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Female-limited colour polymorphism in the crab spider *Synema globosum* (Araneae: Thomisidae)

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Conspicuous colour variation, caused by the influence of the environment on phenotype or by genetic differences among individuals, is frequently observed in nature. If genetic in origin, colour variation can facilitate the study of mechanisms that contribute to the maintenance of true polymorphisms. Here we describe, for the first time, the female-limited colour polymorphism in the crab spider, *Synema globosum*. We looked for associations between life-history traits and female colour morph, and identified potential agents of selection that could influence the maintenance of the polymorphism. Our results showed that the polymorphism is discrete and heritable, and that differences in colour among morphs are likely to be detectable by honeybees, birds, and conspecifics. We found limited evidence of differences among morphs in morphology and ecology, and found no differences in components of reproduction. Based on the lines of evidence obtained in this study, we suggest that selection exerted by prey, predators, and/or mates is likely to influence the maintenance of the polymorphism observed in *S. globosum*.

ADDITIONAL KEYWORDS: genetic variation – heritability – life-history traits – predation – reflectance spectra – sexual selection – signalling.

INTRODUCTION

Polymorphism is a widespread phenomenon in nature that occurs in a broad range of taxa. It is the presence of multiple genetically and phenotypically distinct forms in a population, which do not respond to changes in the environment or body condition and can interbreed to produce fertile hybrids (Roulin, 2004). Polymorphisms have been a subject of great interest in biological research because determining the mechanisms that generate and maintain them can shed light on how natural selection and other evolutionary processes shape both genotypes and phenotypes (Brockmann, 2001; Gray & McKinnon, 2007; Mitchell-Olds, Willis & Goldstein, 2007). Species that show colour variation are good systems with which to study the maintenance of polymorphisms because coloration is easy to score and is known to be involved in processes that influence fitness, such as thermoregulation and inter- and intraspecific communication (Gamberale & Tullberg, 1998; Bush, Yu & Herberstein, 2008; Phifer-Rixey *et al*., 2008; Hampton, Hughes & Houde, 2009).

Variation in colour can be continuous or discrete. Continuous colour variation has often been observed to be condition- or environment dependent and may not reflect a true (genetic) polymorphism (Hill & Montgomerie, 1994; Fitze, Kolliker & Richner, 2003). Discrete variation in colour is usually genetic in origin (Forsman *et al*., 2002; Vercken *et al*., 2007) and thus requires an evolutionary explanation (Gray & McKinnon, 2007; Ajuria Ibarra & Reader, 2013). If coloration does not have a significant effect on fitness, it is possible that a polymorphism is maintained by random genetic drift (Hoffman *et al*., 2006; Mitchell-Olds *et al*., 2007). Where fitness is affected by colour, several selective mechanism(s) could play a role in maintaining colour polymorphisms (Bellido *et al*., 2002; Oxford, 2005). For example, polymorphisms can persist because of heterozygote advantage, where individuals with heterozygous genotypes have higher fitness than do individuals with homozygous-dominant or -recessive genotypes (Vercken, Clobert & Sinervo, 2010). Different morphs can also persist in spatially and/or temporally heterogeneous environments,

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where each morph has an advantage under particular conditions (Todd *et al*., 2006; Forsman *et al*., 2011; Parkash *et al*., 2011). Negative frequency-dependent selection, where rare morphs have a fitness advantage over common morphs, is another mechanism that can explain the maintenance of polymorphisms (Bond & Kamil, 1998; Bleay, Comendant & Sinervo, 2007; Takahashi & Watanabe, 2010). Finally, polymorphisms can be maintained if different morphs exhibit alternative strategies that result in equal mean fitness values (Roulin *et al*., 2003).

Colour variation is common in spiders (Holl, 1987; Oxford & Gillespie, 1998; Gawryszewski & Motta, 2012; Geay *et al*., 2012; Kemp *et al*., 2013). Most studies of the phenomenon have concentrated on species that are able to match their colour to that of the background, in order to appear cryptic or to attract prey (Théry & Casas, 2002; Heiling, Herberstein & Chittka, 2003; Heiling *et al*., 2005; Defrize, Théry & Casas, 2010). In most cases, these species are not truly polymorphic but rather they have the ability to change coloration behaviourally (Théry & Casas, 2002; Heiling *et al*., 2005). Genetic colour polymorphisms in spiders have been most extensively studied in the candy-stripe spider (*Enoplognatha ovata*) and the Hawaiian happy-face spider (*Theridion grallator*) (Oxford & Gillespie, 2001). Evidence implicates both genetic drift and natural selection in the maintenance of the polymorphism in *E. ovata* (Oxford & Shaw, 1986; Reillo & Wise, 1988; Oxford, 2005), whilst selective forces are thought to be most important in *T. grallator* (Gillespie & Tabashnik, 1990; Gillespie & Oxford, 1998). However, regarding the ecological factors that could be generating these evolutionary mechanisms, there is inconclusive evidence in favour of negative-frequency-dependent selection in *T. grallator* (Gillespie & Tabashnik, 1990; Gillespie & Oxford, 1998) and no studies in *E. ovata*. Other less-well-studied species of colour-polymorphic spider appear to show a correlation between colour and environmental factors such as substrate, temperature, and light conditions (Gunnarsson, 1987; Bonte & Maelfait, 2004; Kemp *et al*., 2013), as well as differences in prey capture rates between morphs (Tso *et al*., 2002; Tso, Lin & Yang, 2004; but see also Geay *et al*., 2012).

Although, with the exceptions described above, they are not well studied, we believe that colour-polymorphic spiders represent excellent potential models for investigating the maintenance of genetic and phenotypic diversity, and, in particular, for examining the role played by ecological interactions in generating selection on colour morphs. This potential is highlighted by what is known about the ecological significance of colour in spiders: it has been observed to be important in foraging behaviour (Heiling & Herberstein, 2004; Tso *et al*., 2006; Morse, 2007; Llandres *et al*., 2011; Herberstein & Gawryszewski, 2013), sexual selection (Masta & Maddison, 2002; Hebets & Maddison, 2005; Lim, Li & Li, 2008; Elias *et al*., 2012), and thermoregulation (Robinson & Robinson, 1978), and could potentially serve a function for predator avoidance (Gunnarsson, 1987; Théry & Casas, 2002; Clark *et al*., 2011). To exploit this potential fully, we need, first of all, to characterize colour variation in species of interest, to distinguish between discrete and continuous variation, and to establish whether it reflects a true genetic polymorphism. We then need to look for fitness differences among morphs and relate these to ecological factors in the environment.

In this study, we examine conspicuous colour variation in the crab spider *Synema globosum* (Fabricius 1775). In this species, females can have red, yellow, or white coloration around the margins of the opisthosoma, surrounding a characteristic black marking, whereas males have an almost entirely black opisthosoma with only two very small white areas on the lateral parts (Fig. 1). The colour variation in females appears to be discrete; the three different colours have been found to be produced by different types of pigments in the epithelial cells of the opisthosoma (Théry & Casas, 2009). *Synema globosum* is distributed in southern Europe, Asia, and northern Africa (Preston-Mafham, 1998) and is a common species in our study site (see below) where it conspicuously occupies flowers of many different species and can be seen attacking flower-visiting invertebrates. Adults can be found from the end of March, females with egg sacs can be found from early May, and spiderlings have been observed from early June. The absence of adults in autumn and winter months suggests an annual life cycle. We conducted a survey to establish the frequencies of the three colour morphs in the study site. We then determined if the colour variation observed in female *S. globosum* is discrete or continuous by quantifying the variation among female individuals using reflectance spectra measurements. We then carried out a breeding experiment to evaluate whether the polymorphism is heritable (i.e. has a genetic origin). Finally, we investigated possible fitness differences among morphs and looked for other, more cryptic, traits that might correlate with colour, by examining differences in morphology, behaviour on flowers, and reproductive output among morphs.

MATERIALS AND METHODS

Frequencies of colour morphs

To determine the frequencies of red, yellow, and white female morphs, a survey was carried out at our study

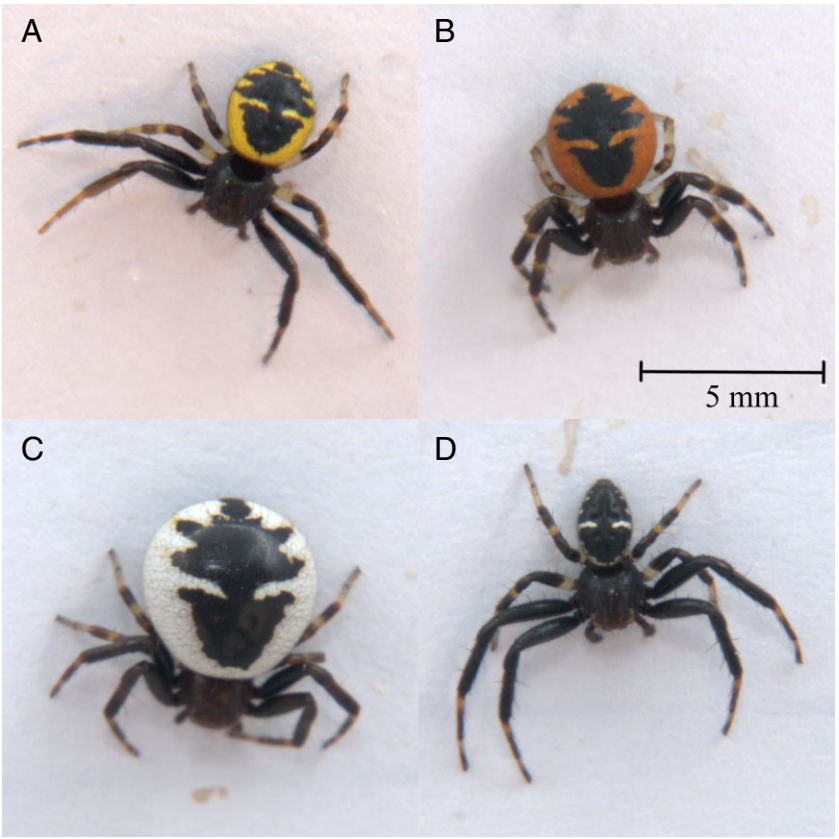

Figure 1. A, yellow female; B, red female; C, white female; and D, male *S. globosum.* All are adult spiders.

site – the Quinta de São Pedro Study Centre and surrounding areas – located in the municipality of Almada in Portugal (38°38′19″ N, 9°11′50″ W). Different species of flower where *S. globosum* is commonly found were surveyed in March and April 2009 over a period of 22 days. A systematic hand search was carried out in which spiders were visually detected. Differences in the detectability of morphs could mean that this sampling method is biased towards particular morphs. However, this method proved the most efficient for *S. globosum* compared with other methods (beating or sweeping branches, and pitfall traps; Ajuria Ibarra, 2013). The possibility of counting the same individual twice was relatively low because females of *S. globosum* only occupy a small number of flowers over several days (see the Results). The colour of adult spiders was scored; however, some juvenile individuals showing intermediate coloration, such as beige, pink, or grey, were found.

Colour measurements

Colour variation in females of *S. globosum* was analysed considering the visual systems of three species which exemplify likely sources of selection on colour in our study population: (1) the honeybee *Apis mellifera* L., which is a common prey species for adult female *S. globosum* in the study site (Reader *et al*., 2006) and is known to use visual cues to detect and avoid spiders on flowers (Herberstein, Heiling & Cheng, 2009); (2) the blue tit (*Parus caeruleus*) as a model predator of invertebrates, which was common at our field site; and (3) male *S. globosum*, which might use colour as a cue during mating.

Adult female *S. globosum* were collected from our study site in spring 2012 ($N = 84$). CO_2 was used to immobilize them for measurements. Reflectance spectra were measured using an Ocean Optics USB2000 spectrometer (Ocean Optics, Inc., Dunedin, FL, USA) connected to a miniature deuterium halogen light source (DT-Mini-2-GS), and a custom-made bifurcated reflection probe (ZFQ-13666) with two 100-μm fibres. The fibreoptic probe was positioned at 45° to the surface of the abdomen of the spiders at a standardized distance of 1 mm. The spectrometer was attached to a personal computer (PC) running Spectra Suite Spectroscopy Software (Ocean Optics, Inc. 2008). All measurements were carried out in a dark room and were generated relative to a white diffuse reflectance standard (Ocean Optics WS-1) and

a dark standard (light source turned off, specimen covered with black cloth). The probe was positioned at three different standardized locations within the coloured area of the opisthosoma of each spider, and the average of the spectra recorded was used for the analyses. Each time the probe was repositioned, ten scans of the spectrum, with an integration time of 250 ms, were taken and averaged to reduce noise from the spectrometer (Llandres & Rodríguez-Gironés, 2011).

To evaluate how honeybees would perceive the different spider colours, photoreceptor excitation estimates were calculated using the colour hexagon model (Chittka, 1992, 1996). To obtain the excitation values (E) of each of the three photoreceptors [ultraviolet (UV), blue, and green] of honeybees, the sensitivity factor of each photoreceptor (R_i) was calculated using the formula:

$$R_i = \frac{1}{\int_{700}^{300} I_B(\lambda) \cdot S(\lambda) \cdot D(\lambda) \cdot d(\lambda)}. \quad (1)$$

$I_B(\lambda)$ is the spectral reflectance of the environmental background to which receptors are adapted; in the case of honeybees this is typically green foliage (Chittka *et al*., 1994), for which the green leaf spectrum provided by Chittka & Kevan (2005) was used. $S(\lambda)$ is the spectral sensitivity function of the photoreceptor (Peitsch *et al*., 1992), $D(\lambda)$ is the illumination spectrum, for which the standard function, D65, for open habitats was employed (Wyszecki & Stiles, 1982), and $d\lambda$ is the wavelength step size. Subsequently, the relative quantum catch absorbed by each spectral photoreceptor type (P_i) was calculated using the formula:

$$P_i = R_i \int_{700}^{300} I_S(\lambda) S(\lambda) \cdot D(\lambda) \cdot d(\lambda), \quad (2)$$

where $I_S(\lambda)$ is the spectral reflectance function of the stimulus (the colour of the spider). The excitation value of each honeybee photoreceptor, E_{UV}, E_{Blue}, and E_{Green}, was then calculated using the formula:

$$E_i = \frac{P_i}{P_i + 1}. \quad (3)$$

To determine the position of the colour of the spiders in the honeybee colour space, the values of E_{UV}, E_{Blue}, and E_{Green} were used to calculate the coordinates in the honeybee colour hexagon using the following equations:

$$x = \frac{\sqrt{3}}{2}(E_{Green} - E_{UV}) \quad (4)$$

$$y = E_{Blue} - 0.5(E_{Green} + E_{UV}). \quad (5)$$

The colour difference among pairs of red, yellow, and white spiders was then calculated by determining the Euclidian distance between them in the colour hexagon:

$$\Delta St = \sqrt{(x_{red} - x_{yellow})^2 + (y_{red} - y_{yellow})^2}, \quad (6)$$

where x and y are the coordinates of the hexagon calculated using eqns (4) and (5) (the subscript text indicates which colours are being compared – red and yellow in this example).

Colour discrimination among *S. globosum* morphs by the blue tit was examined following Théry & Casas (2002), using the visual models of Vorobyev *et al*. (1998). The spectral sensitivities of the four photoreceptors (UV, blue, green, and red) of the blue tit (Hart *et al*., 2000) were used to calculate the sensitivity factor, the relative quantum catch, and the excitation value for each photoreceptor using eqns (1), (2), and (3), respectively. The Euclidian distances among morphs were calculated from their coordinates in the colour tetrahedron (Goldsmith, 1990; Théry *et al*., 2005), obtained from the following equations:

$$x = \frac{2\sqrt{2}}{3}\cos 30^\circ (E_{Green} - E_{Red}) \quad (7)$$

$$y = E_{UV} - \frac{1}{3}(E_{Blue} + E_{Green} + E_{Red}) \quad (8)$$

$$z = \frac{2\sqrt{2}}{3}[\sin 30^\circ (E_{Green} + E_{Red}) - E_{Blue}] \quad (9)$$

Euclidian distances were calculated as:

$$\Delta St = \sqrt{(x_{red} - x_{yellow})^2 + (y_{red} - y_{yellow})^2 + (z_{red} - z_{yellow})^2} \quad (10)$$

Spider colour morphs were plotted in the colour tetrahedron with the 'pavo' package in R 2.15.3 (Maia *et al*., 2013), which calculates the coordinates of stimuli in the tetrahedron using the equations provided by Endler & Mielke (2005).

No data describing the visual system of S. *globosum* are available. Therefore, to evaluate if individuals of *S. globosum* can discriminate among colour morphs, we considered the visual system of a closely related crab spider, *Misumena vatia*. Electrophysiological studies have detected the presence of two classes of photoreceptors in this species: one sensitive in the UV region of the spectrum (around 340 nm); and one sensitive in the green region (around 520 nm) (Defrize *et al*., 2011). Reflectance intensities of the female morphs of *S. globosum* were measured at these

wavelengths and used to assess whether conspecifics are likely to be able to discriminate among them.

Colour development and heritability

In order to evaluate the development and heritability of the female colour trait, spiders were mated and their offspring reared in the laboratory. Specific objectives were to establish at what developmental stage offspring begin to display the trait and whether offspring colour correlates with maternal colour.

Adult females of *S. globosum* were collected in April 2009 ($N = 59$; 25 red, 31 yellow, and 3 white) and kept in the laboratory at 22 °C. They were housed individually in Petri dishes with a piece of moist cotton wool to maintain humidity. Twice a week, each female was fed a hoverfly (Diptera: Syrphidae), a lesser housefly (Diptera: Fannidae), or a blowfly (Diptera: Calliphoridae), caught in the field, before and after producing egg sacs. A total of 30 females (11 red, 16 yellow, and 3 white) produced egg sacs. Some of these females were mated with known males in the laboratory, and some had mated with unknown males in the field. However, it was unknown if females mated in the laboratory were virgin, and thus it is possible that they had been inseminated in the wild before they were collected. As males of *S. globosum* do not show colour variation, their genotype for any heritable component of the female colour trait was unknown.

Upon hatching, spiderlings were placed in separate 7-mL Perspex tubes. A piece of cotton wool was used to cover the tubes and was moistened regularly to maintain humidity. Newly hatched spiderlings were fed on *Drosophila melanogaster* flies. Early and intermediate instar spiders were fed on commercially supplied juvenile black field crickets (*Gryllus bimaculatus*), and houseflies (*Musca domestica*). Late instar and adult spiders were fed on hoverflies and blowflies caught in the field, as well as on houseflies (*M. domestica*). All spiders were fed twice a week. Mothers were kept in Petri dishes and fed regularly until death. Dead spiders were preserved in 70% alcohol. Females were able to produce up to three clutches of eggs. The size and weight of second instar spiderlings from 23-s clutches were measured (spiderlings from first and rare third clutches were not measured owing to time constraints). The length of spiderlings was measured from the anterior end of the cephalothorax to the posterior end of the opisthosoma under a stereomicroscope and using an eye-piece graticule. The total length of spiderlings was measured because they were too small and delicate to immobilize to measure only the length of the cephalothorax. They were weighed using a digital balance. Once spiders started to show signs of development of adult coloration (i.e. showing variation in colour from a previous uniform beige/cream coloration across all spiders), or sexual maturity in the case of the males (i.e. swelling of the palps), sex and coloration were recorded approximately every month until the end of the experiment. There was variation in growth rates among individuals, and many did not reach maturity, even 15 months after the first spiderlings hatched, which was when the experiment ended. Surviving spiders were killed by freezing.

Differences in life-history traits among morphs

To determine if there were differences among morphs in size and body mass, wild-caught spiders with adult coloration were collected in March and April 2009 and measured as described above (in this case, the length from the anterior to the posterior end of the cephalothorax was measured). To look for differences among morphs in behaviour in the field (time spent on flowers and in habitat patches, and the number of flowers occupied), spiders were marked and monitored over a period of 11 days in April and May 2009. Observations were made on all spiders found in two 10-m × 10-m plots in a meadow at the study site, and in two bushes of French lavender (*Lavandula stoechas* L.), upon which *S. globosum* is commonly found. Each 10-m × 10-m plot in the meadow and each French lavender bush was considered to be one 'habitat patch'. A total of 13 spiders in the lavender and 69 spiders in the meadow were marked with three dots of enamel paint on the cephalothorax, behind the ocular area, with each spider having a unique combination of colours. The spiders were then released on the flower where they were found. The plots and bushes were checked two to three times a day, and the location (a single flower or inflorescence in the same or a different plant) of each spider was recorded.

To search for any differences in components of reproduction among morphs, data from the breeding experiment were used. Data considered were: number of clutches laid before death; time from egg sac production to hatching; clutch size; offspring size and mass; sum of offspring size; clutch mass; and number of surviving offspring until the end of the breeding experiment. It was not possible to assess morph-specific survival because the morph of those individuals that did not survive until the end of the experiment was unknown.

Statistical analyses

To test whether colour variation among spiders was discrete, we performed a model-based clustering

analysis, using the Mclust package in R 3.0.1 (see Fraley & Raftery, 2007). This analysis was performed with data from red and yellow spiders only, because insufficient data for white spiders were available. The models assumed that the distribution of spiders in three-dimensional honeybee, four-dimensional blue tit, and two-dimensional crab spider colour-space is a mixture of an unknown number of clusters, each of which has its own multivariate normal distribution. A range of model configurations were considered: clusters were spherical, diagonal, or ellipsoidal in shape, and either equal or variable in volume and shape. The hypothesis that colour variation among spiders is continuous is represented by a modelled distribution containing just one cluster. The hypothesis that spiders belong to one of two common morphs (red and yellow) is represented by a model with two clusters. These hypotheses, and alternatives in which there were between three and nine clusters, were compared using the Bayesian Information Criterion (BIC), as were different model configurations. The performance of the best model was then evaluated by comparing its classification of spiders with our independent a-priori judgement of spider colour (either 'red' or 'yellow'). To ensure that this comparison was unbiased, priors were not used in the analysis.

One-way analysis of variance (ANOVA) and Kruskal–Wallis tests were used to investigate any differences among morphs in the sensitivity of honeybee, blue tit, and *M. vatia* photoreceptors, morphology (cephalothorax length and body mass), and behaviour in the field. Post-hoc multiple comparisons among colour morphs were carried out using Tukey's honest significant difference. A generalized linear model (GLM) with a binomial error structure was conducted to test for differences among morphs in the probability that an individual that was found in a habitat patch at the beginning of the monitoring period was missing at the end. Generalized linear mixed models (GLMMs), with binomial error structures and mother of clutch as a random effect, were used to test the effect of maternal colour morph on the probability of a daughter having red, yellow, or white coloration. Mother of clutch was specified as a random effect to account for the fact that siblings are not statistically independent. Data were pooled across clutches. GLMs and GLMMs, with the appropriate error structures (Gaussian, Poisson, Quasipoisson, or Binomial), and mother of clutch as a random effect in the case of mixed models, were fitted to test for differences among morphs in the measured components of reproduction. Chi-square tests were used to analyse differences among morphs in the number of clutches produced in the laboratory, to test for differences between morph frequencies observed in the laboratory and those observed in the field, and to test for differences in the proportion of females collected in the field and those that produced successful broods in the laboratory.

The normality of the data was tested, and data on honeybee excitation values for the blue photoreceptor, blue tit excitation values for the UV and blue photoreceptors, reflectance values for the crab spider photoreceptor that is most sensitive at 520 nm, spider cephalothorax length, and time spent on a particular flower were Box-Cox transformed to satisfy the assumption of a normal distribution. For GLMs and GLMMs, the significance of each term was assessed by backward deletion from a saturated model using the appropriate test statistic (likelihood ratio, F-ratio, or chi-square) following Crawley (2007) and Zuur *et al*. (2009). Statistics are presented for comparisons of models after each term was removed. All analyses were conducted in R 2.15.3 (R Development Core Team, 2013).

RESULTS

Colour variation among morphs

The coloration of a total of 184 females was classified subjectively. Yellow spiders had the highest frequency (60%), followed by red spiders (37%), whereas white spiders were found at much lower frequencies (3%). Red, yellow, and white females had markedly different reflectance spectra in honeybee (Figs 2, 3), blue tit (Figs 4, 5), and crab spider (Figs 6, 7) colour-space. These differences were confirmed by the clustering analysis, at least in the case of red and yellow spiders. The best model for the distribution of spiders in honeybee (best model with ellipsoidal clusters of equal volume and shape: d.f. = 16, BIC = 551.8), blue tit (best model with ellipsoidal clusters with varying volume, shape, and orientation: d.f. = 29, BIC = 877.4), and crab spider (best model with diagonal clusters of variable volume and shape: d.f. = 8,

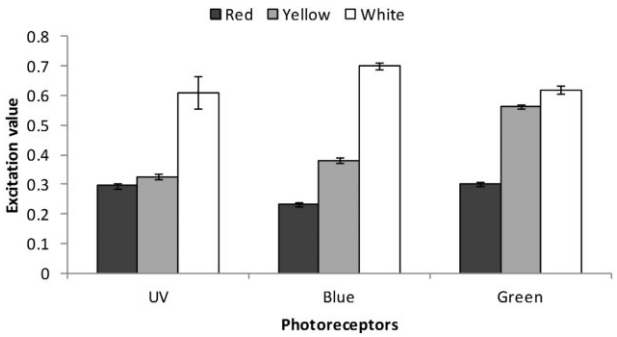

Figure 2. Mean excitation values (± SE) of the three photoreceptors of the honeybee (UV, blue, and green) for red (N = 38), yellow (N = 43), and white (N = 3) females of *S. globosum*.

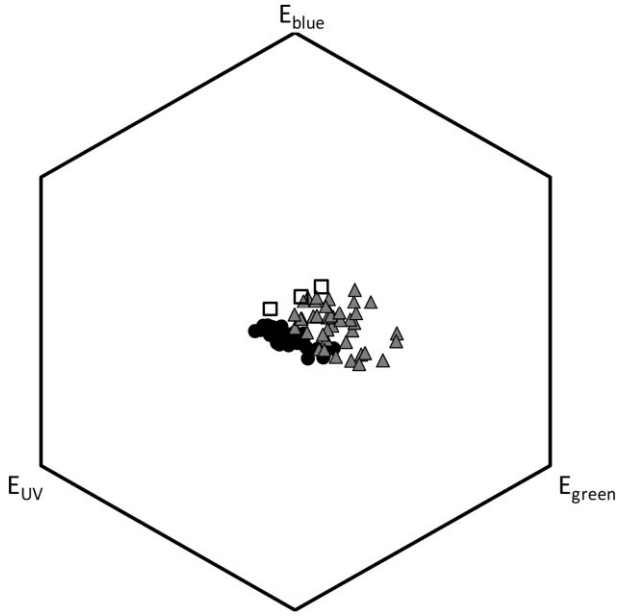

Figure 3. Colour loci of red (black circles, $N = 38$), yellow (grey triangles, $N = 43$), and white (white squares, $N = 3$) females in the honeybee colour hexagon.

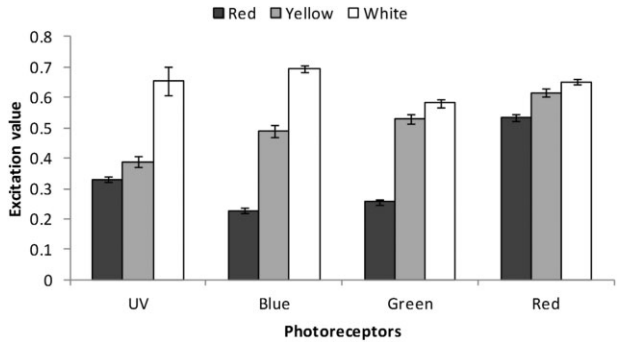

Figure 4. Mean excitation values (± SE) of the four photoreceptors of the blue tit (UV, blue, green, and red) for red ($N = 38$), yellow ($N = 43$), and white ($N = 3$) females of *S. globosum*.

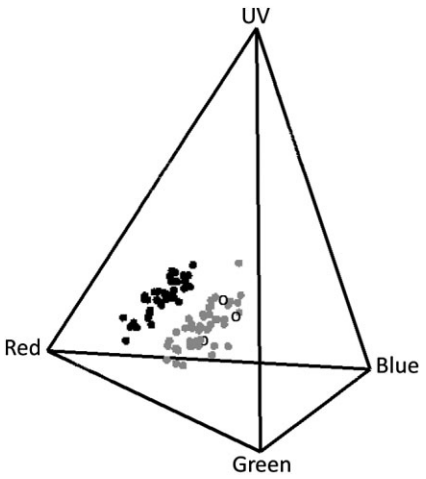

Figure 5. Colour loci of red (black circles, $N = 38$), yellow (grey circles, $N = 43$), and white (white circles, $N = 3$) females in the blue tit colour tetrahedron.

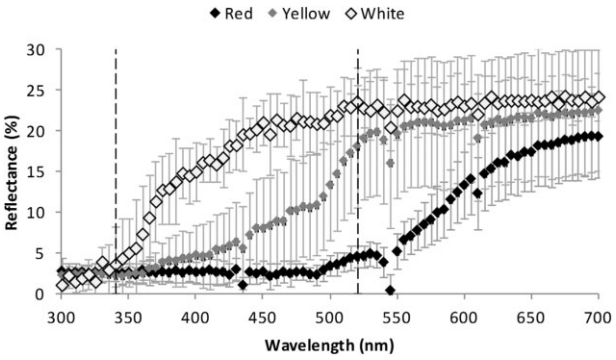

Figure 6. Mean reflectance spectrum values of red ($N = 38$), yellow ($N = 43$), and white ($N = 3$) spiders. Reflectance values were averaged from ten spectra taken at each of three locations on the coloured part of the body for each individual. Error bars represent SD. Vertical dashed lines indicate the peak sensitivities of the two *M. vatia* photoreceptors: 340 and 520 nm.

BIC = –827.7) colour-space contained two clusters. These modelled clusters corresponded closely to the two common colour morphs perceived by human observers: the correct classification rate was 84% (honeybee colour-space model), 99% (blue tit colour-space model), and 96% (crab spider colour-space model). Furthermore, the mean colour contrasts among the three colour morphs in both honeybee and blue tit colour-space were all higher than the colour-discrimination thresholds established for both species: 0.05 for the honeybee and 0.06 for the blue tit (Théry *et al.*, 2005). Mean distances (± SE) in the colour hexagon were: 0.188 ± 0.016 between red and yellow, 0.166 ± 0.04 between red and white, and 0.19 ± 0.083 between yellow and white. Mean distances (± SE) in the colour tetrahedron were: 0.264 ± 0.014 between red and yellow, 0.261 ± 0.028 between red and white, and 0.231 ± 0.116 between yellow and white. Moreover, excitation values were all significantly different among spider morphs for all honeybee photoreceptors (one-way ANOVA: UV: $F_{(DF)} = 20.98_{(2,81)}$, $P < 0.001$; blue: $F_{(DF)} = 66.04_{(2,81)}$, $P < 0.001$; and green: $F_{(DF)} = 104.7_{(2,81)}$, $P < 0.001$; Fig. 2), and for all blue tit photoreceptors (UV: $F_{(DF)} = 13.24_{(2,81)}$, $P < 0.001$; blue: $F_{(DF)} = 125.1_{(2,81)}$, $P < 0.001$; green: $F_{(DF)} = 116.8_{(2,81)}$, $P < 0.001$; and red: $F_{(DF)} = 12.48_{(2,81)}$, $P < 0.001$; Fig. 4). Likewise, significant differences were found among spider morphs

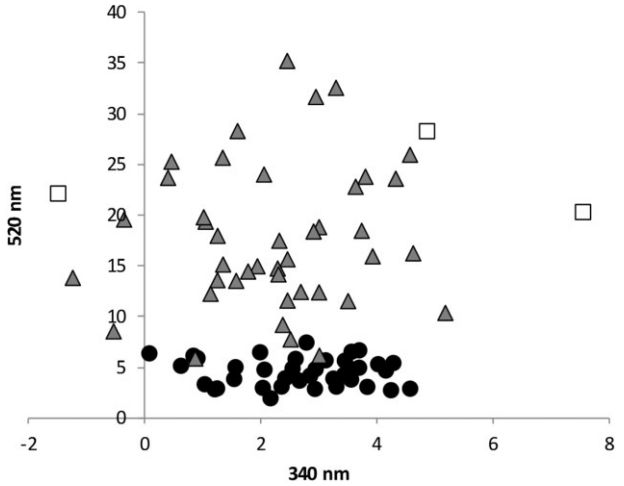

Figure 7. Colour loci of red (black circles, $N = 38$), yellow (grey triangles, $N = 43$), and white (white squares, $N = 3$) females in crab spider colour-space (mean reflectance values for *M. vatia*'s photoreceptor most sensitive at 340 nm and the one most sensitive at 520 nm).

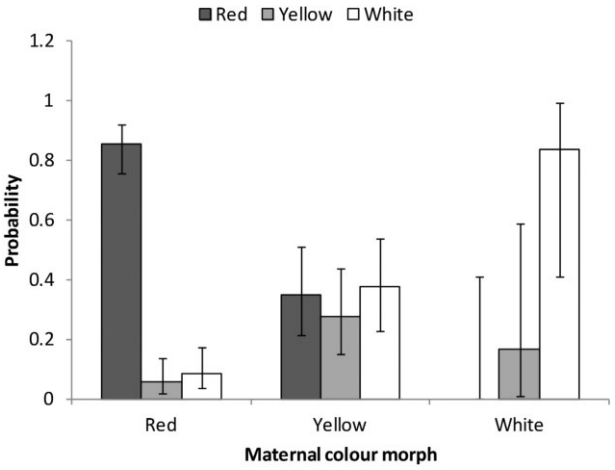

Figure 8. Probability of red, yellow, and white mothers producing red, yellow, or white daughters, across clutches, in the laboratory. Error bars are 95% confidence intervals calculated using the binomial distribution.

in the relative reflectance for the photoreceptor of *M. vatia* that is most sensitive at 520 nm ($F_{(DF)} = 134.4_{(2,81)}$, $P < 0.001$; Fig. 6). However, no significant differences were found for the photoreceptor of *M. vatia* most sensitive at 340 nm ($F_{(DF)} = 1.811_{(2,81)}$, $P = 0.17$; Fig. 6). Post-hoc comparisons indicated a significant difference between all colour morph pairs (Tukey HSD tests, $P < 0.05$), except between yellow and white for the honeybee green photoreceptor, the blue tit green and red photoreceptors, and the crab spider photoreceptor most sensitive at 520 nm. Honeybees and birds are known to use achromatic (brightness), instead of chromatic, contrast for discrimination of objects at long distances or of small size; for this task, honeybees use the signal of the green photoreceptor, and birds use the signal of double cones, the spectral sensitivities of which are closely approximated by the combination of the green and red photoreceptor sensitivities (Osorio, Miklósi & Gonda, 1999; Spaethe, Tautz & Chittka, 2001). Therefore, our results suggest that both honeybees and the blue tit are unable to discriminate between yellow and white spiders using achromatic contrast.

Colour development and heritability

A total of 1864 offspring hatched from 68 broods produced by 30 females in the laboratory. There was no significant difference in the proportions of the different morphs found in samples of adult females from the field, and those found in the group of females which produced successful broods in the laboratory ($\chi^2 = 0.875$, d.f. = 2, $P = 0.646$). Spiderlings that reached maturity did so in approximately 14 months. Of the offspring that hatched, 28.9% survived until the end of the experiment. Only 12.8% ($N = 241$) could be scored as a male or a female of one of the three colour morphs. Of the 12.8% that could be scored, 30.3% were red females, 6.6% were yellow females, 10.8% were white females, and 52.3% were males. Red daughters started to become distinctly red approximately 6 months before it was possible to determine the phenotype of yellow or white daughters. Most yellow and white daughters showed a beige/cream coloration before developing a distinct yellow or white colour. Spiders that could not be assigned to a phenotype or as a male had beige (6.1%), cream (61.7%), grey (8.3%), pink (15%), or almost completely black (8.9%) coloration, and none reached maturity. Individuals that were scored as one of the three morphs always had the same colour every time they were scored. However, we cannot discard the possibility that spiders changed colour and then rapidly reverted to their original colour over the course of a few days (Llandres *et al.*, 2013).

Morph frequencies of daughters for which the phenotype could be determined differed significantly from those of females observed in the field ($\chi^2 = 96.353$, d.f. = 2, $P < 0.001$). Red and white spiders were significantly more common (63% and 23%, respectively) in the laboratory than in the field, and yellow spiders were rarer (14%). Female colour was a heritable trait (Fig. 8). Daughters had a significantly higher probability of having red coloration if their mothers were red (GLMM: $\chi^2 = 22.519$, d.f. = 2, $P < 0.001$), and had a significantly higher probability of having white coloration if their mothers were white ($\chi^2 = 8.721$,

d.f. = 2, $P = 0.013$; Fig. 8). Although yellow offspring were more common amongst the offspring of yellow mothers than amongst the offspring of other mothers, the effect of maternal colour on the probability that a daughter would be yellow was not significant ($\chi^2 = 5.367$, d.f. = 2, $P = 0.068$; Fig. 8).

Differences in life-history traits and behaviour among morphs

A significant difference was found in cephalothorax length among red ($N = 69$), yellow ($N = 109$), and white ($N = 6$) females (one-way ANOVA: $F_{(DF)} = 3.802_{(2,180)}$, $P = 0.024$). Red females had larger cephalothoraxes (mean length ± SE: 1.876 ± 0.043 mm) than did yellow (1.775 ± 0.034 mm) and white (1.59 ± 0.162 mm) females. However, no significant difference in body mass was found among morphs [Kruskal–Wallis test: $\chi^2 = 2.527$, d.f. = 2, $P = 0.283$; median (interquartile range) body mass for red: 0.014 (0.011–0.021) g; yellow: 0.013 (0.01–0.017) g; and white: 0.014 (0.01–0.05) g].

A total of five females were found in the Lavender bushes: one red, one yellow, and three white. In the patches in the meadow, 25 females were observed: 10 red, 13 yellow, and two white. A significant difference among colour morphs was found in the time that a spider spent on a given flower (a single flower or inflorescence in the same or a different plant) ($F_{(DF)} = 4.488_{2,28}$, $P = 0.020$). Yellow females spent significantly more time on a particular flower (mean time ± SE: 97.722 ± 27.654 h) than did red (27.815 ± 3.812 h) or white (28.778 ± 3.226 h) females. However, no significant difference among morphs was found in the time that a spider spent in a particular patch [$\chi^2 = 2.899$, d.f. = 2, $P = 0.235$; median time (interquartile range) for red: 75 (30–152) h; yellow: 244 (48–264) h; and white: 77.5 (28.50–229.25) h]. Likewise, no significant difference among morphs was found in the number of flowers that a spider occupied over the duration of the observations [$\chi^2 = 0.719$, d.f. = 2, $P = 0.698$; median number of flowers (interquartile range) for red: 3 (1–3); yellow: 1 (1–2.75); or white: 2 (1.25–2.75)]. Additionally, there was no significant difference among morphs in the probability that an individual that was found in a habitat patch at the beginning of the observation period was missing at the end (GLM: $\chi^2 = 22.918$, d.f. = 2, $P = 0.186$; proportion of missing red = 0.71, of missing yellow = 0.25, and of missing white = 0.5).

No significant difference was found among morphs in the proportion of females that produced one, two, or three clutches in the laboratory ($\chi^2 = 2.365$, d.f. = 4, $P = 0.669$). A significant effect of clutch number (only the first or the second because white spiders did not produce a third clutch) was found on the number of days that spiderlings took to hatch (GLMM: $\chi^2 = 5.165$, d.f. = 1, $P = 0.023$). Spiderlings from first clutches took longer to hatch than did spiderlings from second clutches (mean number of days ± SE for first clutch: 28.094 ± 1.157 days and for second clutch: 25 ± 0.668 days). However, there was no significant effect of maternal colour morph ($\chi^2 = 0.715$, d.f. = 2, $P = 0.700$) or its interaction with clutch number ($\chi^2 = 3.101$, d.f. = 2, $P = 0.212$) on time to hatching. Similarly, there was no significant main effect of maternal colour morph on clutch size, individual offspring size, individual offspring mass, sum of size of all offspring, total clutch mass, or probability of survival of offspring (Table 1). Likewise, no significant main effect of maternal cephalothorax length was found on any of the variables mentioned earlier. Furthermore, the interaction between maternal colour morph and maternal cephalothorax length was not significant for any of the response variables (Table 1).

DISCUSSION

The results of this study suggest that the colour variation among females of *S. globosum* morphs is discrete and heritable, and hence can be considered to be a true polymorphism. The results also demonstrate that this polymorphism should be detectable to honeybees, birds, and, to a lesser degree, at least to some crab spider species. This implies that prey, predators, and mates have the potential to influence the persistence of the polymorphism via natural selection. We found limited evidence that colour morphs differ in morphology and ecology. These findings establish the nature of the previously unstudied polymorphism observed in *S. globosum* and provide a basis for future research to understand the mechanisms involved in its maintenance. These results should be interpreted with caution because it was necessary to combine approaches in natural conditions and in the laboratory to be able to understand as much as possible about the biology of the system; in natural conditions, bias in sampling caused by variation in detectability of morphs may have influenced our findings, and artificial laboratory conditions (e.g. constraints on diet) may have led to our results being unrepresentative of the natural situation.

Colour variation among morphs

Red, yellow, and white morphs of *S. globosum* clearly differed in the regions of the spectrum in which they showed substantial reflectance. The differences in reflectance spectra, at least between red and yellow morphs, were found to be discrete. These morphs formed distinct clusters in the visual systems of the

Table 1. Results of GLMs and GLMMs testing for an effect of maternal colour morph, maternal cephalothorax length (CL), and the interaction between them on clutch size, individual offspring size, individual offspring mass, sum of size of all offspring per clutch, total clutch mass, and probability of survival from time of hatching to the end of the breeding experiment

Response variable	Maternal morph			Maternal CL			Maternal morph × maternal CL		
	GLM (error structure: Quasipoisson)								
	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Clutch size	6.29	2	0.702	0.005	1	0.982	28.738	2	0.2
	GLMM (error structure: Gaussian, random effect: mother of clutch)								
	LR	d.f.	*P*	LR	d.f.	*P*	LR	d.f.	*P*
Individual offspring size	3.402	2	0.183	0.001	2	0.975	4.371	2	0.112
Individual offspring mass	0.383	2	0.826	0.236	2	0.627	1.560	2	0.458
	GLM (error structure: Gaussian)								
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Sum of offspring size	1.925	2,15	0.18	0.005	1,14	0.948	2.18	2,12	0.156
Total clutch mass	2.028	2,13	0.171	0.014	1,12	0.909	1.051	2,10	0.385
	GLMM (error structure: binomial, random effect: mother of clutch)								
	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Probability of survival	1.745	2	0.418	1.259	1	0.262	0.257	2	0.879

All data analysed were from second clutches, and also across clutches in the case of clutch size and probability of survival. d.f., degrees of freedom; LR, likelihood ratio.

honeybee, the blue tit, and the crab spider, *M. vatia,* and the differences should be detectable by all three taxa. Honeybees and the blue tit should also be able to discriminate between white and red spiders, and, at short distances, between yellow and white. It should be noted that the classification rates from the clustering analysis were not completely accurate (84% for honeybees, 99% for the blue tit, and 96% for crab spiders). This could be caused by the presence of noise in the measurements, a difference in the visual systems of the species which provided the data for the analysis, and the real 'receiver', or some genuine ambiguity. However, without a more powerful study of the heritability and the genetics of the trait, it would be difficult to clarify this further. Nevertheless, these findings, together with several other lines of evidence, suggest that prey, predators, and male conspecifics might play an important role in the maintenance of the polymorphism in female *S. globosum*.

Honeybees are common prey of *S. globosum* in the field (Reader *et al*., 2006), but they try to avoid flowers which harbour crab spiders (Dukas, 2001; Dukas & Morse, 2005; Reader *et al*., 2006). They have been shown to be able to discriminate colours (Hempel de Ibarra, Giurfa & Vorobyev, 2002; Giurfa, 2004; Dyer & Neumeyer, 2005), and spider coloration has been observed to have a significant effect on their behaviour (Heiling *et al*., 2005; Llandres & Rodríguez-Gironés, 2011). Thus, it seems reasonable to postulate that honeybees might respond differently to different *S. globosum* colour morphs, with likely consequences for spider fitness, hence generating selection. It is likely that honeybees have an important impact on *S. globosum*'s fitness because they have been observed to be their most frequent prey at our field site. However, these observations have not been formally quantified, and data on how different types of prey affect *S. globosum*'s fitness are

necessary to establish the importance of honeybees for the maintenance of the polymorphism.

Predation by birds is another factor that could have an important influence on the maintenance of the polymorphism, as differences among all morphs appear to be detectable by the blue tit (at least when using chromatic contrast), and spiders are largely exposed on flower heads in open fields. Spectral sensitivities in the photoreceptors of birds are fairly constant, except for the short-wavelength-sensitive photoreceptor, and thus can be separated into two groups: UV sensitive; and violet sensitive (Hart, 2001). It would be expected that all UV-sensitive species could distinguish among *S. globosum* colour morphs. This could have a significant effect on morph fitness as birds have been observed to respond to differences in coloration in insects (Exnerová *et al*., 2006; Lindstedt *et al*., 2011) and have been shown to have a negative impact on spider populations, at least in forest habitats (Gunnarsson, 2008). Nevertheless, as with honeybees, it is necessary to assess the actual effect of bird predation on populations of *S. globosum* in order to explore formally their impact on the maintenance of the polymorphism. It is worth noting, however, that in 3 years of intensive field observations of *S. globosum* on flowers, the only predators we saw consuming female spiders (rarely) were conspecifics, and birds were very seldom seen foraging on flowers/inflorescences of the species upon which *S. globosum* was most commonly found.

If we assume that the visual system of crab spiders is reasonably consistent across the family, we can infer from the results presented here that male *S. globosum* will be able to discriminate among females of different colours, except between yellow and white. A morphological study of the visual system of *M. vatia* showed the presence of a third type of photoreceptor, which would imply trichromatic colour vision (Insausti *et al*., 2012), and thus it is possible that crab spiders may be able to discriminate between yellow and white morphs. However, even though it has been demonstrated that *M. vatia* has the necessary morphological and physiological characteristics for colour vision, the visual acuity of crab spiders is not well known (Defrize *et al*., 2011; Insausti *et al*., 2012) and, ideally, the visual system of *S. globosum* should be investigated to draw any conclusions about its perception of the three colour morphs. Therefore, it is not possible to know how well male *S. globosum* might distinguish among female colour morphs in the wild. Nevertheless, mate choice could still be a potential factor contributing to the maintenance of the polymorphism (Masta & Maddison, 2002; Hebets & Maddison, 2005; Svensson, Abbott & Härdling, 2005; Lim *et al*., 2008; Takahashi *et al*., 2010).

Although honeybees and crab spiders appear to be able to detect the difference between the female morphs of *S. globosum*, some details of their visual systems argue against a role for either sexual selection or selection by prey in the evolution of the polymorphism. The long-wave photoreceptors of honeybees and crab spiders have the highest sensitivity at wavelengths of 535 nm and 520 nm, respectively, and hence the ability of the two species to be able to perceive red colour on females (which reflects substantial light only above ~550 nm), as distinct from black, which is the colour of the rest of the opisthosoma and the cephalothorax, may be limited. Consequently, the evolution of red colour from a hypothetical black, yellow, or white ancestral state in *S. globosum* as a result of a selective pressure from honeybees or mate choice may be intuitively implausible. Nevertheless, it has been argued that honeybees can actually perceive red colour (Chittka & Waser, 1997). Even if bees and spiders cannot distinguish it from black, red colour in females may still be adaptive; for example, the yellow or the white morph might be ancestral, in which case selection could have favoured a modification to red over a change to black because the transition was either more easily achieved or less costly. This modification would then be adaptive if a mechanism such as frequency-dependent selection was operating, and having a colour morph distinguishable from an ancestral colour was advantageous. Studies that look at morph frequencies in populations with and without the presence of honeybees and potential predators could help to clarify the role of these in the mechanisms maintaining the polymorphism.

Reflectance spectra were only measured in adult females. As selection may act at any life stage in which morphs are phenotypically distinct, it would be useful to measure the reflectance spectra of juvenile spiders that show intermediate coloration in a more accurate and objective way than by human classification to see whether they cluster in the same way as adults.

Heritability of the polymorphism

The results of the breeding experiment provide evidence supporting a genetic origin of the colour polymorphism in *S. globosum*. Maternal colour morph had a significant effect on the probability of daughters having red or white coloration. These results are consistent with the hypothesis that the three colour morphs observed in *S. globosum* are genetically inherited. It is important to note that, without more detailed experiments, it is not possible to identify the exact mode of inheritance, or indeed to rule out completely other possible explanations for the observed

data. For example, it is possible that a maternal effect produced the association between maternal and offspring phenotypes.

Late development of adult coloration in yellow daughters could account for the non-significant effect of maternal colour on the probability of daughters having yellow coloration. Red females developed adult coloration approximately 6 months earlier than did yellow or white females. Therefore, if yellow females took longer than white females to develop adult coloration in the conditions under which they were reared, it is possible that yellow females died or their colour could not be determined before the experiment ended. Such differences in rates of development in coloration have been observed in the colour polymorphic Theridiid spiders *E. ovata* and *T. grallator* (Oxford, 1983; Gillespie & Tabashnik, 1989). An alternative explanation could be that yellow spiders are homozygous recessive, and our statistical power to detect heritability of this morph is therefore lower, owing to the fact that homozygous-recessive yellow mothers would have more variable offspring (with fewer showing the maternal colour) compared with red or white mothers, which were a mixture of heterozygous and homozygous dominant.

There was also a significant difference between the laboratory and the field in the observed frequencies of *S. globosum* colour morphs. Red and white females were more common than yellow females in the laboratory, whereas yellow females were more common than red females, and much more common than white females, in the field. There are several possible explanations to account for the observed differences. First, if yellow coloration takes longer to develop in laboratory conditions, then more yellow spiders may have not been scored at the end of the experiment. Assuming that the field survey occurred relatively later in the developmental period than the laboratory census at the end of the experiment, such a bias against yellow would not be as evident in the field data. Second, the daughters that could be scored at the end of the experiment came only from the subset of females that produced successful broods in the laboratory (30 out of 59 females originally collected). Perhaps, for some reason, mothers that were more likely to have yellow offspring were also more likely to fail to produce offspring in the laboratory. Third, there were undoubtedly differences in prey availability between the laboratory and the field, which may have influenced the survival and development of different morphs in subtly different ways. It has been observed that pigmentation in spiders can be influenced by their diet (Gillespie, 1989; Théry, 2007). If yellow offspring were particularly sensitive to deficiencies in the laboratory diet, this may have hindered their survival or successful maturation. Fourth, the population of *S. globosum* sampled in the field may not be in Hardy–Weinberg equilibrium (Hedrick, 2000). Attempts to fit several different models of Mendelian inheritance to the data from this study were hindered by the small proportion of offspring that could be scored confidently, and failed to show support for any simple hypothesis regarding the number of alleles and the dominance relationship among them (H. Ajuria Ibarra, J. Brookfield & T. Reader, unpubl. data).

Differences in life-history traits among morphs

A significant difference in size was found among *S. globosum* colour morphs. Red females had a larger cephalothorax than did yellow and white females. This difference in size could be explained by a faster development of individuals of this morph than individuals of the yellow and white morphs. At the time of year when the survey was carried out, not all spiders had reached maturity and it was not always possible to differentiate between juveniles and adults. This difficulty was caused by *S. globosum* having an almost completely black coloration in the ventral part of the abdomen and an epigynum that is not as prominent as in other species. As the spiders that were reared in the laboratory were not measured throughout their development, it is not known if there were any differences in size among morphs. However, it is possible that yellow and white females were at an earlier developmental stage (on average) than red females when they were collected in the field and measured, and thus have a smaller size. Red coloration could be correlated with development rate for various reasons (e.g. if colour influences thermoregulation, which influences metabolism – see Robinson & Robinson, 1978; Sweeney & Vannote, 1978; Blanckenhorn, 1997), or it could confer a foraging advantage. Alternatively or additionally, the different morphs could be associated with alternative strategies in which they show different developmental rates under different environmental conditions (Ahnesjo & Forsman, 2003). Proper tests quantifying foraging success and developmental rates of the different morphs are necessary to establish the nature of any variation among them, and between natural and laboratory conditions.

Yellow females remained for significantly longer (on average, 3.5 times longer) on a given flower compared with red or white females. Remaining for longer periods on a particular flower might result from the adoption of alternative foraging strategies. It has been observed that individuals of *M. vatia* which have captured prey remain significantly longer on a flower than do those that have not caught prey (Morse,

2000). Consequently, yellow *S. globosum* might remain for longer on a particular flower if they capture prey more frequently compared with red and white females, which could be a result of the effect of spider colour on prey behaviour or a correlation between colour and some other trait that would enhance prey capture. A correlation between spider colour and flower colour could indicate an advantage of a particular morph when it occupies a flower of a particular colour (Théry, 2007; Llandres *et al*., 2013). However, data forming part of a larger study on morph distributions show no strong association between spider colour and flower colour or species (Ajuria Ibarra, 2013). There might be a lack of selection for crypsis or preference for flower colour in crab spiders because such predators can experience an overabundance of prey or because flower colour is not a reliable cue for prey visitation rate (Brechbühl, Casas & Bacher, 2011; Defrize, Llandres & Casas, 2014). More detailed information about *S. globosum*'s foraging behaviour, diet composition, and the way they are perceived by prey, is necessary to establish whether the differences observed are, in fact, indicative of alternative foraging strategies adopted by females of different morphs.

No difference among morphs was found in the components of reproduction measured in this study. However, our data were obtained in artificial conditions in the laboratory, and we are uncertain about whether the data reflect the situation in the field. Although these results provide some information about particular components of reproduction of the different morphs, complete measures of fitness in natural conditions were not available in this study. Therefore, even though our results do not show evidence of any differences among morphs in reproductive output, they are only a partial representation of the situation. If any adaptive advantage to one particular colour morph over the others is to be confirmed, the effect of colour on other aspects of reproduction (ideally total lifetime reproductive success) and survival (from egg to adult) should be quantified.

CONCLUSION

The great potential of the study of colour-polymorphic animals to inform our understanding of the maintenance of phenotypic and genetic diversity has not been fully exploited, partly because the heritability of observed colour variation is seldom known and because studies often only consider one potential signal receiver. Here, we have shown that a conspicuous natural colour polymorphism is heritable, and that multiple agents of natural selection (prey, predators, and mates) can distinguish among morphs. Hence, our study shows that the polymorphism in *S. globosum* is an ideal candidate for behavioural and ecological studies of factors influencing the maintenance of diversity. As with the few other systems for which we have similar baseline knowledge, if we are to realize the potential of our study system, what is needed now is experimental tests of the impact on individual fitness of differentiation amongst colour morphs by ecologically relevant receivers and long-term studies of morph dynamics in natural populations.

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SHARED DATA

Data deposited in the Dryad digital repository (Ajuria Ibarra & Reader, 2014).