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Color dependent learning in restrained Africanized honey bees

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31 **SUMMARY**

32 Associative color learning has been demonstrated to be very poor using restrained European
33 honey bees unless the antennae are amputated. Consequently, our understanding of proximate
34 mechanisms in visual information processing is handicapped. Here we test learning
35 performance of Africanized honey bees under restrained conditions with visual and olfactory
36 stimulation using the proboscis extension response (PER) protocol. Restrained individuals were
37 trained to learn an association between a color stimulus and a sugar-water reward. We
38 evaluated performance for “absolute” learning (learned association between a stimulus and a
39 reward) and “discriminant” learning (discrimination between two stimuli). Restrained Africanized
40 honey bees (AHBs) readily learned the association of color stimulus for both blue and green
41 LED stimuli in absolute and discriminatory learning tasks within 7 presentations, but not with
42 violet as the rewarded color. Additionally, 24-hour memory improved considerably during the
43 discrimination task, compared to absolute association (15%-55%). We found that antennal
44 amputation was unnecessary and reduced performance in AHBs. Thus color learning can now
45 be studied using the PER protocol with intact AHBs. This finding opens the way toward
46 investigating visual and multimodal learning with application of neural techniques commonly
47 used in restrained honey bees.

48 **INTRODUCTION**

49 Learning and memory mediate numerous behavioral responses to changing environments (e.g.,
50 Thorpe, 1956; Barco et al., 2006; Dukas, 2008). For example, bees optimize foraging efforts by
51 assessing a variable floral market and learning and remembering the stimuli that characterize
52 more profitable flowers, which are preferentially visited (Menzel, 1985). Floral features used by
53 bees include colors (Daumer, 1956; von Frisch, 1967; Menzel, 1985; Galizia et al., 2012), odors
54 (von Frisch, 1967; Galizia et al., 2012; Giurfa and Sandoz, 2012), shapes (Gould, 1985),
55 textures (Kevan and Lane, 1985; Erber et al., 1998), temperature (Whitney et al., 2008), and
56 electrostatic charge (Clarke et al., 2013). Experimentally, perception of floral features can be
57 quantified by conditioning of the proboscis (tongue) extension reflex (PER) of individually
58 restrained bees that are unharmed but prevented from flight. The PER is an unconditioned
59 response (UR) evoked by contact with a sweet substance (the unconditioned stimulus, US) by
60 the antenna, tarsus or proboscis. Given its reliability—up to 80% of bees learn an association
61 between an odor and a sucrose reward after three training repetitions (Bitterman et al., 1983)—
62 PER conditioning has been widely used to study how European honey bees (*Apis mellifera* L.)
63 (EHBs) learn and remember olfactory stimuli (acting as conditioned stimuli, CS) (e.g., Takeda,
64 1961; Daly et al., 2001; Giurfa and Sandoz, 2012).

65 In contrast to olfactory stimuli, PER conditioning has had limited success in the study of
66 honey bee visual learning (Masuhr and Menzel, 1972; Erber and Schildberger, 1980; Menzel,
67 1985; Hori et al., 2006; Mota et al., 2011; Dobrin and Farbach, 2012). After 10-20 training
68 repetitions, fewer than 40% of EHBs learned a conditioned response to visual stimuli (e.g., Hori
69 et al., 2006; Mota et al., 2011; Sakura et al., 2011; Dobrin and Fahrbach, 2012).

70 Excluding olfactory input through antennal amputation leads to higher performance during
71 PER visual learning in EHB, with up to 70% color learning in 10 trials (Niggebrügge et al., 2009;
72 also Kuwabara, 1957; Hori et al., 2006; Mota et al., 2011). In contrast to EHBs, antennal
73 amputation is not required for PER color conditioning of the bumble bee, *Bombus impatiens*
74 (Riveros and Gronenberg, 2012). Antennal amputation precludes any possibility of
75 simultaneously analyzing how visual and olfactory stimuli interact to influence decisions. Here,
76 we evaluate the potential of the PER protocol to study visual learning in Africanized honey bees
77 (feral bees near *Apis mellifera scutellata*; Francoy et al., 2008) (AHB), which were introduced to
78 the Neotropics over 50 years ago (Smith et al., 1989; Spivak et al., 1991; Schneider et al., 2004;
79 Roubik, 2009). They readily learn olfactory cues following the PER protocol (Abramson et al.,
80 1997; McCabe et al., 2007; Couvillon et al., 2010), yet their performance appears to be poorer
81 (Couvillon et al., 2010) and a previous attempt to condition AHBs to light was unsuccessful

82 (Abramson et al., 1997). We relied on absolute and discriminant learning tasks that were
83 evaluated using colored lights of three different wavelengths. We further compared the
84 performance of AHBs to odor *versus* color learning, as well as the effect of antennal amputation
85 on acquisition rates.

86

87

MATERIALS AND METHODS

88

Collecting and restraining bees

89 Our study was conducted in Gamboa, Panama, October 2011 - May 2012. Feral AHBs were
90 attracted to honey-water feeders. After collection, bees were transported to the lab, chilled on
91 ice and then harnessed to plastic tubes (cf. Riveros and Gronenberg, 2012). Following a ~1 hr
92 recovery period, bees were fed to satiation using sugarwater (30% w/w) and kept overnight in a
93 chamber next to a window, to preserve circadian rhythm; a wet cotton ball prevented
94 desiccation. The following morning, bees were tested for their responsiveness to sugar-water.
95 We used only individuals that exhibited a PER after antennal contact with sugar-water.

96

Training apparatus and light stimulation

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

106

Training procedure

107 *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
108 15 s. Then, a needle with a small drop of sugar water was held at short range for 10 s in front of
109 the bee at a position that could not be touched with its antennae. This variation to traditional
110 protocol was introduced because our preliminary observations suggested bees learned the
111 movement of the needle. After 10 s of beginning CS+, we gently stimulated the antennae with
112 sugar water and allowed the bee to drink for 3 s. Hence the CS+ was presented for 13 s
113 followed by the immediate removal of both the CS+ and the reward. One presentation of a
114 stimulus and a reward constituted a trial. All times were recorded with aid of a sound signal
115

116 emitted every 0.5 s by a metronome (application for iPod Metronome! Jacky Ma v.1.1). Memory
117 was tested, approximately 24 hrs after termination of the 7th trial for each experiment, by
118 presenting bees with the trained stimuli. Individuals were not rewarded on the memory test,
119 regardless of proboscis extension. All training was conducted between 9:00 and 14:30 in a dim
120 room.

121
122 *Experiment 1 (Absolute conditioning):* A set of bees was trained to associate one of three colors
123 (CS+: G, V, or B) and a sucrose reward. Those results were compared with a control group in
124 which color and reward were presented in separate trials. Thus, bees in the control group were
125 exposed to seven presentations of one of three colors, alternated with seven presentations of
126 only the US (sugar-water). To maintain the same number of trials for both groups, bees trained
127 to the association were exposed to seven additional blank trials alternated between conditioning
128 trials. During blank trials the bees neither received a CS+ nor were they stimulated with sugar-
129 water. In each training session three out of twelve bees were assigned to each of four
130 treatments. We conducted a total of 15 training sessions. A total of 188 bees were trained to the
131 absolute learning task. Because 22% died prior to memory testing or did not respond, 147 bees
132 were tested for 24-hour memory.

133
134 *Experiment 2 (Discriminant conditioning):* Bees were trained to discriminate between a color
135 (blue [B], violet [V], or green [G]) that was associated with a reward (CS+) and a second color
136 that was not associated with a reward (CS-). During a single session we trained half the bees to
137 solve one discrimination combination (e.g. B+G-; blue rewarded, green unrewarded) and half to
138 solve the alternative combination (e.g. G+B-). Thus, 5-6 training sessions were needed to train
139 30 individual bees for each discrimination pairing (B+G-, B-G+, G+V-, G-V+, B+V-, B-V+). A
140 total of 193 bees were trained to a discrimination task, 12% died prior to memory testing, and
141 169 bees were tested for 24-hr memory.

142
143 *Experiment 3 (Olfactory versus color conditioning):* To compare the performance of AHBs in
144 olfactory and color conditioning, we trained four sets of bees in an absolute conditioning
145 paradigm: two groups were trained to learn colors (blue, green) and two were trained to learn
146 odors (hexanal, 2-nonanone). Odors were selected based on their efficiency as conditioned
147 stimuli in studies of EHBs, and their strong perceptual identity for honey bees, defined by a lack
148 of generalization of one to the other (see Guerrieri et al., 2005). We conducted 10 trials to
149 ensure we could assess differences in response toward stimuli. All other methods remained the

150 same as the absolute training. We assigned three bees to each condition every training session
151 and conducted 10 training sessions. A total of 160 bees were tested for olfactory or color
152 absolute learning; memory training was not compared due to poor color memory.

153

154 *Experiment 4 (Effect of antennal amputation on absolute color learning):* To determine the effect
155 of de-antennation on performance during color conditioning we first compared the two degrees
156 of amputation used in earlier studies, removing the entire antennae (scape, pedicel, and
157 flagellum) (after Hori et al., 2006), or just the flagellum (after Niggebrügge et al., 2009) (N = 15
158 bees each). Bees were then trained to the blue stimulus using methods described above. We
159 also compared amputated and intact bees trained in an absolute conditioning procedure. For
160 the former, we removed the flagellum of each bee 2 hrs before training, excluding any bees that
161 were bleeding hemolymph after 2 hrs.

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

169

Data Analyses

170 All analyses were conducted using JMP 10 (SAS Institute, 2012). Changes over trials were
171 analyzed using a repeated measures ANOVA. Detailed trial by trial analyses were conducted in
172 the same manner with a focus on differences between factors (i.e. color, pairing, etc.) at every
173 trial.

174

Color Space Model

175 LEDs were measured in $\mu\text{mol. Photons m}^{-2} \text{s}^{-1}$ at their peak wavelength using a LI-COR
176 portable spectroradiometer (Model LI-1800) at the Smithsonian Tropical Research Institute in
177 Panama City, Panama. These LED measurements were converted to quantum catch for each of
178 the honey bee photoreceptors using Chittka's Model for trichromatic color vision in (Backhaus,
179 1991; Chittka, 1992; Gomez, 2006). Photoreceptor sensitivities for the European honey bee
180 from Peitch et al. (1992) were used, as data are unavailable for the African subspecies (Chittka
181 and Kevan, 2005). Additionally, we used standard background and ambient light and calculated
182 quantum catch values with the open source light analysis program AVICOL.exe (Gomez, 2006).
183 Relative Quantal catch values were calculated by summing quantum catch values from AVICOL

184 to one. Euclidian coordinates were then calculated according to equations and methods from
185 Endler and Mielke (2005) and Stoddard and Prum (2008). The calculations and the plot in
186 honey bee triangular color space was conducted using the basic R stats package (R Core
187 Team, 2013).

188

189

RESULTS

190 *Experiment 1 (Absolute color conditioning)*: Bees trained to associate a colored light with a
191 reward versus bees (controls) that received the light stimulus and the sucrose in separate trials
192 differed in their responsiveness to light depending upon the wavelength used, as shown across
193 trials with color, pairing of stimuli and their interaction as factors (Repeated Measures analyses:
194 *Trial*: $F_{6,158} = 15.27$, $P < 0.0001$; *Pairing*: $F_{1,163} = 33.99$, $P < 0.0001$; *Color*: $F_{2,163} = 5.76$, $P =$
195 0.004 ; *Color*Pairing*: $F_{2,163} = 10.75$, $P < 0.0001$) (Fig. 1). Trial by trial analyses showed that the
196 effect of pairing was significant after a single rewarding event ($F_{1,163} = 0.09$, $P = 0.0002$).
197 Differences due to color occurred only after the third rewarding event ($F_{2,163} = 0.07$, $P = 0.004$).
198 Within colors, bees successfully learned the association (as shown by significant differences
199 between the “paired” and “unpaired” conditions) when blue (Repeated measures across trials
200 for the effect of “Trial”, “Pairing of stimuli”, and the interaction “Trial*Pairing”: *Trial*: $F_{6,52} = 9.19$, P
201 < 0.0001 ; *Pairing*: $F_{1,57} = 36.8$, $P < 0.0001$; *Trial*Pairing*: $F_{6,52} = 6.56$, $P < 0.0001$) or green
202 (*Trial*: $F_{6,51} = 5.71$, $P = 0.0001$; *Pairing*: $F_{1,56} = 12.96$, $P = 0.0007$; *Trial*Pairing*: $F_{6,51} = 2.83$, $P =$
203 0.019), but not violet (*Trial*: $F_{6,45} = 1.68$, $P = 0.15$; *Pairing*: $F_{1,50} = 0.025$, $P = 0.87$; *Trial*Pairing*:
204 $F_{6,45} = 0.97$, $P = 0.45$) were paired with a sucrose reward. Among colors, the highest percentage
205 of responsiveness was achieved when they were trained to blue (Fig. 1), although this
206 difference is not statistically different from green (*Color- Blue vs. Green*: $F_{6,57} = 2.469$, $P = 0.122$;
207 *Trial*Color*: $F_{6,52} = 0.559$, $P = 0.761$) (Fig. 1).

208 After 24 hrs following training, bees had almost entirely lost the association to blue (2 out of
209 28 remembered the association) and green (2 out of 27 remembered).

210

211 *Experiment 2 (Discriminant conditioning)*: Bees solved the discrimination task after a few trials
212 when either blue (**B+G-**: *Trial*: $F_{6,55} = 6.23$, $P < 0.0001$; *Reward*: $F_{1,60} = 13.82$, $P = 0.0004$;
213 *Trial*Reward*: $F_{6,55} = 4.04$, $P = 0.002$; **B+V-**: *Trial*: $F_{6,49} = 6.83$, $P < 0.0001$; *Reward*: $F_{1,54} =$
214 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
215 $= 0.0001$; *Reward*: $F_{1,60} = 22.04$, $P < 0.0001$; *Trial*Reward*: $F_{6,55} = 4.87$, $P = 0.0005$; **G+V-**: *Trial*:
216 $F_{6,62} = 6.72$, $P < 0.0001$; *Reward*: $F_{1,67} = 28.24$, $P < 0.0001$; *Trial*Reward*: $F_{6,62} = 4.97$, $P =$
217 0.0003) was used as the conditioned stimulus (Fig. 2 A-D). Although the level of response was

218 similar to that observed during absolute conditioning, we found a much greater number of bees
219 correctly responded to stimuli in the memory test after 24 hrs. In particular, the average
220 performance remained the same after 24 hrs when green was rewarded and violet was not
221 rewarded (Fig. 2D).

222 In contrast, the bees could not solve the task when violet was presented as the conditioned
223 stimulus (V+B-: *Trial*: $F_{6,52} = 2.37$, **P = 0.042**, *Reward*: $F_{1,57} = 1.31$, $P = 0.26$; *Trial*Reward*: $F_{6,52}$
224 $= 0.88$, $P = 0.51$; V+G-: *Trial*: $F_{6,53} = 2.64$, **P = 0.026**; *Reward*: $F_{1,58} = 0.065$, **P = 0.80**;
225 *Trial*Reward*: $F_{6,53} = 2.17$, **P = 0.06**; Figs 2 E,F). Although there was a significant effect of trial
226 on bee response when violet was rewarded (see above effect of trial), the average response did
227 not significantly differ between the violet and the unrewarded color responses. Nevertheless,
228 bees exhibited a higher percentage of correct responses to violet (Absolute: 10%;
229 Discrimination: 12-16%) than observed in our absolute conditioning experiment, and we also
230 observed a higher level of memory retention (Figs 1, 2E, 2F).

231
232 *Experiment 3 (Visual versus olfactory learning)*: Bees trained to either odor or color significantly
233 differed in response by stimulus, trial, and their interaction term (*Stimulus*: $F_{3,141} = 27.334$, **P <**
234 **0.0001**; *Trial*: $F_{9,133} = 28.248$, **P < 0.0001**; *Trial*Stimulus (Wilks' Lambda)*: $F_{27,389} = 5.743$, **P <**
235 **0.0001**). Restrained bees correctly responded to odor faster and at a greater percentage,
236 reaching 60-80% by trial 4, whereas bees trained to color only reached approximately 50%
237 (B+) and 30% (G+) after 7 trials (Fig. 3). There was a significant difference between all tested
238 stimuli, with bees learning hexanal best, followed by 2-nonanone, blue, and then green. A more
239 detailed analysis of the odors showed that responses to hexanal were significantly greater after
240 the first trial ($F_{1,69} = 12.730$, **P = 0.0007**). When comparing colors, responses were significantly
241 greater to blue than to green after the 6th trial ($F_{1,72} = 9.257$, **P = 0.0033**). When comparing
242 hexanal to the two colors all responses were significantly greater for hexanal than blue or green
243 after the 1st trial (*Hexanal-Blue*: $F_{1,71} = 27.058$, **P < 0.0001**; *Hexanal-Green*: $F_{1,85} = 49.447$, **P <**
244 **0.0001**). For 2-nonanone responses were significantly greater than green after the 1st trial ($F_{1,70}$
245 $= 7.130$, **P = 0.0094**) and significantly greater than blue after the 2nd trial ($F_{1,56} = 5.874$, **P =**
246 **0.0186**) (Fig. 3).

247
248 *Experiment 4 (Effect of antennal amputation on color learning)*: There were no significant
249 differences in bees' responsiveness comparing partially or fully amputated antennae ($F_{1,58} =$
250 0.518 , **P = 0.474**), thus for the two procedures data were pooled. Overall, bees did not learn the
251 association when any portion of the antennae was removed. Intact bees exhibited performance

252 similar to that observed in our experiment 1, even though stimulation was directed to the
253 proboscis and not the antennae (Figs 1, 4). Intact bees trained to learn blue and green showed
254 similar performance, while bees trained to violet showed very poor performance. In contrast,
255 bees with amputated antennae rarely learned associations, and never surpassed 5% of PER.
256 Generally, performance between antennal amputation and intact sets of bees differed after the
257 first rewarded trial (Fig. 4) when blue ($F_{1,94} = 7.95$, $P = 0.006$) was used as the conditioned
258 color, and after the second rewarded event when green was used ($F_{1,58} = 10.55$, $P = 0.0019$).

259 DISCUSSION

260 Overall Africanized honey bees (AHBs) solved the color conditioning learning tasks
261 differentially, according to wavelength used, although memory retention after 24 hrs was very
262 poor. Performance during color conditioning was significantly lower than olfactory conditioning;
263 performance peaked at 7 trials and then decreased in later trials for the odor trained bees (Fig.
264 3). We speculate that this may be due to the facts that the study was conducted in very dim
265 light, and the lack of additional light stimuli during olfactory training in particular may have led to
266 decreased responsiveness due to lack of stimulation. Furthermore, excluding olfactory input via
267 antennal amputation did not enhance visual learning. It is unclear why AHBs successfully
268 performed tasks that have thus far been quite difficult to achieve in EHBs. Although both
269 subspecies have similar foraging behavior and collect resources from diverse flowers, AHBs
270 have lower sucrose response thresholds than do EHBs in PER protocols (Pankiw, 2003), and
271 response sensitivity can be modified by artificial selection (Page, 2013), so they may differ in
272 other sensory modalities as well, but this remains to be explored.

273 We believe that the most likely cause for our success stems from our methodology that
274 harnessed bees with minimal stress. In fact recently Dobrin and Fahrbach (2012) trained EHBs
275 to learn visual stimuli in a method using pins around the head to harness bees as in Riveros and
276 Gronenberg (2012). Direct comparisons of EHB and AHB using the same methods are needed
277 to assess whether they differ in their sensory ecology, or which methods shape the differential
278 outcomes. Bees solved absolute and discriminant conditioning tasks within three training trials,
279 and about 50% exhibited a conditioned response by the end of the session, following 7 trials (in
280 absolute learning) or 14 trials (discriminant learning). We found a significant effect of trial on
281 individual bees, which reflects a change in responsiveness during training (i.e., learning). A
282 significant effect of 'pairing' reflects between-individual differences in responsiveness due to the
283 association between the colored light and the sucrose reward, consistent with our results
284 indicating association depended upon the color used as the conditioned stimulus. These
285 observations were robust, as similar patterns were observed using different colors and

286 manipulations. Color learning took more trials and did not reach the same level of correct
287 response to stimuli, compared to olfactory learning (Fig. 3). We made three major observations
288 when studying the color learning of AHBs: acquisition of the conditioned association was highly
289 context dependent, memory was affected by the conditioning task, and antennal amputation
290 was not necessary for enhanced visual learning.

291 *Context dependent acquisition*

292 AHBs successfully solved absolute and discrimination learning tasks when blue or green were
293 used as conditioned stimuli, yet failed to solve the same tasks when violet was used. In free
294 flight experiments floral color preferred by naïve bees tends to be of shorter wavelength, such
295 as violet and blue (as perceived by humans) (Menzel, 1985; Giurfa et al., 1995). Honey bees
296 are well known to visit the same flower species within a given period of time (“flower constancy”)
297 (Grant, 1950; Werner et al., 1988). In order to do this visually, the bee must perceive the flowers
298 as the same color through a variety of light conditions throughout the day. According to some
299 color constancy models (e.g., Dyer, 1998; 1999), however, bees may have difficulty identifying a
300 pure UV stimulus. Moreover, Menzel and Erber (1978) argued that bees are maximally sensitive
301 to ultraviolet radiation, but that bees do not encounter pure UV radiation at flowers, where it is
302 mixed with long-wavelength light to form ‘bee purple,’ which in turn is learned faster.

303 The violet LED used is capable of stimulating a UV sensitive photochromic glass lens at the
304 light intensities tested, as well as the short (UV) photoreceptors of the bees. According to a
305 color space model for EHBs it would stimulate the medium (blue) photoreceptor more than the
306 short (UV) and the long (green) photoreceptors (Fig. 5). Thus our violet LED should be a good
307 approximation for the ‘bee purple’ discussed by Menzel and Erber (1978) (Fig. 5). However, the
308 bees did not learn this stimulus (Figs 1, 2, 3).

309 The bees in our experiments perceived violet as a color and were capable of distinguishing it
310 from blue or green, when blue or green were rewarded (Figs 4, 5). All of the colors were
311 presented at the same light intensity measure in lux. The lux system is based upon the human
312 visual system and thus for the relative sensitivity of EHBs violet should have been the most
313 conspicuous among the three colors presented (Backhaus, 1991; Daumer, 1956; Menzel and
314 Blakers, 1976; Menzel and Erber, 1978). While not directly measured this is supported by the
315 quantum catch values calculated in our bee color space; all colors including the violet LED
316 appear to be within normal visual ranges for the bees (Fig. 5). Thus the violet wavelength
317 should have been the most conspicuous and the fastest color learned. However, in light of
318 Dyer's models and our current findings more research is certainly needed. In particular, it would
319 be useful to conduct a study in which bees are tested with a series of wavelengths between blue

320 and UV. Sakura et al. (2011) showed a pattern opposite to our findings when polarized light was
321 used as the conditioned stimulus in the PER protocol. They showed bees solved a
322 discrimination task when polarized ultraviolet light (UV), but not polarized blue or green, was
323 used as the rewarded stimulus. Thus, polarization may give the bees additional information that
324 allows them to discriminate stimuli, unlike the non-directional light presented in our experiments.
325 Thus, the addition of other visual features such as polarization, shape, and patterns may be
326 important for bees to learn and respond to colors.

327 *Memory was affected by the conditioning task*

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

344 *Comparison to previous studies*

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

354 **Conclusions**

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

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377

378

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503 **Figure 1.**

504 The learning curves presented as the percent of individual Africanized honey bees responding
505 to trained stimuli for the absolute conditioning paradigm over 7 learning trials. Conditioned color,
506 blue, green, or violet, was paired with a sucrose reward. Controls had color stimulus and
507 sucrose presented in separate trials. The error bars represent standard error of the mean, and
508 the colored lines represent the stimulus; 24 hour memory is shown to the right of the dashed
509 line.

510 **Figure 2.**

511 The learning performance and memory for the discrimination task, shown as percent of the bees
512 that responded to rewarded stimuli, versus the unrewarded stimuli presented in pseudorandom
513 order over 14 stimulus presentations. Here we present all the possible combinations of
514 rewarded and unrewarded colors, for the three colors used: blue, green, and violet. Solid lines
515 represent the rewarded color while dashed lines represent the unrewarded color. Error bars
516 represent the standard error of the mean for percent individuals responding to the stimulus.
517 Memory after 24 hours is shown to the right of the dashed line.

518 **Figure 3.**

519 The comparative olfactory and visual learning performance in restrained PER experiments.
520 Odors are represented in dashed lines and colors as sold lines; error bars are standard error of
521 the mean over 10 learning trials.

522 **Figure 4.**

523 Color learning performance of bees with and without antennae, using the absolute learning
524 protocol over 7 learning trials. Solid lines represent performance of bees with intact antennae
525 and dashed lines performance of bees without antennae. Bees trained to blue were fully or
526 partially de-antennated (see methods) but the data are pooled due to insignificant differences
527 between those groups. All other antennal removals involved the entire flagellum. Colors of lines
528 correspond to colors used for training. Error bars represent standard error of the mean. Note
529 that inhibiting olfactory stimuli seemed to inhibit responses to rewarded stimuli, and did not
530 improve visual learning.

531 **Figure 5.**

532 The triangular color space as calculated using AVICOL.exe and the basic R stats Package as
533 described in the methods. Each LED used during training is a point in the triangular space
534 determined by the relative stimulation of the three photoreceptors of the European Honey bee.
535 The three vertices of the triangle represent maximal stimulation of the corresponding
536 photoreceptor. Maximal stimulation of each of the photoreceptors occur at wavelengths of 344
537 nm for the short, 436 nm for the medium, and 556 nm for the long. All LED colors should be
538 visible colors that are detectably different to the honey bee according to this model. The violet
539 LED is represented with the light pink point in the middle, the blue LED is to the left represented
540 with the light blue point, and the green LED is represented with the light green point close to
541 maximal stimulation for the long photoreceptor.

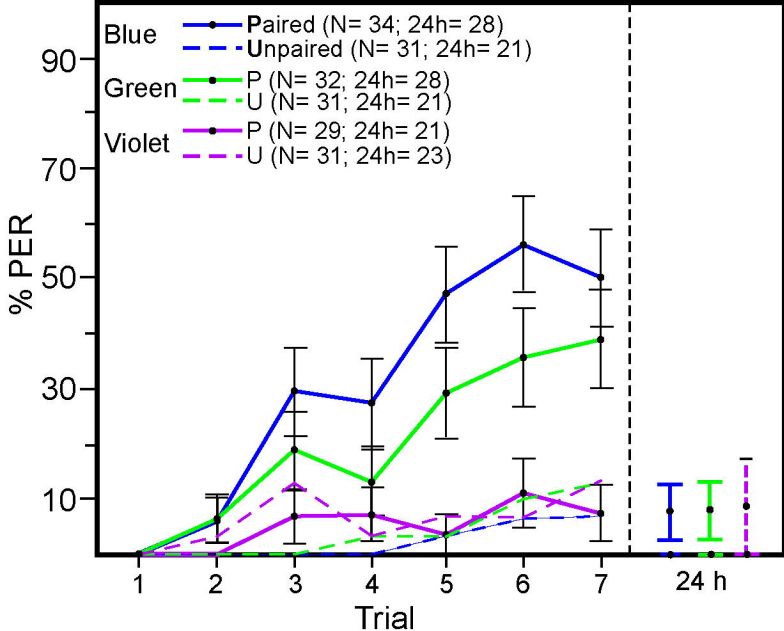
542 **Figure 1S.**

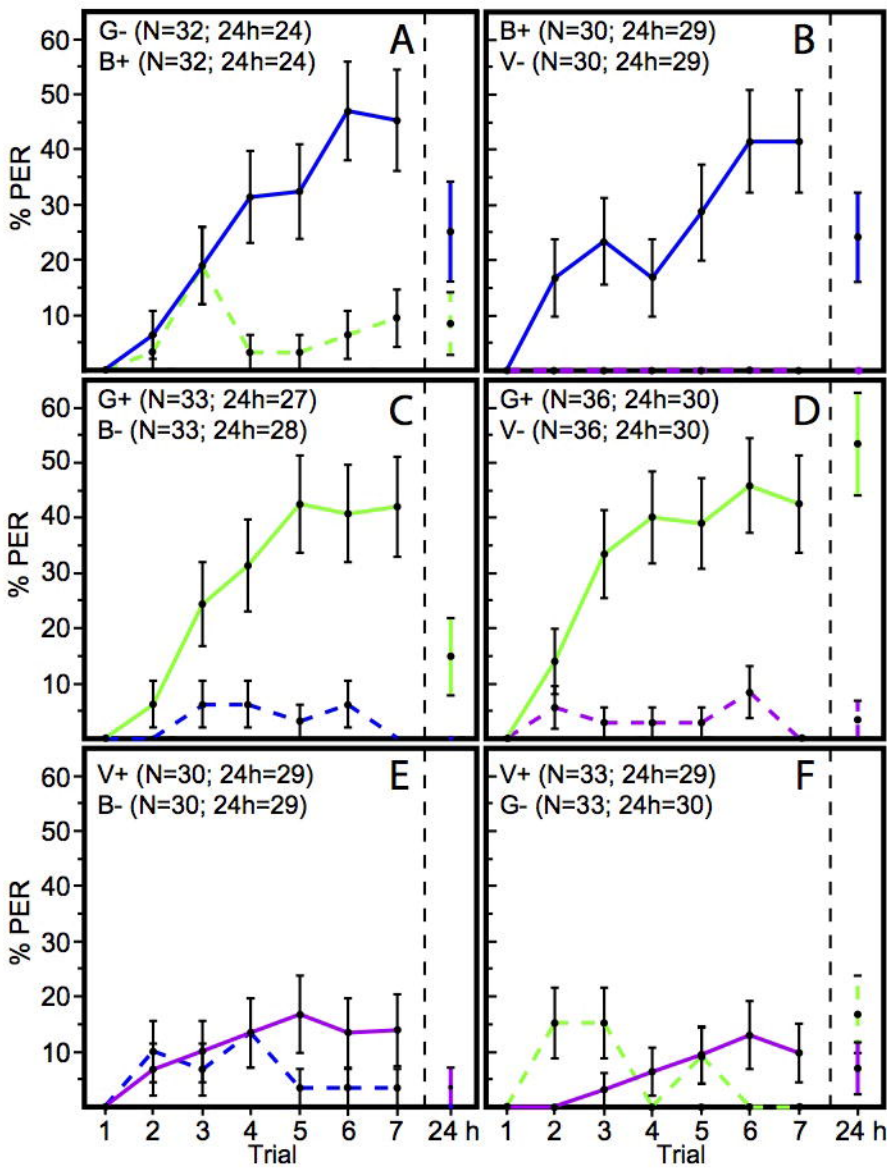
543 The spectral sensitivity of a European honey bee taken from Menzel and Backhaus (1991), with
544 the added peak wavelengths for LEDs used in all experiments.

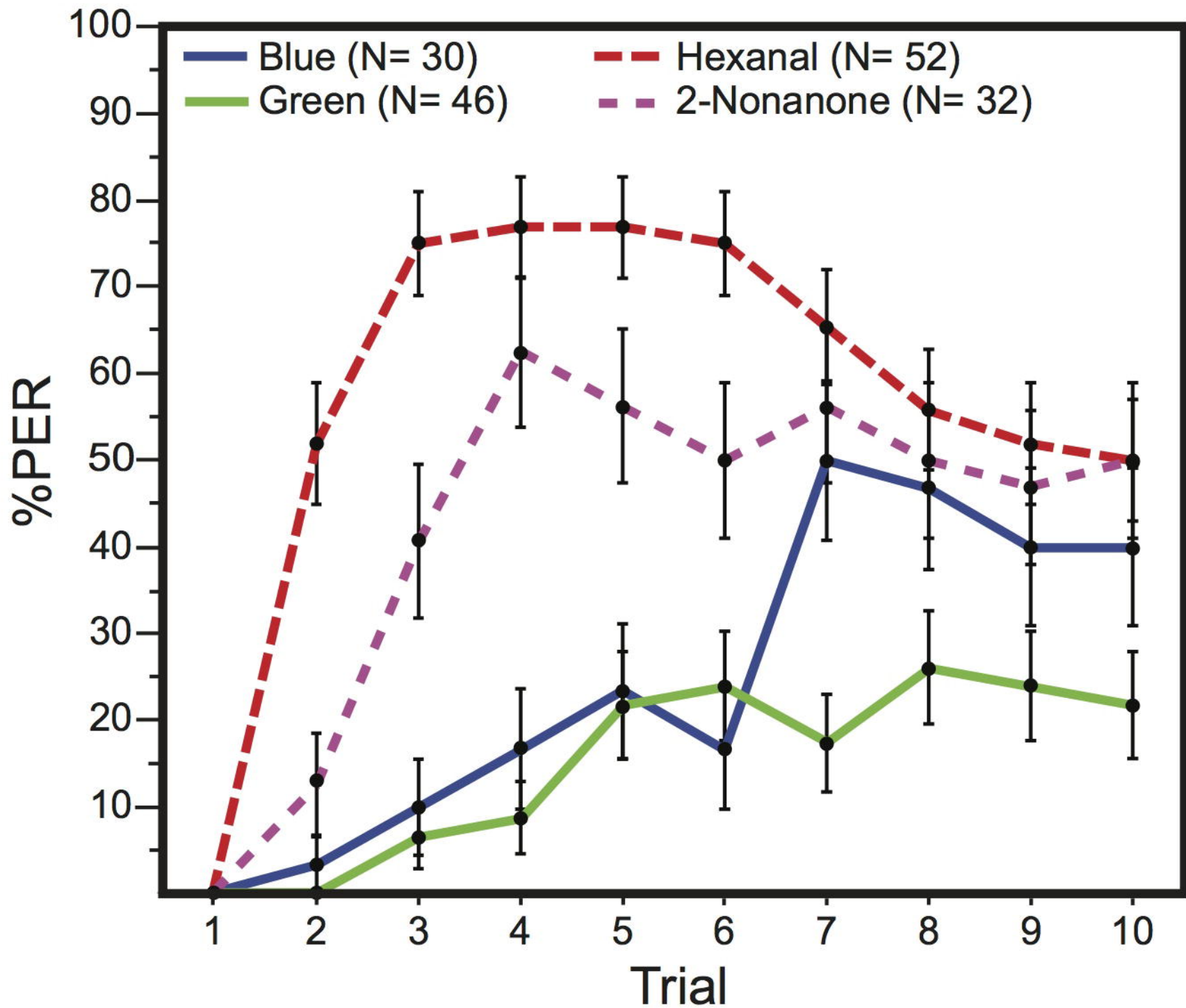
545

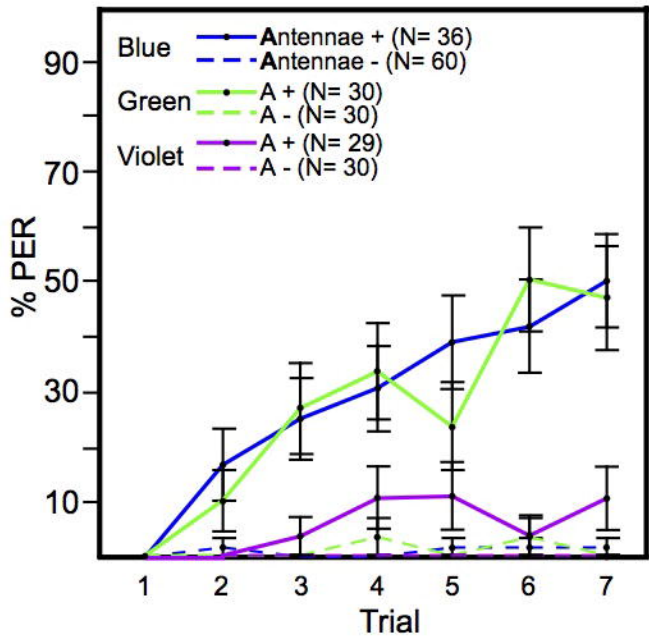
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Bee Color Space

