

1 TITLE: The significance of prey avoidance behaviour for the maintenance of a predator colour polymorphism.

2 AUTHORS: Helena Ajuria Ibarra¹

3 Michael Kinahan¹

4 Julien Marcetteau¹

5 Andrew Mehigan¹

6 Ross Ziegelmeier¹

7 Tom Reader¹

8 ¹ Corresponding author: School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7

9 2RD, UK. Tel ++44 (0)115 9513213; email: tom.reader@nottingham.ac.uk.

10 FUNDING

11 This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT), México (Apoyo para
12 estudios de posgrado en el extranjero, 211023).

13 ACKNOWLEDGEMENTS

14 We thank Armin Pircher and Ben Bödecker from the Quinta de São Pedro Study Centre for facilitating the
15 field work. We are grateful to two reviewers for comments on an earlier version of the manuscript.

16 DATA ACCESSIBILITY STATEMENT

17 Analyses reported in this article can be reproduced using the data provided by Ajuria Ibarra et al (2018).

18

19

20 LAY SUMMARY

21 Why do some animals have variable colour patterns? We show that honeybee behaviour can favour the
22 persistence of red, yellow and white patterns in spider predators. Although it is well known that predators
23 can promote morphological diversity in their prey, our study is one of the first to show that the reverse
24 might also happen. Our results add to our understanding of the ecological processes shaping the appearance
25 of animals in natural populations.

26 TITLE: The significance of prey avoidance behaviour for the maintenance of a predator colour polymorphism.

27 ABSTRACT

28 The existence of conspicuous colour polymorphisms in animals provides an ideal opportunity to examine the
29 mechanisms which determine genetic and phenotypic variation in populations. It is well known that
30 directional and negative frequency-dependent selection by predators can influence the persistence of colour
31 polymorphisms in their prey, but much less attention has been paid to the idea that prey behaviour could
32 generate selection on predator colour morphs. In this study, we examine the role that avoidance behaviour
33 by honeybees might play in selection on a colour-polymorphic sit-and-wait predator, the crab spider *Synema*
34 *globosum*. In two field experiments, we offered flowers harbouring spiders of different colour morphs to
35 foraging honeybees. In the first, we tested for a pre-existing propensity in honeybees to avoid one spider
36 morph over another, and whether this behaviour is influenced by the flower species on which spiders hunt.
37 In the second, we tested the ability of bees to learn to avoid spider morphs associated with a previous
38 simulated attack. Our results suggest that honeybees do not impose strong directional selection on spider
39 morphs in our study population, and that avoidance behaviour is not influenced by flower species. However,
40 we find evidence that honeybees learn to avoid spiders of a colour morph that has previously been
41 associated with a simulated attack. These findings are the first empirical evidence for a mechanism by which
42 prey behaviour might generate negative frequency-dependent selection on predator colour morphs, and
43 hence potentially influence the long-term persistence of genetic and phenotypic diversity in predator
44 populations.

45 KEYWORDS

46 Frequency-dependent selection; pollination; predation; *Synema globosum*; *Apis mellifera*

47 INTRODUCTION

48 Conspicuous colour polymorphisms in animals provide intuitive and tractable study systems with which to
49 explore the origins, maintenance and phenotypic consequences of genetic diversity in natural populations
50 (e.g. recent reviews by Ajuria Ibarra and Reader, 2013; McKinnon and Pierotti, 2010; McLean and Stuart-Fox,
51 2014). For example, the study of morphs with different colour patterns in the peppered moth (*Biston*
52 *betularia* – reviewed in Cook and Saccheri, 2013) and grove snail (*Cepaea nemoralis* – reviewed in Cook,
53 2017), has generated key insights into the genetic and ecological context for adaptive evolution. In many
54 such systems, heritable colour differences among individuals are thought to influence fitness, because of
55 their effect on the behaviour of other animals, and in particular predators. Colour patterns in palatable prey
56 species, for example, can influence their detectability, or how easily they can be discriminated from other
57 less palatable species (Speed et al., 2004). This can lead to directional selection in favour of one particular
58 morph, increasing its frequency in a population, as is thought to happen when predators of the peppered
59 moth select for a melanic morph in polluted environments (Cook, 2017). Alternatively, if the fitness
60 advantage of a morph declines as it becomes relatively common, negative frequency-dependent selection
61 can operate, helping to explain the long-term persistence of phenotypic and genetic diversity. Thus,
62 “apostatic” selection, in which predators form a search image for the most common prey morph, was
63 famously invoked to explain the persistence of colour polymorphism in the grove snail (Clarke, 1962).

64 Despite the existence of a few textbook examples, the mechanisms which permit conspicuous colour
65 polymorphisms to persist in nature are unknown in most cases, and we have limited ability to generalise
66 about the relative importance of predation and frequency-dependent selection (Ajuria Ibarra and Reader,
67 2013). Studies have shown that interactions with species other than predators can generate important
68 patterns of selection on colour polymorphisms. For example, competition among males coupled with female
69 mate choice, and sexual conflict, are thought to generate frequency-dependent selection on colour morphs
70 in lizards (Fitze et al., 2014) and damselflies, respectively (Svensson and Abbott, 2005). We can easily
71 imagine that interspecific competitors or parasites might similarly be involved. In this study, however, we

72 focus on the neglected possibility that selection by prey species may influence the persistence of colour
73 polymorphisms in their predators.

74 We consider the case of a conspicuously polymorphic crab spider, *Synema globosum*, a sit-and-wait predator
75 of pollinators on flowers (Ibarra and Reader, 2014). Female *S. globosum* have a band of either bright white,
76 yellow or red on their opisthosoma, whilst males lack this band and are not polymorphic (They and Casas,
77 2009). The female polymorphism is discrete and heritable, and differences among morphs are detectable by
78 typical prey species, such as the honeybee *Apis mellifera* (Ajuria Ibarra and Reader, 2014). It is not known
79 why the polymorphism is restricted to females, and it is possible that male behaviour (e.g. mate choice or
80 harassment) could play a role in the maintenance of the diversity in female colour (Ajuria Ibarra, 2013).
81 However, males are much smaller than females, and perhaps therefore less conspicuous to their prey, and
82 they seem to spend less time hunting in exposed positions on flowers (Reader, unpublished data). Hence,
83 prey responses to colour may be especially important in determining selection on morphology in females.
84 Here, therefore, we explore the idea that prey behaviour can generate selection on colour patterns in female
85 *S. globosum*, disadvantaging those morphs which are readily detected and avoided before an attack is
86 possible.

87 The effect of prey behaviour towards a predator can lead to directional, frequency-independent selection if
88 it causes one morph to have significantly higher fitness than the alternative morphs. In the case of *S.*
89 *globosum*, if prey show lower aversion towards a particular female morph, that morph may have a higher
90 probability of making a successful attack, and increased survival and/or fecundity as a result. In a panmictic
91 population, assuming this selection is more potent than genetic drift, we would expect it to lead eventually
92 to fixation of the genotype which corresponds to the less aversive morph (Bell, 1997). However, the process
93 of fixation may be slow, and polymorphism may be observed during transition (Mitchell-Olds et al., 2007).
94 Alternatively, directional selection by prey could actively maintain phenotypic diversity in predators. The
95 response of prey to the threat of attack could depend on the local environment, so that different predator
96 morphs are more successful in different habitats or at different times (e.g. if they forage on different flower
97 species). If offspring are more likely to be found in habitats inhabited by their parents, a selection mosaic

98 might exist which favours the persistence of the polymorphism in the population as a whole (Forde et al.,
99 2004; Kondrashov and Mina, 1986). Thus, although there is no evidence for habitat (e.g. flower colour)
100 specialisation in *S. globosum* morphs (Ajuria Ibarra, 2013), directional selection, which may or may not vary
101 with habitat type, could have an important effect on the maintenance of the polymorphism.

102 The potential for negative frequency-dependent selection by prey on predator traits to promote the
103 maintenance of polymorphisms has been recognised by various authors (Arcos, 2007; Hori, 1993; Paulson,
104 1973; Roulin and Wink, 2004), but few empirical studies exist. Perhaps the best evidence supporting the idea
105 comes from the scale-eating cichlid fish *Perissodus microlepis* (Hori, 1993). Populations of *P. microlepis* show
106 a genetically-determined polymorphism for handedness. Right-handed individuals always attack “prey” fish
107 from the left side, while left-handed individuals attack from the right. Frequencies of the two morphs
108 oscillate around unity across generations. This is explained by that fact that prey more effectively guard the
109 side of their body from which they are attacked most frequently, causing the most abundant cichlid morph
110 to feed less often, and the rarer morph appears to have a reproductive advantage as a result. While this
111 system demonstrates the potential for prey to generate frequency-dependent selection on predators, and
112 possibly maintain balanced polymorphisms, few other studies have considered the possibility, and none
113 have tested it empirically (Arcos, 2007; Paulson, 1973; Roulin and Wink, 2004).

114 In theory, negative frequency-dependent selection could occur whenever prey can distinguish between
115 predator morphs, learn to associate the polymorphic trait with a potential attack, and avoid the morph that
116 is encountered more frequently. *S. globosum* is an ideal candidate for such a system, because one of its main
117 prey species is the honeybee (Ajuria Ibarra, 2013; Reader et al., 2006), which has good colour vision (Chittka
118 and Menzel, 1992; Dyer et al., 2011), and is able to learn to associate colour with positive and negative
119 stimuli (e.g. Avargues-Weber et al., 2010; Giurfa, 2007). The response of honeybees to sit-and-wait
120 predators (especially spiders) has been widely investigated. Honeybees show a negative response towards
121 spiders by avoiding flowers or orb-webs (Dukas, 2001; Reader et al., 2006; Tso et al., 2004). Therefore, some
122 spiders have evolved colouration and patterns to appear camouflaged or to attract prey by exploiting their
123 responses to flower signals (Defrize et al., 2010; Heiling et al., 2003; They and Casas, 2002). Different colour

124 morphs within a single spider species can elicit different behavioural responses from honeybees. For
125 example, a melanic morph of the giant wood orb-weaving spider *Nephila pilipes* was shown to intercept
126 significantly fewer prey than a brightly-coloured morph (Tso et al., 2004). This seems to be because the
127 brightly-coloured morph produces visual signals similar to some food resources, while the outline of the
128 melanic morph's body is significantly clearer to honeybees. Although the consequences of such differences
129 in prey capture rates for selection in *N. pilipes* is unknown, these findings underline the potential for
130 honeybees to influence the maintenance of polymorphism in their predators.

131 In the present study, we conducted two experiments to examine how interactions between *S. globosum* and
132 honeybees might generate directional or frequency-dependent selection on female *S. globosum* morphs,
133 which might in turn influence the maintenance of colour polymorphism. The first experiment tested whether
134 honeybees from a population naturally exposed to crab spider predation responded differently to different
135 colour morphs of *S. globosum* on different species of flower. Assuming that prey capture affects fitness, if
136 honeybees in this experiment show a higher propensity to visit flowers harbouring a particular spider colour
137 morph, it would suggest that selection is operating in favour of that morph, either directionally, or in a
138 frequency-dependent way (e.g. because the favoured morph is rare in the study population). Additionally, if
139 honeybee responses to a particular morph depend on the species of flower on which the spider is found, this
140 could indicate habitat-specific directional selection. The second experiment tested for an effect of a recent
141 negative experience with a particular colour morph of *S. globosum* on the subsequent response of
142 honeybees to spiders of the same or a different morph. In this experiment, a decrease in the propensity to
143 visit a flower with a particular morph caused by a recent negative experience with that morph would be
144 consistent with the idea that honeybees can exert frequency-dependent selection on *S. globosum*. The
145 results from our experiments provide the first empirical test of the hypothesis that prey behaviour can
146 favour rare predator colour morphs, and hence potentially contribute to the maintenance of colour
147 polymorphism.

148 METHODS

149 All experiments were carried out between 0930 and 1700 in meadows and open woodland at the Quinta de
150 São Pedro Study Centre, near Lisbon, Portugal (38°38'19" N, 9°11'50" W) in April 2010 and 2011. Spiders
151 were hand-collected from different flowering plants in the area, and killed by leaving them in a Perspex tube
152 filled with CO₂ for one hour, before being glued to flowers for use in the experiments. White female spiders
153 were excluded because they were found at very low frequencies (< 5 %). Honeybees were sampled whilst
154 foraging naturally on patches of flowers spread across an area of several hectares. We assume that most
155 bees encountered came from several apiaries which were maintained at the site by local farmers.

156 **Experiment 1**

157 To investigate if there was an effect of spider colour on the foraging behaviour of honeybees, flowers of
158 sage-leaf rockrose (*Cistus salvifolium*), purple viper's bugloss (*Echium plantagineum*), and lavender
159 (*Lavandula stoechas*) harbouring spiders were offered to wild honeybees. These are species on which *S.*
160 *globosum* is commonly found at the study site (Ajuria Ibarra, 2013). Four spider treatments were used for
161 each flower species: red female, yellow female, male, and control (N = 100 presentations per combination of
162 flower species and spider treatment). Ten newly-collected spiders of each type were used for each of seven
163 days of the experiment. Wheat flour mixed with water was used to glue each spider to a petal (or
164 inflorescence in the case of lavender), such that it was clearly visible to approaching honeybees. The control
165 treatment consisted of flowers with glue added, but no spider. Treatments were performed in temporal
166 blocks, with each combination of flower species and treatment being applied once in each block. Spiders and
167 treatment-flower combinations were chosen randomly within blocks.

168 Each treated flower was offered to a honeybee that was foraging on the same species at the study site. The
169 treated flower was held with a pair of tweezers at arms' length and placed within 5 cm of the flower on
170 which the honeybee was feeding. If the honeybee did not approach it, the flower was repositioned until it
171 did. When offered a flower, one of three responses by the honeybee was recorded, following (Duffield et al.,
172 1993): **Ignored** – the honeybee approached the flower but changed its course without pausing or making
173 contact with it; **Rejected** – the honeybee inspected the flower, hovering in close proximity, sometimes
174 touching it with its antennae or legs, but did not alight; **Accepted** – the honeybee alighted on the flower.

175 **Experiment 2**

176 To test if honeybees respond to spider morphs differently after previously experiencing a simulated attack
177 associated with a spider of a particular colour, individual honeybees were sequentially offered two flowers
178 of *C. salvifolius*, each harbouring a different female spider. *C. salvifolius* was used because it was the species
179 with the highest probability of acceptance in Experiment 1. Spiders were glued to flowers as above, and the
180 flower was attached with sticky tape to a 60 cm garden plant stick, before being offered to foraging
181 honeybees. This method eliminated observer collisions with the vegetation, which occasionally caused us to
182 have to terminate trials in Experiment 1.

183 First, a naïve foraging honeybee was repeatedly presented with a flower harbouring a spider by an observer
184 until the flower was accepted, or until ten rejections had been observed. We refer to these presentations as
185 “Offer 1”. We recorded the number of times the flower was presented before being accepted, as a measure
186 of a honeybee’s willingness to forage despite the presence of a spider. Next, while the honeybee was feeding
187 on the flower in Offer 1, it suffered a simulated spider attack by a second observer. The attack disturbed the
188 honeybee, which invariably flew off to another flower to recommence foraging. The honeybee was followed
189 on its foraging flight by the first observer, and repeatedly presented with a second flower harbouring a
190 different spider until that flower was accepted, or until ten rejections had been observed. We refer to the
191 second set of presentations as “Offer 2”. Again, we recorded the number of times the flower was presented
192 before being accepted, as a measure of willingness to forage. If a naïve honeybee failed to accept a flower at
193 all in Offer 1, the trial was abandoned and a different naïve honeybee was chosen. If a honeybee which had
194 already experienced an attack failed to accept the flower in Offer 2, we recorded the number of rejections as
195 10.

196 To simulate an attack from the spider, bees were prodded with a teasing needle from a dissection kit
197 attached with sticky tape to a plant stick. The attack was conducted with sufficient strength to simulate an
198 attack from a spider (which we often observed occurring naturally at the study site), but without injuring the
199 bee. Honeybees always flew away after they were attacked, but they generally stayed in the same patch of
200 *C. salvifolius* bushes, and they were lost from sight only very occasionally.

201 Four female spider treatments were used for Offer 1 and Offer 2: red followed by yellow, yellow followed by
202 red, red followed by red, and yellow followed by yellow (N = 40 pairs of flowers per treatment). Ten newly-
203 collected spiders of each type were used for each of the four days of the experiment. Spiders and treatments
204 were assigned at random. The behaviour of honeybees in response to the experiment was evaluated in three
205 ways. First, both before and after the simulated attack (Offers 1 and 2), we considered the number of times
206 a flower had to be presented before a bee accepted it. Second, the honeybee's response to a flower
207 presented after the simulated attack (Offer 2) was recorded using the criteria described for Experiment 1,
208 but two types of acceptance were considered: **Landed** – the honeybee landed on the flower, but did not
209 probe it for nectar; **Fed** – the honeybee landed and probed the flower. Third, a subjective index was used to
210 score the speed with which honeybees rejected each flower: 1 = slow (the bee hovered close to the flower
211 for more than approximately 3 sec, often touching the flower with its antennae), 2 = fast (the bee hovered
212 for approximately 2-3 sec, sometimes touching the flower with its antennae), and 3 = very fast (the bee
213 noticeably hesitated close to the flower, but only very briefly (approx. 1 sec) before flying away).

214 **Statistical Analysis**

215 Honeybee responses in Experiment 1 were analysed with generalised linear mixed models (GLMMs) with
216 binomial errors and block as a random effect. Binomial responses were: a) accepted vs. not accepted (reject
217 + ignore), b) accepted vs. rejected (with ignored flowers excluded), and c) inspected (accept + reject) vs. not
218 inspected (ignore). Differences between spider treatments were analysed by comparing a model containing
219 all four treatments to a model where the three spider treatments were collapsed into one and contrasted
220 with the control.

221 For Experiment 2, the differences in number of rejections of flowers by honeybees in Offer 1 (maximum =
222 10) between those harbouring red and yellow spiders, and among the four days on which trials took place,
223 were analysed using a non-parametric two-way analysis of variance. Responses to spider treatments in Offer
224 2 were analysed using generalised linear models (GLMs) with binomial errors. Three binary response
225 variables were used: a) accepted (fed + land) vs. not accepted, b) fed vs. not fed, and c) whether or not the
226 number of presentations which were rejected in Offer 2 was higher than in Offer 1. For the first two of these

227 variables, two separate models were fitted, one considering only the first attempt to present a flower to a
228 honeybee in Offer 2, and the other considering all attempts to present the flower (maximum = 10; i.e. did
229 the bee ever land/feed?). We expected that any effect of learned aversion to spiders encountered in Offer 1
230 would be strongest in the first of these two analyses. Day of study (1 – 4) was included as a fixed factor to
231 account for changes in honeybee behaviour over time. In all models, the significance of each term was
232 assessed using a chi-squared test statistic after backward deletion from a saturated model. A separate chi-
233 squared test was used to assess the difference in the proportion of bee responses in each rejection speed
234 class (slow, fast and very fast) among spider treatments for the first presentation of flowers in Offer 2 in
235 Experiment 2. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

236 RESULTS

237 **Experiment 1**

238 In Experiment 1, significant effects of spider presence and flower species were found on the probabilities
239 that a honeybee would inspect and accept a flower for all response variables (Figure 1 and Table 1). There
240 was a higher probability of a honeybee inspecting and landing on a flower without a spider than a flower
241 with either a red or yellow female, or a male spider, and honeybees were more likely to inspect and accept a
242 flower of *C. salvifolius* than a flower of *E. plantagineum* or an inflorescence of *L. stoechas* (Table 1). No
243 significant differences were found among spider treatments: the effect of collapsing the spider treatments
244 together in the statistical model was not significant for any of the response variables: accepted vs. not
245 accepted ($\chi^2 = 2.718$, $df = 6$, $P = 0.843$), accepted vs. rejected ($\chi^2 = 3.477$, $df = 6$, $P = 0.747$), and inspected vs.
246 not inspected ($\chi^2 = 6.484$, $df = 6$, $P = 0.371$). The interaction between spider type and flower species was not
247 significant for any response variable (Table 1). Hence, the negative effect of the presence of a spider on bee
248 behaviour was similar on all flower species.

249 **Experiment 2**

250 The behaviour of naïve honeybees at the start of Experiment 2, before they received a simulated spider
251 attack, was consistent with the results of Experiment 1. There was no significant overall effect of spider

252 colour on the mean number of times that naïve individual honeybees rejected a flower harbouring the spider
253 before accepting it in Offer 1 (two-way non-parametric ANOVA: $H = 0.40$, $df = 1$ $P = 0.529$) (Figure 2).
254 Similarly, there was no significant effect of the day of study ($H = 3.43$, $df = 3$, $P = 0.331$) on the number of
255 times that honeybees rejected flowers in Offer 1, meaning that naïve bees did not accept flowers more or
256 less quickly as the study progressed. Although there was a tendency for flowers harbouring yellow spiders to
257 be rejected more frequently by naïve bees earlier in the study, the interaction between spider colour and
258 day of study on the number of times that honeybees rejected flowers in Offer 1 was not significant ($H = 7.46$,
259 $df = 3$, $P = 0.060$).

260 There was a significant effect of the spider colour morph harboured by the flower presented to naïve
261 honeybees (in Offer 1) on the proportion of those bees which subsequently fed on a flower the first time it
262 was presented to them after a simulated spider attack (i.e. in Offer 2; see Table 2). A lower proportion of the
263 honeybees which initially encountered a red spider in Offer 1 subsequently fed on flowers in Offer 2 when
264 compared with those honeybees which initially encountered a yellow spider, regardless of spider colour
265 presented in Offer 2 (Figure 3). No such effect was observed, however, on the probability that a honeybee
266 fed on a flower at least once when all presentations of the flower in Offer 2 were considered (Table 2).
267 Similarly, no overall effect of the colour of the spider initially encountered in Offer 1 was found on the
268 probability of a honeybee accepting a flower (accept = fed + land) after a simulated attack (i.e. in Offer 2),
269 considering either just the first attempt to present the flower, or all attempts.

270 Over the four days of Experiment 2, there was no overall significant change in the proportion of honeybees
271 landing or feeding on flowers after a simulated spider attack (i.e. in Offer 2; Table 2). There was, however, an
272 interaction between day and the treatment received by naïve honeybees in Offer 1, when considering the
273 first attempt to present a flower in Offer 2, after the simulated attack. The proportion of honeybees that fed
274 on a flower after experiencing an attack associated with a red spider increased over time, but it decreased in
275 cases where bees had experienced an attack associated with a yellow spider (Figure 3). Although this
276 interaction was not significant when all attempts to present the flower in Offer 2 were considered, a similar

277 pattern was observed for the probability that a honeybee accepted a flower (regardless of whether it fed),
278 considering either just the first attempt to present a flower, or all attempts (Table 2).

279 There were no main effects of the colour of the spider encountered after the simulated attack, or of day, on
280 any aspect of honeybee behaviour towards flowers presented in Offer 2 (Table 2). The key test, however, of
281 whether learnt aversion by bees to spiders of a particular colour could impact on spider foraging success was
282 indicated by the interaction between the treatments applied before and after the simulated attack (i.e. in
283 Offer 1 and Offer 2). Assuming a two-tailed statistical test, this interaction did not have a significant effect on
284 the probability of a honeybee landing or feeding on flowers after the simulated attack, whether or not all
285 attempts to offer a flower in Offer 2 were considered (Table 2 and Figure 4). It is worth noting, however, that
286 a one-tailed test would yield a significant result in the expected direction for the proportion of bees that fed
287 on flowers the first time they were presented: bees presented with a flower harbouring a red spider in Offer
288 1 were about twice as likely to feed on a flower the first time it was presented in Offer 2 if it carried a spider
289 of a different morph, and a similar but weaker effect was observed for bees presented with a yellow spider
290 in Offer 1 (Figure 4). Importantly, there was a significant interaction between the effects of spider colour
291 before and after the simulated attack on the change in the number of rejections of flowers by honeybees
292 between Offer 1 and Offer 2. Honeybees were more likely to reject the flower after the simulated attack (in
293 Offer 2) more often if it harboured a spider of the same colour morph as the spider which they encountered
294 in Offer 1 (Table 2 and Figure 5).

295 Honeybees tended to reject flowers harbouring a particular colour of spider in Offer 2 more quickly if they
296 had previously experienced an attack associated with the same colour spider (56 % responded very fast with
297 red spiders, and 29 % very fast with yellow spiders) than if they had experienced an attack associated with a
298 different colour morph (15 % very fast for those encountering a red spider first, and 13 % very fast for those
299 encountering a yellow spider first). However, this effect of the interaction between the treatments received
300 in Offer 1 and Offer 2 on the frequency with which honeybees rejected flowers carrying spiders in Offer 2 at
301 different speeds was not significant with a two-tailed test (Chi-squared: $\chi^2 = 11.802$, $df = 6$, $P = 0.067$).

302 DISCUSSION

303 The results of these experiments showed no evidence that wild-caught honeybees discriminated between
304 the different colour morphs of *S. globosum* on different species of flowers when they first encountered them
305 in our study. However, there was some evidence that honeybees are able to learn to avoid spiders of a
306 colour morph associated with a recent simulated attack. These results suggest that, while prey in the study
307 population may not exert directional selection on predator colour patterns, they do have the potential to
308 generate frequency-dependent selection in favour of rare morphs, which could help to explain the long-term
309 persistence of the polymorphism.

310 **Directional selection**

311 The presence of spiders on flowers had significant effects on the responses of honeybees. Honeybees
312 avoided flowers where *S. globosum* was present, no matter if it was a red female, a yellow female, or a male.
313 These results support previous findings where honeybees have been observed to avoid flowers that harbour
314 spiders or cues associated with their presence (Dukas, 2001; Reader et al., 2006; Robertson and Maguire,
315 2005), and suggest that in general spiders should be under selection to develop traits which help them evade
316 detection. However, honeybees did not discriminate among spider types in Experiment 1, or between
317 female spider colour morphs at the start of Experiment 2. Furthermore, there was no effect of flower species
318 on the tendency of honeybees to avoid spiders, or on their response to different spider types.

319 These results are not consistent with directional selection favouring one *S. globosum* morph, or habitat-
320 specific selection on morphs. Combined with the fact that *S. globosum* morphs do not favour particular
321 flower types at our study site (Ajuria Ibarra, 2013), this suggests that the polymorphism in *S. globosum* is not
322 transient (Mitchell-Olds et al., 2007) and is not maintained by gene flow between sub-populations of spiders
323 which are adapted to different flower-specific niches (King and Lawson, 1995). Nevertheless, there may be
324 other forms of niche-specific selection operating in this system, such as bees being better able to avoid
325 certain colours of spider in relation to levels of light or other environmental factors. It is therefore impossible
326 to rule out spatial (selection mosaics) or temporal variation in selection affecting the persistence of the
327 polymorphism. Such processes have been observed to occur in other polymorphic species, such as the

328 marine snails of the genus *Littoraria*, where certain morphs are found at higher frequencies in different parts
329 of mangrove trees and at different positions at different times of the year (Parsonage and Hughes, 2002).

330 The results suggest that, when foraging at a site like the one studied here, where red and yellow female *S.*
331 *globosum* were roughly equal in frequency (Ajuria Ibarra, 2013), honeybee behaviour does not have strong
332 effects on the relative fitness of these different colour morphs. This is not strongly indicative of negative
333 frequency-dependent selection, but could be consistent with it, if the observed ratio of red to yellow females
334 is close to equilibrium in this population. Ultimately, time series data on morph frequencies, and ideally
335 measurements of morph fitness from a population experiencing experimental manipulation of morph
336 frequencies, are required to establish whether frequency-dependent selection is operating. Such an
337 approach has been successful in other systems: patterns of variation in fitness that are consistent with
338 frequency-dependent selection have been observed in polymorphic damselflies and lizards, both in natural
339 populations that vary in morph frequencies, and in populations where morph frequencies have been
340 manipulated (Bleay et al., 2007; Sinervo et al., 2001; Van Gossum et al., 1999). Observations of morph
341 frequencies and fitness in damselflies agree with simple genetic models that predict frequency-dependent
342 dynamics (Svensson et al., 2005; Takahashi et al., 2010).

343 **Learning and the potential for frequency-dependent selection**

344 Honeybees rejected flowers in Offer 2 of Experiment 2 relatively more often if they harboured a spider of
345 the same colour morph as one which had previously been associated with a simulated attack. Similar (but
346 non-significant) patterns were observed when considering the propensity of honeybees to feed on flowers
347 the first time they were presented in Offer 2, and the speed with which these flowers were rejected. Thus,
348 honeybees appear to have learned to associate spider colour with a simulated attack, and changed their
349 behaviour to avoid such attacks in future. These results are consistent with previous studies where bees
350 have been observed to avoid a colour associated with a negative experience (Avargues-Weber et al., 2010)
351 and to behave in a negative frequency-dependent way with respect to colour (Gigord et al., 2004; Smithson
352 and Macnair, 1997). Based on these findings, it is plausible that in a population of *S. globosum* where one
353 female morph is found at a higher frequency than the others, honeybees would experience attacks from

354 spiders of this morph more often, and would avoid them more than rarer morphs. To help confirm this,
355 supporting evidence could be obtained from studies where honeybees are exposed to flower patches with
356 varying *S. globosum* morph frequencies. Ultimately, we would also need to evaluate the effect of prey
357 avoidance behaviour on spider foraging success and fitness: we know that honeybees are common in the
358 diet of *S. globosum* (Ajuria Ibarra, 2013), but we do not know how reduced encounter/capture rates might
359 translate into reduced survival or fecundity. Nevertheless, our experiment provides the first empirical
360 evidence supporting the idea that frequency-dependent selection caused by prey behaviour could help to
361 maintain colour polymorphism in a predator. Taken together with findings of other studies of this kind
362 (Arcos, 2007; Hori, 1993), and contrasted with textbook examples in which predator search images generate
363 selection on prey, our results suggest that there is more than one way that behavioural interactions between
364 predators and prey can influence the maintenance of genetic and phenotypic diversity.

365 **Differences in learnt responses to red and yellow spiders**

366 Although wild-caught honeybees did not discriminate between spider morphs in either experiment, results
367 from Experiment 2 suggested that honeybees that had previously experienced a simulated attack from a red
368 spider were less likely to land and feed on flowers in Offer 2 than those that had previously experienced a
369 simulated attack from a yellow spider, regardless of the treatment received in Offer 2. This result could be
370 explained by the different ways in which red and yellow spiders reflect light and the different chromatic
371 contrasts of the spiders and flowers. Yellow colouration in spiders has been shown to reflect UV light (Heiling
372 et al., 2005; They and Casas, 2009; Tso et al., 2004), and measurements of *S. globosum* have shown that this
373 is true for yellow females (Ajuria Ibarra and Reader, 2014). Some spiders have been observed to use UV
374 reflection to produce visual signals similar to those produced by flowers to attract honeybees (Heiling et al.,
375 2003; Herberstein et al., 2009; Tso et al., 2004). Because UV-containing signals may more typically be used as
376 cues associated with positive stimuli (i.e. food), honeybees might not be as efficient at learning to associate
377 them with danger. Consequently, after a honeybee has experienced an attack from a red spider, it might be
378 relatively more cautious about feeding on a flower with anything that might resemble a spider than would
379 be the case after an experience with a yellow spider.

380 This effect of spider colour in Offer 1 on honeybee behaviour in Offer 2 reduced in magnitude over the four
381 days of our experiment, until the difference between the effects of experiences with red and yellow spiders
382 in Offer 1 was small and in the opposite direction (Figure 5). The change over time might be owing to the use
383 of the same population of honeybees during the experiment. It has been observed that honeybees show
384 patch fidelity (Franzen et al., 2009; Osborne and Williams, 2001; Slaa et al., 2003), and because we revisited
385 some patches on different days, individual honeybees may have been exposed to spider treatments more
386 than once over the four days. Therefore, it is possible that honeybees eventually learned to associate yellow
387 colour with predation risk, thus reducing the probability that they would accept a second flower with a
388 spider after experiencing an attack from a yellow spider. However, we sampled many patches of flowers
389 spread around a large field site which contained several apiaries, and we think that the likelihood of
390 individuals being repeatedly encountered was low. Unfortunately, because we did not mark sampled
391 individuals, it is impossible to be sure the extent to which individual learning impacted on the temporal
392 patterns in our results.

393 **Conclusion**

394 The findings presented here contribute to our understanding of how the presence of predators might affect
395 the foraging behaviour of their prey, and how these predator-prey interactions might influence the
396 maintenance of polymorphism in a population of predators. However, clearly further research is needed to
397 understand the general importance of this mechanism in the maintenance of polymorphisms in *S. globosum*
398 and other similar species, and its relative importance compared with other diversifying mechanisms.

399 REFERENCES

- 400 Ajuria Ibarra H, 2013. Maintenance of a female-limited colour polymorphism in the crab spider *Synema*
401 *globosum* (Araneae: Thomisidae). University of Nottingham.
- 402 Ajuria Ibarra H, Reader T, 2013. Reasons to be different: do conspicuous polymorphisms in invertebrates
403 persist because rare forms are fitter? *Journal of Zoology* 290:81-95. doi: 10.1111/jzo.12034.
- 404 Ajuria Ibarra H, Reader T, 2014. Female-limited colour polymorphism in the crab spider *Synema globosum*
405 (Araneae: Thomisidae). *Biological Journal of the Linnean Society* 113:368-383. doi:
406 10.1111/bij.12338.
- 407 Arcos JM, 2007. Frequency-dependent morph differences in kleptoparasitic chase rate in the polymorphic
408 arctic skua *Stercorarius parasiticus*. *Journal of Ornithology* 148:167-171. doi: 10.1007/s10336-006-
409 0114-0.
- 410 Avargues-Weber A, de Brito Sanchez MG, Giurfa M, Dyer AG, 2010. Aversive Reinforcement Improves Visual
411 Discrimination Learning in Free-Flying Honeybees. *Plos One* 5. doi: 10.1371/journal.pone.0015370.
- 412 Bell G, 1997. *The basics of selection.*: Chapman & Hall, , New York, NY.
- 413 Bleay C, Comendant T, Sinervo B, 2007. An experimental test of frequency-dependent selection on male
414 mating strategy in the field. *Proceedings of the Royal Society B-Biological Sciences* 274:2019-2025.
415 doi: 10.1098/rspb.2007.0361.
- 416 Chittka L, Menzel R, 1992. The evolutionary adaptation of flower colors and the insect pollinators color-
417 vision. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology* 171:171-181.
- 418 Clarke B, 1962. Natural selection in mixed populations of two polymorphic snails. *Heredity* 17:319-345. doi:
419 10.1038/hdy.1962.35.
- 420 Cook LM, 2017. Reflections on molluscan shell polymorphisms. *Biological Journal of the Linnean Society*
421 121:717-730. doi: 10.1093/biolinnean/blx033.
- 422 Cook LM, Saccheri IJ, 2013. The peppered moth and industrial melanism: evolution of a natural selection
423 case study. *Heredity* 110:207-212. doi: 10.1038/hdy.2012.92.

424 Defrize J, They M, Casas J, 2010. Background colour matching by a crab spider in the field: a community
425 sensory ecology perspective. *Journal of Experimental Biology* 213:1425-1435. doi:
426 10.1242/jeb.039743.

427 Duffield GE, Gibson RC, Gilhooly PM, Hesse AJ, Inkley CR, Gilbert FS, Barnard CJ, 1993. Choice of flowers by
428 foraging honey-bees (*Apis mellifera*) - possible morphological cues. *Ecological Entomology* 18:191-
429 197. doi: 10.1111/j.1365-2311.1993.tb01089.x.

430 Dukas R, 2001. Effects of perceived danger on flower choice by bees. *Ecology Letters* 4:327-333. doi:
431 10.1046/j.1461-0248.2001.00228.x.

432 Dyer AG, Paulk AC, Reser DH, 2011. Colour processing in complex environments: insights from the visual
433 system of bees. *Proceedings of the Royal Society B-Biological Sciences* 278:952-959. doi:
434 10.1098/rspb.2010.2412.

435 Fitze PS, Gonzalez-Jimena V, San-Jose LM, Heulin B, Sinervo B, 2014. Frequency-dependent sexual selection
436 with respect to progeny survival is consistent with predictions from rock-paper-scissors dynamics in
437 the European common lizard. *Frontiers in Ecology and Evolution*. doi:
438 <https://doi.org/10.3389/fevo.2014.00077>.

439 Forde SE, Thompson JN, Bohannan BJM, 2004. Adaptation varies through space and time in a coevolving
440 host-parasitoid interaction. *Nature* 431:841-844. doi: 10.1038/nature02906.

441 Franzen M, Larsson M, Nilsson S, 2009. Small local population sizes and high habitat patch fidelity in a
442 specialised solitary bee. *Journal of Insect Conservation* 13:89-95. doi: 10.1007/s10841-007-9123-4.

443 Gigord LDB, Macnair MR, Smithson A, 2004. Negative frequency-dependent selection maintains a dramatic
444 flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo (vol 98, pg
445 6253, 2001). *Proceedings of the National Academy of Sciences of the United States of America*
446 101:7839-7839. doi: 10.1073/pnas.0308117101.

447 Giurfa M, 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the
448 magic well. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral*
449 *Physiology* 193:801-824. doi: 10.1007/s00359-007-0235-9.

450 Heiling AM, Cheng K, Chittka L, Goeth A, Herberstein ME, 2005. The role of UV in crab spider signals: effects
451 on perception by prey and predators. *Journal of Experimental Biology* 208:3925-3931. doi:
452 10.1242/jeb.01861.

453 Heiling AM, Herberstein ME, Chittka L, 2003. Pollinator attraction - Crab-spiders manipulate flower signals.
454 *Nature* 421:334-334. doi: 10.1038/421333a.

455 Herberstein ME, Heiling AM, Cheng K, 2009. Evidence for UV-based sensory exploitation in Australian but not
456 European crab spiders. *Evolutionary Ecology* 23:621-634. doi: 10.1007/s10682-008-9260-6.

457 Hori M, 1993. Frequency-dependent natural selection on the handedness of a scale-eating cichlid fish.
458 *Science* 260:216-219. doi: 10.1126/science.260.5105.216.

459 Ibarra HA, Reader T, 2014. Female-limited colour polymorphism in the crab spider *Synema globosum*
460 (Araneae: Thomisidae). *Biological Journal of the Linnean Society* 113:368-383. doi:
461 10.1111/bij.12338.

462 King RB, Lawson R, 1995. Color-pattern variation in lake-Erie water snakes - the role of gene flow. *Evolution*
463 49:885-896. doi: 10.2307/2410411.

464 Kondrashov AS, Mina MV, 1986. Sympatric speciation - when is it possible. *Biological Journal of the Linnean*
465 *Society* 27:201-223. doi: 10.1111/j.1095-8312.1986.tb01734.x.

466 McKinnon JS, Pierotti MER, 2010. Colour polymorphism and correlated characters: genetic mechanisms and
467 evolution. *Molecular Ecology* 19:5101-5125. doi: 10.1111/j.1365-294X.2010.04846.x.

468 McLean CA, Stuart-Fox D, 2014. Geographic variation in animal colour polymorphisms and its role in
469 speciation. *Biological Reviews* 89:860-873. doi: 10.1111/brv.12083.

470 Mitchell-Olds T, Willis JH, Goldstein DB, 2007. Which evolutionary processes influence natural genetic
471 variation for phenotypic traits? *Nature Reviews Genetics* 8:845-856. doi: 10.1038/nrg2207.

472 Osborne JL, Williams IH, 2001. Site constancy of bumble bees in an experimentally patchy habitat.
473 *Agriculture Ecosystems & Environment* 83:129-141. doi: 10.1016/s0167-8809(00)00262-0.

474 Parsonage S, Hughes J, 2002. Natural selection and the distribution of shell colour morphs in three species of
475 *Littoraria* (Gastropoda : Littorinidae) in Moreton Bay, Queensland. *Biological Journal of the Linnean*
476 *Society* 75:219-232. doi: 10.1111/j.1095-8312.2002.tb01423.x.

477 Paulson DR, 1973. Predator polymorphism and apostatic selection. *Evolution* 27:269-277. doi:
478 10.1111/j.1558-5646.1973.tb00672.x.

479 Reader T, Higginson AD, Barnard CJ, Gilbert FS, Behavioural Ecology Field C, 2006. The effects of predation
480 risk from crab spiders on bee foraging behavior. *Behavioral Ecology* 17:933-939. doi:
481 10.1093/beheco/arl027.

482 Robertson IC, Maguire DK, 2005. Crab spiders deter insect visitations to slickspot peppergrass flowers. *Oikos*
483 109:577-582. doi: 10.1111/j.0030-1299.2005.13903.x.

484 Roulin A, Wink M, 2004. Predator-prey polymorphism: relationships and the evolution of colour a
485 comparative analysis in diurnal raptors. *Biological Journal of the Linnean Society* 81:565-578. doi:
486 10.1111/j.1095-8312.2004.00308.x.

487 Sinervo B, Bleay C, Adamopoulou C, 2001. Social causes of correlational selection and the resolution of a
488 heritable throat color polymorphism in a lizard. *Evolution* 55:2040-2052.

489 Slaa EJ, Tack AJM, Sommeijer MJ, 2003. The effect of intrinsic and extrinsic factors on flower constancy in
490 stingless bees. *Apidologie* 34:457-468. doi: 10.1051/apido:2003046.

491 Smithson A, Macnair MR, 1997. Negative frequency-dependent selection by pollinators on artificial flowers
492 without rewards. *Evolution* 51:715-723. doi: 10.2307/2411148.

493 Speed MP, Ruxton GD, Sherratt TN, 2004. *Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning*
494 *Signals and Mimicry.*: Oxford University Press, Oxford, U. K., Oxford.

495 Svensson EI, Abbott J, 2005. Evolutionary dynamics and population biology of a polymorphic insect. *Journal*
496 *of Evolutionary Biology* 18:1503-1514. doi: 10.1111/j.1420-9101.2005.00946.x.

497 Svensson EI, Abbott J, Hardling R, 2005. Female polymorphism, frequency dependence, and rapid
498 evolutionary dynamics in natural populations. *American Naturalist* 165:567-576. doi:
499 10.1086/429278.

500 Takahashi Y, Yoshimura J, Morita S, Watanabe M, 2010. Negative frequency-dependent selection in female
501 color polymorphism of a damselfly. *Evolution* 64:3620-3628. doi: 10.1111/j.1558-5646.2010.01083.x.

502 They M, Casas J, 2002. Predator and prey views of spider camouflage - Both hunter and hunted fail to notice
503 crab-spiders blending with coloured petals. *Nature* 415:133-133. doi: 10.1038/415133a.

- 504 Thery M, Casas J, 2009. The multiple disguises of spiders: web colour and decorations, body colour and
505 movement. *Philosophical Transactions of the Royal Society B-Biological Sciences* 364:471-480. doi:
506 10.1098/rstb.2008.0212.
- 507 Tso IM, Lin CW, Yang EC, 2004. Colourful orb-weaving spiders, *Nephila pilipes*, through a bee's eyes. *Journal*
508 *of Experimental Biology* 207:2631-2637. doi: 10.1242/jeb.01068.
- 509 Van Gossum H, Stoks R, Matthysen E, Valck F, De Bruyn L, 1999. Male choice for female colour morphs in
510 *Ischnura elegans* (Odonata, Coenagrionidae): testing the hypotheses. *Animal Behaviour* 57:1229-
511 1232. doi: 10.1006/anbe.1999.1100.

512

513

514

515

516 FIGURE LEGENDS

517 Figure 1. Proportion of flowers of *C. salvifolius*, *E. plantagineum*, and inflorescences of *L. stoechas*, that were
518 accepted (upper two panels) or inspected (lower panel) by honeybees. Accepted flowers are shown as a
519 proportion of all flowers (upper panel) or a proportion of only those flowers which were inspected (middle
520 panel). Flowers harboured a red or yellow female spider, or a male spider, or no spider (control). Error bars
521 are 95 % confidence intervals calculated using the binomial distribution.

522 Figure 2. Mean (\pm S.E.) number of times a flower harbouring a spider was presented to, and rejected by,
523 naïve foraging honeybees before it was accepted in Experiment 2. Spiders were of two different colour
524 morphs (red and yellow), and data are shown for trials which were conducted on four different days.

525 Figure 3. Proportion of honeybees which fed on flowers harbouring a spider on the first occasion they were
526 presented after the honeybee had experienced a simulated attack associated with either a red or a yellow
527 spider in Experiment 2 (i.e. in Offer 2 – see main text). Data are shown for trials which were conducted on
528 four different days. Error bars are 95 % confidence intervals calculated using the binomial distribution.

529 Figure 4. The effect of the spider colour morphs encountered by honeybees in Experiment 2 on the
530 proportion of honeybees which landed (response = land) and fed upon (response = feed) flowers the first
531 time they were presented to them following a simulated attack by a spider (i.e. in Offer 2 – see main text).
532 Naïve honeybees were initially presented with a flower harbouring either a red or yellow spider (“First spider
533 colour”), before being subject to a simulated attack, and then presented with a spider of the same or a
534 different colour (“Second spider colour”). Error bars are 95 % confidence intervals calculated using the
535 binomial distribution.

536 Figure 5. The effect of the spider colour morph encountered by honeybees in Experiment 2 on the
537 proportion of honeybees that rejected a flower harbouring a spider more often after a simulated attack by a
538 spider (i.e. in Offer 2 – see main text) than before the simulated attack (in Offer 1). Naïve honeybees were
539 initially presented with a flower harbouring either a red or yellow spider (“First spider colour”), before being
540 subject to a simulated attack, and then presented with a spider of the same or a different colour (“Second

541 spider colour”). Both before and after the attack, flowers were presented up to 10 times to each honeybee
542 until they were accepted. Error bars are 95 % confidence intervals calculated using the binomial distribution.

543

544 TABLES

545 Table 1. Results of binomial GLMMs testing the effect of spider treatment, flower species, and the
 546 interaction between them on honeybee responses to flowers offered in the field in Experiment 1. The effect
 547 of spider treatment (present versus control) was assessed after collapsing factor levels for female colours
 548 (see text).

Response	Spider			Flower species			Spider x flower species		
	χ^2	d.f.	<i>p</i>	χ^2	d.f.	<i>p</i>	χ^2	d.f.	<i>p</i>
Accepted vs not accepted	26.869	1	< 0.001	119.210	2	< 0.001	1.392	1	0.500
Accepted vs rejected	17.770	1	< 0.001	77.606	2	< 0.001	1.640	1	0.440
Inspected vs not inspected	8.957	1	0.003	48.010	2	< 0.001	3.174	1	0.205

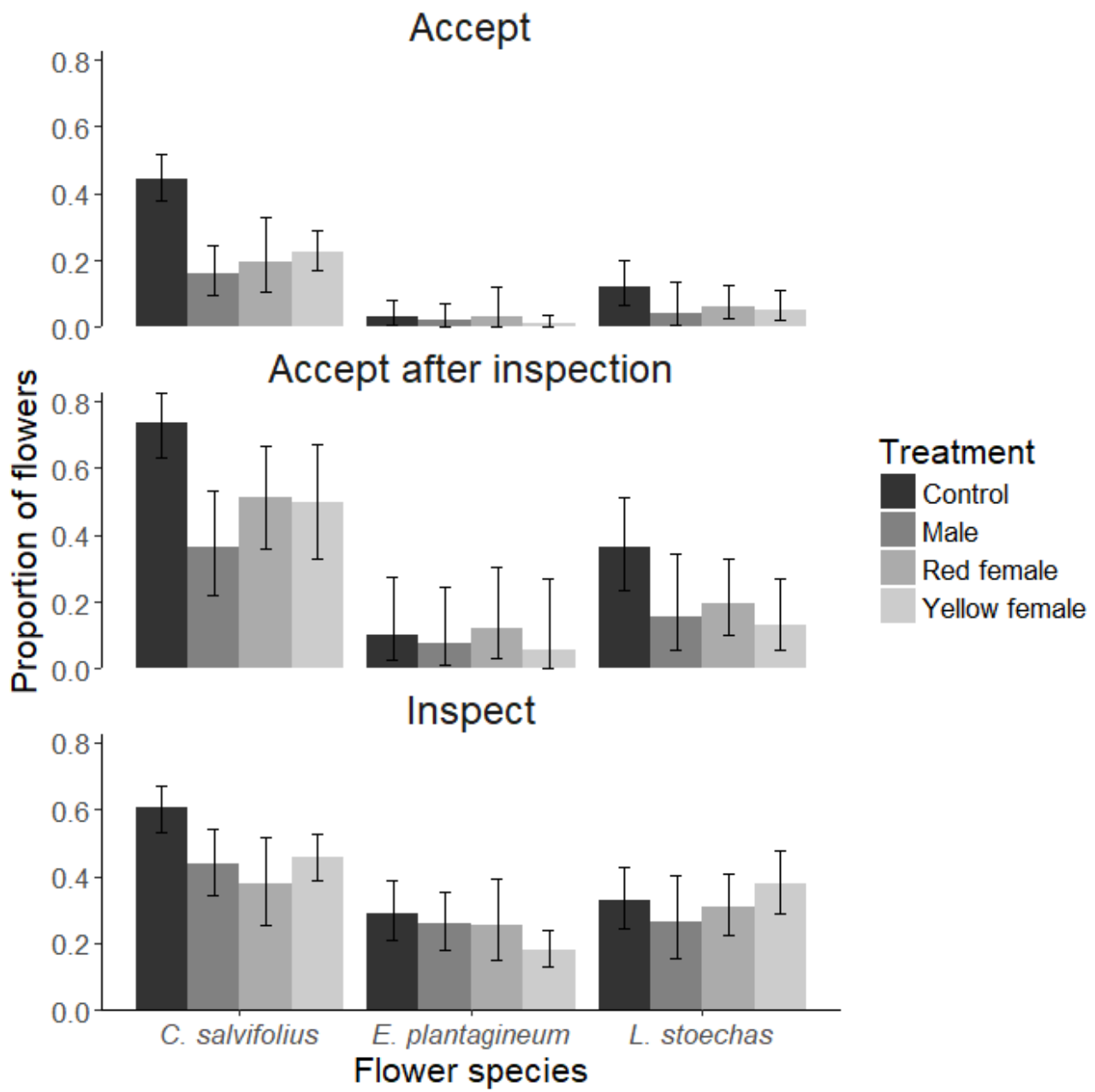
549

550

551 Table 2. Results of binomial GLMs testing the effects in Experiment 2 of the spider treatment in Offer 1, the spider treatment in Offer 2, and day, on honeybee
 552 responses to flowers presented in Offer 2. Variation in the proportion of honeybees landing and feeding on flowers were considered separately for the first
 553 presentation in Offer 2, and for all presentations in Offer 2 combined. The other response considered was the proportion of honeybees that rejected a flower more
 554 often in Offer 2 than in Offer 1. All main effects and significant interactions are shown; interactions that were not significant for any response variable are not
 555 shown.

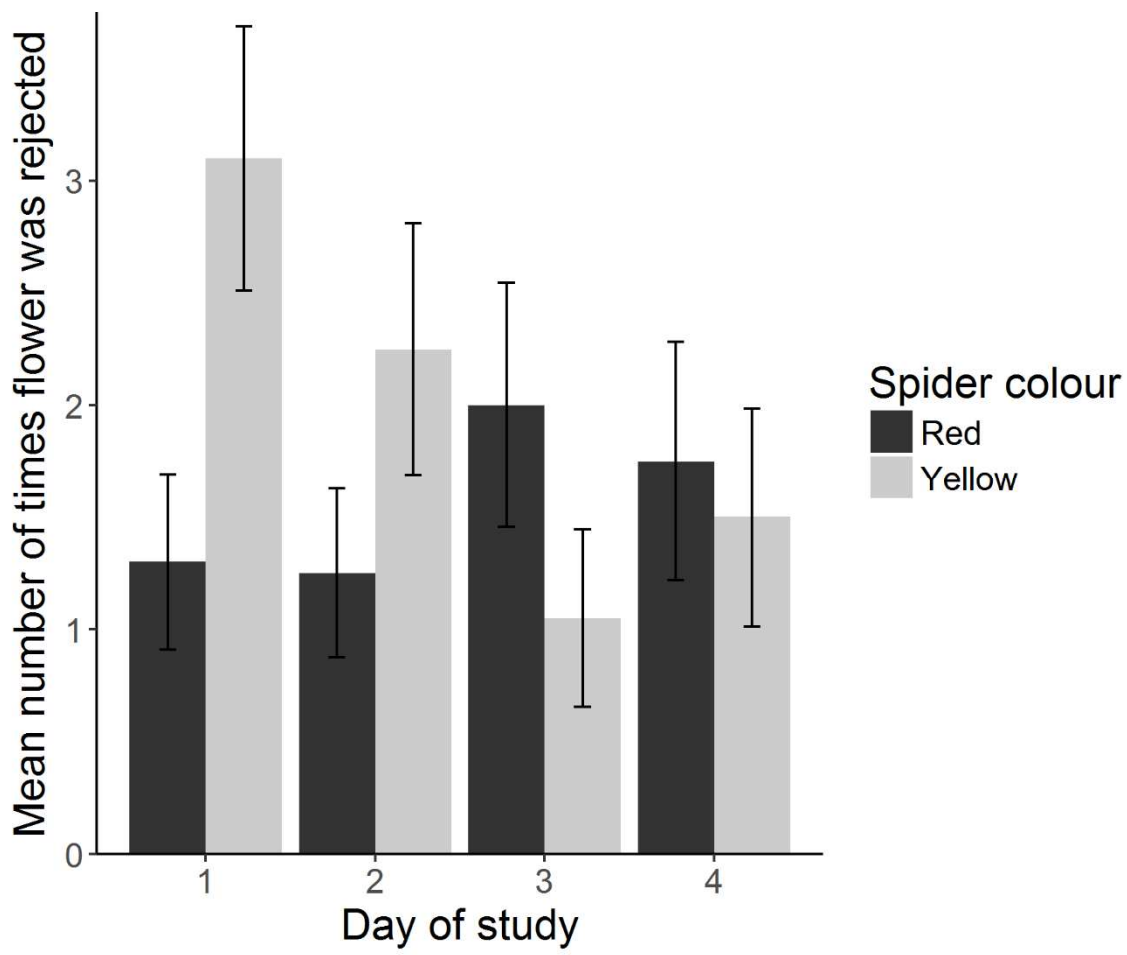
Term	First presentation in Offer 2						All presentations in Offer 2						Difference in number of rejections between Offer 1 and Offer 2		
	Land		Feed				Land		Feed				χ^2	d.f.	p
	χ^2	d.f.	p	χ^2	d.f.	p	χ^2	d.f.	p	χ^2	d.f.	p	χ^2	d.f.	p
Offer 1	3.747	1	0.053	4.361	1	0.037	0.280	1	0.597	0.008	1	0.928	0.826	1	0.364
Offer 2	1.038	1	0.308	0.466	1	0.495	0.144	1	0.704	0.479	1	0.489	0.232	1	0.630
Day	0.016	1	0.900	0.170	1	0.680	0.395	1	0.530	0.973	1	0.324	0.541	1	0.462
Offer 1 x Offer 2	0.188	1	0.665	2.955	1	0.086	1.207	1	0.272	0.234	1	0.629	6.721	1	0.010
Offer 1 x Day	9.433	1	0.002	0.014	1	0.014	5.530	1	0.019	1.238	1	0.266	2.216	1	0.137

556



557

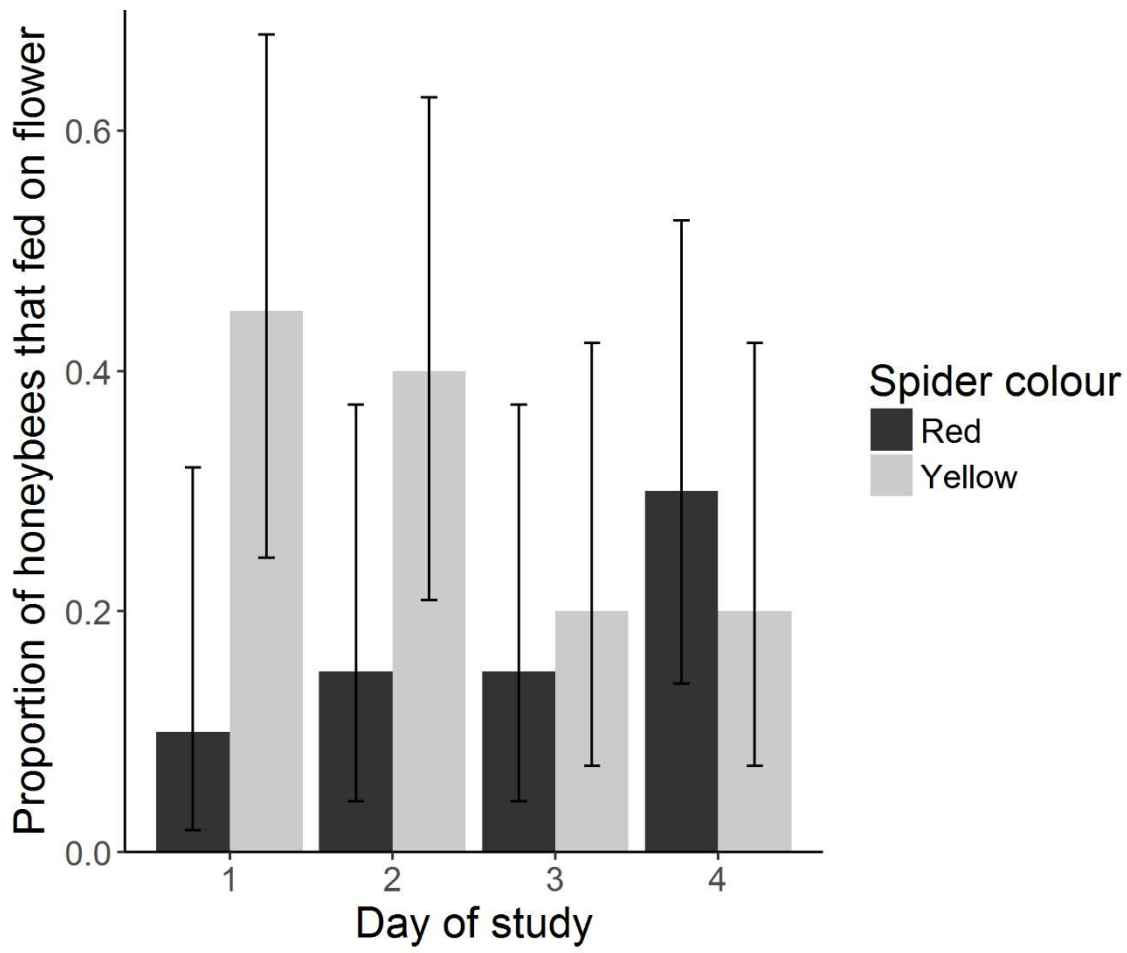
558 Figure 1



559

560 Figure 2

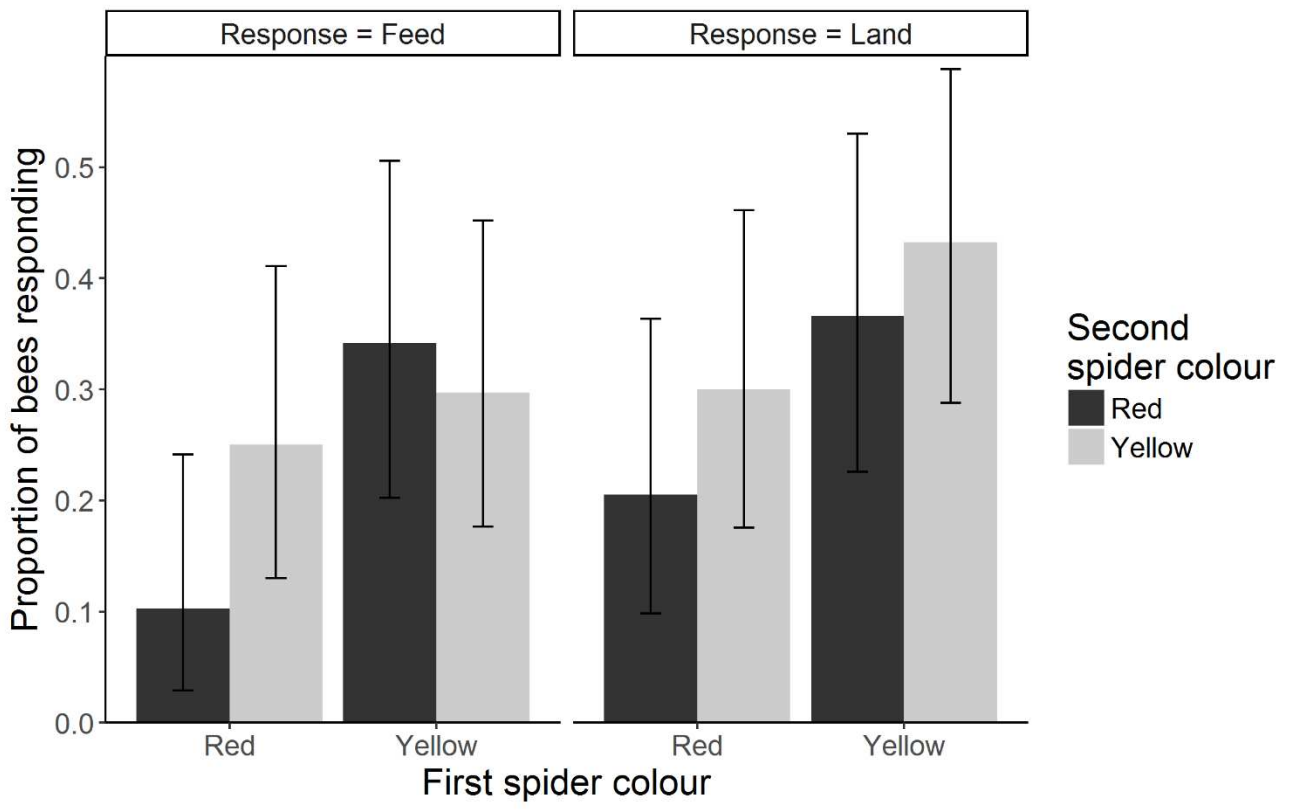
561



562

563 Figure 3

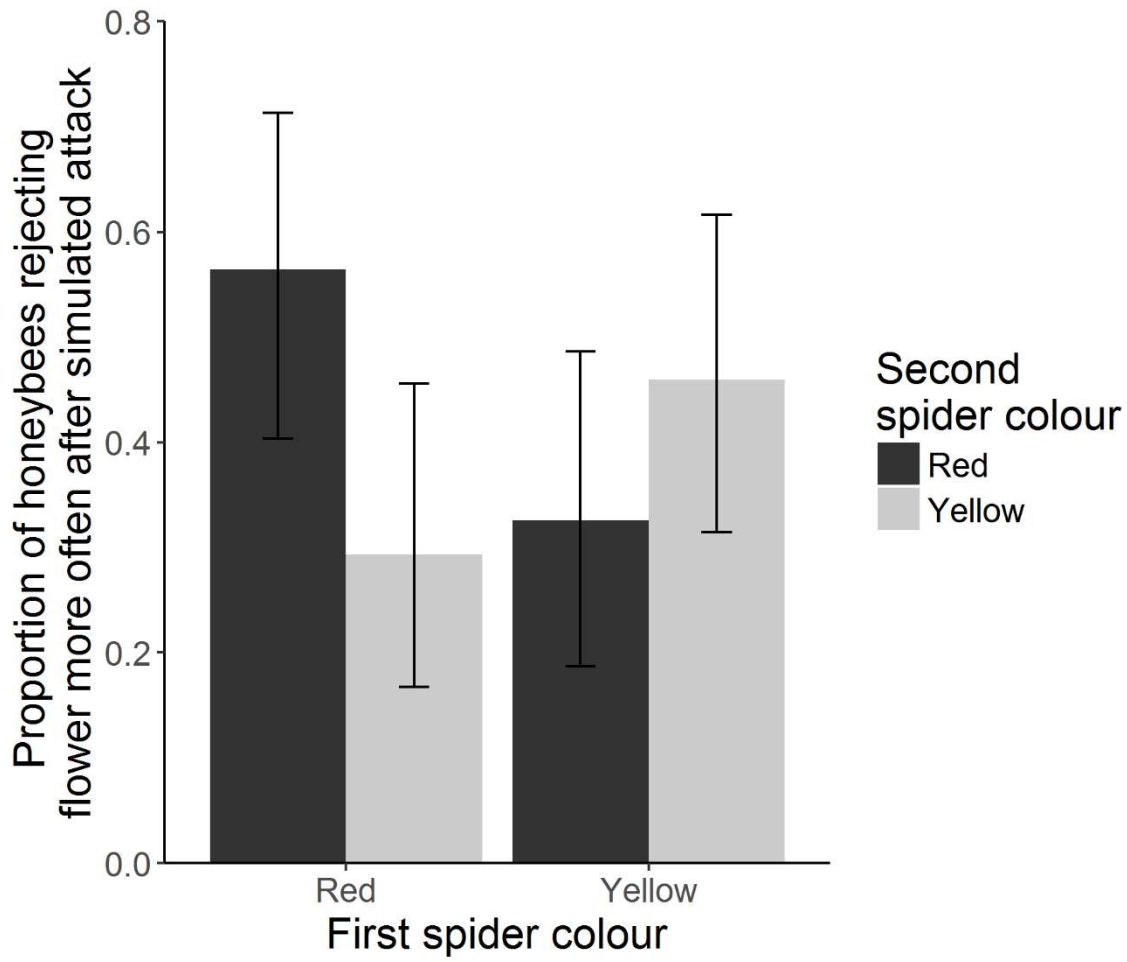
564



565

566 Figure 4

567



568

569 Figure 5

570