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# Appetitive reversal learning differences of two honey bee subspecies with different foraging behaviors

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We aimed to examine mechanistically the observed foraging differences across two honey bee, *Apis mellifera*, subspecies using the Proboscis Extension Response (PER) assay. Specifically, we compared differences in appetitive reversal learning ability between honey bee subspecies: *Apis mellifera caucasica* (Pollman), and *Apis mellifera syriaca* (Skorikov) in a “common garden” apiary. It was hypothesized that specific learning differences could explain previously observed foraging behavior differences of these subspecies: *A.m. caucasica* switches between different flower color morphs in response to reward variability, and *A.m. syriaca* does not switch. We suggest that flower constancy allows reduced exposure by minimizing search and handling time, whereas plasticity is important when maximizing harvest in preparation for long winter is at a premium. In the initial or *Acquisition* phase of the test we examined specifically discrimination learning, where bees were trained to respond to a paired conditioned stimulus with an unconditioned stimulus and not to respond to a second conditioned stimulus that is not followed by an unconditioned stimulus. We found no significant differences among the subspecies in the *Acquisition* phase in appetitive learning. During the second, *Reversal* phase of the experiment, where flexibility in association was tested, the paired and unpaired conditioned stimuli were reversed. During the *Reversal* phase *A. mellifera syriaca* showed a reduced ability to learn the reverse association in the appetitive learning task. This observation is consistent with the hypothesis that *A.m. syriaca* foragers cannot change the foraging choice because of lack of flexibility in appetitive associations under changing contingencies. Interestingly, both subspecies continued responding to the previously rewarded conditioned stimulus in the reversal phase. We discuss potential ecological

correlates and molecular underpinnings of these differences in learning across the two subspecies. In addition, in a supplemental experiment we demonstrated that these differences in appetitive reversal learning do not occur in other learning contexts.

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12 **Appetitive reversal learning differences of two honey bee subspecies with different foraging**  
13 **behaviors.**  
14

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36 **Abstract:**

37 We aimed to examine mechanistically the observed foraging differences across two honey  
38 bee, *Apis mellifera*, subspecies using the Proboscis Extension Response (PER) assay. Specifically,  
39 we compared differences in appetitive reversal learning ability between honey bee  
40 subspecies: *Apis mellifera caucasica* (Pollman), and *Apis mellifera syriaca* (Skorikov) in a  
41 “common garden” apiary. It was hypothesized that specific learning differences could explain  
42 previously observed foraging behavior differences of these subspecies: *A.m. caucasica* switches  
43 between different flower color morphs in response to reward variability, and *A.m. syriaca* does not  
44 switch. We suggest that flower constancy allows reduced exposure by minimizing search and  
45 handling time, whereas plasticity is important when maximizing harvest in preparation for long  
46 winter is at a premium. In the initial or *Acquisition* phase of the test we examined specifically  
47 discrimination learning, where bees were trained to respond to a paired conditioned stimulus with  
48 an unconditioned stimulus and not to respond to a second conditioned stimulus that is not followed  
49 by an unconditioned stimulus. We found no significant differences among the subspecies in the  
50 *Acquisition* phase in appetitive learning. During the second, *Reversal* phase of the experiment,  
51 where flexibility in association was tested, the paired and unpaired conditioned stimuli were  
52 reversed. During the *Reversal* phase *A. mellifera syriaca* showed a reduced ability to learn the  
53 reverse association in the appetitive learning task. This observation is consistent with the  
54 hypothesis that *A.m. syriaca* foragers cannot change the foraging choice because of lack of  
55 flexibility in appetitive associations under changing contingencies. Interestingly, both subspecies  
56 continued responding to the previously rewarded conditioned stimulus in the reversal phase. We  
57 discuss potential ecological correlates and molecular underpinnings of these differences in learning

58 across the two subspecies. In addition, in a supplemental experiment we demonstrated that these  
59 differences in appetitive reversal learning do not occur in other learning contexts.

## 60 **Introduction**

61 A honey bee colony shifts its foraging effort as the floral resources come and go in the  
62 environment (see Seeley, 1995). This dynamic allocation of foragers is thought to be adaptive  
63 since resources are harvested maximally. The basis of this constant response to changes in floral  
64 resources is the preference and foraging decisions of individual honey bees. Several mechanisms  
65 involving learning have been shown to be important in decisions of individual foragers (e.g.  
66 Ferguson, Cobey & Smith, 2001). We examined whether plasticity in appetitive learning will  
67 differentiate bees of *A.m. caucasica* subspecies that switch foraging preferences with ease from  
68 bees of *A.m. syriaca* subspecies that do not switch even when reward contingencies change (see  
69 Cakmak et al. 2010).

70 Both specialist strategy of *A.m. syriaca*, and generalist strategy of *A.m. caucasica* could  
71 be adaptive in their respective environments. The hypothesis is that specializing on a single  
72 flower type makes the bee faster both in finding the flower and in handling the flower, and thus  
73 decreases the time spent outside, at risk, or exposure to predators. Therefore, appetitive learning  
74 flexibility in the specialist subspecies, *A.m. syriaca* should be reduced to keep the bee focused on  
75 a single flower type. Alternately, in low risk environment, a fully plastic foraging choice  
76 towards the most rewarding resources is the best solution, and favors greater learning plasticity  
77 in the generalist subspecies, *A.m. caucasica*. Then predation risk sets limits to plasticity in  
78 foraging choice (DeWitt, Sih & Wilson, 1998; Murren et al., 2015).

79 Honey bees live in a wide range of habitats, extending from tropical to subarctic, either  
80 because of human intervention or because of evolutionary history of the populations (Whitfield  
81 et al., 2006; Wallberg et al., 2014). These genetically distinct populations are recognized as  
82 subspecies or races. Bringing members of different subspecies together for experiments revealed  
83 many genetic differences in behavior and its regulation (Giray et al., 2000; Brillet et al., 2002;  
84 Alaux et al., 2009; Çakmak et al., 2009; Cakmak et al., 2010; Kence et al., 2013; Büchler et al.,  
85 2014). Foraging choice differences across two subspecies from Turkey provides the ideal  
86 situation to test the underlying learning plasticity differences across specialists and generalists.  
87 Previously, *Apis mellifera syriaca* and *A.m. caucasica* bees have been studied for genetic, colony  
88 and behavioral differences (genetics: Bodur, Kence & Kence, 2007; foraging behavior: Çakmak  
89 et al., 2009; colony traits: Cakmak et al., 2010; Kence et al., 2013).

90 The bees from the subspecies *A.m. syriaca* inhabit southeast Anatolia, a generally dry  
91 habitat with longer seasonal foraging periods constrained by periodic blooms of one or few  
92 flowers (Kandemir, Kence & Kence, 2000; Kandemir et al., 2006). For foraging *A.m. syriaca*  
93 bees, minimizing predation risk is important. In this region there is a predatory wasp that can  
94 capture foraging honey bees, and bees of this region are demonstrated to have specific behavioral  
95 adaptations against this *Vespa* species, such as reducing foraging activity (Ishay, Bytinski-Salz &  
96 Shulov, 1967; Butler, 1974; Ruttner, 1988; Roubik, 1992; Çakmak, Wells & Firatli, 1998). This  
97 response is absent in *A. m. mellifera* (Matsuura & Sakagami, 1973). In contrast, the bees from  
98 the subspecies *A.m. caucasica* inhabit temperate deciduous forests in the northeast of Anatolia  
99 and the eastern Black Sea coast regions of Turkey. Weather in these regions limits foraging to a  
100 short, three month seasonal period, making it important to maximize collection rate.

101           One specific type of plasticity in learning, reversal learning, has been examined because  
102 of its potential relevance to tracking changing foraging resources (e.g Ferguson, Cobey & Smith,  
103 2001). The bees learn to associate a stimulus (a floral odor) with a reward and learn to  
104 discriminate this from a second odor not associated with reward. Later bees are asked to switch  
105 the odor associations. Reversal learning measures behavioral flexibility, and either single or  
106 multiple reversions, and either two or more choices are utilized to examine the extent of  
107 flexibility (Izquierdo et al. 2016). In comparison of bees of different ages (Ben-Shahar et al.,  
108 2000), selected lines (Ferguson, Cobey & Smith, 2001) and subspecies (Abramson et al. 2015),  
109 rate of reversal appears to differ, albeit the shape of reversal appears to remain similar (see  
110 Supplement Figure 1).

111           In the context of foraging behavior, reversal learning is similar to when a bee visits one  
112 flower providing nectar at that time, and later in the day switch to a different flower that is  
113 providing nectar then (Wagner et al., 2013). In addition, response of bees to variability in nectar  
114 availability is similar to response of other organisms such as vertebrates to variable reward or  
115 resources under experimental or natural conditions (Commons, Kacelnik & Shettleworth, 1987).  
116 For instance, if constant forage rate would provide energetic needs, organisms are likely to  
117 abandon variable reward for constant reward (Caraco, 1981; Zalocusky et al., 2016). In previous  
118 work we have demonstrated that bees from the temperate subspecies *A.m. caucasica* is more  
119 likely to switch to a different flower color morph. In contrast, bees from the subtropical  
120 subspecies, *A.m. syriaca* are not sensitive to variability in reward, and continue to visit the same  
121 flower morph even when rate of reward is 1 in 3 visits (Cakmak et al., 2010 and Figure 1).

122           We hypothesized that flower constancy even when faced with variable reward could be  
123 due to learning and memory differences of *A.m. syriaca* bees from other bees, including *A.m.*



124 *caucasica*. We used Proboscis Extension Response (PER) conditioning (Abramson et al., 2015)  
125 assay to examine differences in appetitive learning behavior across bees from colonies of both  
126 subspecies maintained in a “common garden” apiary (Kence et al., 2013).

127

## 128 **Materials and Methods**

### 129 **Experimental Design:**

130 Proboscis Extension Response Conditioning experiments were performed between June  
131 and July 2014 at the Middle East Technical University in Ankara, Turkey. In a preliminary work  
132 we also examined reversal in a non-appetitive aversive learning test, Electric Shock Avoidance  
133 conditioning (ESA, Agarwal et al., 2011; Dinges et al., 2013). To control for calendar variables  
134 associated with weather and field conditions, both PER and ESA (supplement) conditioning  
135 assays were run simultaneously. In the ESA series we investigated the reversal of spatial  
136 avoidance learning in honey bees confined to a shuttle box.

137 Foragers of two subspecies populations in Turkey were used. One subspecies was *Apis*  
138 *mellifera caucasica*, and the other subspecies was *Apis mellifera syriaca*. Both subspecies were  
139 maintained in a common garden under similar environmental conditions. Great care is taken to  
140 ensure that the subspecies lines are maintained and this is confirmed by use of genetic and  
141 morphological measurements, and acquiring new colonies or naturally mated queens from the  
142 geographically separated (>600 miles) locations (Kence et al. 2013). We used three colonies  
143 from each honey bee subspecies to increase genetic variation within the samples for a total of  
144 261 individuals that were tested in learning and memory assays. One hundred thirty-seven bees  
145 (137), divided in two equal groups (but for one bee), one for each subspecies, were recruited for

146 the PER assays where each experimental group consisted of 12 individuals, except in occasion  
147 one or two bees were eliminated when not responsive. One hundred twenty-four bees (124),  
148 divided in four equal groups, two for each subspecies, were recruited for the supplemental ESA  
149 assays where each experimental group consisted of up to 34 individuals.

150 Proboscis Extension Response (PER) Reversal Learning:

151 In these experiments there are two phases, acquisition and reversal. In the acquisition  
152 phase we examined differential conditioning, where we trained the honey bees to discriminate  
153 between two conditioned stimuli (CS) – one paired with a sucrose feeding (CS+) and the other  
154 not (CS-). Following this, in the reversal phase, we reversed the CS+ and CS- roles such that the  
155 CS+ is now the CS- and the CS- is now the CS+.

156 One CS consisted of lavender odor (Gilbertie's, Southampton, NY) and the other  
157 cinnamon odor (Gilbertie's, Southampton, NY). The rationale behind the use of these odors is  
158 that we have found them effective in our previous discrimination experiments in Turkey  
159 (Abramson et al., 2008, 2010, 2015). The CS odor was applied to a 1 cm<sup>2</sup> piece of Whatman (#4)  
160 filter paper using a wooden dowel and then secured to the plunger of a 20 cc plastic syringe with  
161 an uncoated metal thumbtack. Our earlier work demonstrated this procedure produces reliable  
162 results consistent with automated methods (Abramson & Boyd, 2001).

163 To remain consistent with our previous work: 1) a non-overlap procedure was used in  
164 which the CS terminated before the US (Abramson et al., 1997), 2) the CS duration was 3  
165 seconds and the US duration was 2 seconds, and 3) the intertrial interval (ITI) between CS  
166 presentations was a fixed 5-minute interval. During the initial discrimination learning phase,  
167 each bee received 6 trials each with lavender and cinnamon for a total of 12 trials. During the

168 reversal phase in which the role of the CSs were reversed, bees received 6 trials each with  
169 lavender and cinnamon for an additional 12 trials. The order of CS+ and CS- presentations were  
170 pseudorandom and identical for each bee. We used the order: Initial Discrimination training:  
171 CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-, CS+, CS-, CS-, CS+, Reversal Training: CS-, CS+,  
172 CS+, CS-, CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS- for a total of 24 trials (12 CS+ and 12 CS-).

173 Honey bees from both subspecies were captured one day before the experiment. They  
174 were captured in glass vials and placed in ice. While sedated they were harnessed in metal tubes  
175 with a piece of duct tape placed between the head and thorax. Once awake they were fed 1.5 M  
176 sucrose solution in water until satiated and set aside in a fume hood. On the day of the  
177 experiment, the bees were removed from the fume hood and were placed in batches consisting of  
178 about 12 bees.

179 A conditioning trial was initiated by picking up a bee from its position in the batch and  
180 placing it in the fume hood. The purpose of the fume hood was to eliminate any lingering CS  
181 odors. After a few seconds, but never immediately upon placement, the CS was administered for  
182 3 seconds and was immediately followed by the US. This procedure was necessary as bees can  
183 associate the “placement” with a feeding. The US was presented by touching the bee’s antennae  
184 with a filter paper strip containing 1.5 M sucrose and bees were allowed to lick the filter paper  
185 for 2 seconds after extending their proboscis. At the end of the 2-second feeding, the bee was  
186 removed from fume hood and returned to its place in the batch at which time the next bee in the  
187 batch was placed in fume hood for its trial. This process continued until all the subjects in the  
188 batch received the required number of conditioning trials. During each trial, responses to the CS  
189 were recorded visually. If the bee extended its proboscis during the CS presentation, a positive  
190 response was recorded. If the bee did not extend its proboscis during the CS presentation, a “0”

191 response was recorded. The experiment was run blind as the experimenter did not know what  
192 subspecies was being trained. This was assured by using a code for source colony, and by using  
193 help of individuals who would not run the experiment in fixing bees into holders in preparation  
194 for PER conditioning.

195 Each experiment consisted of two phases. The stage where memory of the paradigm was  
196 being acquired for the first time was termed *Acquisition* Phase. The step where we reverse the  
197 paradigm was termed *Reversal* Phase. During each trial we presented a CS+ and a CS-, each CS  
198 was a different odor. We used a model with two sets of experiments where each odor had the  
199 role of acquisition phase CS+ (Initial CS+) or acquisition phase CS- (Initial CS-) thus creating a  
200 counterbalance. The measured value was the PER response.

201

202 Supplemental Electric shock avoidance assay (ESA):

203 This experiment had two phases of 5 minutes each for a total of 10 minutes. During Acquisition  
204 phase, individuals were presented two colors, one as the punishment conditioned stimulus (CS+),  
205 this color was paired with electric shock (unconditioned stimuli), and the other as the no  
206 punishment conditioned stimulus (CS-), this color was not paired with electric shock. Here  
207 individuals learn to avoid punishment or one of the colors. That is to say, the bee learns to stay  
208 on one side of the box and not on the other. During the second or Reversal phase, the colors for  
209 the CS+ and CS- were switched. Now the acquisition phase CS+ is the reversal phase CS- and  
210 the acquisition phase CS- is the reversal phase CS+. We do the switch by changing the  
211 side/color of the box that receives shock, and not by moving the colors, this way we avoid  
212 confounding position and color effects. Moreover, by moving the shock from one side of the box

213 to another, the bee can only avoid the shock by making an active response; by moving from one  
214 side to the other. We omitted the test phase (period of time without shock) that is usually  
215 performed after a trial or phase that demonstrates memory (Agarwal et al., 2011; Dinges et al.,  
216 2013; Giannoni-Guzmán et al., 2014). This was done to prevent the memory extinction process  
217 from interfering with the reversal phase.

218 To analyze the results from these experiments we first confirmed there is no color  
219 preference by bees from either subspecies when either blue or yellow was the CS- during  
220 Acquisition and Reversal Phases. Because we did not observe significant differences (see results  
221 in the supplement) Color was not included as a variable in subsequent analyses. Instead, the first  
222 color associated with punishment is A+, and the second or Reversal phase this is A-, whereas the  
223 alternate color becomes B+.

224 We used a shuttle box apparatus as described before (Agarwal et al., 2011; Giannoni-  
225 Guzmán et al., 2014). The shuttle box measured 15 cm long by 2 cm wide and contained an  
226 electric shock grid with wires spaced .35 cm apart. The shock was presented to only one side of  
227 the apparatus identified by a specific color. Shock intensity was 6 V 50 mA DC from an analog  
228 power supply and was low enough not to produce a sting reflex. In one half of the shuttle box a  
229 color (CS) is paired with electric shock (US) to create a CS+, on the other half another color  
230 (CS) is not paired with the electric shock (US) to create a CS-. Time spent on the shock side was  
231 recorded by an observer, one observer for each individual. We used blue and yellow as we know  
232 from our previous experiments that bees can readily distinguish between them. We measured  
233 the mean amount of time spent on the shock side in sets of 60 seconds for a total of 5 sets or 300  
234 seconds as was done previously (Agarwal et al., 2011).

235

236 **Statistical Analysis:**

237 Statistical analyses were performed using the GraphPad Prism 6 statistical software  
238 program. Analyses of the data from PER and the ESA assays were done with: two-way repeated  
239 measures ANOVA, Wilcoxon- matched-pairs signed rank test, and Student's T test. We tested  
240 the data for significant phase (Acquisition vs Reversal), subspecies, and interaction effects. In the  
241 case of ANOVA, a post-hoc Tukey-HSD test was used to examine trial to trial differences. We  
242 verified fit to a normal distribution using the Shapiro-Wilk's W test.

243 **Results:**

244 Two-way ANOVA comparison shows *A. m. caucasica* has no significant odor preference  
245 between lavender and cinnamon for the Initial CS+ ( $F_{(1,54)} = 0.6779$ ,  $\omega^2 = 0.2454$ ,  $p = 0.4139$ ;  
246  $N_{1(\text{Lavender})} = 27$ ,  $N_{2(\text{Cinnamon})} = 29$ ) or the Initial CS- ( $F_{(1,54)} = 0.04922$ ,  $\omega^2 = 0.01582$ ,  $p =$   
247  $0.8253$ ;  $N_{1(\text{Cinnamon})} = 27$ ,  $N_{2(\text{Lavender})} = 29$ ). Likewise, *A. m. syriaca* showed no significant odor  
248 preference between lavender and cinnamon for the Initial CS+ ( $F_{(1,54)} = 0.2687$ ,  $\omega^2 = 0.0628$ ,  $p =$   
249  $0.6063$ ;  $N_{1(\text{Lavender})} = 27$ ,  $N_{2(\text{Cinnamon})} = 29$ ) or the Initial CS- ( $F_{(1,54)} = 1.626$ ,  $\omega^2 = 0.6175$ ,  $p =$   
250  $0.2077$ ;  $N_{1(\text{Cinnamon})} = 27$ ,  $N_{2(\text{Lavender})} = 29$ ). As a result, type of odor was excluded from further  
251 consideration, and the first CS+ odor is simply coded as A+, and the second CS+ as B+, the  
252 odors that are CS- are then B- in the Acquisition phase, and A- in the reversal phase.

253 The learning rates for the A+ in the Acquisition phase for both subspecies members are  
254 described in Figure 2, Panel A+. The fewer response of proboscis extension by members of both  
255 subspecies to B- in the acquisition phase is plotted in Figure 2, Panel B-. The Reversal Phase  
256 responses are shown in Figure 2, Panel B+. The Reversal Phase extinction of odor A (A-)  
257 showed that after 6 trials of where no reward was presented following odor A (A-), bees of both

258 subspecies continued to present PER response above 50% of the trials (Figure 2, Panel A-).  
259 During this phase *A.m. syriaca* reached significantly lower response rates in comparison to *A.m.*  
260 *caucasica* ( $F_{(1,110)} = 4.777$ ,  $\omega^2 = 1.607$ ,  $p = 0.0310$ ;  $N_{1(\text{Caucasica})} = 56$ ,  $N_{2(\text{Syriaca})} = 56$ ).

## 261 Discussion

262 The most significant finding of this study is that appetitive olfactory reversal learning  
263 differences across honey bee subspecies match differences in their foraging plasticity. In  
264 appetitive olfactory reversal learning, bees from the subtropical subspecies *A.m. syriaca* do not  
265 show reversal, specifically they do not form association for the odor that is rewarded in the  
266 reversal phase. Unlike the typical reversal response of other organisms, such as other bee  
267 subspecies (see below), bees in this study continued to respond to the previously rewarded but  
268 now unrewarded odor in the reversal phase. Should these responses occur in the context of  
269 foraging, *A.m. syriaca* bees are expected to visit only flowers similar to a first learned flower.  
270 *A.m. caucasica* bees would be expected to visit an expanding repertoire of flowers with different  
271 features. These results suggest molecular substrates of learning and memory to be candidates for  
272 selection in adaptation to specific ecological conditions.

273

### 274 *Specific learning differences across populations*

275 This study is, to our knowledge, the first to demonstrate specific learning plasticity  
276 differences across genetically distinct populations of the same species. This could be due both to  
277 comparison of populations from contrasting environmental conditions and to use of a complex  
278 learning paradigm. We found that bees from both subspecies has a similar learning rate for the  
279 A+ in the Acquisition phase (see Figure 2, Panel A+). We also found that both subspecies

280 showed discrimination and did not respond by proboscis extension to B- in the acquisition phase  
281 (see Figure 2, Panel B-). Surprisingly we found that during Reversal Phase *A. m. syriaca*'s  
282 acquisition of B+ is impaired (Figure 2, Panel B+). This is unique to *A. m. syriaca* as can be seen  
283 when our results are compared with those of similar experiments in the European honey bee  
284 from North America ( a mix of the European *A. mellifera* subspecies, Ben-Shahar et al., 2000,  
285 Figure S1) or *A. m. anatoliaca* (Abramson et al. 2015). In contrast, in this study especially the  
286 Reversal Phase extinction of odor A (A-) was different, in that complete extinction did not occur,  
287 and extinction was slower for both *A. m. caucasica* and *A. m. syriaca* in comparison to bees from  
288 other subspecies (Figure S1, also see Figure 2, Panel A-). Yet another difference was for *A.*  
289 *syriaca* in the Reversal Phase conditioning of odor B (B+), where *A. m. caucasica* showed the  
290 typical learning curve and responded with PER to B+, the *A. m. syriaca* continued withholding  
291 PER (Figure 2, Panel B+).

292 In summary, the behavior of both of these subspecies, living at near extremes of honey  
293 bee distribution, differ from other subspecies such as *A. m. ligustica*, *caronica*, and *anatoliaca*  
294 (Ben-Shahar et al., 2000; Hadar & Menzel, 2010; Abramson et al., 2015). In these other  
295 subspecies similar paradigms result in complete switch from proper response to A+B- to proper  
296 response to A-B+, similar to other organisms (Izquierdo et al 2016).

297

### 298 *The complexity of learning challenge*

299 Using simple conditioning, differences can be observed across drug treatment and control  
300 groups (Abramson et al. 2010, Giannoni-Guzman et al. 2014), but this simple paradigm cannot  
301 differentiate age and job-related differences; for instance, across nurse and forager honey bees,



302 or younger and older foraging bees (Ben-Shahar et al. 2000). In these situations, reversal  
303 learning paradigms are used to better differentiate the learning abilities that change with age or  
304 disease. For example, only during the reversal phase of a reversal learning paradigm could it be  
305 shown that dogs and primates exhibit impaired spatial navigation as they age (Lai et al., 1995;  
306 Mongillo et al., 2013). In another recent study, reversal learning was necessary to show that an  
307 animal model of anorexia nervosa (rat) has impaired cognitive-flexibility, just like the human  
308 counterpart (Tchanturia et al., 2011; Allen et al., 2017).

309         Reversal learning paradigms can probe deeper than its simple conditioning counterpart  
310 because it combines two related yet distinct conditioning phases: discrimination and reversal.  
311 Thus, we suggest the use of reversal learning paradigms could also be more appropriate when  
312 small differences in cognitive performance are expected in other organisms.

313

#### 314 *Neural substrates of reversal learning*

315         In studies targeting mechanistic understanding of reversal learning, it is shown that in the  
316 first acquisition of rewarded vs non-rewarded stimuli, a type of discrimination learning, vs the  
317 second or reversal phase are shown to depend on different neural substrates (Izquierdo et al.  
318 2016, in bees Devaud et al. 2007). The acquisition phase does not require the mushroom body,  
319 yet the reversal phase requires the alpha-lobes of the mushroom bodies; as demonstrated by the  
320 effects of anesthetics applied directly to this region which only interfere with the reversal phase  
321 but not with the acquisition phase (Devaud et al., 2007). Because neuropharmacological studies  
322 demonstrate the role of dopamine in reversal learning (Costa et al., 2015), it will be interesting to

323 examine correlates of dopaminergic signaling in the mushroom bodies of *A.m. syriaca* and *A.m.*  
324 *caucasica* bees.

325

326 *A.m. caucasica* versus *A.m. syriaca*

327 In this study, using the appetitive reversal learning paradigm we demonstrate that *A.m.*  
328 *caucasica* learns new associations, and keeps the previous associations. This is consistent with a  
329 highly plastic, generalist foraging behavior. *A.m. syriaca* shows very low plasticity in foraging  
330 choice (Cakmak et al. 2010, see Figure 1), and *A.m. syriaca* does not learn to respond to the  
331 reversal CS+ in the appetitive reversal learning paradigm. This is consistent with specialization  
332 to one or few resources. Specialization provides for speed of foraging and may reduce exposure  
333 to predators during foraging episodes. Foraging modeling (Becher et al., 2014) can help us  
334 further dissect the ecological importance of these observed differences.

335

336 *Appetitive vs aversive learning*

337 One interpretation of differences across *A.m. syriaca* and *A.m. caucasica* could have been  
338 greater learning ability in one vs the other subspecies. However, in that case learning effects  
339 would have been expected to be general, such as performance differences in all tasks across the  
340 two subspecies. This would be similar to comparing bees treated orally with ethanol and control  
341 group bees. For these two groups, both in appetitive and aversive learning tasks the 10% or  
342 higher ethanol treatment group performed poorly (Giannoni-Guzmán et al., 2014). However, in  
343 a supplemental study we demonstrated in an aversive learning paradigm, ESA conditioning, both  
344 *A.m. syriaca* and *A.m. caucasica* demonstrated complete reversal of punishment learning. This

345 difference across aversive vs appetitive reversal learning also supports ecological relevance of  
346 differences in appetitive reversal learning across subspecies. It is important to note that  
347 modality of association cues did not make a difference for the acquisition phase, and  
348 demonstrated both subspecies to establish associations for color or odor equally well.

349

### 350 *Conclusion*

351 In this study we demonstrated a match between the ecology of foraging behavior and  
352 learning and memory differences of two honey bee subspecies. As a result we conclude neural  
353 substrates of foraging differences may extend beyond modulation of the reward pathway (Giray  
354 et al., 2015, Agarwal et al. 2011), and involves learning and memory centers in the brain of the  
355 honey bee. In the future, it will be important to compare neurons such as in mushroom bodies  
356 and olfactory lobes in the two subspecies, in relation to differences in acquisition and reversal  
357 phases in reversal learning (Devaud et al. 2007). Finding the neural substrates linked with the  
358 obsessive-like behavior of *A.m. syriaca* will be relevant for other learning contexts and  
359 organisms.

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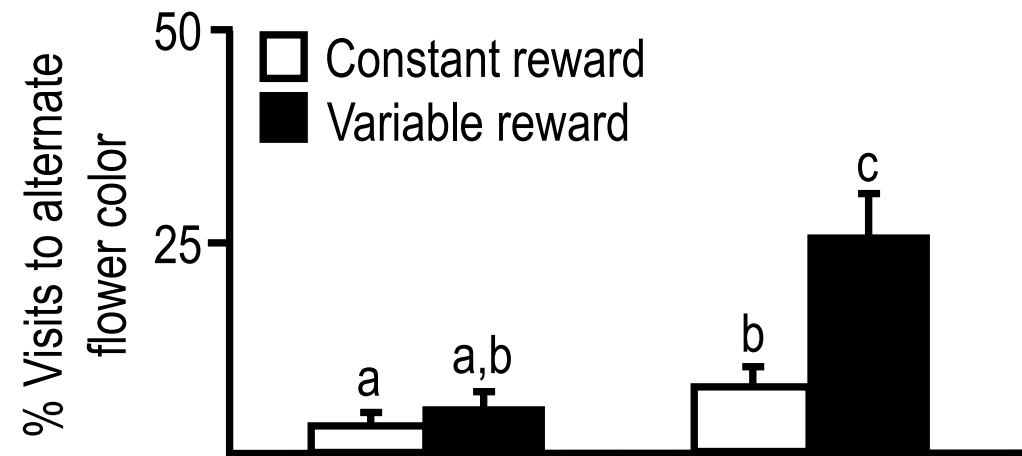
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**Figure 1**(on next page)

Foraging visits of bees from two subspecies to alternate flowers when preferred flower provides constant or variable amounts of nectar reward.

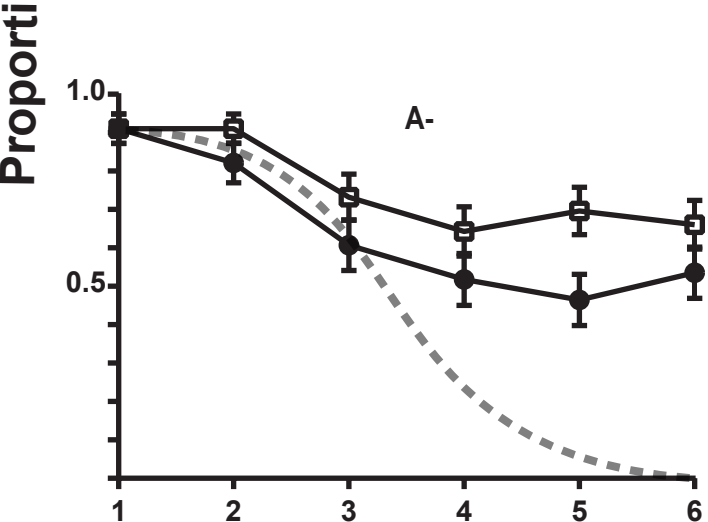
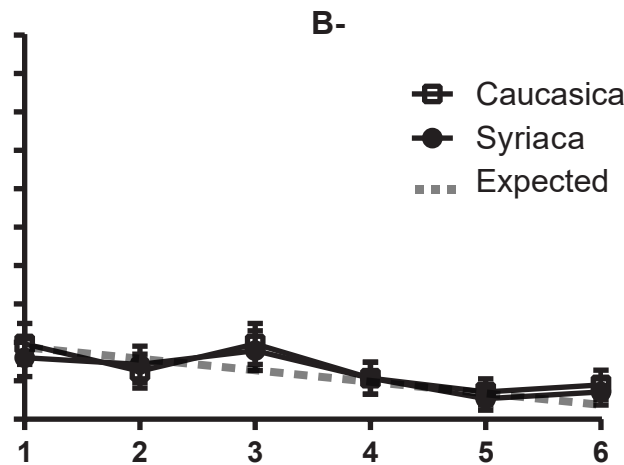
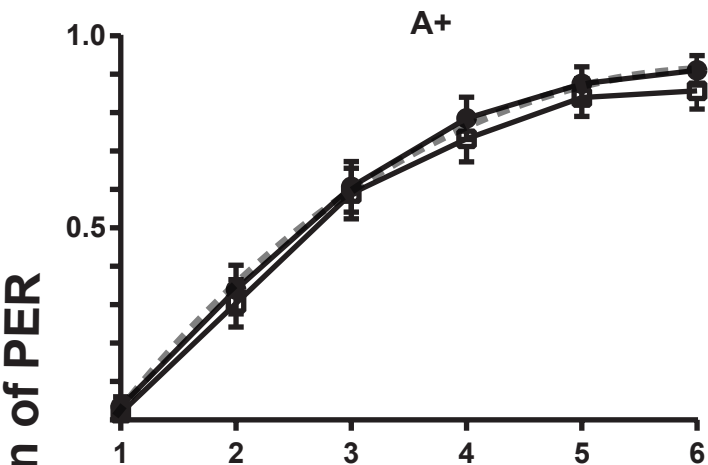
Average percent visits to alternate flower color was significantly less for *A.m. syriaca* than *caucasica*. Bees first visited blue, white or yellow flowers. Later they visited alternates or initial preferred flowers with either constant reward (2 $\mu$ l 1M sucrose) or variable reward (only 1 of 3 flowers with 6  $\mu$ l reward). Sample size: 6 colonies / subspecies, 30-35 bees /colony, 30-40 choices/bee. Error bars = SE. Factorial ANOVA indicated significant subspecies differences. Groups with different letters above bars are different at  $P < 0.05$ . (Cakmak et al., 2010) .



**Figure 2**(on next page)

Proboscis Extension Response of *A.m. caucasica* and *A.m syriaca* during a Reversal Learning test.

Comparison of responses to odors A and B between honey bee subspecies *A.m. caucasica* and *A.m. syriaca* during a proboscis extension response (PER) assay. Each data point shows the percentage ( $\pm$  standard error) of bees that showed PER during the assay. During the **Reversal for A-**, ANOVA test shows differences at the subspecies level in the extinction rate (P-value = 0.0310,  $F(1,110) = 4.777$ ). During the **Reversal for B-**, ANOVA test shows differences in the learning rate at the subspecies level (P-value $<0.0001$ ,  $F(1,110) = 44.43$ ).



## Reversal

