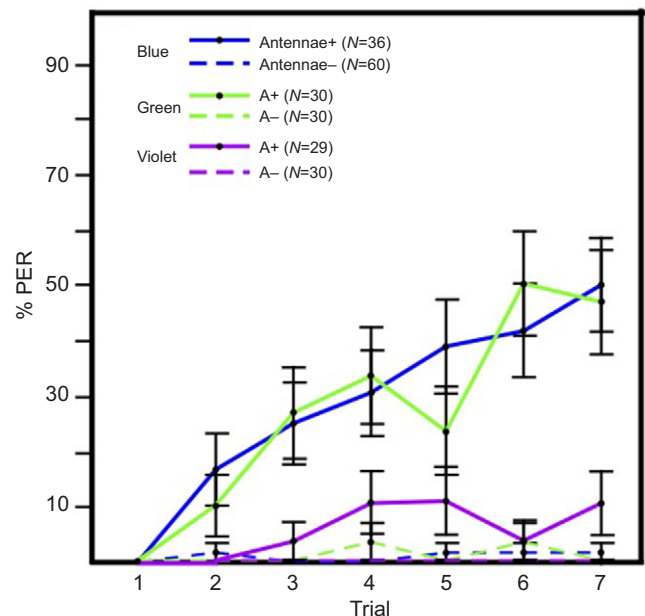


Fig. 4. Color learning performance of bees with and without antennae, using the absolute learning protocol over seven learning trials.

Continuous lines represent performance of bees with intact antennae and dashed lines represent performance of bees without antennae. Bees trained to blue were fully or partially de-antennated (see Materials and methods), but the data are pooled because of insignificant differences between those groups. All other antennal removals involved the entire flagellum. Colors of lines correspond to colors used for training. Error bars represent standard errors of the mean. Note that inhibiting olfactory stimuli seemed to inhibit responses to rewarded stimuli, and did not improve visual learning.

give the bees additional information that allows them to discriminate stimuli, unlike the non-directional light presented in our experiments. Addition of other visual features such as polarization, shape and patterns may be important for bees to learn and respond to colors.

Memory was affected by the conditioning task

A second observation and potential difference between AHBs and EHBs is that memory retention was very poor after 24 h. We found a dramatic decrease in performance compared with free-flight studies using the EHB (Menzel, 1968). This observation highlights the fact that color learning under restrained conditions not only leads to lower acquisition performance, but also to shorter memory retention than that observed during olfactory conditioning of the proboscis extension (Menzel, 1999). Longer inter-trial intervals (>10 min) generally lead to long-term memory formation after a few trials of olfactory conditioning in the EHB (Smith, 1991; Menzel, 1999). Thus our results cannot be explained by the lack of an appropriate inter-trial interval to form long-term memory. Remarkably, however, we did find an enhancement in memory retention in the discrimination-learning task, which was strongest when rewarded green was paired with unrewarded violet. These wavelengths are the furthest from one another in the light spectra of tested colors, but may also represent abundant and generally uninformative visual stimuli in the outdoors. Since the levels of acquisition remained as high as in the absolute conditioning tasks, the improved memory suggests that the additional information provided by an alternative color facilitated the process of consolidation and longer or more accurate memory retention.

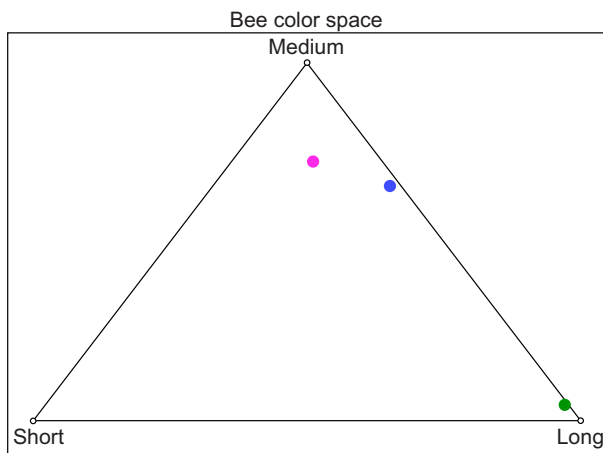


Fig. 5. Triangular color space as calculated using AVICOL.exe and the basic R package as described in Materials and methods. Each LED used during training is a point in the triangular space determined by the relative stimulation of the three photoreceptors of the European honey bee. The three vertices of the triangle represent maximal stimulation of the corresponding photoreceptor. Maximal stimulation of each of the photoreceptors occurs at wavelengths of 344 nm for the short, 436 nm for the medium and 556 nm for the long. All LED colors should be visible colors that are detectably different to the honey bee according to this model. The violet LED is represented by the pink point in the middle, the blue LED is to the left represented by the blue point, and the green LED is represented by the green point close to maximal stimulation for the long photoreceptor.

Comparison with previous studies

In our set-up, antennal amputation was detrimental, as almost none of the amputated bees correctly responded to stimuli (Fig. 4). Antennal amputation in EHB facilitated greater color learning in restrained bees using a PER method (Hori et al., 2006; Niggebrügge et al., 2009). However, these studies are difficult to compare with the current study or even each other because the training methods varied. EHB and AHB are known to differ in some aspects of sensory physiology (e.g. response thresholds to sucrose), which suggest further exploration is warranted. Thus systematic studies comparing different conditions and stimuli types, using different races of bees, would likely be very helpful in teasing apart why results appear to be so varied.

Conclusions

Foragers of Africanized *A. mellifera* can be successfully trained to learn colors using the conditioning of the PER and classical taping methods, and their learning performance depends upon the color used as a conditioned stimulus. Furthermore, additional visual features such as polarization may be very important for bees to learn and respond to these stimuli, but more research into these phenomena are certainly needed. Next, memory does not necessarily depend upon only the stimulus but rather upon the learning task presented to the bee (absolute *versus* discriminant learning). Notably, visual learning in AHB does not require antennal amputation, which opens new possibilities for the study of bimodal learning and memory. Last, this study highlights potential sensory and cognitive differences between European and Africanized honey bees, which warrant further investigation.

MATERIALS AND METHODS

Collecting and restraining bees

Our study was conducted in Gamboa, Panama, from October 2011 to May 2012. Feral AHBs were attracted to honey-water feeders. After collection,

bees were transported to the laboratory, chilled on ice and then harnessed to plastic tubes (cf. Riveros and Gronenberg, 2012). Following a ~1 h recovery period, bees were fed to satiation using sugar-water (30% w/w) and kept overnight in a chamber next to a window, to preserve circadian rhythm; a wet cotton ball prevented desiccation. The following morning, bees were tested for their responsiveness to sugar water. We used only individuals that exhibited a PER after antennal contact with sugar water.

Training apparatus and light stimulation

The training apparatus included 12 individual chambers attached to a rotatory platform (see Riveros and Gronenberg, 2012). Each chamber held an individual bee and was coated with a sheet of aluminum foil to increase stimulation around the bee. We trained a maximum of 12 bees per session. Colored light was shone from below into the chamber by an array of LEDs of three colors: green (wavelength peak=520 nm), violet (wavelength peak=406 nm), and blue (wavelength peak=462 nm) (supplementary material Fig. S1). Light intensity could be adjusted by varying the voltage and was set to 110 lx for each color. The ventral part of the eye received direct light and the other parts received diffused light, but we did not measure the homogeneity of light intensity throughout the chamber.

Training procedure

General protocol

Bees were trained to associate a conditioned stimulus (color or odor; CS+) and a reward (sugar water, 50% w/w). Prior to each training trial, individuals were acclimated for 15 s. Then a needle with a small drop of sugar water was held at short range for 10 s in front of the bee at a position that could not be touched with its antennae. This variation to traditional protocol was introduced because our preliminary observations suggested that bees learned the movement of the needle. After 10 s of beginning CS+, we gently stimulated the antennae with sugar-water and allowed the bee to drink for 3 s. Hence the CS+ was presented for 13 s followed by the immediate removal of both the CS+ and the reward. One presentation of a stimulus and a reward constituted a trial. All times were recorded with aid of a sound signal emitted every 0.5 s by a metronome (using the iPhone App 'Metronome!', v.1.1, Jacky Ma). Memory was tested, ~24 h after termination of the seventh trial for each experiment, by presenting bees with the trained stimuli. Individuals were not rewarded on the memory test, regardless of proboscis extension. All training was conducted between 09:00 and 14:30 h in a dim room.

Experiment 1 (absolute conditioning)

A set of bees was trained to associate one of three colors (CS+: G, V or B) and a sucrose reward. Those results were compared with a control group in which color and reward were presented in separate trials. Thus bees in the control group were exposed to seven presentations of one of three colors, alternated with seven presentations of only the US (sugar water). To maintain the same number of trials for both groups, bees trained to the association were exposed to seven additional blank trials alternated between conditioning trials. During blank trials the bees neither received a CS+ nor were they stimulated with sugar water. In each training session, three out of 12 bees were assigned to each of four treatments. We conducted a total of 15 training sessions. A total of 188 bees were trained to the absolute learning task. Because 22% died prior to memory testing or did not respond, 147 bees were tested for 24-h memory.

Experiment 2 (discriminant conditioning)

Bees were trained to discriminate between a color (B, V or G) that was associated with a reward (CS+) and a second color that was not associated with a reward (CS-). During a single session we trained half the bees to solve one discrimination combination (e.g. B+G-; blue rewarded, green unrewarded) and half to solve the alternative combination (e.g. G+B-). Thus five to six training sessions were needed to train 30 individual bees for each discrimination pairing (B+G-, B-G+, G+V-, G-V+, B+V-, B-V+). A total of 193 bees were trained to a discrimination task, 12% died prior to memory testing, and 169 bees were tested for 24-h memory.

Experiment 3 (olfactory versus color conditioning)

To compare the performance of AHBs in olfactory and color conditioning, we trained four sets of bees in an absolute conditioning protocol: two

groups were trained to learn colors (blue, green) and two were trained to learn odors (hexanal, 2-nonanone). Odors were selected based on their efficiency as conditioned stimuli in studies of EHBs, and their strong perceptual identity for honey bees, defined by a lack of generalization of one to the other (see Guerrieri et al., 2005). We conducted 10 trials to ensure we could assess differences in response towards stimuli. All other methods remained the same as the absolute training. We assigned three bees to each condition every training session and conducted 10 training sessions. A total of 160 bees were tested for olfactory or color absolute learning; memory training was not compared due to poor color memory.

Experiment 4 (effect of antennal amputation on absolute color learning)

To determine the effect of de-antennation on performance during color conditioning we first compared the two degrees of amputation used in earlier studies, removing the entire antennae (scape, pedicel and flagellum) (see Hori et al., 2006), or just the flagellum (see Niggebrügge et al., 2009) ($N=15$ bees each). Bees were then trained to the blue stimulus using the methods described above. We also compared amputated and intact bees trained in an absolute conditioning procedure. For the former, we removed the flagellum of each bee 2 h before training, excluding any bees that were bleeding hemolymph after 2 h.

In this experiment we directly stimulated the proboscis, even if bees belonged to the treatment group with intact antennae, to exclude known differential effects of stimulation on antenna or proboscis (Scheiner et al., 2005). Thus the only difference between these groups was the surgical procedure. We trained bees to all three color stimuli (B, G and V), and assigned three bees to each group every training session ($N=20$ training sessions). A total of 215 bees were trained, 120 without antennae (30 without scape or flagellum) and 95 with intact antennae; again, memory was not compared because of poor color memory.

Data analyses

All analyses were conducted using JMP 10 (SAS Institute, Cary, NC, USA). Changes over trials were analyzed using a repeated-measures ANOVA. Detailed trial-by-trial analyses were conducted in the same manner with a focus on differences between factors (i.e. color, pairing, etc.) at every trial.

Color space model

LEDs were measured in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at their peak wavelength using a LI-COR portable spectroradiometer (Model LI-1800, Lincoln, NE, USA) at the Smithsonian Tropical Research Institute in Panama City, Panama. These LED measurements were converted to quantum catch for each of the honey bee photoreceptors using Chittka's model for trichromatic color vision (Backhaus, 1991; Chittka, 1992; Gomez, 2006). Photoreceptor sensitivities for the European honey bee from Peitsch et al. (Peitsch et al., 1992) were used, as data are unavailable for the African subspecies (Chittka and Kevan, 2005). Additionally, we used standard background and ambient light and calculated quantum catch values with the open source light analysis program AVICOL.exe (Gomez, 2006). Relative quantal catch values were calculated by summing quantum catch values from AVICOL to one. Euclidian coordinates were then calculated according to equations and methods from Endler and Mielke (Endler and Mielke, 2005) and Stoddard and Prum (Stoddard and Prum, 2008). The calculations and the plot in honey bee triangular color space was conducted using the basic R statistics package (R Development Core Team, 2013).

Acknowledgements

We thank Wulfila Gronenberg and Sophia Riveros for their help with the construction of the training set-up, Milton Garcia for helping to measure the LED spectrum, and Smithsonian Tropical Research Institute staff for logistical support. A tremendous amount of help in creating the color space plot was provided by Russell A. Ligon, Nikolas N. Lessios and Brett M. Seymoure from the School of Life Sciences at Arizona State University. The Autoridad Nacional del Medio Ambiente of the Republic of Panama provided research permits.

Competing interests

The authors declare no competing financial interests.

Author contributions

C.M.J. contributed to the experimental setup, experimental design, collection of the data, statistical analysis, discussion of results and writing the manuscript. D.W.R. contributed with advice during experimental design, discussion of results and comments on the manuscript. W.T.W. contributed to experimental design, discussion of results and writing the manuscript. A.J.R. contributed to the experimental setup, developing the protocol, experimental design, statistical analysis, discussion of results and writing the manuscript.

Funding

C.M.J. was supported by a collaborative internship of the Smithsonian Tropical Research Institute (STRI) and Butler University, and by generous support of the Office of Education of STRI. A.J.R. was supported by a Latin American Scholar fellowship of the STRI and by a Scholarly Studies Grant of the Smithsonian Institution (PI: William Eberhard). This project was generally supported by funds of the Smithsonian Institution to W.T.W. and C.M.J.

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.091355/-/DC1>

References

- Abramson, C. I., Aquino, I. S., Silva, M. C. and Price, J. M. (1997). Learning in the Africanized honey bee: *Apis mellifera* L. *Physiol. Behav.* **62**, 657-674.
- Backhaus, W. (1991). Color opponent coding in the visual system of the honeybee. *Vision Res.* **31**, 1381-1397.
- Barco, A., Bailey, C. H. and Kandel, E. R. (2006). Common molecular mechanisms in explicit and implicit memory. *J. Neurochem.* **97**, 1520-1533.
- 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.
- Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* **170**, 533-543.
- Chittka, L. and Kevan, P. G. (2005). Flower colour as an advertisement. In *Practical Pollination Biology* (ed. A. Dafni, P. G. Kevan and B. C. Husband), pp. 157-196. Cambridge, ON: Enviroquest Ltd.
- Clarke, D., Whitney, H., Sutton, G. and Robert, D. (2013). Detection and learning of floral electric fields by bumblebees. *Science* **340**, 66-69.
- Couvillon, M. J., DeGrandi-Hoffman, G. and Gronenberg, W. (2010). Africanized honeybees are slower learners than their European counterparts. *Naturwissenschaften* **97**, 153-160.
- Daly, K. C., Chandra, S., Durtschi, M. L. and Smith, B. H. (2001). The generalization of an olfactory-based conditioned response reveals unique but overlapping odour representations in the moth *Manduca sexta*. *J. Exp. Biol.* **204**, 3085-3095.
- Daumer, K. (1956). Reizmetrische untersuchungen des farbensehens der bienen. *Z. Vergl. Physiol.* **38**, 413-478.
- Dobrin, S. E. and Fahrback, S. E. (2012). Visual associative learning in restrained honey bees with intact antennae. *PLoS ONE* **7**, e37666.
- Dukas, R. (2008). Evolutionary biology of insect learning. *Annu. Rev. Entomol.* **53**, 145-160.
- Dyer, A. G. (1998). The colour of flowers in spectrally variable illumination and insect pollinator vision. *J. Comp. Physiol. A* **183**, 203-212.
- Dyer, A. G. (1999). Broad spectral sensitivities in the honeybee's photoreceptors limit colour constancy. *J. Comp. Physiol. A* **185**, 445-453.
- Endler, J. A. and Mielke, P. W., Jr (2005). Comparing entire colour patterns as birds see them. *Biol. J. Linn. Soc. Lond.* **86**, 405-431.
- Erber, J. and Schildberger, K. (1980). Conditioning of an antennal reflex to visual stimuli in bees (*Apis mellifera* L.). *J. Comp. Physiol.* **135**, 217-225.
- Erber, J., Kierzek, S., Sander, E. and Grandy, K. (1998). Tactile learning in the honeybee. *J. Comp. Physiol. A* **183**, 737-744.
- Francoy, T. M., Wittmann, D., Drauschke, M., Müller, S., Steinhage, V., Bezerra-Laure, A. F., Jong, D. D. and Goncalves, L. S. (2008). Identification of Africanized honey bees through wing morphometrics: two fast and efficient procedures. *Apidologie (Celle)* **39**, 488-494.
- Galizia, C. G., Eisenhardt, D. and Giurfa, M. (2012). *Honeybee Neurobiology and Behavior: A Tribute to Randolph Menzel*. New York, NY: Springer.
- Giurfa, M. and Sandoz, J. C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* **19**, 54-66.
- Giurfa, M., Nunez, J., Chittka, L. and Menzel, R. (1995). Colour preferences of flower-naïve honeybees. *J. Comp. Physiol. A* **177**, 247-259.
- Gomez, D. (2006) AVICOL, a program to analyze spectrometric data. Available at <http://sites.google.com/site/avicolprogram/>.
- Gould, J. L. (1985). How bees remember flower shapes. *Science* **227**, 1492-1494.
- Grant, V. (1950). The flower constancy of bees. *Bot. Rev.* **15**, 379-398.
- Guerrieri, F., Schubert, M., Sandoz, J. C. and Giurfa, M. (2005). Perceptual and neural olfactory similarity in honeybees. *PLoS Biol.* **3**, e60.
- 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.

- 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.
- Kuwabara, M.** (1957). Bildung des bedingten reflexes von Pavlovs typus Honigbiene, *Apis mellifera*. *J. Fac. Sci. Hokkaido Univ. (Zool.)* **13**, 458-464.
- Masuhr, T. and Menzel, R.** (1972). Learning experiments on the use of side-specific information in the olfactory and visual system in the honey bee (*Apis mellifica*). In *Information Processing in the Visual System of Arthropods* (ed. R. Wehner), pp. 315-321. Berlin; Heidelberg: Springer.
- McCabe, S. I., Hartfelder, K., Santana, W. C. and Farina, W. M.** (2007). Odor discrimination in classical conditioning of proboscis extension in two stingless bee species in comparison to Africanized honeybees. *J. Comp. Physiol. A* **193**, 1089-1099.
- Menzel, R.** (1968). Das gedächtnis der honigbiene für spektralfarben: On the honey bee's memory of spectral colours. *Z. Vgl. Physiol.* **60**, 82-102.
- Menzel, R.** (1985). Learning in honey bees in an ecological and behavioral context. *Experimental Behavioral Ecology and Sociobiology* (ed. K. von Frisch, B. Hölldobler and M. Lindauer), pp. 55-74. Sunderland, MA: Sinauer Associates.
- Menzel, R.** (1999). Memory dynamics in the honeybee. *J. Comp. Physiol. A* **185**, 323-340.
- Menzel, R. and Backhaus, W.** (1991). Color vision in insects. In *Vision and Visual Dysfunction. The Perceptions of Colour* (ed. P. Gouras), pp. 262-293. London: Macmillan Press.
- Menzel, R. and Blakers, M.** (1976). Colour receptors in the bee eye – morphology and spectral sensitivity. *J. Comp. Physiol.* **108**, 11-13.
- Menzel, R. and Erber, J.** (1978). Learning and memory in bees. *Sci. Am.* **239**, 102-111.
- Mota, T., Roussel, E., Sandoz, J. C. and Giurfa, M.** (2011). Visual conditioning of the sting extension reflex in harnessed honeybees. *J. Exp. Biol.* **214**, 3577-3587.
- Niggebrügge, C., Lebouille, 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.
- Page, R.** (2013). *The Spirit of the Hive: the Mechanisms of Social Evolution*. Cambridge, MA: Harvard University Press.
- Pankiw, T.** (2003). Directional change in a suite of foraging behaviors in tropical and temperate evolved honey bees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* **54**, 458-464.
- Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F. and Menzel, R.** (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* **170**, 23-40.
- R Development Core Team** (2013). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. Available at: <http://www.R-project.org/>
- Riveros, A. J. and Gronenberg, W.** (2012). Decision-making and associative color learning in harnessed bumblebees (*Bombus impatiens*). *Anim. Cogn.* **15**, 1183-1193.
- Roubik, D. W.** (2009). Ecological impact on native bees by the invasive Africanized honey bee. *Acta Biolo. Colomb.* **14**, 115-124.
- Sakura, M., Okada, R. and Aonuma, H.** (2012). Evidence for instantaneous e-vector detection in the honeybee using an associative learning paradigm. *Proc. Biol. Sci.* **279**, 535-542.
- Scheiner, R., Kuritz-Kaiser, A., Menzel, R. and Erber, J.** (2005). Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees. *Learn. Mem.* **12**, 626-635.
- Schneider, S. S., DeGrandi-Hoffman, G. and Smith, D. R.** (2004). The African honey bee: factors contributing to a successful biological invasion. *Annu. Rev. Entomol.* **49**, 351-376.
- Smith, B. H.** (1991). The olfactory memory of the honeybee *Apis mellifera*: I. odorant modulation of short- and intermediate-term memory after single-trial conditioning. *J. Exp. Biol.* **161**, 367-382.
- Smith, D. R., Taylor, O. R. and Brown, W. M.** (1989). Neotropical Africanized honey bees have African mitochondrial DNA. *Nature* **339**, 213-215.
- Spivak, M., Fletcher, D. J. C. and Breed, M. D.** (1991). *The 'African' Honey Bee*. Boulder, CO: Westview Press Inc.
- Stoddard, M. C. and Prum, R. O.** (2008). Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of new world buntings. *Am. Nat.* **171**, 755-776.
- Takeda, K.** (1961). Classical conditioned response in the honey bee. *J. Insect Physiol.* **6**, 168-179.
- Thorpe, W. H.** (1956). *Learning and Instinct in Animals*. London: Methuen and Co. Ltd.
- von Frisch, K.** (1967). *The Dance Language and Orientation of Bees*. Cambridge, MA: Belknap Press
- Werner, A., Menzel, R. and Wehrhahn, C.** (1988). Color constancy in the honeybee. *J. Neurosci.* **8**, 156-159.
- Whitney, H. M., Dyer, A., Chittka, L., Rands, S. A. and Glover, B. J.** (2008). The interaction of temperature and sucrose concentration on foraging preferences in bumblebees. *Naturwissenschaften* **95**, 845-850.