1   TITLE: White flowers finish last: Pollen-foraging bumble bees show biased learning in a floral

2   color polymorphism

3

4   RUNNING TITLE: Learned but not innate biases in a color polymorphism

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31   *preference*

32 ABSTRACT

33

34 Pollinator-driven selection is thought to drive much of the extraordinary diversity of flowering

35 plants. Plants that produce floral traits preferred by particular pollinators are more likely to

36 receive conspecific pollen and to evolve further adaptations to those pollinators that enhance

37 pollination and ultimately generate floral diversity. Two mechanisms in particular, sensory bias

38 and learning, are thought to explain how pollinator preference can contribute to divergence and

39 speciation in flowering plants. While the preferences of pollinators, such as bees, flies, and birds,

40 are frequently implicated in patterns of floral trait evolution, the role of learning in generating

41 reproductive isolation and trait divergence for different floral types within plant populations is

42 not well understood. Floral color polymorphism in particular provides an excellent opportunity

43 to examine how pollinator behavior and learning might maintain the different floral morphs. In

44 this study we asked if bumble bees showed innate preferences for different color morphs of the

45 pollen-only plant *Solanum tridynamum*, whether bees formed preferences for the morphs with

46 which they had experience collecting pollen from, and the strength of those learned preferences.

47 Using an absolute conditioning protocol, we gave bees experience collecting pollen from a color

48 polymorphic plant species that offered only pollen rewards. Despite initially-naïve bees showing

49 no apparent innate bias toward human-white versus human-purple flower morphs, we did find

50 evidence of a bias in learning. Specifically, bees learned strong preferences for purple corollas,

51 but learned only weak preferences for hypochromic (human-white) corollas. We discuss how our

52 results might explain patterns of floral display evolution, particularly as they relate to color

53 polymorphisms. Additionally, we propose that the ease with which floral visual traits are learned

54 – i.e. biases in learning – can influence the evolution of floral color as a signal to pollinators.

55

INTRODUCTION

Pollinator-mediated selection on floral traits is held to be a common process driving the evolution and diversity of flowering plants (Johnson 2006; Van der Niet & Johnson 2012). As a consequence of adaptation to pollinators, plants often exhibit suites of floral traits that closely match the traits of their pollinators (Anderson et al. 2009; Newman et al. 2014). Accordingly, shifts in floral traits, such as floral form, scent, or color, are often associated with pollinator shifts and with reproductive isolation in plants (Briscoe Runquist & Moeller 2014; Van der Niet et al. 2014). Two mechanisms underlying pollinator behavior in particular are thought to contribute to plant evolution: innate bias and learning. Some innate biases are thought to have evolved due to pollinator adaptation to flowers (termed adaptive preference); other biases evolved in a context separate from foraging on flowers (i.e.; pre-existing sensory bias; Schaefer & Ruxton 2010; Schiestl & Johnson 2013). Behaviors formed through associative learning are particularly intriguing because they are thought to contribute to rapid diversification of floral cues and rewards (Gegear & Burns 2007).

Learning is ubiquitous in animals and has been studied in a variety of pollinators (e.g., Fukushi 1989; Lunau 1992; Kelber & Pfaff 1997; Weiss 1997; Hurly & Healy 2002; Chittka & Thomson 2005). Pollinators learn to associate a variety of floral cues, including color, scent, texture, heat and iridescence, with floral rewards, such as nectar and pollen (Muth et al. 2016). Because learned behaviors are often durable, quickly formed (Giurfa 2007), and may be exhibited in response to evolutionarily novel stimuli, learning has the potential to rapidly alter floral

78   preferences. In fact, pollinators typically exhibit strong preferences for species with which they

79   have had experience (e.g., Weiss 1997; Chittka et al. 1999; Russell et al. 2015). These behavioral

80   changes increase pollen transfer between conspecific plants, while reducing receipt of pollen

81   from heterospecific plants (Gegear & Laverty 1995; Hopkins & Rausher 2012). Learning could

82   thus contribute to reproductive isolation between flowering plant species and evolutionary

83   divergence (Gegear & Burns 2007; Schiestl & Johnson 2013). Accordingly, patterns of floral

84   trait evolution are thought to reflect learned pollinator preferences in part (Schiestl & Johnson

85   2013).

86

87   In this study, we focus on how learning might shape variation in floral traits within plant

88   populations. Plants often exhibit intraspecific variation in their floral display traits – floral color

89   polymorphisms are a common example (Weiss 1995; Rausher 2008). Color polymorphisms in

90   plant populations often arise from single loss-of-function mutation, resulting in a species with

91   both highly pigmented and hypochromic (often human-white) flower morphs (e.g. Fig. 1)

92   (Rausher 2008; Malerba & Nattero 2012). These simple shifts in floral visual display traits are

93   thought to favor the formation of reproductive barriers between flower morphs, and thus

94   divergent evolution, as a consequence of strong pollinator visual preferences (Gegear & Burns

95   2007). Learned preferences for floral visual traits have been studied in taxonomically diverse

96   pollinators, including bees, flies, and birds (Lunau & Maier 1995). However, how learning might

97   affect the maintenance of genetic floral color polymorphisms is still poorly understood.

98   Pollinators learn some floral features more easily than others (e.g., Hempel de Ibarra & Giurfa

99   2003; Guerrieri et al. 2005); one might expect that the strength of resulting preferences or the

100   speed with which these preferences are formed would therefore vary among flower morphs.

101   Accordingly, pollinators should transfer more conspecific pollen between strongly preferred

102   flower morphs and show less fidelity to weakly preferred morphs (Gegear & Burns 2007).

103   Differences in the efficacy of learning could thus have immediate and profound fitness

104   consequences for novel floral forms.

105

106   While a number of studies have investigated pollinator preferences for particular morphs (e.g.,

107   Waser & Price 1981; Epperson & Clegg 1987; Bradshaw & Schemske 2003), the role of learning

108   in formation of these preferences is unclear. In this study we asked whether pollen-foraging

109   bumble bees (*Bombus impatiens*) expressed innate and learned preferences for a pigmented

110   versus a hypochromic (human-white) floral morph. We examined the degree to which floral

111   experience affected preferences for each morph and additionally explored the role of specific

112   flower parts, corolla versus anther, in mediating differences in learning performance. We studied

113   the pollen-only species, *Solanum tridynamum*. Populations of *S. tridynamum* exhibit a naturally-

114   occurring color polymorphism (J. Wiens pers. comm.) composed of a pigmented, human-purple

115   morph and a hypochromic, nearly human-white morph (Fig. 2). Here we will refer to the human-

116   purple morph as purple and the human-white morph as white. Both morphs conceal their pollen

117   rewards within the anthers, which release their pollen when vibrated ('sonicated') by bees. A

118   previous study demonstrated that bumble bees given experience collecting pollen from *S.*

119   *tridynamum* rapidly formed durable preferences for the species over other pollen-only species

120   (Russell et al. 2015). That study employed only the more common purple morph; here we used

121   both morphs to examine patterns of innate and learned preference.

122

123   METHODS

124

125   *Outline of Experiment*

126

127   We conducted an experiment with 5 experience treatments (1 no-experience treatment and 4

128   morph-experience treatments). The no-experience treatment involved an assessment of responses

129   by flower-naïve bees to 4 types of flowers. In that treatment, flower-naïve bees were presented

130   with equal numbers of the 2 naturally-occurring color morphs of *Solanum tridynamum* (purple

131   and white) as well as 2 mosaic morphs. Mosaic morphs consisted of corollas from one natural

132   morph and anthers from the other natural morph (purple corolla/white anther and white

133   corolla/purple anther morphs). All flowers were manipulated such that pollen could not be

134   collected from them. In the remaining 4 treatments, flower-naïve bees were given experience

135   with flowers in a training phase; specifically, they were allowed to collect pollen from 1 of the 4

136   morphs. In a subsequent test phase, they were allowed to forage in an array of 4 unrewarding

137   morphs identical to that in which bees in the no-experience treatment foraged. The no-experience

138   treatment evaluates innate preference in terms of what morphs bees first visited, and also the

139   overall preference resulting beyond what was observed in first visits. The 4 morph-experience

140   treatments evaluate how a bee's early experience in collecting pollen from one morph affects its

141   preference among morphs subsequently. The use of mosaic morphs allowed us to determine

142   which flower part affected preference. Details of system and protocol follow.

143

144   *Bees*

145   We used 52 workers from 3 colonies of *Bombus impatiens* in experiments conducted between

146   May 2015 and September 2015. Colonies were purchased from Koppert Biological Systems

147   (Howell, MI, USA). We used approximately equal numbers of bees from each colony and for

148   each treatment. We allowed bees to forage daily for sucrose and pollen in arenas constructed of

149   plywood (LxWxH 82 x 60 x 60 cm). The arenas had clear acrylic ceilings and were lit from

150   above by 40W 60Hz fluorescent lights (Lithonia Lighting). The colony was kept dark while

151   lights in the arenas were set to a 14:10 light:dark cycle. Colonies had access to *ad libitum* 2M

152   sucrose solution and pulverized honeybee-collected pollen (Koppert Biological Systems, MI,

153   USA) within the foraging arena. Sucrose solution was dispensed via braided cotton wicks (6 inch

154   Braided Cotton Rolls, Richmond Dental) that extended into 40 dram vials (BioQuip Products,

155   Inc., USA). Pollen was presented using custom-made feeders (Russell & Papaj 2016) consisting

156   of chenille fibers, glued to the inside walls of 40 dram vials (BioQuip Products, Inc., USA).

157

158   *Plants and Floral Morphs*

159

160   In behavioral assays, we used freshly clipped flowers from 13 *Solanum tridynamum* plants raised

161   in a university greenhouse. Eight plants produced white morph flowers and 5 plants produced

162   purple morph flowers. Ten *S. tridynamum* plants were provided by a local museum (Arizona-

163   Sonora Desert Museum, Tucson, AZ) and a further 3 plants were raised from seeds. Plants were

164   fertilized weekly (Miracle Gro, NPK = 15-30-15) and grown under natural light in a portable

165   field cage (1.8 x 1.8 x 1.8 m, BioQuip Products, Inc., USA). Approximately 1444 flowers were

166   used in experiments.

167

168   Each of 4 floral morphs used in our experiment was constructed from 2 freshly clipped flowers

169   (see Russell et al. 2015). One flower had its anthers excised where the filament joined with the

170   corolla (leaving the "corolla"). The other flower had its perianth mostly removed, leaving a circle

171   of corolla tissue to which the stamens, including their anthers, were joined. This circle of tissue

172   was hot-glued into the center of the flower that had had its anthers removed. Four morphs were

173   produced in this way (Fig. 3). Two were mosaic morphs of contrasting color combinations: white

174   morph anthers glued to purple morph corolla (mosaic 1: $P_C W_A$) and purple morph anthers glued

175   to white morph corolla (mosaic 2: $W_C P_A$). The other 2 were sham morphs of the naturally-

176   occurring color combination: white morph anthers glued to white morph corolla (white sham:

177    $W_C W_A$) and purple morph anthers glued to purple morph corolla (purple sham: $P_C P_A$). We did

178   not observe any wilting or browning in these morphs. Control assays comparing sham and intact

179   purple flowers confirmed that cutting and gluing the tissue in this way did not affect bee

180   behavior (see Russell et al. 2015).

181

182   In test assays, we manipulated receipt of a pollen reward using glue. To create unrewarding

183   flowers, drops of glue (Elmer's Glue All, Elmer's Products, Inc.) were applied to the tip of each

184   poricidal anther with a clean toothpick and allowed to dry for 5 minutes. The glue sealed the

185   anther pore, preventing release of pollen.

186

187   *Experimental Protocol*

188   All trials took place in a foraging arena (LxWxH, 82cm x 60cm x 60cm) painted gray on floor

189   and sides. For both training and testing, freshly clipped flowers were horizontally displayed

190   (their natural orientation) on custom-built water tubes (see Russell et al. 2015), to prevent

191   desiccation. The water tubes were Velcro mounted on the arena wall, facing the flight chamber's

192   nest entrance. Flowers were arranged on the board in a Cartesian grid with each water tube

193   spaced 7 cm apart in the horizontal and vertical axes of the grid. Fresh flowers were used at the

194   start of every trial and for every bee. Flowers were never reused across training and testing, or

195   across bees. We systematically alternated the 5 treatments in time to control for effects of day

196   and time of day on behavior.

197

198   Our experiment consisted of 5 experience treatments (1 no-experience treatment and 4 morph-

199   experience treatments). In the no-experience treatment, we assayed innate preference in terms of

200   first visits to the 4 floral morphs as well as preference beyond the first visits. We presented

201   flower-naïve bees with an array composed of 2 flowers of each of the 4 morphs, all of which

202   were unrewarding. Flowers were arranged in a 3x3 grid without a central flower (8 total targets).

203   Targets of different types were assigned to positions such that all position–target–type

204   combinations were equally represented across all trials and no single type of target appeared

205   more than once in a row or column within a given array. To initiate testing, a single flower-naïve

206   individual was introduced into the arena. We recorded landings made by the test bee on the

207   various morphs, a landing being defined as the bee touching the flower with at least 3 of its legs

208   simultaneously. Landings consisted of 2 types: landings with sonication buzzes and landings

209   without sonication buzzes. Sonication buzzes were identified by their distinctive sound and

210   occurred only after a bee had landed. Bees were allowed to make up to 20 landings with

211   sonication buzzes after which the trial was terminated.

212

213 The 4 morph-experience treatments evaluated the degree to which preference for a floral morph

214 was shaped by collection of pollen. We used an absolute conditioning (S+) protocol (Giurfa

215 2007). Bees were individually trained and tested. No pre-training of any kind was used. Each

216 conditioning treatment corresponded to training on 1 of the 4 morphs described above. To

217 initiate training, a vertical array of 9 flowers of a single flower morph in a 3 x 3 grid was set up

218 in the arena. One to 4 flower-naïve individuals were then introduced into the arena

219 simultaneously. When a bee landed on a flower in a training array, the other bees were quickly

220 removed from the arena by catching them with vials and returned to the colony. While these bees

221 were being captured, the test bee continued to visit flowers and did not exhibit signs of being

222 threatened by our activity, such as aggressive behavior or attempts to escape from the arena.

223 Bees in these treatments were allowed to collect pollen for 20 flower visits in a single training

224 trial. We used the occurrence of sonication buzzes as a proxy for pollen collection, because

225 sonication is the only behavior these bees use to extract pollen from the flower's poricidal

226 anthers. It is a consistent and reliable indicator of pollen collection. By this criterion, all bees in

227 all conditioning treatments successfully collected pollen in 20 visits.

228

229 After the training trial, the test subject was labeled with a unique color combination of acrylic

230 paint and returned to the colony. When the test subject emerged from the nest, always within 20-

231 40 minutes after training, it was allowed to forage in a test array identical to that used in the no-

232 experience treatment (2 unrewarding flowers of each of the 4 morphs; see above). Bees were

233 allowed to make up to 20 landings with sonication buzzes after which the test trial was

234 terminated. A testing trial was occasionally terminated before 20 visits were made, if the bee did

235 not forage on the array for a period of 5 minutes. We used all landings, with or without

236   sonication buzzes, to estimate preference, and to understand how pollen rewards affected

237   preference.

238

239   During training and testing, bees made repeated visits to the same flowers within an array.

240   Although floral pollen would have diminished in flowers over time as a bee collected it during

241   training, no bees appeared to deplete individual flowers of their pollen. To confirm that bees

242   visited multiple flowers in a given trial, both training and testing, we counted the number of

243   unique flowers landed upon in the trial. All bees in all treatments, both in training (if any) and

244   testing, visited multiple flowers. We included all visits (including revisits) in our analyses.

245

246   To facilitate recording of behavior, video for all tests was captured at 30fps with a high-

247   definition digital camcorder (Canon VIXIA HF R400) positioned in front of the array. Audio

248   was input to the camcorder using an external microphone (33-3013 Lavaliere Microphone,

249   RadioShack) attached to the center of floral arrays. A Zoom H2 Handy Recorder (ZOOM

250   Corporation) was used to amplify and verify sonication buzzes in training and test trials.

251

252   *Data Analyses*

253

254   All behavioral data from the experiment were analyzed using R v.3.2.0 (R Development Core

255   Team). We used all landings on various flower morphs to estimate preference.

256

257   We used an exact multinomial test (EM) to analyze whether naïve bees had a preference for any

258   of the 4 flower morphs with their first landing choice that was different from the expected (25%;

259   i.e. random expectation for an assay with 4 choices). We report the likelihood ratio via the

260   xmulti() function in the XNomial package (Engels 2015). In addition we ran chi-square (χ^2) tests

261   via the chisq.test() function in R to analyze whether naïve bees had a preference for the purple

262   sham morph over others and/or whether they preferred the white sham morph over others, with

263   their first landing choice. Lastly we used a χ^2 test to analyze whether naïve bees had a preference

264   for mosaic flowers (P_CW_A and W_CP_A pooled) versus pure sham flowers (P_CP_A and W_CW_A

265   pooled).

266

267   To analyze whether the proportion of landings by initially flower-naïve bees differed between

268   mosaic flowers (P_CW_A and W_CP_A pooled) and pure sham flowers (P_CP_A and W_CW_A pooled), we

269   ran paired *t*-tests via the aov() function in R.

270

271   We used a hierarchical Bayesian model (BayesPref package) designed for multinomial count

272   data to analyze differences in preference across the 4 morphs (a detailed description of this

273   analysis can be found in Fordyce et al. 2011; Forister & Scholl 2012). MCMC runs were

274   conducted for 40,000 generations with the first 10,000 generations discarded as burn-in for all

275   analyses. Using the 'plot' diagnostic tool, MCMC samples were examined to confirm even

276   sampling of the posteriors.

277

278   We utilized pairwise comparisons of posterior probabilities (i.e., 'PP') to identify significant

279   differences among estimates of preference for each of the 4 morphs (BayesPref package). When

280   preference for a particular morph is greater than preference for another morph in more than 95%

281   of the sampled MCMC steps, preference estimates are considered to be significantly different

282   (Fordyce et al. 2011). Posterior probabilities can be interpreted similarly to P-α (where α = 0.05)

283   in a frequentist approach. Because pairwise comparisons give values for both choice A over B

284   and choice B over A (values that are complementary: A over B is equal to 1-[B over A]), we

285   report only the smaller value. We use a Bayesian approach (rather than MANOVAs or GLMERs,

286   for instance), because to our knowledge it is the only statistical approach that reports pairwise

287   differences and simultaneously does not suffer from inflated type I/II error rates when analyzing

288   differences between categories that (a) are not independent, (b) lack moderate correlation

289   between dependent variables, (c) have outliers, (d) and do not have homogeneity of variances.

290

291   To analyze the effect of experience on corolla or anther morph preference we used binomial

292   generalized linear mixed effect models (GLMERs), specifying type II Wald chisquare (χ^2) tests

293   via the Anova() function in the car package (Fox 2015). For these models we included 'BeeID'

294   as a random factor and visits as repeated measures within BeeID and the response variable

295   'morph choice' (white or purple corolla; white or purple anther) and the factor 'treatment'

296   (Naïve, P_CP_A, P_CW_A, W_CW_A, W_CP_A). GLMERs were carried out using the glmer() function in

297   the lme4 package (Bates et al. 2015). In cases of significant effects, we ran Tukey's post hoc test

298   using the glht() function in the multcomp package (Hothorn et al. 2015) to determine which pairs

299   were significant.

300

301   For all GLMERs, maximal models were run first. For each analysis, we performed 2 rounds of

302   backward elimination (as described in Fox 2015). We checked first whether any interaction

303   terms should be eliminated from the model and then whether any main effects should be

304   removed. We used the anova() function in R to examine significance for each of these effects

305   relative to the full model.

306

307   To analyze potential interactions between corolla and anther morphs, we used mixed

308   multinomial logit models (MMNLs). We included 'BeeID' as a random factor and the fixed

309   factors 'treatment' (P_CP_A, P_CW_A, W_CW_A, W_CP_A, and Naïve), 'anther choice' (purple or white),

310   and 'corolla choice' (purple or white). We also ran MMNLs for each treatment separately, to

311   examine interactions within a treatment. MMNLs were carried out using the mlogit() function in

312   the mlogit package (Henningsen & Toomet 2011, Croissant 2012).

313

314   *Reflectance and Irradiance Spectra and Bee Color Space*

315

316   We characterized the color of flower morphs, leaves, and the background against which the

317   flowers were presented in experiments, using reflectance and irradiance spectra. Each reflectance

318   spectrum consists of the mean of 5 measurements. Each measurement was taken from a different

319   leaf or flower (or part of the foraging arena). Reflectance spectra for all samples were measured

320   using an UV-VIS spectrophotometer (Ocean Optics USB2000) with tungsten-deuterium light

321   source (Ocean Optics DH2000) and a fluoropolymer white standard (USRS-99-010 Spectralon;

322   Labsphere, NH, USA). An RPH reflectance probe (Ocean Optics) was held at constant height

323   and angle above the samples using a holder that shielded the probe from extraneous light.

324   Reflectance measurements were taken using a 5ms integration time in the same session.

325

326   Irradiance within the flight arena was measured at the center of the foraging array using a P600

327   UV/VIS optical fiber (Ocean Optics), a CC-3-UV-T cosine-corrected (180 degrees) irradiance

328   probe (Ocean Optics), and a tungsten-deuterium calibration light source (Ocean Optics

329   DH2000). Irradiance measurements were taken using a 50ms integration time.

330

331   To characterize what bees perceived, we used our reflectance and irradiance measurements to

332   plot color morphs within a color space for *B. impatiens*. The color space diagram (i.e. color

333   hexagon) and table were made in accordance with Chittka (1992), using data on receptor spectral

334   sensitivities for *B. impatiens* from Skorupski & Chittka (2010). Chromatic contrast was

335   measured as the Euclidean distance between stimuli in the color hexagon (Chittka 1992).

336   Achromatic contrast was measured as the green contrast between stimuli (Giurfa & Vorobyev

337   1997). We used the arena wall on which the flowers were displayed as the background stimulus

338   for the color hexagon and the irradiance of the overhead arena lights in calculations of receptor

339   excitation values. For visual contrast measurements presented in Table 1, we additionally used *S.*

340   *tridynamum* leaves, corolla, and anthers as the background stimulus.

341

342

343   RESULTS

344

345   *Initially-flower-naïve bees did not express preferences for one flower morph over another*

346

347   In the no-experience treatment, there was no significant difference in the number of flower-naïve

348   bees that made their first landing on any of the 4 flower morphs, compared to the expected

349   number of first landings (EM: P = 0.859; % bees that made their first landing on P_CP_A: 16.7;

350   P_CW_A: 33.3; W_CW_A = 22.2; W_CP_A = 27.8, N = 18). In particular, bees did not innately prefer the

351   purple sham morph over others; neither did they prefer the white sham morph over others (χ^2-

352   tests: P_CP_A versus P_CW_A: χ^2 = 1, P = 0.317; P_CP_A versus W_CW_A: χ^2 = 0.1429, P = 0.706; P_CP_A

353   versus W_CP_A: χ^2 = 0.5, P = 0.480; W_CW_A versus P_CW_A: χ^2 = 0.4, P = 0.527; W_CW_A versus W_CP_A:

354   χ^2 = 0.0909, P = 0.763). Bees tended to make more first landings on the mosaic morphs than on

355   the sham morphs, but the difference did not approach statistical significance (χ^2-test: χ^2 = 0.889, P

356   = 0.346; % bees that made their first landing on shams: 38.9; or on mosaics: 61.1, N = 18).

357

358   Because bees in the no-experience treatment foraged exclusively on unrewarding flowers, we

359   might expect their overall preference to reflect the pattern shown in first landings. This was

360   indeed the case. These bees showed no significant landing preference for any 1 of the 4 morphs

361   over another (Fig. 4; differences in pairwise posterior probabilities: P_CP_A versus P_CW_A, PP =

362   0.2718; P_CP_A versus W_CW_A, PP = 0.2381; P_CP_A versus W_CP_A, PP = 0.3548; P_CW_A versus

363   W_CW_A, PP = 0.0927; P_CW_A versus W_CP_A, PP = 0.4036; W_CP_A versus W_CW_A, PP = 0.1269). In

364   particular, they did not prefer the purple sham morph over others; neither did they prefer the

365   white sham morph over others (Fig. 4). In fact, initially flower-naïve bees showed a significant

366   landing preference for the mosaic morphs (P_CW_A and W_CP_A pooled) over the sham morphs (P_CP_A

367   and W_CW_A pooled) (paired t-test: t_{17} = -2.9619, P < 0.009; mean % landing choice for mosaic

368   flowers ± SE: 56.5 ± 2.2). In other words, flower-naïve bees foraging on unrewarding flowers

369   showed a landing preference for morphs of *S. tridynamum* that do not, to our knowledge, occur

370   in nature.

371

372 *Bees expressed stronger learned preferences when given experience with flowers with purple*

373 *corollas*

374

375 Bees given experience on flower morphs with purple corollas and then tested in the mixed array

376 after 20-40 minutes expressed strong, significant landing preferences for morphs with purple

377 corollas relative to morphs with white corollas (Fig. 5a, 5b). The anther-corolla combination of

378 experience did not significantly affect landing preferences (Fig. 5a, 5b; exact differences in

379 pairwise posterior probabilities are found in Table S1, S2).

380

381 In contrast, bees given experience on flowers with white corollas showed weak landing

382 preferences for flowers with white corollas in the test condition (Fig. 5c, 5d). Specifically, bees

383 given experience on W_CP_A exhibited no significant preference and bees given experience W_CW_A

384 exhibited a small, but significant preference for W_CW_A flowers relative to P_CP_A flowers, but to

385 neither of the other 2 combinations (Fig. 5c, 5d; exact differences in pairwise posterior

386 probabilities are found in Table S3, S4).

387

388 *Learned preferences were mediated by the corolla, but not the anther*

389

390 To examine the role of corolla and anther in learned preferences, we use the same datasets used

391 above (results from all 5 treatments' test condition), but we pooled data across morphs to

392 estimate corolla morph preference and anther morph preference. Overall there were differences

393 among treatments in their percent landing response to morphs with a purple corolla (Fig. 6a;

394 GLMER overall effect: Type II Wald χ^2 test: $\chi^2 = 32.501$, df = 4, P < 0.0001). Specifically,

395   treatments where bees were trained to morphs with a purple corolla showed a significant landing

396   preference in the test condition for either morph with purple corolla; treatments where bees were

397   trained to morphs with a white corolla however showed no significant landing preference

398   (according to Tukey's post hoc tests; Fig. 6a). Overall there were no differences among

399   treatments in percent landing response to morphs with a purple anther (Fig. 6b; GLMER overall

400   effect: Type II Wald χ^2 test: $\chi^2 = 5.4494$, df = 4, P = 0.2442). Likewise, bees in none of the

401   morph-experience treatments showed significant landing preferences in the test condition for the

402   anther morph of experience (according to Tukey's post hoc tests; Fig. 6b).

403

404   *Anther and corolla generally did not interact to affect preference*

405

406   Across the 5 treatments, there was a significant effect of corolla morph, but not anther morph, on

407   floral landing preference in the test condition (MMNL: corolla morph effect: $t = 2.8457$, P <

408   0.0045; anther morph effect: $t = 0.2823$, P = 0.7777). In addition there was a trend for an overall

409   interaction between anther and corolla morph on landing preference in the test condition which

410   was not significant (MMNL; anther morph x corolla morph effect: coefficient estimate = -

411   0.4797, $t = -1.6467$, P = 0.0996). Specifically, there was a tendency for bees to prefer mosaic

412   morphs over sham morphs. This effect was larger with respect to morphs with white anthers than

413   morphs with purple anthers.

414

415   Furthermore, using MMNLs to examine each treatment independently, we found no significant

416   interaction between effects of anther and corolla morph on landing preference in the test

417   condition of any treatment. However, for bees in the no-experience treatment, as well as bees

418 that had been given experience with P_CW_A flowers, there was a trend for an interaction in the test

419 condition; as above, there was a tendency for bees to prefer mosaic morphs over sham morphs

420 (MMNL: naïve treatment: coefficient estimate = -0.43507, t = -1.8172, P = 0.0692; P_CW_A

421 training morph treatment: coefficient estimate = -0.72249, t = -1.6563, P = 0.0977).

422

423 *Spectra and Color Space*

424

425 The 2 pure morphs exhibit similar chromatic contrast from the arena background and should be

426 readily discriminable (Fig. 2, Fig. 7). The white morph however exhibits much less achromatic

427 contrast from the arena background, compared to the purple morph; against a more natural

428 background, a *S. tridynamum* leaf, these patterns change little (Table 1). Against the *S.*

429 *tridynamum* leaf the purple morph, however, exhibits even greater chromatic contrast, while the

430 white morph exhibits much less chromatic contrast (Table 1).

431

432 *Results summary*

433

434 Summing up, the results show that initially-naïve bees exhibited no landing preference for any 1

435 morph either on their first landing (when bees were truly flower-naïve) or after foraging in an

436 array where no flowers were rewarding. These bees did not prefer the morphs that occur in

437 nature. In fact, after searching in an array of all-unrewarding flowers, the bees showed an overall

438 landing preference for the mosaic morphs, preferring patterned- over unpatterned (sham)

439 morphs.

440

441   Despite the apparent absence of an innate preference, bees showed a bias in terms of how

442   experience shaped preference: experience with the purple morph had a greater effect on

443   preference than experience with the white morph. Learned preferences, and the bias in learning,

444   were mediated by corolla identity and not anther identity. Finally, bees did not adopt a stronger

445   preference for the specific anther-corolla combination with which they had been given

446   experience.

447

448

449   DISCUSSION

450

451   Pollinator preference can reflect innate dispositions (including sensory bias and adaptive innate

452   preference) as well as effects of experience. Both innate and learned responses can shape the

453   evolution of the floral display (Schiestl & Johnson 2013). The results presented here suggest that

454   the evolution of floral color signals may conceivably reflect how readily particular colors are

455   learned. While we did not find evidence of innate bias in color responses for naïve bees, we did

456   find a bias in the outcome of experience. Specifically, bees learned strong preferences for a

457   purple morph of *Solanum tridynamum*, but learned only weak ones for a co-occurring white

458   (hypochromic) morph. To our knowledge, this is the first demonstration that pollinators show a

459   bias in learning particular color morphs within a naturally-occurring floral color polymorphism.

460   It is likewise the first evidence that pollinators can learn a preference for a color morph in the

461   context of pollen collection; virtually all studies of pollinator behavior and floral color

462   polymorphism involve nectar as the floral reward.

463

464   Floral color polymorphisms involving hypochromic morphs are common, perhaps because such

465   morphs are relatively readily generated by loss-of-function mutations in genes for floral pigment

466   production (Rausher 2008; Wessinger & Rausher 2012). These morphs, which are generally

467   human-white in color, are often found in low frequencies in color polymorphic populations

468   (Table S5). Our findings here suggest that biases against learning the color of these morphs

469   might curtail the success of such morphs and perhaps even contribute to the low frequencies in

470   which they occur. To further address these possibilities, it would be interesting to examine

471   preference learning in more pollinator species for more plant species that exhibit color

472   polymorphisms involving hypochromic morphs.

473

474   Why might bees have difficulty forming a preference for hypochromic morphs? One possibility

475   is that biased learning reflects a difference in reward quality between the 2 morphs. In our study,

476   pollen was the sole reward. If the white morph offered less or poorer pollen, we would expect

477   learning of flowers with white anthers to be less effective than learning of flowers with purple

478   anthers. This was not the case. For example, bees learned purple corollas paired with white

479   anthers just as well as they learned purple corollas paired with purple anthers, which we would

480   not expect if anthers of different colors offered rewards of different value. Biased learning was

481   more likely due to differences in the distinctiveness of each morph's floral visual cues. Mapped

482   onto bee color space (Chittka 1992), the 2 morphs exhibit similar chromatic contrast from the

483   arena background and should be readily discriminable (Fig. 7, Table 1). However, compared to

484   the pigmented morph, the hypochromic morph exhibits very little achromatic green contrast with

485   the arena background. Furthermore, we would expect differences in more natural conditions:

486   when we examined achromatic and chromatic contrast against a *S. tridynamum* leaf, we found

487 that while achromatic contrast changes little, the purple morph exhibited much greater chromatic

488 contrast, while the white morph exhibited even less chromatic contrast (Fig. 7, Table 1). As a

489 consequence, we expect that the hypochromic *S. tridynamum* morph is likely even less

490 discriminable in real-world foraging conditions. Although bees use achromatic contrast to both

491 detect and learn floral colors, achromatic contrast is much more important in the latter task (Dyer

492 2008), which could explain why bees in our naïve preference test showed equal preference for

493 the 4 flower types.

494

495 While bees did not innately prefer any of the morphs, initially-naïve bees foraging on

496 unrewarding flowers surprisingly showed a preference for the mosaic morphs, the equivalent of

497 which is not found in nature, over the naturally-occurring morphs. The mosaic morphs show

498 greater contrast in color between corolla and anther, which might explain the preference. A

499 number of studies have demonstrated that foraging bees exhibit innate preferences for artificial

500 flowers with strong center-surround ('bulls-eye') color contrast and furthermore, are able to learn

501 such patterns when paired with a nectar or pollen reward (Lunau et al. 1996; Hempel de Ibarra et

502 al. 2001; Muth et al. 2016).

503

504 Plants frequently evolve floral display traits that cause flowers and floral rewards to be more

505 attractive or easier to locate for pollinators (Lunau 1991; Leonard & Papaj 2011; Hempel de

506 Ibarra et al. 2015). Given that initially-naïve bees exhibited significant preferences for patterned

507 (mosaic) flowers in our study, we might expect to see similarly-patterned flowers in nature. That

508 we have not seen these patterned (mosaic-like) morphs invites speculation as to why. Possibly,

509 anthocyanin pigmentation in *S. tridynamum*'s anther and corolla is controlled by a single

510   pathway and mutations in the function or regulation of the pathway necessarily involve its

511   complete disruption throughout both floral structures. Alternatively, mutations giving rise to

512   patterned morphs occur, but selection may not favor a contrasting pattern for various reasons.

513   For example, a cryptic anther might reduce attack by a florivore. In addition, bees in our study

514   did not adopt a significantly stronger preference for the specific anther-corolla combination with

515   which they had been given experience, even for the mosaics. As such, there may be less selection

516   for a visually-contrasting anther than the overall results of the naïve test might imply.

517

518   In conclusion, pollen-foraging bees can learn preferences for flower morphs of a particular plant

519   species. Furthermore, the strength of these preferences is dependent on the corolla morph that the

520   bee was conditioned to. Although both sensory biases and learning undoubtedly contribute to

521   floral trait evolution generally, our results suggest learning may influence the evolution of floral

522   color polymorphisms. In addition, biases in learning may be a key, yet often overlooked,

523   mechanism driving the evolution of floral display traits generally (ten Cate & Rowe 2007) and

524   the maintenance of floral color polymorphisms specifically. We predict that much as innate

525   preferences are often conserved within a pollinator group (e.g. Lunau & Maier 1995), biases in

526   learning may also be conserved within a given pollinator functional group. We suggest that

527   future experiments should test this prediction directly by examining and comparing the learning

528   performance of bees (not just well-studied honey- and bumble bee species) and other important

529   pollinator taxa, such as flies and butterflies, on hypochromic and pigmented floral morphs.

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682

683 TABLES, FIGURES & FIGURE LEGENDS (figures are low-resolution versions for review)

684

685 **Table 1**: Visual contrast between flower structures and background stimuli

Background stimulus	Chromatic contrast					Achromatic contrast				
	Purple corolla	Purple anther prox.	Purple anther distal	White corolla	White anther	Purple corolla	Purple anther prox.	Purple anther distal	White corolla	White anther
Arena	0.137	0.220	0.074	0.283	0.369	0.318	0.031	0.344	0.013	0.009
Leaf	0.425	0.101	0.226	0.117	0.210	0.254	0.064	0.288	0.081	0.102
Purple corolla	NA	0.291	0.123	0.350	0.424	NA	0.298	0.047	0.309	0.323
White corolla	0.350	0.087	0.222	NA	0.108	0.309	0.018	0.337	NA	0.022

686

687

688 **Figure 1**

689

690 **Figure 1:** Many color polymorphic species exhibit both highly pigmented and hypochromic

691 flower morphs, as in (a, b) *Geranium thunbergii*, (c, d) *Aquilegia coerulea*, and (e, f) *Antennaria*

692 *dioica*. *G. thunbergii* offers pollen and nectar rewards and is pollinated by *Hymenoptera*

693 (especially bees), but also some *Diptera* and *Lepidoptera* (Kandori 2002). *A. coerulea* offers

694 pollen and nectar rewards and is pollinated by *Hymenoptera* (bees) and *Lepidoptera*

695 (hawkmoths) (Brunet 2009). *A. dioica* offers a nectar reward and is pollinated by a variety of

696 *Coleoptera*, *Diptera*, *Hymenoptera*, and *Lepidoptera* (Willis & Burkill 1903). Photographs: (a, b)

697 Alpsdake, (c) Josef F. Stuefer, (d) Alvesgaspar, (e) Biodehio, (f) Isidre blanc. Licensing: (a, b, d)

698 CC BY-SA 3.0 CC, (c) CC BY 2.0, (e, f) BY-SA 4.0.

699
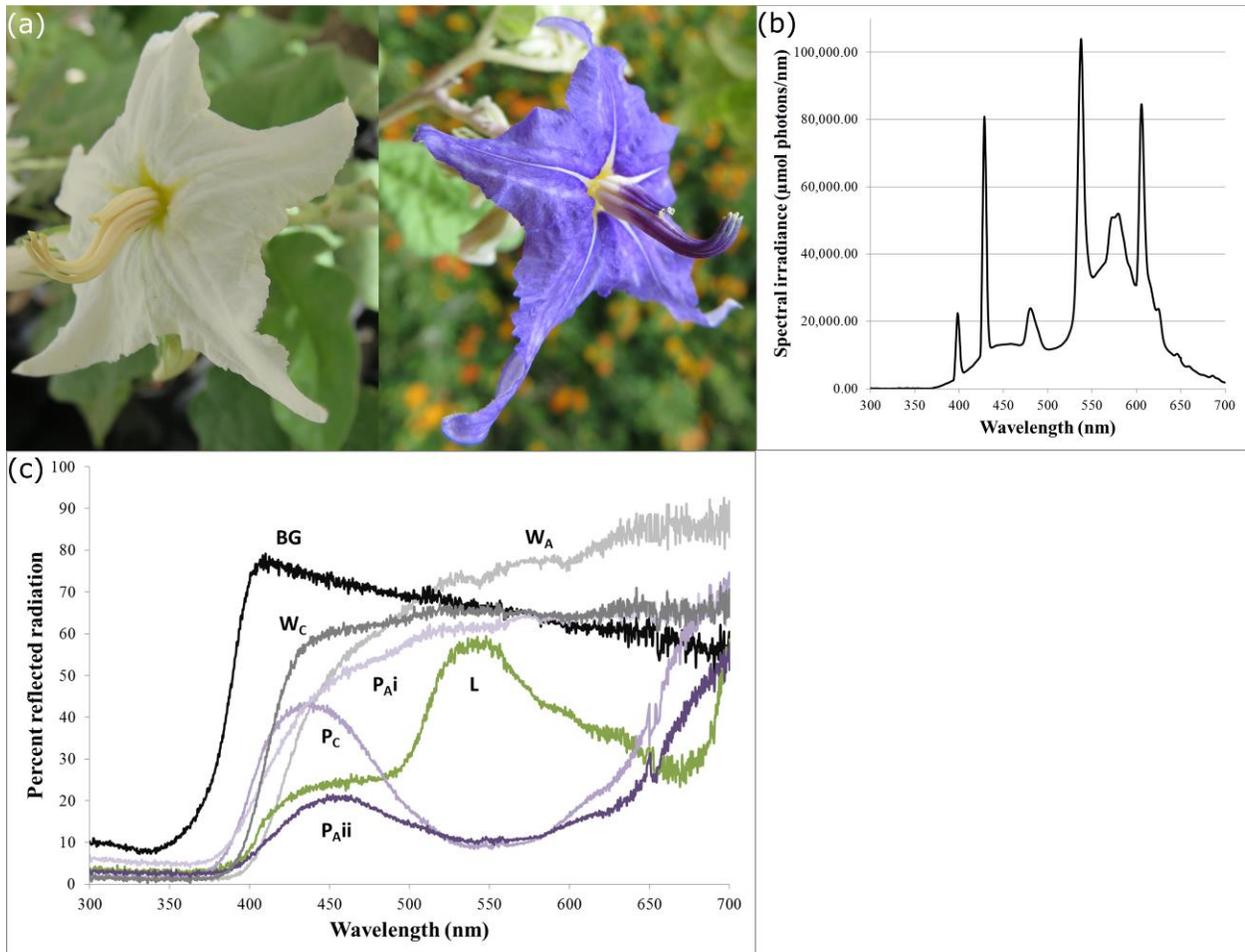
Figure 2

**Figure 2.** (a) Human-white and human-purple forms of color polymorphic *Solanum tridynamum*. (b) The irradiance of the florescent lights illuminating the foraging arena. (c) The reflectance spectra of the foraging arena background (BG) and the color polymorphic *S. tridynamum*: purple morph corolla (P_C), proximal (P_Ai) and distal ($P_A$ii) areas of anthers, white morph corolla (W_C) and anthers (P_A), and leaf (L). Spectra of the corollas of all species were made from the peripheral tissue and not the yellowish central tissue.
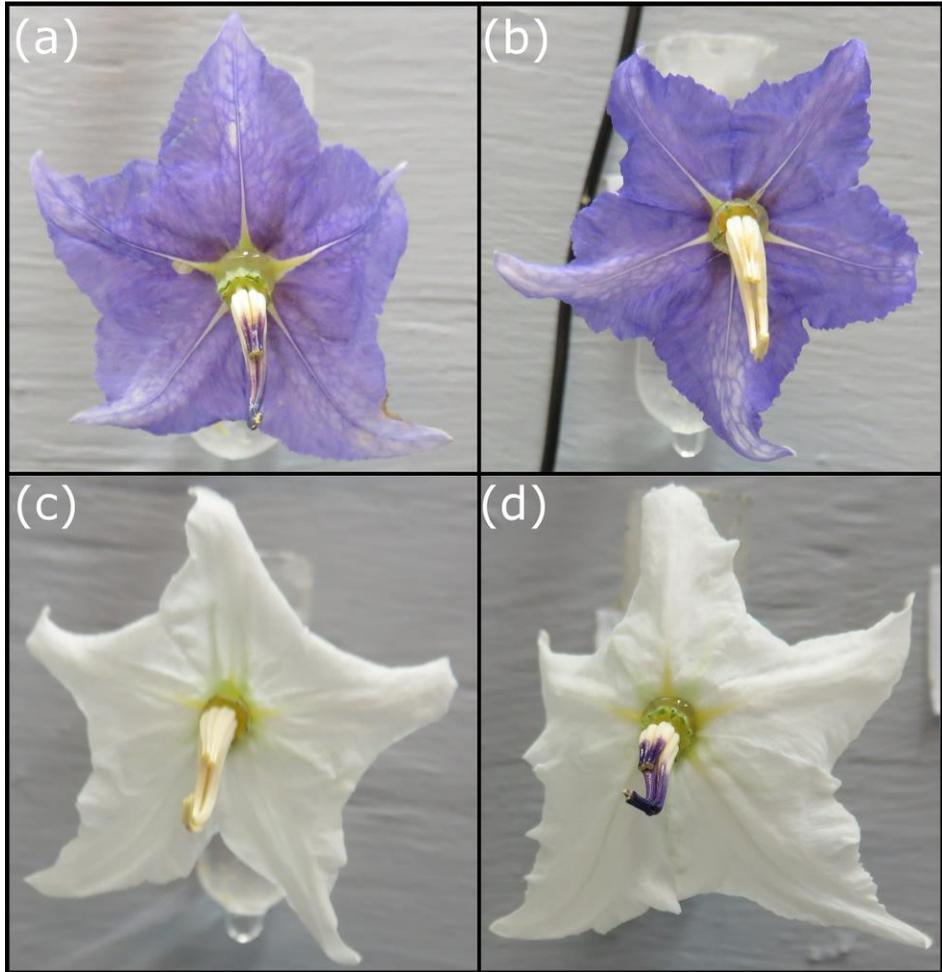
Figure 3

Figure 3: Sham and mosaic *S. tridynamum* flowers used in the study. (a) Purple morph anthers glued to purple morph corolla (purple sham: P_CP_A), (b) white morph anthers glued to purple morph corolla (mosaic 1: P_CW_A), (c) white morph anthers glued to white morph corolla (white sham: W_CW_A), (d) purple morph anthers glued to white morph corolla (mosaic 2: W_CP_A).

715 **Figure 4**
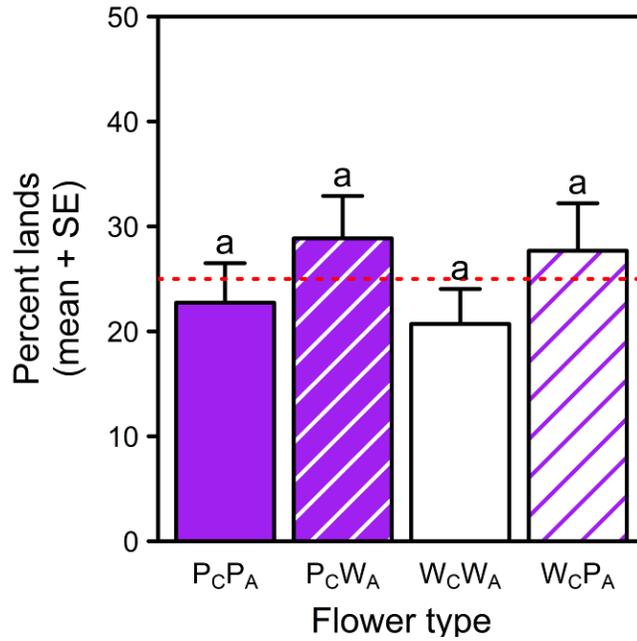
716

717 **Figure 4**. Floral preference for initially-naïve bees visiting mixed arrays in the no-experience

718 treatment. N=18 bees. The hatching color indicates the anther morph color and the background

719 color indicates the corolla morph color. A solid fill indicates that the anther and corolla are from

720 the same color morph. Letters indicate pairwise differences at posterior probabilities <0.05.

721 Dashed line at 25% indicates random expectation for an assay with 4 choices.

722

723 **Figure 5**.
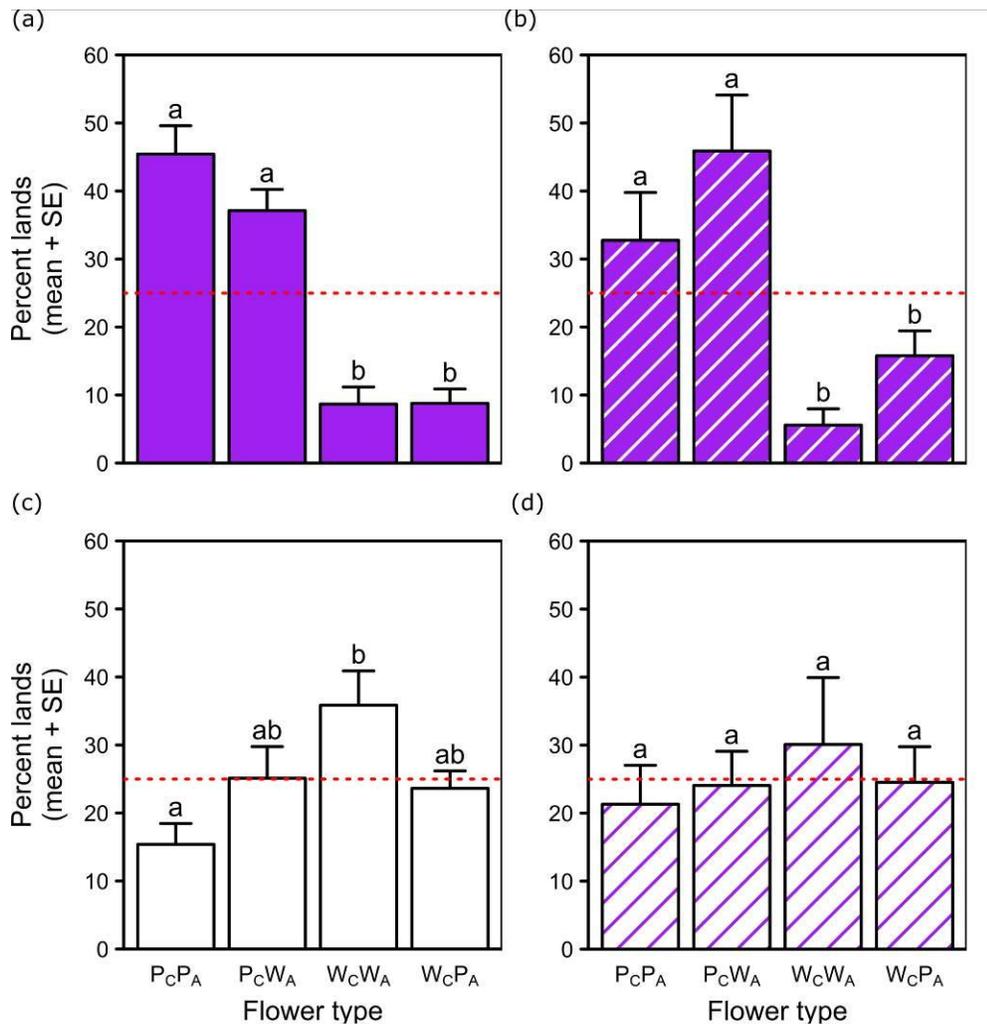
724

725 **Figure 5**. (a) Floral preference for bees experienced on (a) $P_C P_A$, (b) $P_C W_A$, (c) $W_C W_A$, and (d)

726 $W_C P_A$. $N=9$ for each treatment, aside from $N=7$ for bees given experience on $W_C W_A$. The

727 hatching color indicates the anther morph color the bee experienced and the background color

728 indicates the corolla morph color that the bee experienced during training. A solid fill indicates
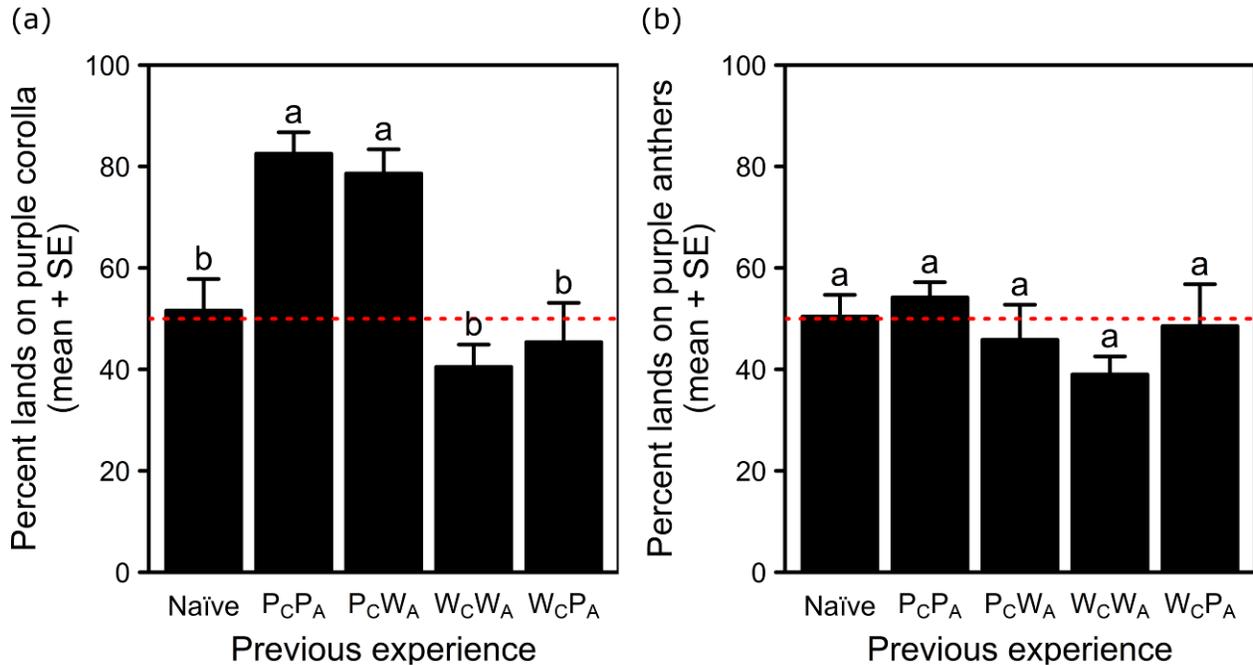
729 that the anther and corolla combination used in training were from the same color morph. Letters

730 indicate pairwise differences at posterior probabilities <0.05 within a morph-experience

731 treatment. Dashed line at 25% indicates random expectation for an assay with 4 choices.

732

733 **Figure 6**.

734

735 **Figure 6**. We use a combined dataset, presented previously in Figs. 4 and 5, but here we analyze

736 corolla morph choice independently of anther morph choice. (a) Corolla morph and (b) anther

737 morph preference, for initially-naïve and experienced bees visiting mixed arrays. $N=9$ for each

738 morph-experience treatment, aside from $N=7$ for bees given experience on W_CW_A. $N=18$ bees

739 for the no-experience (naïve) treatment. Differences in letters above bars indicate significant

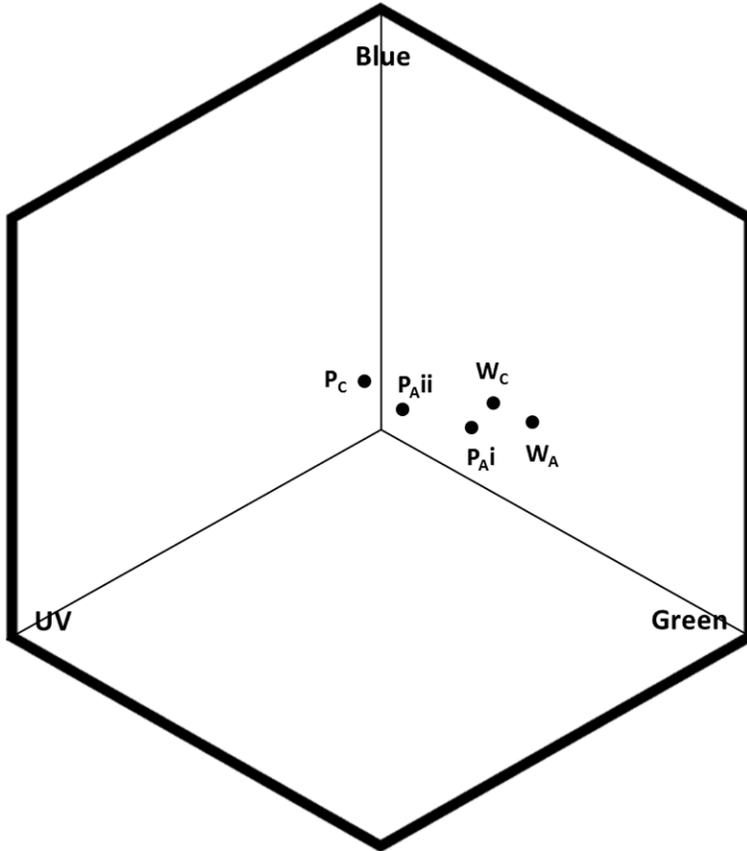740 pairwise differences at $p<0.05$ according to a Tukey's post hoc test. Dashed line at 50% indicates

741 random expectation for an assay with 2 color choices (purple versus white): consequently, bar

742 values >50% indicate a preference for purple, while bar values <50% indicate a preference for

743 white.

744

745   **Figure 7**

746

747   **Figure 7.** The loci in *B. impatiens* color space of color polymorphic *S. tridynamum*: purple

748   morph corolla (P_C), proximal (P_Ai) and distal (P_Aii) areas of anthers, white morph corolla (W_C)

749   and anthers (P_A). Spectra of the corollas of all species were made from the peripheral tissue and

750   not the yellowish central tissue.

751