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The significance of prey avoidance behavior for the maintenance of a predator color polymorphism

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The existence of conspicuous color polymorphisms in animals provides an ideal opportunity to examine the mechanisms which determine genetic and phenotypic variation in populations. It is well known that directional and negative frequency-dependent selection by predators can influence the persistence of color polymorphisms in their prey, but much less attention has been paid to the idea that prey behavior could generate selection on predator color morphs. In this study, we examine the role that avoidance behavior by honeybees might play in selection on a color-polymorphic sit-and-wait predator, the crab spider *Synema globosum*. In 2 field experiments, we offered flowers harboring spiders of different color morphs to foraging honeybees. In the first, we tested for a preexisting propensity in honeybees to avoid one spider morph over another, and whether this behavior is influenced by the flower species on which spiders hunt. In the second, we tested the ability of bees to learn to avoid spider morphs associated with a previous simulated attack. Our results suggest that honeybees do not impose strong directional selection on spider morphs in our study population, and that avoidance behavior is not influenced by flower species. However, we find evidence that honeybees learn to avoid spiders of a color morph that has previously been associated with a simulated attack. These findings are the first empirical evidence for a mechanism by which prey behavior might generate negative frequency-dependent selection on predator color morphs, and hence potentially influence the long-term persistence of genetic and phenotypic diversity in predator populations.

Key words: **Apis mellifera**, **frequency-dependent selection, pollination, predation, *Synema globosum*.**

INTRODUCTION

Conspicuous color polymorphisms in animals provide intuitive and tractable study systems with which to explore the origins, maintenance and phenotypic consequences of genetic diversity in natural populations (e.g., recent reviews by McKinnon and Pierotti 2010; Ajuria Ibarra and Reader 2013; McLean and Stuart-Fox 2014). For example, the study of morphs with different color patterns in the peppered moth (*Biston betularia*—reviewed in Cook and Saccheri 2013) and grove snail (*Cepaea nemoralis*—reviewed in Cook 2017), has generated key insights into the genetic and ecological context for adaptive evolution. In many such systems, heritable color differences among individuals are thought to influence fitness, because of their effect on the behavior of other animals, and in particular predators. Color patterns in palatable prey species, for example, can influence their detectability, or how easily they can be discriminated from other less palatable species (Speed et al. 2004). This

can lead to directional selection in favor of one particular morph, increasing its frequency in a population, as is thought to happen when predators of the peppered moth select for a melanic morph in polluted environments (Cook 2017). Alternatively, if the fitness advantage of a morph declines as it becomes relatively common, negative frequency-dependent selection can operate, helping to explain the long-term persistence of phenotypic and genetic diversity. Thus, "apostatic" selection, in which predators form a search image for the most common prey morph, was famously invoked to explain the persistence of color polymorphism in the grove snail (Clarke 1962).

Despite the existence of a few textbook examples, the mechanisms which permit conspicuous color polymorphisms to persist in nature are unknown in most cases, and we have limited ability to generalize about the relative importance of predation and frequency-dependent selection (Ajuria Ibarra and Reader 2013). Studies have shown that interactions with species other than predators can generate important patterns of selection on color polymorphisms. For example, competition among males coupled with female mate choice, and sexual conflict, are thought to generate

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frequency-dependent selection on color morphs in lizards (Fitze et al. 2014) and damselflies, respectively (Svensson and Abbott 2005). We can easily imagine that interspecific competitors or parasites might similarly be involved. In this study, however, we focus on the neglected possibility that selection by prey species may influence the persistence of color polymorphisms in their predators.

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

The effect of prey behavior towards a predator can lead to directional, frequency-independent selection if it causes one morph to have significantly higher fitness than the alternative morphs. In the case of *S. globosum*, if prey show lower aversion towards a particular female morph, that morph may have a higher probability of making a successful attack, and increased survival and/or fecundity as a result. In a panmictic population, assuming this selection is more potent than genetic drift, we would expect it to lead eventually to fixation of the genotype which corresponds to the less aversive morph (Bell 1997). However, the process of fixation may be slow, and polymorphism may be observed during transition (Mitchell-Olds et al. 2007). Alternatively, directional selection by prey could actively maintain phenotypic diversity in predators. The response of prey to the threat of attack could depend on the local environment, so that different predator morphs are more successful in different habitats or at different times (e.g., if they forage on different flower species). If offspring are more likely to be found in habitats inhabited by their parents, a selection mosaic might exist which favors the persistence of the polymorphism in the population as a whole (Kondrashov and Mina 1986; Forde et al. 2004). Thus, although there is no evidence for habitat (e.g., flower color) specialization in *S. globosum* morphs (Ajuria Ibarra 2013), directional selection, which may or may not vary with habitat type, could have an important effect on the maintenance of the polymorphism.

The potential for negative frequency-dependent selection by prey on predator traits to promote the maintenance of polymorphisms has been recognized by various authors (Paulson 1973; Hori 1993; Roulin and Wink 2004; Arcos 2007), but few empirical studies exist. Perhaps the best evidence supporting the idea comes from the scale-eating cichlid fish *Perissodus microlepis* (Hori 1993). Populations of *P. microlepis* show a genetically-determined polymorphism for handedness. Right-handed individuals always attack "prey" fish from the left side, whereas left-handed individuals attack from the right. Frequencies of the 2 morphs oscillate around unity across generations. This is explained by that fact that prey more effectively guard the side of their body from which they are attacked most frequently, causing the most abundant cichlid morph to feed less often, and the rarer morph appears to have a reproductive advantage as a result. While this system demonstrates the potential for prey to generate frequency-dependent selection on predators, and possibly maintain balanced polymorphisms, few other studies have considered the possibility, and none have tested it empirically (Paulson 1973; Roulin and Wink 2004; Arcos 2007).

In theory, negative frequency-dependent selection could occur whenever prey can distinguish between predator morphs, learn to associate the polymorphic trait with a potential attack, and avoid the morph that is encountered more frequently. *S. globosum* is an ideal candidate for such a system, because one of its main prey species is the honeybee (Reader et al. 2006; Ajuria Ibarra 2013), which has good color vision (Chittka and Menzel 1992; Dyer et al. 2011), and is able to learn to associate color with positive and negative stimuli (e.g., Giurfa 2007; Avargues-Weber et al. 2010). The response of honeybees to sit-and-wait predators (especially spiders) has been widely investigated. Honeybees show a negative response towards spiders by avoiding flowers or orb-webs (Dukas 2001; Tso et al. 2004; Reader et al. 2006). Therefore, some spiders have evolved coloration and patterns to appear camouflaged or to attract prey by exploiting their responses to flower signals (Thery and Casas 2002; Heiling et al. 2003; Defrize et al. 2010). Different color morphs within a single spider species can elicit different behavioral responses from honeybees. For example, a melanic morph of the giant wood orb-weaving spider *Nephila pilipes* was shown to intercept significantly fewer prey than a brightly-colored morph (Tso et al. 2004). This seems to be because the brightly-colored morph produces visual signals similar to some food resources, whereas the outline of the melanic morph's body is significantly clearer to honeybees. Although the consequences of such differences in prey capture rates for selection in *N. pilipes* is unknown, these findings underline the potential for honeybees to influence the maintenance of polymorphism in their predators.

In the present study, we conducted 2 experiments to examine how interactions between *S. globosum* and honeybees might generate directional or frequency-dependent selection on female *S. globosum* morphs, which might in turn influence the maintenance of color polymorphism. The first experiment tested whether honeybees from a population naturally exposed to crab spider predation responded differently to different color morphs of *S. globosum* on different species of flower. Assuming that prey capture affects fitness, if honeybees in this experiment show a higher propensity to visit flowers harboring a particular spider color morph, it would suggest that selection is operating in favor of that morph, either directionally, or in a frequency-dependent way (e.g., because the favored morph is rare in the study population). Additionally, if honeybee responses to a particular morph depend on the species of flower on which the spider is found, this could indicate habitat-specific directional selection. The second experiment tested for an effect of a recent negative experience with a particular color morph of *S. globosum* on the subsequent response of honeybees to spiders of the same or a different morph. In this experiment, a decrease in the propensity to visit a flower with a particular morph caused by a recent negative experience with that morph would be consistent with the idea that honeybees can exert frequency-dependent selection on *S. globosum*. The results from our experiments provide the first empirical test of the hypothesis that prey behavior can favor rare predator color morphs, and hence potentially contribute to the maintenance of color polymorphism.

METHODS

All experiments were carried out between 09:30 AM and 05:00 PM in meadows and open woodland at the Quinta de São Pedro Study Centre, near Lisbon, Portugal (38°38′19″ N, 9°11′50″ W) in April 2010 and 2011. Spiders were hand-collected from different flowering plants in the area, and killed by leaving them in a Perspex tube filled with CO_2 for 1 h, before being glued to flowers for use in the experiments. White female spiders were excluded because they were found at very low frequencies (<5%). Honeybees were sampled while foraging naturally on patches of flowers spread across an area of several hectares. We assume that most bees encountered came from several apiaries which were maintained at the site by local farmers.

Experiment 1

To investigate if there was an effect of spider color on the foraging behavior of honeybees, flowers of sage-leaf rockrose (*Cistus salvifolium*), purple viper's bugloss (*Echium plantagineum*), and lavender (*Lavandula stoechas*) harboring spiders were offered to wild honeybees. These are species on which *S. globosum* is commonly found at the study site (Ajuria Ibarra 2013). Four spider treatments were used for each flower species: red female, yellow female, male, and control ($N = 100$ presentations per combination of flower species and spider treatment). Ten newly-collected spiders of each type were used for each of 7 days of the experiment. Wheat flour mixed with water was used to glue each spider to a petal (or inflorescence in the case of lavender), such that it was clearly visible to approaching honeybees. The control treatment consisted of flowers with glue added, but no spider. Treatments were performed in temporal blocks, with each combination of flower species and treatment being applied once in each block. Spiders and treatment-flower combinations were chosen randomly within blocks.

Each treated flower was offered to a honeybee that was foraging on the same species at the study site. The treated flower was held with a pair of tweezers at arms' length and placed within 5 cm of the flower on which the honeybee was feeding. If the honeybee did not approach it, the flower was repositioned until it did. When offered a flower, 1 of 3 responses by the honeybee was recorded, following (Duffield et al. 1993): *Ignored*—the honeybee approached the flower but changed its course without pausing or making contact with it; *Rejected*—the honeybee inspected the flower, hovering in close proximity, sometimes touching it with its antennae or legs, but did not alight; *Accepted*—the honeybee alighted on the flower.

Experiment 2

To test if honeybees respond to spider morphs differently after previously experiencing a simulated attack associated with a spider of a particular color, individual honeybees were sequentially offered 2 flowers of *C. salvifolius*, each harboring a different female spider. *C. salvifolius* was used because it was the species with the highest probability of acceptance in Experiment 1. Spiders were glued to flowers as above, and the flower was attached with sticky tape to a 60-cm garden plant stick, before being offered to foraging honeybees. This method eliminated observer collisions with the vegetation, which occasionally caused us to have to terminate trials in Experiment 1.

First, a naïve foraging honeybee was repeatedly presented with a flower harboring a spider by an observer until the flower was accepted, or until 10 rejections had been observed. We refer to these presentations as "Offer 1". We recorded the number of times

the flower was presented before being accepted, as a measure of a honeybee's willingness to forage despite the presence of a spider. Next, while the honeybee was feeding on the flower in Offer 1, it suffered a simulated spider attack by a second observer. The attack disturbed the honeybee, which invariably flew off to another flower to recommence foraging. The honeybee was followed on its foraging flight by the first observer, and repeatedly presented with a second flower harboring a different spider until that flower was accepted, or until 10 rejections had been observed. We refer to the second set of presentations as "Offer 2". Again, we recorded the number of times the flower was presented before being accepted, as a measure of willingness to forage. If a naïve honeybee failed to accept a flower at all in Offer 1, the trial was abandoned and a different naïve honeybee was chosen. If a honeybee which had already experienced an attack failed to accept the flower in Offer 2, we recorded the number of rejections as 10.

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

Four female spider treatments were used for Offer 1 and Offer 2: red followed by yellow, yellow followed by red, red followed by red, and yellow followed by yellow ($N = 40$ pairs of flowers per treatment). Ten newly-collected spiders of each type were used for each of the 4 days of the experiment. Spiders and treatments were assigned at random. The behavior of honeybees in response to the experiment was evaluated in 3 ways. First, both before and after the simulated attack (Offers 1 and 2), we considered the number of times a flower had to be presented before a bee accepted it. Second, the honeybee's response to a flower presented after the simulated attack (Offer 2) was recorded using the criteria described for Experiment 1, but 2 types of acceptance were considered: *Landed*—the honeybee landed on the flower, but did not probe it for nectar; *Fed*—the honeybee landed and probed the flower. Third, a subjective index was used to score the speed with which honeybees rejected each flower: 1 = slow (the bee hovered close to the flower for more than approximately 3 s, often touching the flower with its antennae), 2 = fast (the bee hovered for approximately 2–3 s, sometimes touching the flower with its antennae), and 3 = very fast (the bee noticeably hesitated close to the flower, but only very briefly [approx. 1 s] before flying away).

Statistical analysis

Honeybee responses in Experiment 1 were analyzed with generalized linear mixed models (GLMMs) with binomial errors and block as a random effect. Binomial responses were: 1) accepted versus not accepted (reject + ignore), 2) accepted versus rejected (with ignored flowers excluded), and 3) inspected (accept + reject) versus not inspected (ignore). Differences between spider treatments were analyzed by comparing a model containing all 4 treatments to a model where the 3 spider treatments were collapsed into one and contrasted with the control.

For Experiment 2, the differences in number of rejections of flowers by honeybees in Offer 1 (maximum = 10) between those harboring red and yellow spiders, and among the 4 days on which trials took place, were analyzed using a non-parametric 2-way analysis of variance. Responses to spider treatments in Offer 2 were

analyzed using generalized linear models (GLMs) with binomial errors. Three binary response variables were used: 1) accepted (fed + land) versus not accepted, 2) fed versus not fed, and 3) whether or not the number of presentations which were rejected in Offer 2 was higher than in Offer 1. For the first 2 of these variables, 2 separate models were fitted, one considering only the first attempt to present a flower to a honeybee in Offer 2, and the other considering all attempts to present the flower (maximum = 10; i.e., did the bee ever land/feed?). We expected that any effect of learned aversion to spiders encountered in Offer 1 would be strongest in the first of these 2 analyses. Day of study (1–4) was included as a fixed factor to account for changes in honeybee behavior over time. In all models, the significance of each term was assessed using a chi-squared test statistic after backward deletion from a saturated model. A separate chi-squared test was used to assess the difference in the proportion of bee responses in each rejection speed class (slow, fast and very fast) among spider treatments for the first presentation of flowers in Offer 2 in Experiment 2. All analyses were conducted in R 2.12.2 (The R Development Core Team 2017).

RESULTS

Experiment 1

In Experiment 1, significant effects of spider presence and flower species were found on the probabilities that a honeybee would inspect and accept a flower for all response variables (Figure 1 and Table 1). There was a higher probability of a honeybee inspecting and landing on a flower without a spider than a flower with either a red or yellow female, or a male spider, and honeybees were more likely to inspect and accept a flower of *C. salvifolius* than a flower of *E. plantagineum* or an inflorescence of *L. stoechas* (Table 1).

No significant differences were found among spider treatments: the effect of collapsing the spider treatments together in the statistical model was not significant for any of the response variables: accepted versus not accepted ($\chi^2 = 2.718$, df = 6, $P = 0.843$), accepted versus rejected ($\chi^2 = 3.477$, df = 6, $P = 0.747$), and inspected versus not inspected ($\chi^2 = 6.484$, df = 6, $P = 0.371$). The interaction between spider type and flower species was not significant for any response variable (Table 1). Hence, the negative effect of the presence of a spider on bee behavior was similar on all flower species.

Experiment 2

The behavior of naïve honeybees at the start of Experiment 2, before they received a simulated spider attack, was consistent with the results of Experiment 1. There was no significant overall effect of spider color on the mean number of times that naïve individual honeybees rejected a flower harboring the spider before accepting it in Offer 1 (2-way nonparametric Anova: $H = 0.40$, df = 1 $P = 0.529$) (Figure 2). Similarly, there was no significant effect of the day of study ($H = 3.43$, df = 3, $P = 0.331$) on the number of times that honeybees rejected flowers in Offer 1, meaning that naïve bees did not accept flowers more or less quickly as the study progressed. Although there was a tendency for flowers harboring yellow spiders to be rejected more frequently by naïve bees earlier in the study, the interaction between spider color and day of study on the number of times that honeybees rejected flowers in Offer 1 was not significant ($H = 7.46$, df = 3, $P = 0.060$).

There was a significant effect of the spider color morph harbored by the flower presented to naïve honeybees (in Offer 1) on the proportion of those bees which subsequently fed on a flower the first time it was presented to them after a simulated spider attack (i.e., in Offer 2; see Table 2). A lower proportion of the honeybees
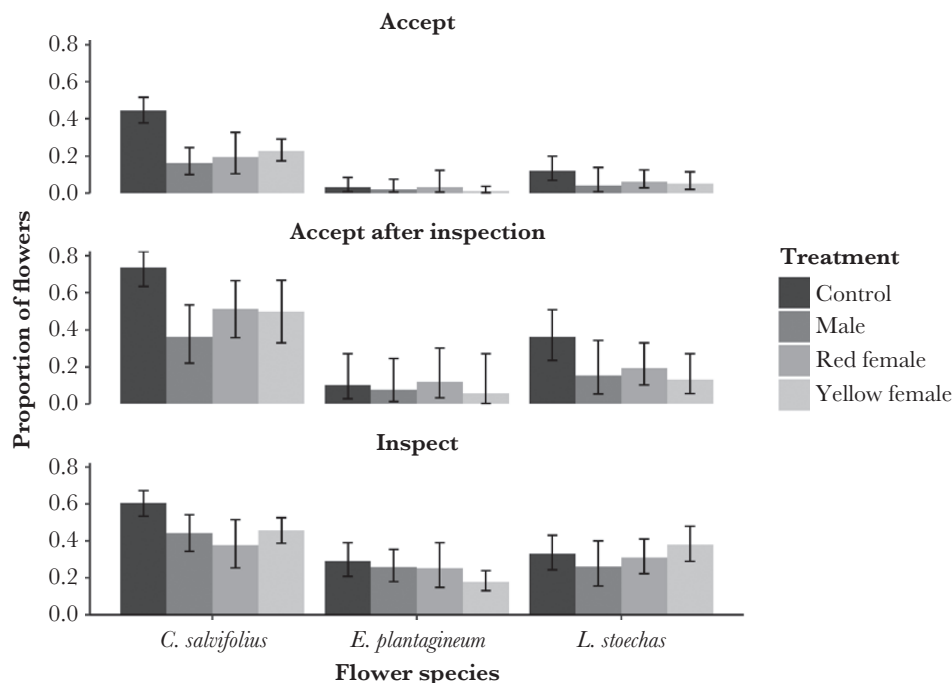

Figure 1
Proportion of flowers of *C. salvifolius*, *E. plantagineum*, and inflorescences of *L. stoechas*, that were accepted (upper 2 panels) or inspected (lower panel) by honeybees. Accepted flowers are shown as a proportion of all flowers (upper panel) or a proportion of only those flowers which were inspected (middle panel). Flowers harbored a red or yellow female spider, or a male spider, or no spider (control). Error bars are 95% confidence intervals calculated using the binomial distribution.

Table 1

Results of binomial GLMMs testing the effect of spider treatment, flower species, and the interaction between them on honeybee responses to flowers offered in the field in Experiment 1

Response	Spider			Flower species			Spider × flower species		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
Accepted versus not accepted	26.869	1	**<0.001**	119.210	2	**<0.001**	1.392	1	0.500
Accepted versus rejected	17.770	1	**<0.001**	77.606	2	**<0.001**	1.640	1	0.440
Inspected versus not inspected	8.957	1	**0.003**	48.010	2	**<0.001**	3.174	1	0.205

Figures in bold indicate significant effects ($P < 0.05$).

The effect of spider treatment (present versus control) was assessed after collapsing factor levels for female colors (see text).
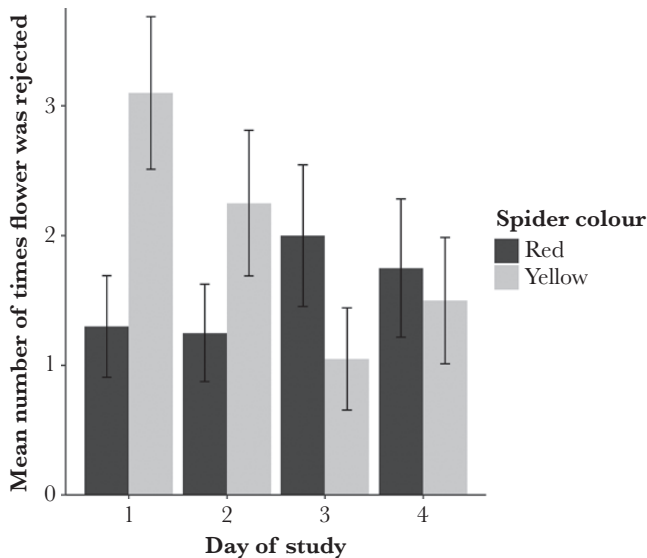

Figure 2

Mean (±SE) number of times a flower harboring a spider was presented to, and rejected by, naïve foraging honeybees before it was accepted in Experiment 2. Spiders were of 2 different color morphs (red and yellow), and data are shown for trials which were conducted on 4 different days.

which initially encountered a red spider in Offer 1 subsequently fed on flowers in Offer 2 when compared with those honeybees which initially encountered a yellow spider, regardless of spider color presented in Offer 2 (Figure 3). No such effect was observed, however, on the probability that a honeybee fed on a flower at least once when all presentations of the flower in Offer 2 were considered (Table 2). Similarly, no overall effect of the color of the spider initially encountered in Offer 1 was found on the probability of a honeybee accepting a flower (accept = fed + land) after a simulated attack (i.e., in Offer 2), considering either just the first attempt to present the flower, or all attempts.

Over the 4 days of Experiment 2, there was no overall significant change in the proportion of honeybees landing or feeding on flowers after a simulated spider attack (i.e., in Offer 2; Table 2). There was, however, an interaction between day and the treatment received by naïve honeybees in Offer 1, when considering the first attempt to present a flower in Offer 2, after the simulated attack. The proportion of honeybees that fed on a flower after experiencing an attack associated with a red spider increased over time, but it decreased in cases where bees had experienced an attack associated with a yellow spider (Figure 3). Although this interaction was not significant when all attempts to present the flower in Offer 2 were considered, a similar pattern was observed for the probability that a honeybee accepted a flower (regardless of whether it fed),

considering either just the first attempt to present a flower, or all attempts (Table 2).

There were no main effects of the color of the spider encountered after the simulated attack, or of day, on any aspect of honeybee behavior towards flowers presented in Offer 2 (Table 2). The key test, however, of whether learned aversion by bees to spiders of a particular color could impact on spider foraging success was indicated by the interaction between the treatments applied before and after the simulated attack (i.e., in Offer 1 and Offer 2). Assuming a 2-tailed statistical test, this interaction did not have a significant effect on the probability of a honeybee landing or feeding on flowers after the simulated attack, whether or not all attempts to offer a flower in Offer 2 were considered (Table 2 and Figure 4). It is worth noting, however, that a one-tailed test would yield a significant result in the expected direction for the proportion of bees that fed on flowers the first time they were presented: bees presented with a flower harboring a red spider in Offer 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 of a different morph, and a similar but weaker effect was observed for bees presented with a yellow spider in Offer 1 (Figure 4). Importantly, there was a significant interaction between the effects of spider color before and after the simulated attack on the change in the number of rejections of flowers by honeybees between Offer 1 and Offer 2. Honeybees were more likely to reject the flower after the simulated attack (in Offer 2) more often if it harbored a spider of the same color morph as the spider which they encountered in Offer 1 (Table 2 and Figure 5).

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

DISCUSSION

The results of these experiments showed no evidence that wild-caught honeybees discriminated between the different color morphs of *S. globosum* on different species of flowers when they first encountered them in our study. However, there was some evidence that honeybees are able to learn to avoid spiders of a color morph associated with a recent simulated attack. These results suggest that, while prey in the study population may not exert directional selection on

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 responses to flowers presented in Offer 2

| | 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 | | | | | |
Term	χ^2	df	P	χ^2	df	P	χ^2	df	P	χ^2	df	P	χ^2	df	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 × 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 × 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

Figures in bold indicate significant effects ($P < 0.05$).
Variation in the proportion of honeybees landing and feeding on flowers were considered separately for the first 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 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 shown.
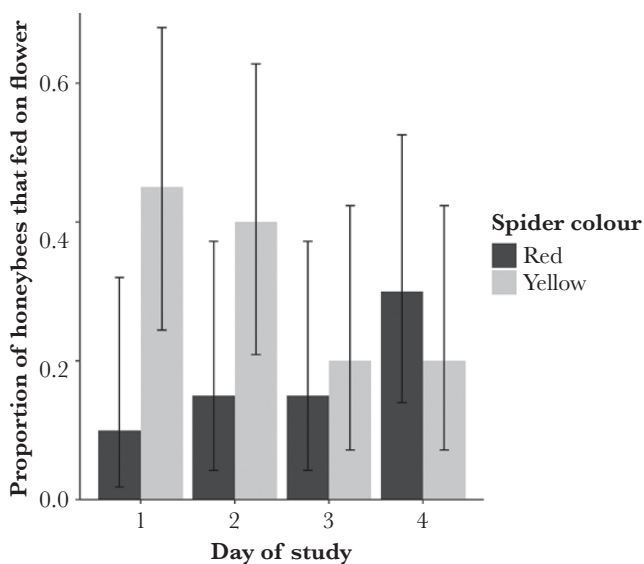
Figure 3
Proportion of honeybees which fed on flowers harboring a spider on the first occasion they were presented after the honeybee had experienced a simulated attack associated with either a red or a yellow spider in Experiment 2 (i.e., in Offer 2—see main text). Data are shown for trials which were conducted on 4 different days. Error bars are 95% confidence intervals calculated using the binomial distribution.

predator color patterns, they do have the potential to generate frequency-dependent selection in favor of rare morphs, which could help to explain the long-term persistence of the polymorphism.

Directional selection

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

effect of flower species on the tendency of honeybees to avoid spiders, or on their response to different spider types.

These results are not consistent with directional selection favoring one *S. globosum* morph, or habitat-specific selection on morphs. Combined with the fact that *S. globosum* morphs do not favor particular flower types at our study site (Ajuria Ibarra 2013), this suggests that the polymorphism in *S. globosum* is not transient (Mitchell-Olds et al. 2007) and is not maintained by gene flow between sub-populations of spiders which are adapted to different flower-specific niches (King and Lawson 1995). Nevertheless, there may be other forms of niche-specific selection operating in this system, such as bees being better able to avoid certain colors of spider in relation to levels of light or other environmental factors. It is therefore impossible to rule out spatial (selection mosaics) or temporal variation in selection affecting the persistence of the polymorphism. Such processes have been observed to occur in other polymorphic species, such as the marine snails of the genus *Littoraria*, where certain morphs are found at higher frequencies in different parts of mangrove trees and at different positions at different times of the year (Parsonage and Hughes 2002).

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

Learning and the potential for frequency-dependent selection

Honeybees rejected flowers in Offer 2 of Experiment 2 relatively more often if they harbored a spider of the same color morph as one
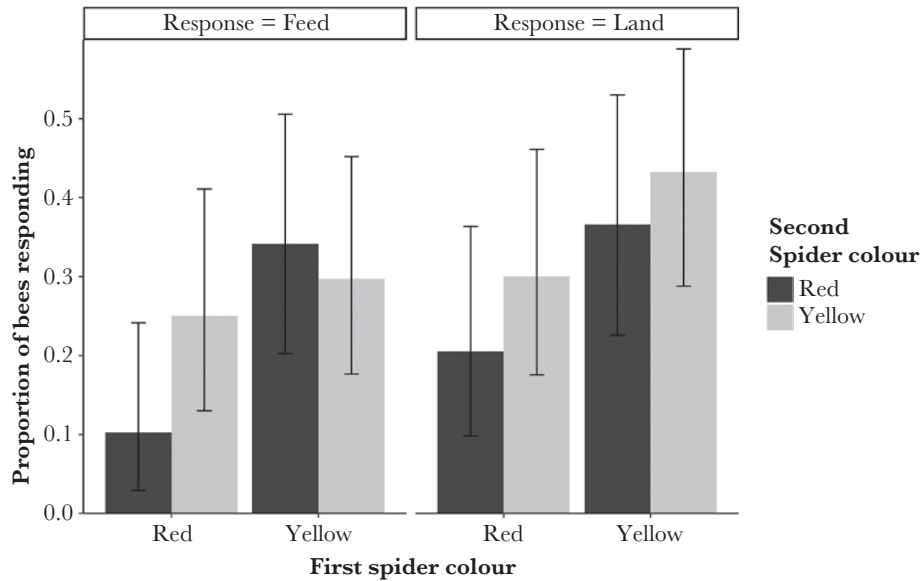
Figure 4

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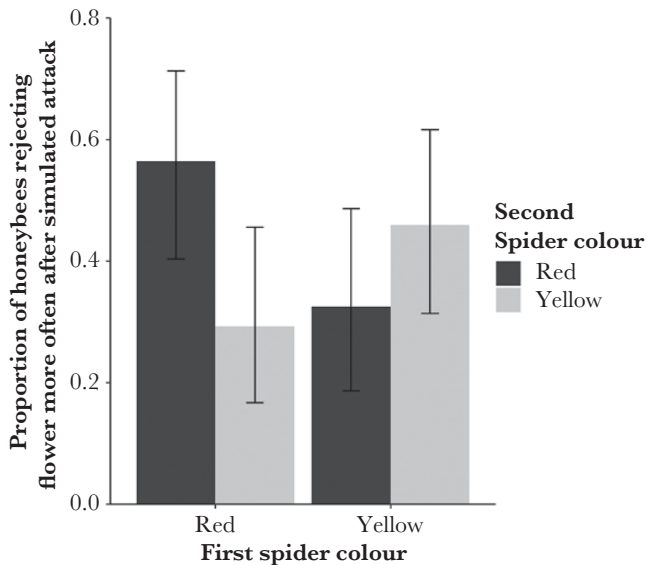

Figure 5

The effect of the spider color morph encountered by honeybees in Experiment 2 on the proportion of honeybees that rejected a flower harboring a spider more often after a simulated attack by a spider (i.e., in Offer 2—see main text) than before the simulated attack (in Offer 1). Naïve honeybees were initially presented with a flower harboring either a red or yellow spider ("First spider color"), before being subject to a simulated attack, and then presented with a spider of the same or a different color ("Second spider color"). Both before and after the attack, flowers were presented up to 10 times to each honeybee until they were accepted. Error bars are 95% confidence intervals calculated using the binomial distribution.

which had previously been associated with a simulated attack. Similar (but nonsignificant) patterns were observed when considering the propensity of honeybees to feed on flowers the first time they were

presented in Offer 2, and the speed with which these flowers were rejected. Thus, honeybees appear to have learned to associate spider color with a simulated attack, and changed their behavior to avoid such attacks in future. These results are consistent with previous studies where bees have been observed to avoid a color associated with a negative experience (Avargues-Weber et al. 2010) and to behave in a negative frequency-dependent way with respect to color (Smithson and Macnair 1997; Gigord et al. 2004). Based on these findings, it is plausible that in a population of *S. globosum* where one female morph is found at a higher frequency than the others, honeybees would experience attacks from spiders of this morph more often, and would avoid them more than rarer morphs. To help confirm this, supporting evidence could be obtained from studies where honeybees are exposed to flower patches with varying *S. globosum* morph frequencies. Ultimately, we would also need to evaluate the effect of prey avoidance behavior on spider foraging success and fitness: we know that honeybees are common in the diet of *S. globosum* (Ajuria Ibarra 2013), but we do not know how reduced encounter/capture rates might translate into reduced survival or fecundity. Nevertheless, our experiment provides the first empirical evidence supporting the idea that frequency-dependent selection caused by prey behavior could help to maintain color polymorphism in a predator. Taken together with findings of other studies of this kind (Hori 1993; Arcos 2007), and contrasted with textbook examples in which predator search images generate selection on prey, our results suggest that there is more than one way that behavioral interactions between predators and prey can influence the maintenance of genetic and phenotypic diversity.

Differences in learned responses to red and yellow spiders

Although wild-caught honeybees did not discriminate between spider morphs in either experiment, results from Experiment 2 suggested that honeybees that had previously experienced a simulated attack from a red spider were less likely to land and feed on flowers in Offer

2 than those that had previously experienced a simulated attack from a yellow spider, regardless of the treatment received in Offer 2. This result could be explained by the different ways in which red and yellow spiders reflect light and the different chromatic contrasts of the spiders and flowers. Yellow coloration in spiders has been shown to reflect UV light (Tso et al. 2004; Heiling et al. 2005; Thery and Casas 2009), and measurements of *S. globosum* have shown that this is true for yellow females (Ajuria Ibarra and Reader 2014). Some spiders have been observed to use UV reflection to produce visual signals similar to those produced by flowers to attract honeybees (Heiling et al. 2003; Tso et al. 2004; Herberstein et al. 2009). Because UV-containing signals may more typically be used as cues associated with positive stimuli (i.e., food), honeybees might not be as efficient at learning to associate them with danger. Consequently, after a honeybee has experienced an attack from a red spider, it might be relatively more cautious about feeding on a flower with anything that might resemble a spider than would be the case after an experience with a yellow spider.

This effect of spider color in Offer 1 on honeybee behavior in Offer 2 reduced in magnitude over the 4 days of our experiment, until the difference between the effects of experiences with red and yellow spiders in Offer 1 was small and in the opposite direction (Figure 5). The change over time might be owing to the use of the same population of honeybees during the experiment. It has been observed that honeybees show patch fidelity (Osborne and Williams 2001; Slaa et al. 2003; Franzen et al. 2009), and because we revisited some patches on different days, individual honeybees may have been exposed to spider treatments more than once over the 4 days. Therefore, it is possible that honeybees eventually learned to associate yellow color with predation risk, thus reducing the probability that they would accept a second flower with a spider after experiencing an attack from a yellow spider. However, we sampled many patches of flowers spread around a large field site which contained several apiaries, and we think that the likelihood of individuals being repeatedly encountered was low. Unfortunately, because we did not mark sampled individuals, it is impossible to be sure the extent to which individual learning impacted on the temporal patterns in our results.

CONCLUSION

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

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