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RESEARCH ARTICLE

Environmental and hormonal factors controlling reversible colour change in crab spiders

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SUMMARY

Habitat heterogeneity that occurs within an individual's lifetime may favour the evolution of reversible plasticity. Colour reversibility has many different functions in animals, such as thermoregulation, crypsis through background matching and social interactions. However, the mechanisms underlying reversible colour changes are yet to be thoroughly investigated. This study aims to determine the environmental and hormonal factors underlying morphological colour changes in *Thomisus onustus* crab spiders and the biochemical metabolites produced during these changes. We quantified the dynamics of colour changes over time: spiders were kept in yellow and white containers under natural light conditions and their colour was measured over 15 days using a spectrophotometer. We also characterised the chemical metabolites of spiders changing to a yellow colour using HPLC. Hormonal control of colour change was investigated by injecting 20-hydroxyecdysone (20E) into spiders. We found that background colouration was a major environmental factor responsible for colour change in crab spiders: individuals presented with white and yellow backgrounds changed to white and yellow colours, respectively. An ommochrome precursor, 3-OH-kynurenine, was the main pigment responsible for yellow colour. Spiders injected with 20E displayed a similar rate of change towards yellow colouration as spiders kept in yellow containers and exposed to natural sunlight. This study demonstrates novel hormonal manipulations that are capable of inducing reversible colour change.

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INTRODUCTION

Pigment patterns are classic traits used to study adaptation and the generation and maintenance of phenotypic diversity (Endler, 1980; Sheppard, 1951). Colour patterns are among the most visually obvious evolutionary adaptations in the natural world and play an essential role in the most basic pursuits of an animal's life, such as camouflage or sexual signalling. Decades of research focusing on natural history, ecology and behaviour have revealed the function of colour patterns in a wide range of animals (e.g. Cott, 1940; Endler, 1980; Stevens and Merilaita, 2009). For instance, cryptic colouration allows some animals to escape predators by matching their background (Endler, 2006). Other animals use pigmentation for sexual display (Allen et al., 2011; Badyaev and Hill, 2003; Kodric-Brown, 1998) or social communication (Rohwer, 1975; Tibbetts and Dale, 2004). In some animals, conspicuous colouration through aposematism has evolved to act as a warning signal and advertise defences to predators (Stevens and Ruxton, 2012).

Most colouration studies have been conducted on animals that establish a fixed colour pattern that is retained through their lifespan (Reed et al., 2008; Steiner et al., 2008; but see Whiteley et al., 2011). Temporal and spatial habitat heterogeneity that occurs within an individual's lifetime may favour the evolution of reversible plasticity by enabling phenotypic changes in response to environmental variation. Many fish, reptiles and invertebrates exhibit reversible colour plasticity during their lifetime (e.g. Hanlon et al., 1999; Stuart-Fox et al., 2006; Whiteley et al., 2011). Colour reversibility has many different functions in animals, including an involvement in thermoregulation (Brown and Sandeen, 1948), crypsis through background matching to avoid predator detection (Stuart-Fox et al., 2008), and social interactions (Stuart-Fox and Moussalli, 2008). Speculation as to the evolution of phenotypic plasticity should be based upon comprehensive knowledge of the molecular and cellular mechanisms by which individual organisms express flexibility in a changing environment. However, the mechanisms underlying phenotypic plasticity have, to date, been poorly studied (Schlichting and Smith, 2002).

Recent work has greatly improved our understanding of the genetic, developmental and physiological mechanisms underlying colour pattern formation in insects (Nijhout, 2010). For insects that undergo a morphological ontogenetic or non-reversible seasonal colour change, the change is regulated internally by hormones. It is associated with adaptations to particular environments and/or with transitions in developmental stages and is usually mediated by the endocrine system through a pulse of 20-hydroxyecdysone (20E) (reviewed by Nijhout, 2010). In the buckeye butterfly, *Precis coenia*, the pale beige linea form develops if ecdysteroids are present during a critical period in the early pupal stage. In the absence of ecdysteroids, the dark reddish-brown rosa form develops. In brainless individuals that develop the rosa phenotype irrespective of environmental conditions, an injection of ecdysone during the ecdysone-sensitive period induced the development of linea phenotype (Rountree and Nijhout, 1995). Koch et al. (Koch et al.,

1996) found similar effects of 20E injection on the wing colour patterns of the seasonal polyphonic butterfly *Bicyclus anynana*. Juvenile hormone titres and ecdysone titres have been shown not only to control moulting and metamorphosis, but also to regulate species-specific pigment variation in several species of butterflies (Gäde et al., 1997; Koch, 1996). These studies provide a solid foundation for our understanding of the mechanisms regulating colour pattern formation in insects that show a non-reversible colour change.

The mechanisms underlying reversible colour changes have been less well investigated [but see the recently published review by Sköld et al. (Sköld et al., 2012)]. Crab spiders can change colour over several days to match the colour of the flower on which they sit (Morse, 2007), and so provide a good system in which to study the mechanisms underlying reversible colour plasticity. The crab spider *Misumena vatia* can reversibly change its colour from white to yellow (Morse, 2007; Oxford and Gillespie, 1998; Théry and Casas, 2009). Whilst some of the metabolites involved in *M. vatia* colour production have been identified (Riou and Christidès, 2010), nothing is known about the internal hormonal mechanisms controlling colour production. Prey and light reflected by the background have been shown to be important external factors in determining colour change in crab spiders (Gabritschevsky, 1927; Rabaud, 1919; Théry, 2007): individuals presented with a yellow background and fed red-eyed flies better matched the yellow background, whilst individuals submitted to white background and fed white-eyed flies better matched the white background (Théry, 2007). However, variability in response was very high as many individuals were found to remain white despite being submitted to yellow stimuli. Furthermore, spiders that responded to the yellow background treatment did not change to the typical yellow colour that we observe in individuals collected from the field (Théry, 2007).

The study aims to determine the environmental and hormonal factors underlying morphological colour changes, and the biochemical metabolites produced during these changes, in *Thomisus onustus* Walckenaer 1805 crab spiders. We quantified the dynamics of colour change over time by keeping spiders in yellow and white containers under natural light conditions and measuring their colour over 15 days using a spectrophotometer. In the laboratory, we characterised the chemical metabolites of spiders undergoing colour change to yellow using HPLC. We also investigated the hormonal basis of colour change using 20E injections.

MATERIALS AND METHODS

Study species

A total of 160 female *T. onustus* crab spiders were collected between 9 and 13 April 2012, on *Chrysanthemum segetum* yellow flowers in Extremadura, Spain. Spiders were brought to a rearing room and kept in plastic containers with a white paper and a piece of wet cotton. Spiders were kept in plastic containers as they change towards white colour under these laboratory conditions (A.L.L., personal observations); we wished to ensure that they would change towards a whitish colouration before the start of our experiments. Spiders were fed bees once per week and kept at 18°C to 20°C with a relative humidity of 50 to 62%.

Spider colour measurements

We measured the reflectance of spiders using a spectroradiometer (Avantes Avaspec 256, Eerbeek, The Netherlands), which was connected to a deuterium-halogen lamp (Avantes Avalight D/H-S) that emitted light of wavelengths between 350 and 1000 nm. Reflectance was expressed relative to a 99% reflectance (white) and a 0% reflectance (black) standard. The dark spectrum was taken from a black tape that absorbed all wavelengths and was also used as the background for spider measurements. We used an optic fibre sensor (1.5 mm in diameter) equipped with a quartz window (cut at a 45 deg angle) to take measurements. A bifurcated fibre was attached to the spectrometer and to the light source. Ten spectra, with an integration period of 500 ms, were averaged for each spider to reduce spectrometer noise. We collected spiders that were large enough for accurate colour measurements (prosoma width: 2.00–3.52 mm). Individuals were placed on a flat mounting stand and colour measurements were taken on the opisthosoma (approximately double the size of the prosoma). Only one spider was measured at a time. We fixed the spectrometer's fibre within the probe holder, 2 mm above the surface of the spider, so that a series of colour measurements could be taken from the same distance.

Evaluation of spider colour

We decided not to use a visual model to evaluate colouration as we wanted to avoid making any assumptions about colouration from the perspective of a particular receiver. Our calculations of reflectance provide an indicator of colour that is independent of any visual model. We normalised each reflectance spectrum to its maximum, as the height of a reflectance curve taken from a curved surface (such as a spider) depends on the distance between the surface and the optic fibre sensor used to take the measurement: the height of the reflectance spectrum is greater at small distances. Although we followed the same measurement protocol for every individual, the distance between the spider and the optic fibre may have varied between spiders. The height of the reflectance spectrum represents spider brightness, and normalising each reflectance spectrum to its maximum also eliminated variation due to brightness. We focused on changes in the colour (spectral shape) of each reflectance spectrum (in the same way that we would compare histograms using relative frequencies). We used spiders ranging from white to yellow colour, collected from the field and measured the day of collection, to compute our colour index. The 'colour index' we refer to was calculated as the area below the spider reflectance curve between 400 and 500 nm. This measure was used because the reflectance curve of yellow spiders is sigmoidal in shape, with a steeply increasing slope at approximately 500 nm, and that of white spiders has a marked slope increase at approximately 400 nm. The spectral files collected from measurements gave us the percent reflectance value for each 5 nm of wavelength; the 'colour index' is therefore percent reflectance measured in 5 nm intervals. For simplicity we will use '%' instead of '% reflectance×5 nm' as units of the colour index throughout this article. The reflectance curve (between 400 and 500 nm) of white spiders typically gives a wider colour index than that of yellow spiders (Fig. 1). Our colour index ranged from 12.38 to 1468.8%, the values for the 'most yellow' and 'most white' spider measured during the experiments, respectively. We measured the reflectance spectra of a range of yellow papers and a white paper from Canson Mi-Teintes (reference nos 553, 400, 101 and 335, Tours, France; supplementary material Fig. S1). The change between yellow and white colour in spiders can also be well described using the inflection point of an estimated sigmoid curve fitting their reflectance curve; we found a high correlation between colour index and the wavelength of the inflection point of the estimated sigmoid curve of white and yellow spiders ($P<0.0001$, $F_1=52{,}120$, $R^2=0.99$, d.f.=574; see supplementary material Fig. S2). However, we used colour index as our descriptor as it is a measured

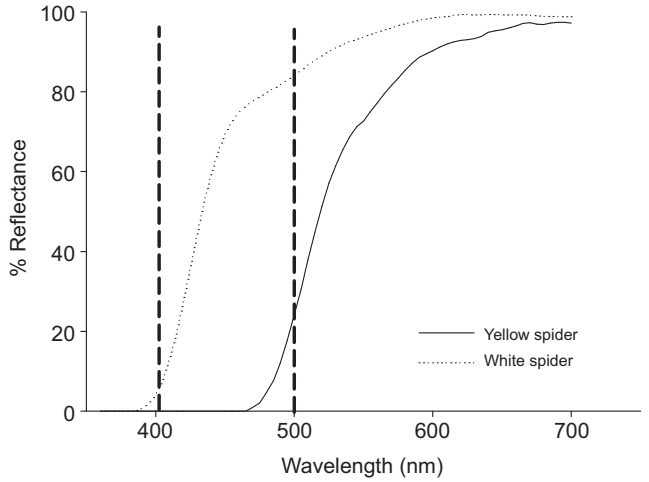


Fig. 1. Normalised reflectance spectra of crab spiders. Reflectance spectra of a white (dotted line) and a yellow spider (solid line). The area under the reflectance curve located between the vertical dashed lines indicates the estimator used for spider colour. A larger area indicates a whiter spider.

value taken from reflectance spectrum data. The value of the inflection-point wavelength is an estimated value from the sigmoid curve fit of the reflectance spectrum data.

Effect of moulting on spider colour change in the laboratory

To determine the effect of moulting on colour change from yellow to white, we used 80 field-collected spiders that were initially yellow (mean ± s.d. of colour index: 134±69.31%). Spider colour was measured on the day of collection and several times over the following 23 days before the start of Experiment 1 (see supplementary material Fig. S3). We recorded the date of moulting and prosoma width after the moult; prosoma widths were measured to control for size effects.

Experiment 1: background colour manipulation

We kept spiders in yellow (N=32) and white (N=18) containers for 15 days during May 2012 to study the dynamics of reversible colour change. Spiders in white and yellow containers had initial mean colour indices of 927.2±260.5 and 983.6±274.6%, respectively. We consider these spiders to have been a 'whitish' colour at the start of the experiment. A colour index value of approximately 900% may be described as 'whitish' as its tonality is slightly whiter than the midpoint value when considering the range of individuals collected in the field (colour index range: 12.38 to 1301.7%). The containers were made of coloured cardboard and metal wire mesh. The reflectance spectra of the white (reference no. 335) and yellow (reference no. 400) papers used to build the containers are shown in supplementary material Fig. S1 and a diagrammatical representation showing container dimensions is given in supplementary material Fig. S4. The experiment was set up in a naturally illuminated outdoor garden, which allowed the full spectrum of light to enter the containers. We protected spiders from direct exposure to sunlight using a thin bamboo mesh, which gave the containers a half-sun/half-shade area; the mesh allowed approximately 50% of the light to pass through. Treatment groups did not significantly differ in spider colour (F=0.49, P=0.48) or size (F=0.25, P=0.61) at the start of the experiment.

We measured the colour of the spiders at the start of the experiment and every day until its completion. Spiders were fed bees once a week and were rinsed with water daily during the experiment.

Chemical determination of metabolites

At the beginning of Experiment 1, and every 3 days until its completion, a number of spiders from the yellow background were dissected to study the pigments produced during the colour change to yellow. We dissected 4, 4, 4, 4, 4 and 7 spiders on days 0, 3, 6, 9, 12 and 15, respectively, and measured opisthosoma width and length. Spider tissues were dissected into two sample types (leg-prosoma and opisthosoma). The internal tissue and eyes were discarded so that only the pigments responsible for the external colouration of spiders (cuticle and hypodermis) remained. Each sample was crushed, using a Wheaton plastic tissue grinder (VWR International, Fontenay-sous-Bois, France), in a 2 ml Eppendorf tube containing 0.5 ml acidified methanol (0.5% HCL). We made a sequence of three extractions for chemical analysis. In the first extraction, the Eppendorf tube containing the crushed sample was vortexed for 20 s, centrifuged for 15 min at 4°C and 4000 rpm and the supernatant was transferred to a second 2 ml Eppendorf tube. For the second and third extractions we added 0.5 ml acidified methanol to the original Eppendorf tube containing the crushed sample (again vortexed and centrifuged for 15 min at 4°C and 4000 rpm). The supernatant from each extraction was transferred to the second 2 ml Eppendorf tube. This liquid was passed through a 0.22 μm filter (Millex CV, Millipore, Saint-Quentin, France) and condensed using a speedvac (Thermo Savant, Holbrook, NY, USA) followed by a lyophilisator (Fisher Bioblock Scientific, Illkirch, France). Sample compounds were separated by HPLC [see Riou and Christidès (Riou and Christidès, 2010) for methodology]. At a given wavelength, we measured the peak surface absorbance of compounds for which we had standards: tryptophan, kynurenine, 3-hydroxy-kynurenine, kynurenic acid, xanthurenic acid, dihydroxy-xanthomatin and ommatin D. We also recorded the peak absorbance of other relevant unidentified substances (those having an ommochrome or ommochrome precursor-like spectrum).

Experiment 2: hormonal manipulation

We carried out hormonal injections during July 2012 to determine whether 20E triggers colour change in crab spiders. 20E dissolved in insect Ringer's solution (86 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 3 mmol l^{-1} $CaCl_2$ $2H_2O$) was used for the manipulation. As it was not possible to directly inject individuals without risking high mortality due to hemolymph loss, we used the natural ability of spiders to autotomise their legs when under threat from predators. Spiders were immobilised by covering them with a meshed tissue, and were anaesthetised for a few minutes at –20°C and maintained on a support made of foam. The left front leg was removed under a microscope and a solution was injected into the prosoma by introducing a sharpened Hamilton syringe to the spiders' coxa. Three treatments used injections of 20E diluted in Ringer's solution (1 ng μl^{-1}, weak treatment, N=19; 10 ng μl^{-1}, intermediate treatment, N=19; 100 ng μl^{-1}, strong treatment, N=20) and an injection of Ringer's solution alone was used as a control treatment (N=15). Vitellogenesis is related to fluctuations in hemolymphatic ecdysteroid levels in some spider species (Trabalon et al., 1992). Our injection concentrations were therefore chosen based on a study of *Tegenraria atrica* spiders that tested the role of 20E on vitellogenesis (Pourié and Trabalon, 2003), taking into account the relative sizes of the two species. Our highest concentration dose,

Table 1. Comparisons of spider colour change over time for the background and hormonal changing colour experiments

Experiment	Treatment	Slope ± s.e.m.	*P*-value	
			H_0: slope not different from 0	H_0: slope not different from spiders from yellow containers
1	Yellow	–63.8±22.1	**0.0054**	–
	White	29.3±20.0	0.1526	**0.0039**
2	Control	3.3±29.2	0.9117	**0.0491**
	Strong	–52.7±17.6	**0.0053**	0.6937
	Medium	–63.8±21.5	**0.0052**	0.9977
	Weak	–89.5±29.2	**0.0041**	0.4123

Results of the linear mixed effects model presented under two null hypotheses: the first H_0 states that spiders showed no colour change over time while the second H_0 states that spiders showed a colour change not significantly different from that of spiders kept in yellow containers in the background changing colour experiment.
Bold indicates $P<0.05$.

100 ng µl^{-1}, was half the highest concentration dose used by Pourie and Trabalon. We adjusted the volume injected based on spider mass: individuals were assigned to a category (0.02–0.04, 0.04–0.08 or 0.08–0.12 g) and were injected according to their size (with 0.2, 0.6 or 0.8 µl of solution, respectively). Prosoma width and spider body mass prior to injection were measured to control for size. We measured spider colour at the start of the experiment, and on each of the following 2 days, to study the dynamics of colour change in individuals that underwent hormonal treatments. We could only inject one pulse of 20E because of the invasive procedure requiring leg removal; in the analysis we focused on colour change during the first 2 days after injection as injected and ingested ecdysteroid hormones are known to be quickly removed from haemolymph (Connat et al., 1988). We also measured spider colour after the experiment had finished (on the sixth day after hormone injection) to investigate long-term effects of one-pulse hormone injections. At the start of the experiment, spiders from each treatment group were not significantly different in mass (F=0.72, P=0.39), colour (F=0.57, P=0.63) or size (F=1.28, P=0.26). The mean colour index values were 1157.2±161.7, 1228.9±239.9, 1151.20±267.9 and 1235.6±309.6% for the control, weak, medium and strong treatments, respectively. Spiders used for this experiment were not used for Experiment 1, but were kept in a laboratory in white containers after their collection in April. As a result, these spiders were whiter (higher colour index values) at the start of the experiment than those used for Experiment 1. Spiders were kept in the same yellow containers as those used for Experiment 1 (see supplementary material Fig. S4) but Experiment 2 was performed under laboratory conditions.

Data analysis

Linear mixed effects models were performed using R (R Development Core Team, 2008) to analyse spider colour change. Spider size was included as a covariate, and spider identity was treated as a random factor, in all analyses.

We used a linear mixed effects model with colour index as the dependent variable to determine the effect of moulting on colour change in spiders that had been kept in the laboratory before the start of the experiments. Moult (1 if the spider moulted or 0 if the spider did not moult during the 23 days before Experiment 1), time (23 days) and the interaction between moult and time were used as independent variables.

To study the effect of background on spider colour change we used a linear mixed effects model with colour index as the dependent variable and background (white or yellow), time (15 days), moult (1 if the spider moulted or 0 if the spider did not moult during the 15 days) and their interaction as the independent variables. Two-way and three-way interactions were included. The interaction between background and time tells us whether spiders responded differently to background colour over time. However, it does not give us information about differences in the speed of colour change because the two groups changed colour in different directions (see Results for the positive and negative slope for the white and yellow treatments, respectively). We compared the absolute slope values for both treatments using another linear mixed effects model to investigate differences in the speed of changes. We did this by converting the negative slope measured for spiders kept in yellow containers to a positive slope (see Results), using negative values of colour index instead of positive values for spiders kept in yellow containers. Under this transformation, a significant background × time interaction would show that spiders do not change colour at the same speed in both treatments. We confirmed this analysis was correct by checking that the absolute values of the slopes calculated by the model were the same before and after the colour index transformation.

To quantify the effect of 20E injection on colour change we used a linear mixed effects model with colour index as the dependent variable and hormonal treatment (weak, medium, strong and control), time (3 days) and their interaction as the independent variables. Moult was not included as a variable as only two spiders moulted during the experiment. We tested whether the slopes of colour change over time were different as a result of hormonal treatment (whether they were significantly different from 0). To compare the colour change dynamics of spiders from the first and second experiments, we used a linear mixed effects model that included colour index as the dependent variable and treatment (weak, medium, strong, control, yellow and white), time (3 days) and their interaction as the independent variables. The treatment variable had six levels because including the data of spiders collected during the first 3 days of the Experiment 1 added two more levels (yellow and white). We compared colour change speed (i.e. the slopes calculated by the model for each treatment) from days 1 to 3 for both experiments. Our null hypothesis was that the slopes of colour change over time, for all hormonal treatments, were not significantly different from the slope obtained for spiders kept in yellow containers during Experiment 1 (i.e. not different from a slope of –63.8; see results from Table 1).

Spider metabolite composition was analysed by principal components analysis (PCA). Colour index and spider size were added as supplementary variables and hence were not part of the construction of axes. Two independent PCAs were carried out for the prosoma-leg cuticle and the opisthosoma cuticle samples. PCA allowed us to identify the metabolites that explained colour differences best; these metabolites were then used as independent

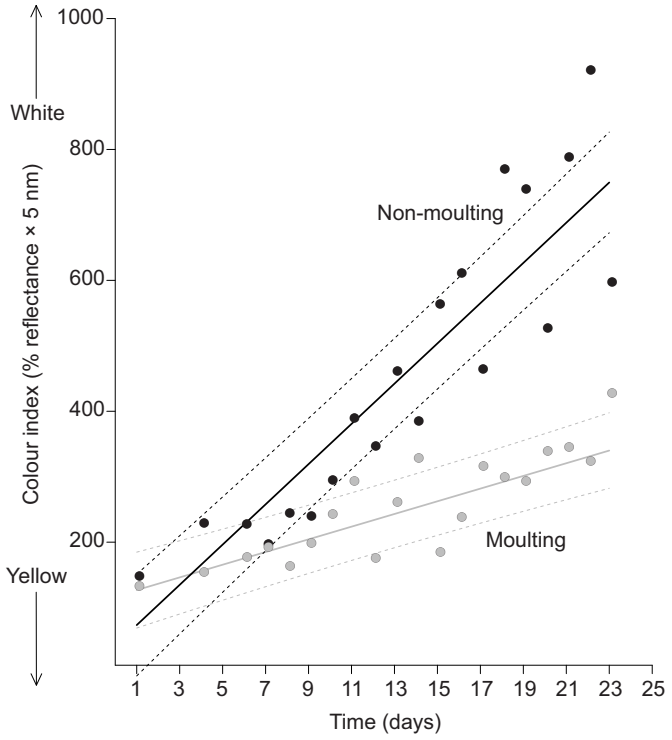


Fig. 2. Effect of moulting on spider colour change under laboratory conditions. Estimated linear mixed effects models for spiders that moulted (grey line) and spiders that did not moult (black line). Points represent mean observed values and dashed lines indicate the 95% confidence intervals.

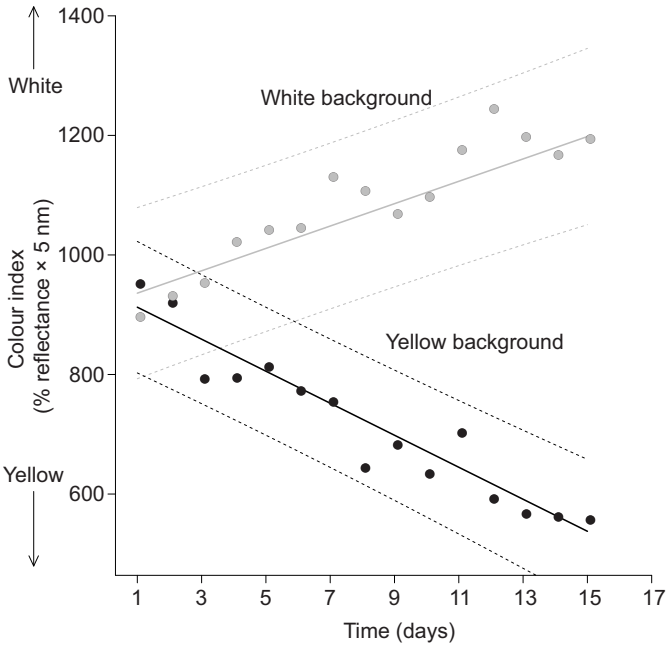


Fig. 3. Effect of background colour on spiders' dynamics colour change. Estimated linear models for spiders kept in white containers (grey line) and yellow containers (black line). Points represent mean observed values for each treatment and dashed lines indicate the 95% confidence intervals based on all values.

variables in a partial least squares regression (PLSR) to determine the contribution of each compound to the formation of spider colour. We chose PLSR rather than multiple regression analysis as our independent variables were highly correlated (Carrascal et al., 2009).

RESULTS

Effect of moulting on spider colour change in the laboratory

There was a highly significant interaction between moult and time after spider collection influencing the colour change dynamics of individuals kept in the laboratory before the start of the experiments (t=–10.48, d.f.=478, P<0.001; see supplementary material Table S1). Spiders that moulted during the observation period (N=51) tended to change from yellow to white at a slower rate than those that did not moult (N=29; Fig. 2). Spider size did not influence colour change (t=0.647, d.f.=77, P=0.519; see supplementary material Table S1). Time had a significant effect on the colour of spiders stored in the laboratory: spiders changed from yellow to a more white colour over time, as seen by an increase in the colour index (t=19.20, d.f.=478, P<0.001; Fig. 2, supplementary material Table S1). We confirmed that spiders from the non-moulting group also had the ability to moult, as most spiders from this group (19 of the 29 spiders) were observed after data collection and moulted at some stage.

Experiment 1: background colour manipulation

Our results showed a statistically significant effect of the interaction between time and background on spider colour (t=7.571, d.f.=367, P<0.001; see supplementary material Table S1). Spiders changed colour according to the colour of the background in which they were placed: spiders kept in white containers tended to increase their colour index whilst spider kept in yellow containers tended to decrease their colour index (Fig. 3). Spider size, spider moult and the interaction between background, time and moult did not affect colour change dynamics (all P>0.347). There was non-significant trend in the speed of colour change for individuals kept in yellow (–26.77±2.93) and white (18.71±3.12) containers (difference=8.06±4.28, t=1.88, d.f.=369, P=0.060; see supplementary material Table S1). On average, the speed of colour change was 1.43 times faster for spiders in yellow containers than for those in white containers.

Experiment 2: hormonal manipulation

Hormonal treatment affected colour change over time. Spiders injected with Ringer's solution (control group) did not show a significant colour change when stored under laboratory conditions (slope not significantly different from 0; see Table 1, Fig. 4). In contrast, spiders submitted to the strong, medium and weak hormonal treatments all showed a yellowing over the first 3 days after injection (see Table 1, Fig. 4). Colour measurements taken 6 days after the injection showed that one-pulse hormone treatment does not last for a long time: spiders injected with the hormone had reverted to a whiter colour by day six (see Fig. 4). The slope for spiders injected with Ringer's solution was significantly different to that of the spiders kept in yellow containers and used in Experiment 1. This difference was not statistically significant between groups of spiders injected with a high, medium or weak dose of the hormone (see Table 1). Spider size did not affect colour change dynamics (t=–1.209, d.f.=68, P=0.231). The speed of colour change from 'whitish' to yellow was 2.14 times faster (slope yellow treatment=–63.8) than the change from yellow to white (slope white treatment=29.3; Table 1).

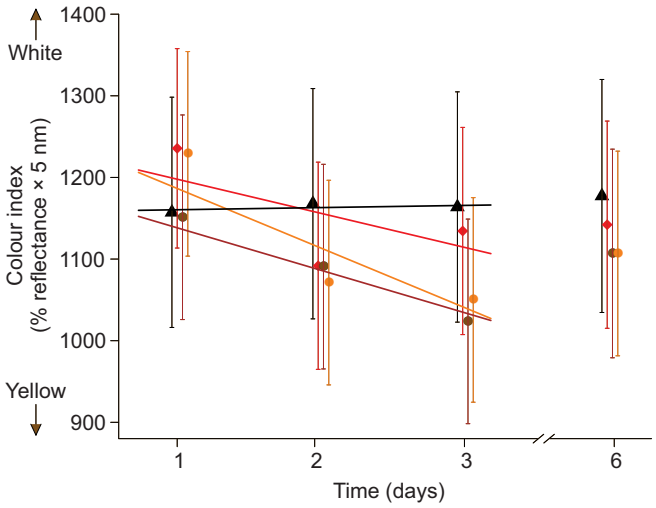


Fig. 4. Role of 20-hydroxyecdysone (20E) on the dynamics of colour change in crab spiders. Estimated linear model for spiders injected with a high (red line), medium (brown line) or weak (orange line) dose of 20E, and for those given a Ringer's solution dose (black line), at the start of Experiment 2. Vertical lines represent 95% confidence intervals for each treatment group based on all values. Symbols represent mean observed values for high dose (red diamonds), medium dose (brown circles), weak dose (orange circles) and control (black triangles) treatments.

Chemical determination of metabolites

Seven metabolites were identified and used for multivariate analyses (Table 2). Ommochromes were rarely detected and were therefore not considered for the PCAs. The first axis accounted for most of the variability: 46.5 and 37.27% for that in spider prosoma and opisthosoma, respectively (Fig. 5A,B). This axis can be interpreted as the colour axis because it separates the more yellow spiders (on the right of the graphs) from the more white spiders (on the left of the graphs) for both samples of spider prosoma and opisthosoma. Colour index correlated with the first axis, and with the chemical compounds that correlated with the axis (Fig. 5C,D). Relative amounts of 3-hydroxykynurenine and an unknown compound, S4a, were highly positively correlated with this axis. In contrast, kynurenine and tryptophan were negatively correlated with this axis. S1 and S5 unknown metabolites were anti-correlated with the axis that explained spider colour (Fig. 5C,D).

PLSR results showed that three compounds (3-hydroxykynurenine, kynurenine and tryptophan) were able to explain spider colour (Table 3). The first components from prosoma and opisthosoma analyses explained 65 and 67% of observed colour variance, respectively. Other PLSR components did not explain more than 5% of the variance and are therefore not discussed. Kynurenine, 3-hydroxykynurenine and tryptophan explained 100% of the first component variance (Table 3). S4a did not contribute to explaining the first component variance (Table 3) and S4b was not considered in the PLSR analysis as the PCA showed that it did not explain spider colour differences well (especially for the opisthosoma samples). Using the calculated regression coefficients (Table 3), our analysis can be used as a model to calculate spider colour, based on its composition of 3-hydroxykynurenine, kynurenine and tryptophan. PLSR results were consistent between prosoma and opisthosoma samples.

DISCUSSION

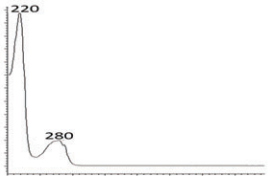
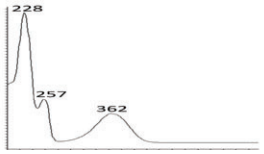
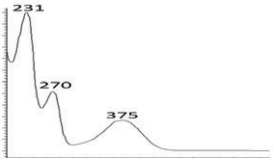
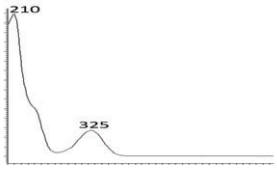
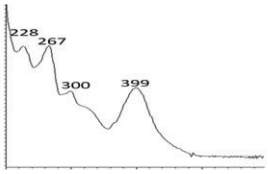
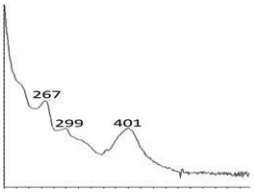
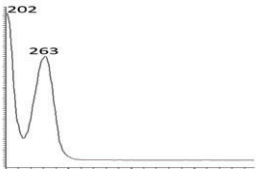
This study identifies environmental and hormonal factors underlying the morphological colour change of crab spiders, an animal that shows reversible plasticity in its colouration. It also identified key biochemical metabolites produced during this change.

Environmental factors influencing reversible colour change: the role of background and light

Background colour was the external factor responsible for spider colour change under direct exposure to sunlight: spiders kept on white and yellow backgrounds changed in colour to match their environments over time. Other animals that can undergo reversible changes in colour (such as fish, reptiles, amphibians, crustaceans and cephalopods) are also known to exhibit background matching (Hanlon et al., 1999; Norris and Lowe, 1964; Stuart-Fox et al., 2008; Thurman, 1988; Whiteley et al., 2011). Background colour alone was not sufficient to trigger colour change in *T. onustus* under laboratory conditions: control spiders from the injection experiment that were also submitted to yellow background did not change colour over time under laboratory conditions. Spiders injected with the hormone also became whiter again 3 days after the experiment ended. This suggests that once the effect of the hormone injection had worn off, background colour alone was not sufficient for triggering the colour change towards yellow. This could also explain why some previous studies have not successfully manipulated colour change towards yellow in crab spiders under non-natural light conditions [either under laboratory (Théry, 2007) or greenhouse conditions (Gawryszewski et al., 2012)]. This second study found that spiders became whiter (a higher area under the reflectance curve between 400 and 500 nm) after 4 weeks of being submitted to different backgrounds (white and yellow), irrespective of the background on which they were initially placed [see supplementary material fig. S1 in Gawryszewski et al., 2012 (Gawryszewski et al., 2012)]. Melanin dispersion, the process causing skin darkening in the gecko *Tarentola mauritanica*, is triggered by both background colouration and surrounding illumination (see Vroonen et al., 2012). In the case of crab spiders, further work is needed to understand which other factors, together with background colour, trigger colour change towards yellow under natural conditions.

Previous studies with *M. vatia* spiders have successfully manipulated their colouration in response to background (Gabritschevsky, 1927; Rabaud, 1919) but explain very little about the details of colour change dynamics. Colour changes were found to take 10–25 days (white to yellow) or 5–6 days (yellow to white), suggesting that the average speed of change from yellow to white is 3.18 times faster than from white to yellow. In contrast to our study, the authors described spider colour according to human vision, a subjective measure. We found that the speed of colour change in spiders changing from 'whitish' to yellow colour was 1.43 times faster than the speed in spiders changing to become a white colour. When we separately analysed colour change observations from the first 3 days of Experiment 1, we observed that the difference was even greater: the speed of colour change from 'whitish' to yellow was 2.14 times faster than the speed for spiders changing towards a white colour. Our results are not directly comparable with previous studies as we used a different species of crab spiders and because spider colour was not objectively measured in previous work. However, our study shows that colour change from white to yellow in some species of crab spider can occur much more rapidly than previously expected.

Table 2. Characteristics of the metabolites measured in the HPLC analyses of spiders kept in yellow containers in the background changing colour experiment

Metabolite	Retention time (min)	Wavelength of absorbance peaks (nm)	Wavelength of surface measure (nm)	Spectrum (*X*: nm; *Y*: absorbance)
Tryptophan (Tryp)	17.8	220, 280	280	
Kynurenine (Kyn)	15.0	228, 257, 362	360	
3-hydroxy-kynurenine (OHKyn)	12.8	231, 270, 375	370	
S1	25.2	210, 325	325	
S4a	23.5	228, 267, 300, 399	400	
S4b	25.5	267, 299, 401	400	
S5	12	202, 263	263	

Hormonal factors influencing reversible colour change: the role of moulting and ecdysone

Our results showed a relationship between spider moulting events and colour change: spiders that moulted showed a slower rate of change from yellow to white than those that did not. Studies on animals that undergo non-reversible ontogenetic colour changes (such as locusts, grasshoppers and many lepidopterans) have shown a link between the development of colour patterns and animal growth by moulting and metamorphosis, which occurs *via* the endocrine system (Nijhout, 2010; Pener, 1991; Tanaka, 2004). In many cases, the change is regulated by hormones and is associated with adaptations to particular environments and/or with transitions in developmental stages (Nery and Castrucci, 1997; Nijhout, 2010). Non-reversible ontogenetic colour change in insects is influenced by the accumulation of pigments inside granules, a phenomenon also observed in crab spiders (Insausti and Casas, 2008; Insausti and Casas, 2009). The colour change is usually mediated by the endocrine system *via* a pulse of 20E (reviewed by Nijhout, 2010). In contrast to non-reversible ontogenetic colour change (for which colour determination is associated with growing events), we found that *T. onustus* colour change could also occur independently of moulting. Although colour change in crab spiders has been the subject of several studies (Gabritschevsky, 1927; Rabaud, 1919; Théry, 2007), our work is the first to show an association between colour change and moulting events in this species.

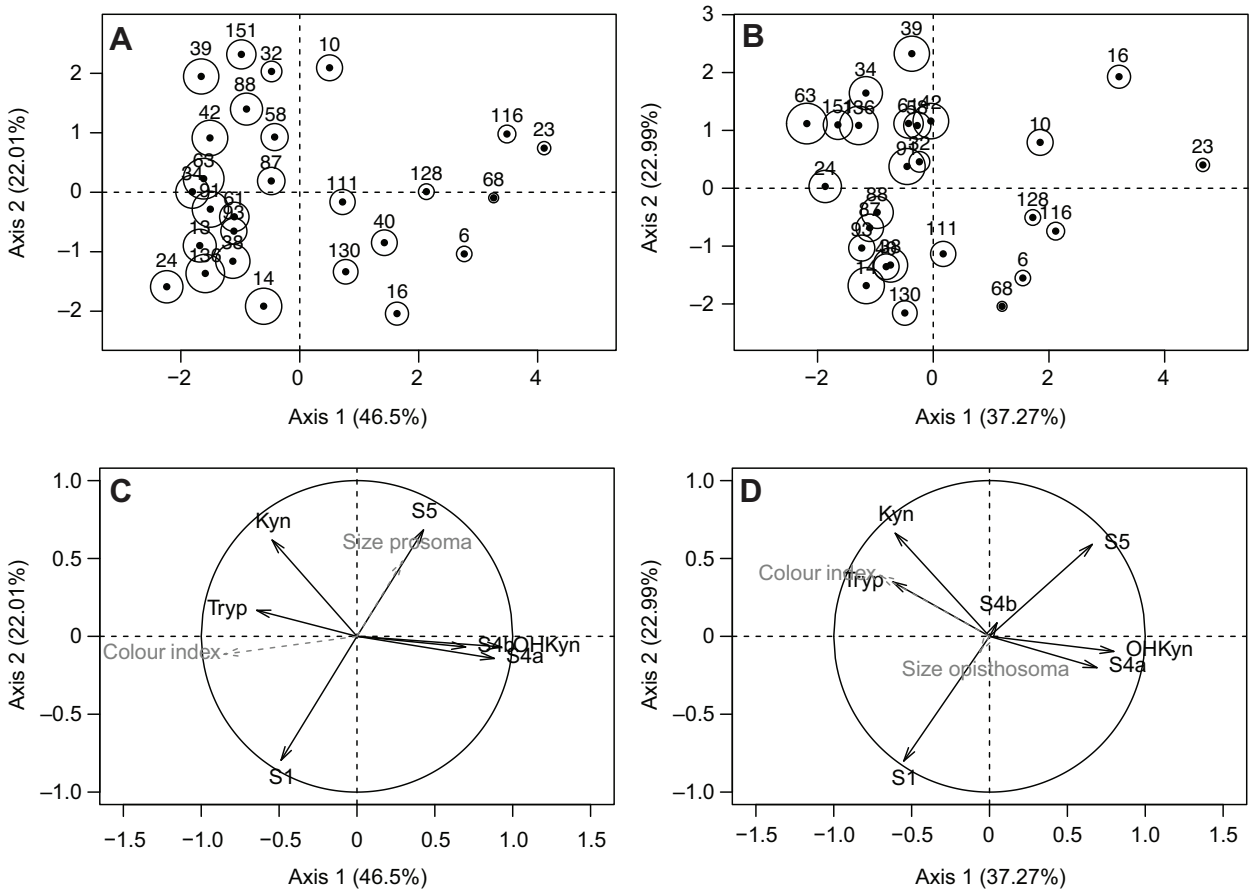


Fig. 5. Multivariate principal component analyses of the chemical metabolites of crab spiders kept in yellow containers during the background colour change experiment. The top row represents the multivariate PCA for samples of (A) prosoma-legs and (B) opisthosoma. A single number represents each spider (the same for samples A and B). The radii of circles are proportional to square root of colour measures, so that the biggest circles represent the whitest spiders. The bottom row shows the relative proportion of metabolites for samples of (C) prosoma and (D) opisthosoma. Solid black arrows represent variables that were used to build the axes. Grey dashed arrows represent supplementary variables (colour index and spider size). Metabolite acronyms are given in Table 2.

Our results regarding the effect of moulting on colour change are in accordance with previous studies of animals that change colour reversibly. The prawn *Caridina weberi* shows variation in colour change speed that relates to its moulting cycle: slow responses were most common soon after the moult and in the in the premoult stage (Nagabhushanam and Vasantha, 1971). In several arthropods, 20E titres reach a maximum at, or slightly after, apolysis (i.e. when a new epicuticle is being secreted) (Gäde et al., 1997). Because the hormone responsible for moulting in spiders and in other arthropods is 20E, we suggest that this hormone may also be responsible for triggering colour change. Our injection experiment supports this notion, as spiders injected with 20E in the laboratory displayed a rate of colour change towards yellow similar to that of spiders placed in yellow containers and exposed to natural sunlight. This suggests that one pulse of 20E is sufficient to trigger colour change in *T. onustus* crab spiders.

Elucidating the biochemical metabolites of colour change

Our results in *T. onustus* are consistent with previous chemical and histological studies of pigment composition in *M. vatia* crab spiders, which suggest that white colour is related to the presence of tryptophan and kynurenine pigments in the epidermis (Insausti and Casas, 2008; Insausti and Casas, 2009; Riou and Christidès, 2010). However, these studies did not support the notion that ommochrome pigments were responsible for yellow colouration in *T. onustus*. A precursor of ommochromes, 3-OH-kynurenine, was the main pigment responsible for yellow colours. This was found to be responsible for yellow pigmentation in *M. vatia* (Seligy, 1972). Similarly, Riou and Christidès (Riou and Christidès, 2010) showed 3-OH-kynurenine to be the most common chemical compound in yellow *M. vatia* and the only compound that showed significantly higher quantities in the integument of yellow spiders than in that of white spiders. Studies of the ommochrome pathway in flies and butterflies show that the yellow integument pattern is due to the presence of 3-OH-kynurenine in cell granules (Reed et al., 2008; Reed and Nagy, 2005). In these species, binding proteins may protect 3-OH-kynurenine from condensation into xanthommatin, which could stabilise 3-OH-kynurenine as the final pigment within the cell granules (Ferguson and Jiggins, 2009). Nevertheless, our results should be interpreted with caution, as pigment granules from epidemic cells in yellow spiders are filled with substances that, unlike 3-OH-kynurenine, do not fluoresce under UV light. Osmophilic, electron-dense content has been observed by electron microscopy in *M. vatia* (Insausti and Casas, 2008) and *T. onustus* (T. C. Insausti, personal communication).

It may be suggested that the chemical analyses used here and in other studies may have not been sensitive enough to detect

Table 3. Results of partial least squares regressions (PLSR) between colour and four metabolites presented in the integument of spiders kept in yellow containers in the background changing colour experiment

	Explained variance of the first component (%)		Regression coefficient	
Metabolite	Prosoma	Opisthosoma	Prosoma	Opisthosoma
3-hydroxykynurenine	76.6	67.4	–21.93	–22.28
Kynurenine	4.1	17.6	5.07	11.36
Tryptophan	19.3	15	11.00	10.50
S4a	0	0	–0.60	–1.01
Intercept	824.38	839.88		

Only the results from the first component for the PLSR analyses of prosoma and opisthosoma are shown because other components did not explain more than 5% of colour variability.

ommochrome pigments. However, as previous chemical analyses of the internal content of spiders have detected ommochrome pigments (Riou and Christidès, 2010), this is unlikely. Further work is needed to understand the conflicting evidence provided so far as we have no reason to favour one metabolite over the other. Ommochromes are known to be very stable and define the ultimate colour pattern of insects (Nijhout, 2010); using precursors as pigments could therefore be a good strategy in enabling reversible colour change.

Adaptive significance of colour change

Substantial evidence suggests that the degree of background matching correlates with the risk of predator detection for animals that change colour, such as fish, chameleons and cephalopods (Stevens and Merilaita, 2009). In the case of crab spiders, the benefits of a reversible colour change remain unknown. Predation events by vertebrates have rarely been observed in the field (Brechbühl et al., 2010; Morse, 2007), although predation by mud-dauber and spider wasps has often been documented (Morse, 2007). Experimental evidence with crab spiders is still lacking, despite predator avoidance being the most widely accepted notion to explain the evolution of colour changing in other animal taxa. Alternatively, colour change evolution in crab spiders may be related to the fact that ommochrome pigmentation present beneath the transparent cuticle could protect against photodestruction by UV light (Théry and Casas, 2009). These authors suggest that this protection may be necessary as the spiders have a transparent cuticle and are exposed to direct sunlight for long periods of time. However, if ommochrome pigmentation is related to protection against photodestruction, we would expect that all spiders exposed to sunlight would synthesise pigments to protect themselves against radiation and thus change to a yellow colour independently of their background. We found no evidence of this: only spiders placed in yellow containers changed colour to yellow colour and synthesised yellow pigments. Spiders in white containers changed to a whiter colour, suggesting that spider crypsis may play a greater role than protection against radiation. It was thought that crab spiders evolved the ability to match their background to be cryptic from their prey (Gabritschevsky, 1927; Rabaud, 1919). However, three recent studies showed that, at least in some systems, background colour matching does not seem to play a role for pollinators (Brechbühl et al., 2010; Defrize et al., 2010; Llandres and Rodriguez-Gironés, 2011). It is nevertheless important to highlight that plasticity might be favoured globally, even when fitness is not highest at any particular site.

Our study of colour plasticity in *T. onustus* sheds light onto signal transduction pathways, from the environmental factors affecting their induction to the biochemical mechanisms. Reversible, morphological colour change is thought to be controlled by the endocrine system, as is the case in animals that show non-reversible changes. The endocrine system may control the transduction of environmental cues (such as background colour) into physiological responses, altering the timing or the level of hormone secretion, which in turn triggers pigmentation synthesis. Animals that undergo reversible and non-reversible morphological colour changes could therefore have a common transduction pathway linking environmental signals and phenotypic responses. After more than a century of observational studies under varying environmental conditions, this is the first study using targeted *in vivo* hormonal manipulations to show plasticity in colouration.

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AUTHOR CONTRIBUTIONS

A.L.L., F.F., J.-P.C., N.M. and J.C. conceived and designed the experiments. A.L.L. and F.F. performed the experiments. A.L.L., F.F. and J.C. analysed the data. A.L.L., F.F. and J.C. wrote or revised the paper.

COMPETING INTERESTS

No competing interests declared.

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