RESEARCH ARTICLE

Spiders have rich pigmentary and structural colour palettes

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ABSTRACT

Elucidating the mechanisms of colour production in organisms is important for understanding how selection acts upon a variety of behaviours. Spiders provide many spectacular examples of colours used in courtship, predation, defence and thermoregulation, but are thought to lack many types of pigments common in other animals. Ommochromes, bilins and eumelanin have been identified in spiders, but not carotenoids or melanosomes. Here, we combined optical microscopy, refractive index matching, confocal Raman microspectroscopy and electron microscopy to investigate the basis of several types of colourful patches in spiders. We obtained four major results. First, we show that spiders use carotenoids to produce yellow, suggesting that such colours may be used for condition-dependent courtship signalling. Second, we established the Raman signature spectrum for ommochromes, facilitating the identification of ommochromes in a variety of organisms in the future. Third, we describe a potential new pigmentary–structural colour interaction that is unusual because of the use of long wavelength structural colour in combination with a slightly shorter wavelength pigment in the production of red. Finally, we present the first evidence for the presence of melanosomes in arthropods, using both scanning and transmission electron microscopy, overturning the assumption that melanosomes are a synapomorphy of vertebrates. Our research shows that spiders have a much richer colour production palette than previously thought, and this has implications for colour diversification and function in spiders and other arthropods.

KEY WORDS: Arthropod, Carotenoid, Ommochrome, Melanosome, Raman spectroscopy, Silk

INTRODUCTION

Colour has diverse and important functions in animals, including spiders (Cott, 1940). Crypsis, aposematism, prey attraction, courtship signalling and thermoregulation are all influenced by spider coloration (Oxford and Gillespie, 1998). Furthermore, many spiders have diverse colour polymorphisms that make them useful systems to study adaptive radiation (Yim et al., 2014) and genotype–phenotype relationships (Oxford and Gillespie, 2001). New functional hypotheses about colours in spiders are constantly formed and tested through behavioural and ecological analyses (Anderson and Dodson, 2015; Blamires et al., 2014; Painting et al., 2016; Taylor et al., 2016, 2014). In contrast, colour production mechanisms in spiders have so far received little attention, even though these mechanisms are important for understanding both

colour evolution and function, such as how melanins and carotenoids function as condition-dependent/honest signals in other animals (Jawor and Breitwisch, 2003; MacDougall and Montgomerie, 2003).

Colours are commonly produced either by selective light absorption by pigment molecules or by scattering from nanostructures with periodic interfaces between materials of different refractive indices (structural colours). Pigmentary colours are independent of viewing angle (non-iridescent), while structural colours are usually angle dependent (iridescent). The nanomorphology of structural colours has been investigated recently for some spiders (Foelix et al., 2013; Hsiung et al., 2015b; Ingram et al., 2011; Land et al., 2007; Simonis et al., 2013; Stavenga et al., 2016). However, our knowledge of spider pigment biochemistry has remained stagnant for almost 30 years (Holl, 1987, but see Hsiung et al., 2015a). Thus, we still know very little about pigments and the colour production mechanisms of spiders in general. The cumulative body of literature suggests that spiders lack some of the most common pigments that are widespread in other organisms, such as melanins and carotenoids, and only three pigments have been identified in spiders: ommochromes, bilins and eumelanin (Hsiung et al., 2015a; Oxford and Gillespie, 1998). However, given the recent discovery of eumelanin in spiders (see below), it is time to reassess pigmentary colours in spiders.

Modern techniques such as mass spectrometry (MS) and high-performance liquid chromatography (HPLC) have greatly improved sensitivity and made pigment identification easier. However, these techniques are still laborious and require pigment extraction from large amounts of material, making them impractical for spiders and other small arthropods. In contrast, Raman spectroscopy provides a non-destructive, fast and easy way to identify pigments *in situ* with almost no sample preparation. With Raman spectroscopy, pigmentary colours can be readily categorized into one of the few more common groups of pigments by comparing their Raman signature signals to expedite further biochemical analyses using HPLC and/or MS. Raman spectroscopy has identified many different types of pigments in bird feathers (Galván et al., 2013; Thomas and James, 2016; Thomas et al., 2013) but has rarely been applied to spiders. Thus, we developed a workflow that begins with Raman spectroscopy to evaluate long-standing assumptions about pigmentary colours in spiders.

Initial development of the workflow led to our discovery of eumelanin in spiders using Raman spectroscopy (Hsiung et al., 2015a), a pigment that is nearly ubiquitous in animals but was previously assumed to be absent in spiders. This unexpected finding motivated us here to look for another common pigment, carotenoids, in spiders using Raman spectroscopy. Many animals produce their yellow, orange and red body colorations through carotenoids. Almost no animals can synthesize carotenoids *de novo* and instead acquire the pigments through their diet (Fox, 1976), making carotenoids honest signals in some species (MacDougall and Montgomerie, 2003). Pea aphids (Moran and Jarvik, 2010), spider mites (Grbić et al., 2011) and gall midges (Cobbs et al., 2013)

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are the only known exceptions. Therefore, we sampled several species of spiders showing common carotenoid colours – yellow, orange and red.

The golden silk orb weavers (*Nephila* spp.) are famous for their large and strong webs that are often golden/yellow. Almost all literature mentioning *Nephila*'s striking yellow webs state that the yellow colour is produced by quinones (mostly benzoquinone and naphthoquinone) and xanthurenic acid (Craig et al., 1996; Putthanarat et al., 2004). However, this claim is based on a study using a rudimentary analytical technique – thin film chromatography (TLC) (Holl and Henze, 1988). Hence, here we first used Raman spectroscopy to identify the origin of the yellow colour from *Nephila* silk and other yellow patches of spiders.

We then sampled orange/red patches in spiders, including the iconic red hourglass from a black widow spider (*Latrodectus hesperus*), which was previously hypothesized to be produced by ommochromes (Seligy, 1972), and three species of jumping spiders. We also included the colour black, because ommochromes are able to produce a series of different colours from yellow to orange, red and black (Seligy, 1972).

Melanin is deposited in organelles called melanosomes in vertebrates, but is diffusely deposited in all arthropods examined so far, potentially preventing them from producing the organized melanosome stacks that create brilliant iridescent colours in feathers (Shawkey et al., 2009). However, the deposition of melanin in spiders has never been investigated. Hence, in this study, we also examined how melanin is deposited in spiders using electron microscopy.

Additionally, we suggest a workflow (Fig. 1) to efficiently categorize the colour production mechanisms in spiders (or any colourful organisms) by using Raman spectroscopy, assisted with refractive index matching and optical microscopy. This workflow is designed to identify the types of pigments involved, but can also distinguish whether the colour under investigation is pigmentary, structural or a combination of the two.

Using our suggested workflow, we report: (1) the discovery of the presence of carotenoids in spiders, (2) the establishment of the Raman signature spectrum for ommochromes, and (3) the discovery of a new kind of pigmentary–structural colour interaction. Moreover, we also present, to the best of our knowledge, the first anatomical evidence for the existence of melanosomes in arthropods using both scanning and transmission electron microscopy.

MATERIALS AND METHODS

Fig. 1 shows our suggested workflow, which begins with Raman spectroscopy as a quick and easy way to screen for several types of pigments. The workflow then uses a combination of microscopy and refractive index matching to screen for the possible role of structural mechanisms in colour production. Finally, more detailed pigmentary information can be acquired after pigment extraction, using a combination of surface enhanced Raman spectroscopy (SERS) and HPLC/MS. Contrary to conventional approaches that usually start with pigment extraction, our workflow shows how a great deal of information about the mechanism of colour production can be quickly obtained before extracting pigments. Hence, pigment extraction can be treated as a final step to identification of a small subset of 'difficult' pigments. More importantly, most of the workflow can be applied to very tiny colour patches, making it ideal to investigate colour in small arthropods. The principles and rationale for the workflow are explained below in detail. In addition, we also used electron microscopy to look for melanosomes – organelles consisting of melanin pigments.

Chemical standards

β-Carotene (C9750), xanthurenic acid (D120804), *p*-benzoquinone (B10358) and 1,4-naphthoquinone (152757) (purchased from Sigma-Aldrich, St Louis, MO, USA) were used as references in Raman spectroscopy. Xanthurenic acid, *p*-benzoquinone and 1,4-naphthoquinone were previously thought to produce the yellow colour of *Nephila* silks, and were thus used as positive standards. β-Carotene was added later, after we found the Raman spectrum from the yellow silk did not match any of the spectra from xanthurenic acid, *p*-benzoquinone or 1,4-naphthoquinone.

Feathers

We used several types of bird feathers as positive controls for Raman spectroscopy. Raman spectra of the classes of pigments (carotenoids, psittacofulvins and turacoverdin) in birds are already established. Individual feathers from different birds with previously identified pigments were examined using Raman spectroscopy to show that our results were consistent with previous literature. They include: American goldfinch (*Spinus tristis*, carotenoids), Knysna turaco (*Tauraco corythaix*, turacoverdin – a unique pigment in the group porphyrins) and blue-and-gold macaw (*Ara ararauna*,
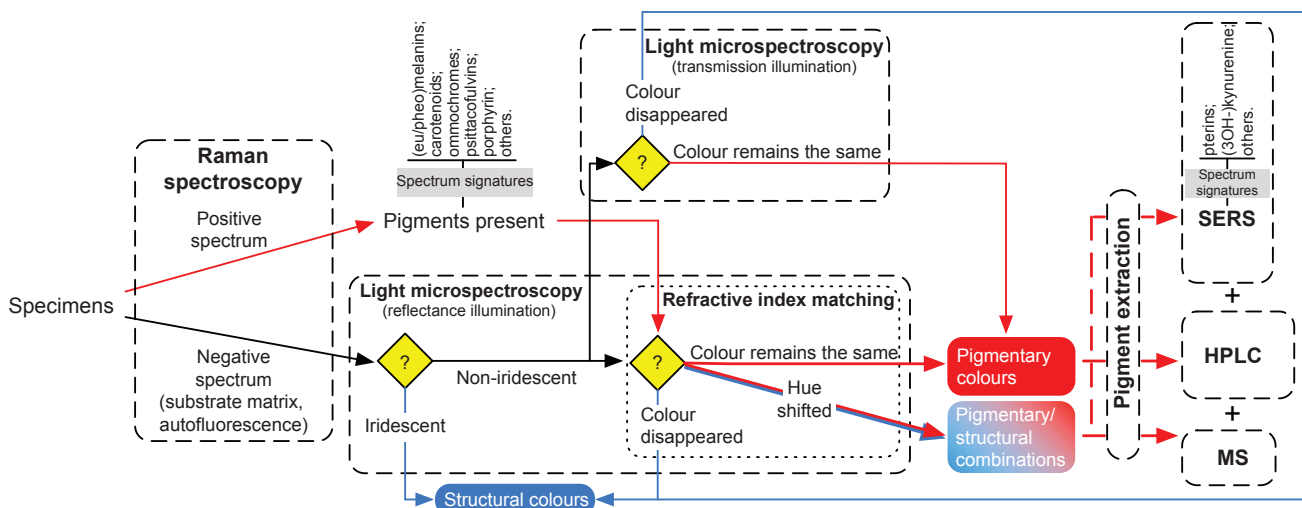

Fig. 1. A suggested workflow for identifying colour production mechanisms. Researchers without access to Raman spectrometry can still use this flowchart by following the black solid arrows. SERS, surface enhanced Raman spectroscopy; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

psittacofulvin). The feathers were collected from study skins in The University of Akron collections.

Spiders, silks and egg sacs

We chose samples based on their colour (common pigmentary colours: yellow, orange, red and black) and accessibility. Yellow silks of *Nephila pilipes* (Fabricius 1793) (Nephilidae) were collected from Miaoli, Taiwan. Yellow and orange/red scales and black opisthosoma cuticles of *Maratus* spp. Karsch 1878 (Salticidae) were collected from New South Wales, Australia. White silks and haemolymph of *Nephila clavipes* (Linnaeus 1767) (Nephilidae), orange/red scales of *Menemerus bivittatus* (Dufour 1831) (Salticidae), yellow opisthosoma patches of *Lyssomanes viridis* (Walckenaer 1837) (Salticidae) and orange/red scales of *Habronattus brunneus* (Peckham & Peckham 1901) (Salticidae) were collected around Gainesville, FL, USA. White silks and yellow opisthosoma patches of *Argiope trifasciata* (Forsskål 1775) (Araneidae), orange/red scales of *Phidippus audax* (Hentz 1845) (Salticidae) and black *Hyptiotes cavatus* (Hentz 1847) (Uloboridae) egg sacs were collected around Akron, OH, USA. White silks and red hourglass patches of *Latrodectus hesperus* Chamberlin and Ivie 1935 (Theridiidae) were purchased from bugsofamerica.com. Yellow hairs from the moults of *Poecilotheria metallica* Pocock 1899 (Theraphosidae) and *Pterinochilus murinus* Pocock 1897 (Theraphosidae) were purchased from Etsy.com.

Spider silks

Nephila silks range from pure white to bright golden yellow (Putthanarat et al., 2004). Therefore, we tested silks with different shades of yellow from *N. pilipes* and compared their Raman spectra with those of the chemicals previously suggested to produce the yellow – xanthurenic acid, *p*-benzoquinone and 1,4-naphthoquinone (positive controls) – using white silks from other spiders as negative controls.

Major ampullate silks from *A. trifasciata*, *L. hesperus*, *N. pilipes* and *N. clavipes* were collected in the lab by reeling silks from restrained spiders onto glass capillary tubes using electric motors. The tubes were then stored for up to 3 months under ambient conditions until further Raman spectroscopy characterization.

Raman spectroscopy

Raman spectroscopy detects the change in polarizability when certain molecular bonds vibrate. Each type of bond scatters light at a different frequency (wavenumber) in response to laser excitation, allowing its identification. Larger changes in polarizability result in stronger Raman signals (Albrecht, 1961; Garozzo, 1976). When highly polar bonds (such as C–O, C=O, C–N, O–H, N–H) vibrate, the change in polarizability is small, resulting in weak Raman signals (more Raman inert). In contrast, when non-polar bonds (such as C–C, C=C, C–H) vibrate, the change in polarizability is larger, resulting in strong Raman signals (more Raman active). Thus, Raman spectroscopy is most efficient at identifying the signatures of pigments with mostly non-polar bonds.

Raman spectra of samples of interest were collected using the LabRAM HR Evolution Raman spectroscopy system (HORIBA Scientific, Edison, NJ, USA), with a 50 mW, 532 nm laser excitation light source, through an Olympus BX41 confocal microscope with a ×50 objective lens, a slit aperture of 100 μm, a pinhole of 400 μm and a grating of 1200 lines mm^{-1}. These conditions produced an average spectral resolution around 1 cm^{-1} in the wavelength range of 500 to 2000 cm^{-1}. We tested both 532 and 785 nm excitation lasers and chose the former because it gave

us better signal-to-noise ratio for our specimens. The laser beam cross-sectional diameter was around 40 μm. The system was operated with LabSpec 5 software with an integration time of 5 s×2 accumulations. Spectra were collected from three different locations for each sample of interest. Samples were visually inspected under the microscope after spectrum acquisition to ensure that they were not burnt. Replicate spectra were smoothed, normalized, averaged and plotted using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA) without baseline correction.

Refractive index matching

Structural colour is produced by selective light reflection from structures with periodic interfaces between materials of different refractive indices. Matching the refractive indices across materials destroys the periodic interfaces, and hence eliminates the reflection and the colour. Therefore, structural colours should disappear when the specimen is immersed in an index-matching solution, while pigmentary colours remain unaffected (D'Alba et al., 2011; Vukusic et al., 1999).

We previously determined that the refractive index (n_r) for spider cuticle is ∼1.60 (Hsiung et al., 2015b). Therefore, either ethyl cinnamate (n_r≈1.56, W243019, Sigma-Aldrich) or quinoline (n_r≈1.63, 241571, Sigma-Aldrich) was used as an index-matching solution.

Reflected/transmitted light microscopy

In reflected light, both pigmentary and structural colours are visible, but only pigmentary colours are visible under transmission illumination (Land et al., 2007; Stavenga et al., 2014; Vukusic et al., 1999).

Thus, specimens were viewed under a dissecting stereoscope (Leica S8 APO, Leica Microsystems, Wetzlar, Germany), equipped with white LEDs from both above (reflected light) and below (transmitted light) the specimens. We observed and compared any difference in the specimens' colour under reflected versus transmitted illumination.

Transmission electron microscopy (TEM)

A *Maratus anomalus* opisthosoma cuticle fragment was dehydrated and washed, followed by epoxy resin infiltration and embedding based on a previously reported protocol (Hsiung et al., 2015b). The cured epoxy block was trimmed with a Leica EM TRIM2 (Leica Microsystems) and microtomed into 80 nm thin sections using a Leica EM UC6 (Leica Microsystems) with a DiATOME diamond knife (Electron Microscopy Sciences, Hatfield, PA, USA). Sections were mounted onto 100 mesh copper grids (EMS FCF100-Cu, Electron Microscopy Sciences) and observed under a transmission electron microscope (JSM-1230, Japan Electron Optics Laboratory Co. Ltd, Akishima, Tokyo, Japan) without sample contrast staining.

Scanning electron microscopy (SEM)

A *Maratus splendens* opisthosoma cuticle fragment was attached to a sample stub using carbon tape. The sample was sputter-coated with gold-palladium for 3 min under 20 mA, 1.4 kV, and observed under a scanning electron microscope (JEOL 7401, Japan Electron Optics Laboratory Co. Ltd) with 8 mm operating distance and 5 kV accelerating voltage.

RESULTS
Yellow

Fig. 2A shows the yellow coloured silk of *Nephila*. Raman spectra of all white silks are similar to each other (Table S1), and may
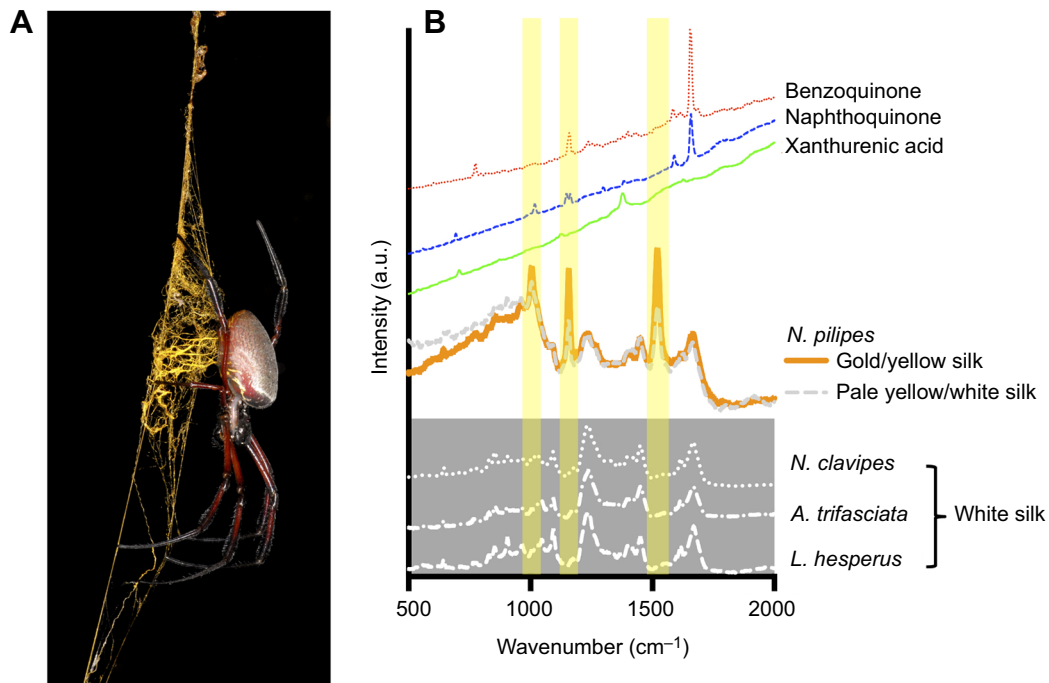
Fig. 2. The colour of golden *Nephila* silks is not produced by previously hypothesized pigments. (A) A *Nephila* and its golden silk. Credit: M. Gregorič. (B) Raman spectra of previously suggested pigments in *Nephila* silks (red, blue and green), two different shades of yellow silks from *N. pilipes*, and white silks from three different species of spiders. The yellow silks from *N. pilipes* have three extra peaks around wavenumber 1005, 1160 and 1520 cm^{-1} (yellow shaded area) that are not present in the white silks. None of the previously suggested pigments have the peak around wavenumber 1520 cm^{-1}.

represent the spectra of the major ampullate spidroins. The coloured silks from *N. pilipes* show similar spectra overall to white silks, but with three additional sharp and intense peaks around wavenumbers 1005, 1160 and 1520 cm^{-1} (Fig. 2B; Table S2). These three peaks appear only in coloured *N. pilipes* silks and not in normal white silks, including white *N. clavipes* silk, and the peak intensity correlates with the intensity of the yellow colour from the silks, suggesting that these three peaks are from a yellow pigment. Moreover, the peaks do not match with any of the spectra from the chemicals in the positive controls xanthurenic acid, *p*-benzoquinone and 1,4-naphthoquinone (which all lack the peak at ~1520 cm^{-1}; Table S3), strongly suggesting that the main pigment responsible for the yellow *Nephila* silks is not any of these previously hypothesized chemicals (Fig. 2B).

Instead, the 1005, 1160 and 1520 cm^{-1} peaks are the signature peaks of carotenoids (Arcangeli and Cannistraro, 2000). Fig. 3A shows the Raman spectra from β-carotene, and from feathers of an American goldfinch (*Spinus tristis*) that contain carotenoids (McGraw et al., 2002) which match perfectly with the three peaks from yellow *Nephila* silks (Table S3). This suggests that the pigment responsible for the yellow coloration of *Nephila* silks is a carotenoid, a group of pigments previously thought to be absent from spiders (Oxford and Gillespie, 1998). Fig. 3B shows the Raman spectra from feathers with identified yellow pigments other than carotenoids, and neither of these spectra matches with the signature spectra of carotenoids shown in Fig. 3A (Table S3). We subsequently surveyed additional yellow patches, and discovered carotenoids in the haemolymph of *Nephila*, and in epidermal chromatophores (which are under the cuticle) from the banded garden spider (*A. trifasciata*) and a magnolia green jumper spider (*L. viridis*) (Fig. 3C; Table S2).

However, not all yellow in spiders appears to be produced by carotenoids. We did not detect any positive Raman signal from the yellow integumental setae (i.e. yellow hairs and yellow scales) of spiders, even when refractive index matching and optical microscopy analyses (Fig. 4) both indicated that such a coloration has a pigmentary origin (but see Foelix et al., 2013). Whatever pigments were responsible for producing yellow in the integumental setae of *P. murinus*, *P. metallica*, *M. bivittatus* and *Maratus volans*, the signal of the pigments may be too Raman inert to be detected (e.g. kynurenine and/or pterins).

Orange/red

The red hourglass of *Latrodectus* is hypothesized to be produced by ommochromes (Seligy, 1972), but the Raman signature of this pigment class is currently unknown. Using Raman spectroscopy, we detected four peaks from the red hourglass of *L. hesperus* around wavenumbers 595, 1255, 1430 and 1490 cm^{-1} (Table S4). Consequently, we tentatively assign these four peaks as the signatures of ommochromes, as Raman spectra of ommochromes have never been reported before and ommochrome standards are not commercially available. We were able to detect these exact four peaks in all orange/red spider patches that we sampled (Fig. 5; Table S4), although the signals from the red opisthosoma scales of *M. volans* were very weak. Our results are in line with previous reports that the orange/red colour is produced by ommochromes in nine species of spiders across four different families (Salticidae, Tetragnathae, Theridiidae and Thomisidae) (Insausti and Casas, 2008; Riou and Christidès, 2010; Seligy, 1972). However, the colour of jumping spider scales changes from red to orange (Fig. 6) or almost disappears (Fig. 4D,H) after immersion of the scales in an index-matching solution. This suggests that the colour of some red scales may have a structural rather than pigmentary origin, with ommochromes producing the orange colour in these scales. Following the same reasoning, almost all *M. volans* red scales disappeared after immersion in an index-matched solution (Fig. 4H)
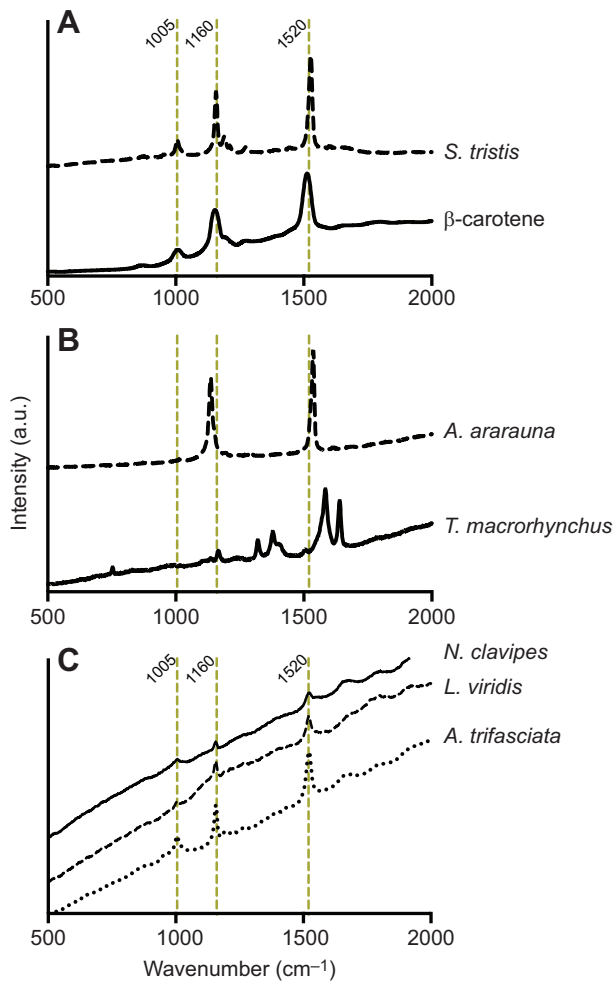
Fig. 3. Raman spectra of β-carotene and pigments from bird feathers and spiders. (A) Raman spectra of β-carotene, and carotenoids from feathers of American goldfinch (*Spinus tristis*). (B) Raman spectra of turacoverdin from feathers of yellow-billed turaco (*Tauraco macrorhynchus*) and psittacofulvin from blue-and-gold macaw (*Ara ararauna*). (C) Raman spectra from the yellow patches (chromatophores) on the ventral opisthosoma of *Argiope trifasciata*, dorsal opisthosoma of *Lyssomanes viridis* and the abdominal internal mass (haemolymph) of *Nephila clavipes*. All spectra show three peaks around wavenumber 1005, 1160 and 1520 cm^{-1}, indicating the presence of carotenoids.

because of the structural origin of the colour red and the small amount of ommochromes present (Fig. 5).

Black

Under the colourful scales, the dorsal side of the peacock spiders (*Maratus* spp.) opisthosoma is usually pitch black (Fig. 4D). We previously showed that this black colour is produced by eumelanin using Raman spectroscopy (Hsiung et al., 2015a). The Raman signals that we detected must be from eumelanin, not false-positives due to carbonization of burnt specimens, because we only obtained positive Raman signals from black spider body patches, not from nearby regions that were not black (Hsiung et al., 2015a). Here, we found anatomical evidence that the eumelanin is contained within discrete melanosomes rather than dispersed throughout the cuticle. By examining the opisthosoma using electron microscopy, we observed highly electron-dense spherical pigment granules at the epidermis region immediately beneath the cuticle (Fig. 7). We assign these pigment granules as melanosomes based on their

colour (black), location (epidermal), size (starting from ∼200 nm to just over 1 µm) and morphology (spherical) (Li et al., 2014). This not only corroborates our previous findings that eumelanin is present in spiders but also, to the best of our knowledge, is the first documentation of the presence of melanosomes in arthropods.

However, not all black colour in spiders is produced by eumelanin. The surface of the egg sacs from *H. cavatus* is covered by black spikes made out of silk (Fig. 8A,B). The Raman spectra of these black silk spikes show the signature peaks of ommochromes (Fig. 8C; Table S5), with wavenumbers around 595, 1255, 1430 and 1490 cm^{-1}. This is also in line with previous reports that the colour black could be produced in spiders by ommochromes (Seligy, 1972).

Raman signature peaks of the *H. cavatus* black egg sac (Fig. 8C; Table S5) are shifted slightly from those of the red ommochromes (Fig. 5; Table S4), probably because ommochrome molecules are in different chemical environments. In the egg sac, the ommochromes are in a spidroin protein matrix, while in other instances, the ommochromes are in the cuticle, which is mostly composed of chitin. Furthermore, the colour black is produced by ommins, while red is produced by ommatins (Seligy, 1972). The two molecular structures vary slightly (Fig. S1), and may cause the peaks to shift.

DISCUSSION

Using Raman spectroscopy, we identified the primary pigments producing yellow colour in *Nephila* silks as carotenoids. However, this does not exclude the potential co-existence of the previously suggested quinones and xanthurenic acid in these silks (Holl and Henze, 1988). Carotenoids are highly Raman active, so signals from other pigments present in trace amounts could simply be overshadowed. Determining whether quinones and xanthurenic acid are present, and identifying the specific carotenoids in *Nephila* silks will require detailed analyses using HPLC/MS. However, the intensity of the Raman signals correlates positively with the intensity of the yellow coloration of the silks (Fig. 2), suggesting that *Nephila* silks range from white to golden yellow simply because of different carotenoid concentrations.

Lutein and zeaxanthin are common dietary carotenoids directly deposited to produce yellow coloration. However, canary xanthophylls A and B are also common yellow carotenoids in avian yolk and feathers, as well as fish eggs and integument (LaFountain et al., 2015). These xanthophylls are thought to be synthesized via one simple dehydration reaction from their dietary precursor lutein, making them a potential candidate for the yellow carotenoids detected in Araneae if this conversion can be completed (LaFountain et al., 2015). Therefore, lutein, zeaxanthin and xanthophylls could be used as references for future HPLC/MS investigation.

There are two possible reasons why we could not detect any positive Raman signal from the yellow integumental setae of spiders, even when observations from optical microscopy and refractive index matching experiments clearly show the presence of yellow pigments (Fig. 4). The first is that carotenoids may be present in the yellow integumental setae, but the spiky setae interfere with and inhibit the successful detection of the pigments. However, this is unlikely, as in most cases the orange/red setae have very similar morphologies to the yellow setae, and we are able to detect signals from ommochromes in the orange/red setae (Fig. 5). The second, more likely, explanation is that carotenoids are not present in these setae, and other yellow pigments produce the colour. However, the responsible pigments probably have weak intrinsic Raman signals (i.e. they are more Raman inert), and are undetectable because of the auto-fluorescent background noise. There are many C–C and C–H
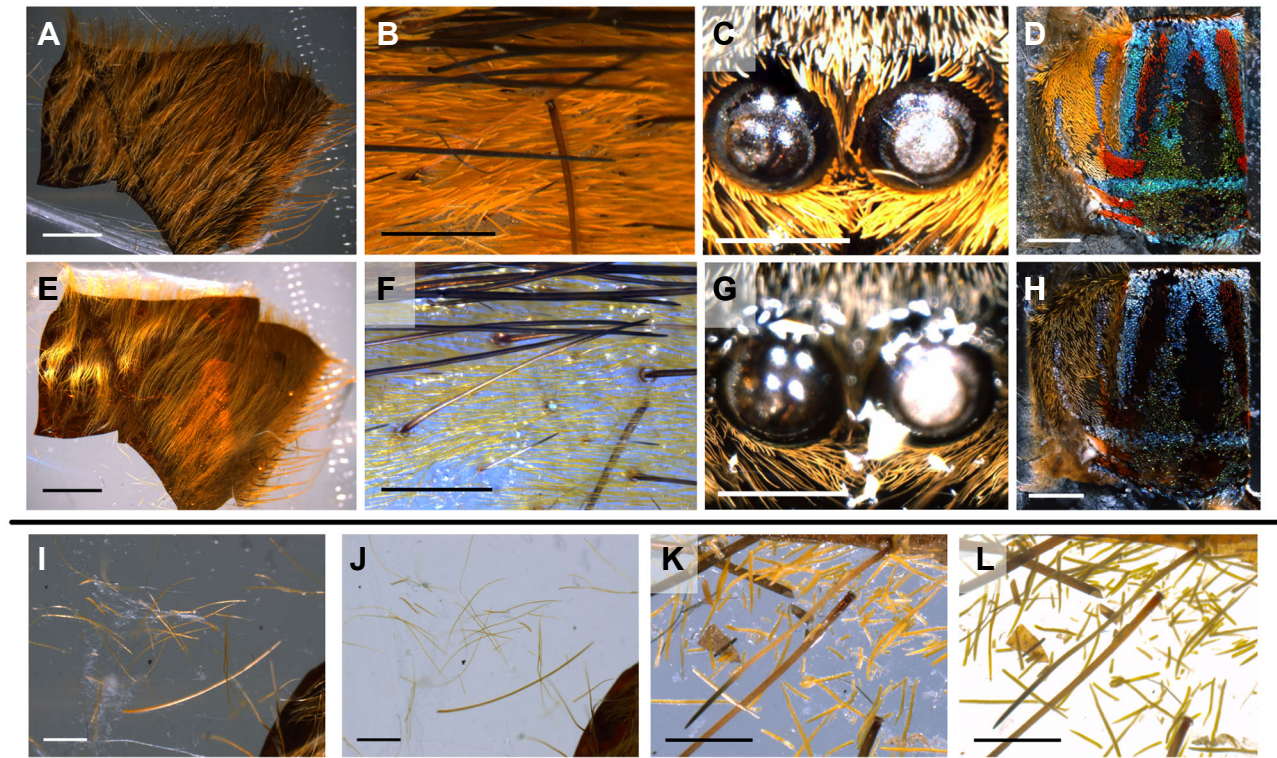
Fig. 4. Refractive index matching and optical microscopy analyses of spider yellow integumental setae. A cuticle fragment with yellow hairs from orange bamboo tarantula (*Pterinochilus murinus*) was observed before (A) and after (E) immersion in an index-matched solution, or observed under reflectance (I) and transmission (J) illumination. Hairs of a yellow patch from a Gooty sapphire tarantula (*Poecilotheria metallica*) were observed before (B) and after (F) immersion in an index-matched solution, and observed under reflectance (K) and transmission (L) illumination. The clypeus of *Menemerus bivittatus* was observed before (C) and after (G) immersion in an index-matched solution. The abdominal fan of *Maratus volans* was observed before (D) and after (H) immersion in an index-matched solution. Pigmentary origin is supported when the yellow colour remains after immersion in the index-matched solution, or under transmission illumination (e.g. E–H, J and L). Scale bars: A and E, 1 mm; B–D and F–H, 500 µm; I–L, 250 µm.

bonds (Raman active bonds) in melanins and carotenoids (Fig. S1), and hence they have strong Raman signals in general. Ommochromes have fewer C–C and C–H bonds relative to melanins and carotenoids (Fig. S1). Ommochrome Raman signals are comparably lower, but still detectable if they are present on their
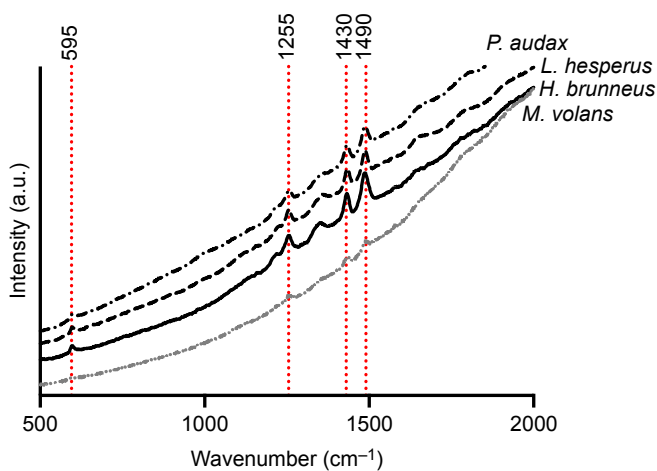

Fig. 5. Raman spectra of orange/red patches from different spiders. Raman spectra are from *Phidippus audax* (scale/opisthosoma), *Latrodectus hesperus* (chromatophore), *Habronattus brunneus* (scale/prosoma) and *Maratus volans* (scale/opisthosoma). All spectra show four peaks at wavenumber 595, 1255, 1430 and 1490 cm^{-1}, although the signals from the opisthosoma red scales of *M. volans* are very weak.

own, but as previously mentioned, they will be overshadowed if they co-exist with melanins and/or carotenoids. Kynurenine and 3OH-kynurenine (intermediates in the tryptophan metabolic pathway, and precursors for ommochrome synthesis; Riou and Christidès, 2010) were reported to cause the yellow coloration in the colour-changing crab spider *Misumena vatia* (Insausti and Casas, 2008), and were recently suggested to be present in the yellow scales of *M. splendens* (Stavenga et al., 2016). Kynurenine and 3OH-kynurenine have even fewer C–C and C–H bonds than ommochromes (Fig. S1). Therefore, we can hypothesize that kynurenine and 3OH-kynurenine produce the yellow colour in the integumental setae of spiders but are not detectable by Raman. This could be tested in the future by extracting pigments from the yellow setae, then depositing the pigment extract onto specially designed metal substrates, and performing SERS. SERS utilizes the surface plasma resonance of the metal substrates to enhance Raman signals up to 10^{11}-fold. Even molecules that are more Raman inert, such as 3OH-kynurenine, should be able to be detected by SERS. Indeed, pterins are another group of pigments that can produce yellow, and have similar or fewer C–C bonds (Fig. S1) than 3OH-kynurenine and cannot be detected by Raman, but have been successfully detected by SERS (Smyth et al., 2011). Pterins are abundant in insects (Shamim et al., 2014), and a previous genomic study suggested that pterins could be synthesized *de novo* in spiders (Croucher et al., 2013). Therefore, pterins could also be responsible for the colour production of these yellow setae.

Most animals cannot synthesize carotenoids *de novo*, and acquire carotenoids from their diets (Fox, 1976). These dietary carotenoids are
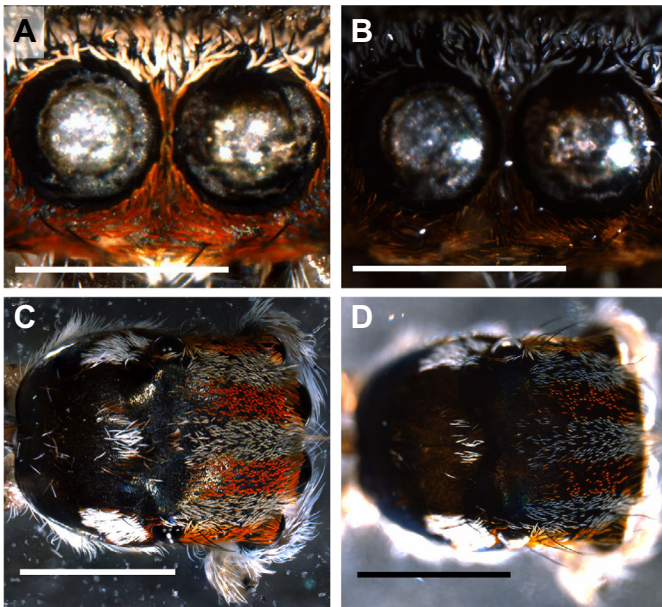
Fig. 6. Refractive index matching changes the colour of the scales from red to orange. The clypeus of *H. brunneus* before (A) and after (B) immersion in an index-matched solution. The carapace of *M. volans* before (C) and after (D) immersion in an index-matched solution. In both cases, the scales look red originally, but change to orange after immersion in the index-matched solution. This suggests that orange is a pigmentary colour, but red is a structural colour in these specimens. Scale bars: A and B, 500 μm; C and D, 1 mm.

mostly yellow. Based on our data, the chromatophores containing carotenoids may be responsible for many of the yellow colour markings in the opisthosoma of spiders, and the varying concentration of carotenoids in the chromatophores may explain the different colour morphs, ranging from white to yellow, in species such as *Gasteracantha kuhli* and *Verrucosa arenata*, as well as in some species of *Cyrtarachne*. It also suggests that in highly visual jumping spiders, the yellow coloration could function as an honest signal for sexual selection, as carotenoid-based colours do in many birds (MacDougall and Montgomerie, 2003) and fishes (Price et al., 2008).

The few animals that produce orange/red carotenoid colours either evolved carotenoid conversion enzymes, like crustaceans, insects, fishes (Goodwin, 1984; 1986) and birds (Twyman et al., 2016), or acquired genes for *de novo* production of carotenoids through horizontal gene transfer from fungi, such as in mites (Grbić et al., 2011). We did not find carotenoids in orange/red spiders, although our sampling was limited. This suggests that spiders may not possess such carotenoid-conversion enzymes and instead solely

utilize ommochromes to produce the orange/red colour as suggested in the literature (Nijhout, 1997; Riou and Christidès, 2010; Seligy, 1972; Stavenga et al., 2014). Although ommochromes can produce red (for example, the iconic red hourglass in most *Latrodectus*), it appears that in many red jumping spiders, ommochromes only produce orange, and that their vibrant reds are primarily structural colours. This pigmentary–structural colour combination is unique in that both are long-wavelength colours. The pigmentary orange may facilitate light scattering, and make the setae appear less iridescent than setae with solely structural red coloration (see Fig. S2 for more details of this hypothesis). We previously categorized pigmentary–structural colour interactions into three different outcomes: (1) enhancement of saturation (structural colours on top of a highly absorbing pigmentary layer that is usually eumelanin), (2) increasing brightness by using a broad-spectrum structural reflector beneath a pigment layer, and (3) colour mixing that combines a short-wavelength structural colour with a long-wavelength pigmentary colour to produce a mid-wavelength colour (e.g. mixing structural blue and pigmentary yellow to produce green) (Hsiung et al., 2014). The pigmentary orange and structural red interaction described here is a new category because the structural colour produces a longer wavelength colour than the pigmentary colour. Other evidence to support this hypothesis is that: (1) the colour red is weakly iridescent and (2) the red remains pristine even in specimens that have been preserved in alcohol.

Most current research on spider coloration focuses on answering functional questions through behavioural and/or ecological studies. However, the mechanistic approach to understanding the biochemical basis of colours we describe here adds a novel dimension to these functional hypotheses. For example, it could help us understand the evolutionary origin of similar colour hues among related species, or help determine that a particular colour is condition dependent and therefore useful as an honest signal in sexual selection. Understanding colour production mechanisms may also reveal potential non-visual functions. For example, finding eumelanin in spiders hints at functions like photoprotection (Brenner and Hearing, 2008), microbial resistance (Justyn et al., 2017; Krams et al., 2016) and mechano-enhancement of cuticle sclerization (Riley, 1997). Similarly, *Nephila*'s golden web is sometimes hypothesized to attract insects to webs through visual cues (Craig et al., 1996). We found that *Nephila*'s golden silk contains carotenoids. While carotenoids certainly produce yellow coloration, they also degrade naturally through oxidative cleavage or by carotenoid cleavage dioxygenases to produce volatile apocarotenoids (Heath et al., 2012). Apocarotenoids are associated with the scents of flowers and ripe fruits and the pheromones of Hymenoptera/Lepidoptera; hence, they might attract insects to the
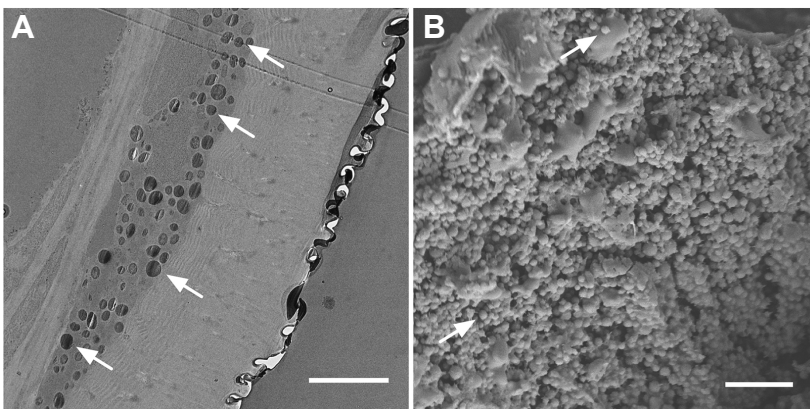

Fig. 7. Electron micrographs of melanosomes. (A) A TEM micrograph from *Maratus anomalus* showing a layer of highly electron-dense granules at the epidermal region just beneath the cuticle. (B) A SEM micrograph from *Maratus splendens* showing many densely packed spherical granules. This morphology is similar to melanosomes found in extant lizard, turtle and crocodilian skin (Li et al., 2014). Scale bars, 5 μm.
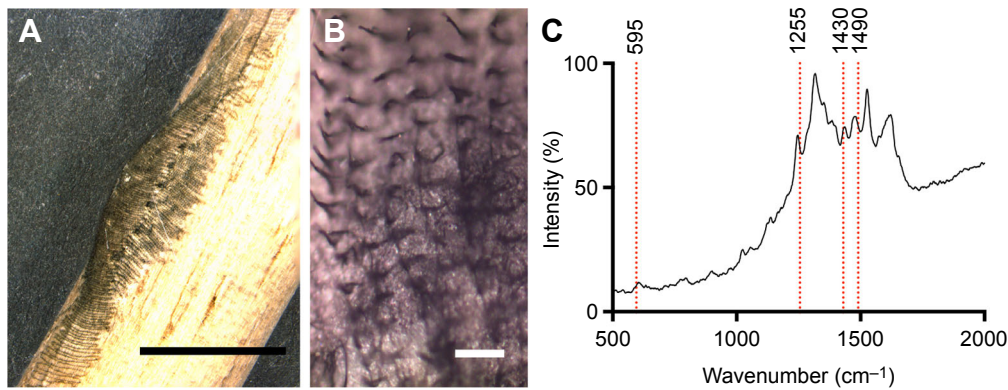
Fig. 8. The colour of black *Hyptiotes cavatus* egg sac is produced by ommochromes. A black *H. cavatus* egg sac on a wooden dowel rod under low (A) and high (B) magnification. The egg sac would be very well camouflaged on a natural twig. (C) A representative Raman spectrum from the black tufts on the egg sac showing the signature Raman signals for ommochromes around wavenumber 595, 1255, 1430 and 1490 cm^{-1}. Scale bars: A, 5 mm; B, 100 μm.

webs as olfactory cues (Heath et al., 2012). While speculative, carotenoid cleavage dioxygenases are active under high light (Scherzinger and Al-Babili, 2008), and this could explain why *Nephila* sometimes weave golden webs under high light intensity environments, but 'normal' white webs in dim environments (Craig et al., 1996).

Using our suggested workflow, we have: (1) discovered evidence of carotenoids in spiders, (2) established the signature Raman signals for ommochromes, and (3) revealed a new type of pigmentary–structural colour interaction that produces long-wavelength colours. We have also presented the first anatomical evidence of melanosomes in arthropods. New hypotheses await investigation following the finding that the biochemical basis for some of the yellow colours in spiders is carotenoids. For example, are carotenoid colours honest signals for jumping spiders that might predict mating success? And do yellow *Nephila* silks have non-visual olfactory functions? With modern instrumental analytical techniques and our suggested workflow, the door is now open to investigate the colour production mechanisms in small animals, leading to a better understanding of the evolution and function of colours in spiders and other arthropods.

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carotenoids

pterins

kynurenine

3-OH kynurenine

ommochromes

ommatins

ommin A

Common molecular structure between ommins and ommatins
is shown in black, the rest in red.

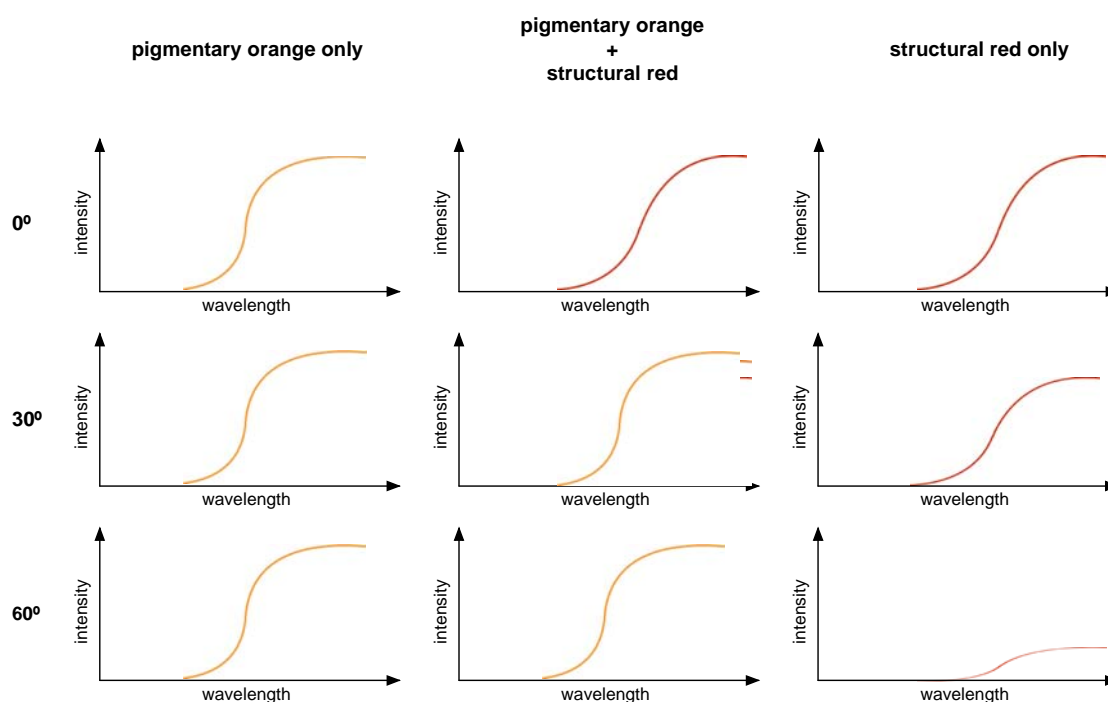**Fig. S1. Molecular structures of common pigments.**

Fig. S2. Hypothetical pigmentary orange/structural red interaction and their reflectance spectra. These setae are too small that their reflectance spectra can only be acquired through a microspectrophotometer at the normal direction, hence no experimental spectra were measured. The pigmentary colour is non-iridescent, therefore the reflectance spectra remain the same no matter the viewing directions. The structural coloured setae remain the same hue due to their complex morphology. However, the intensity is significantly affected by the viewing directions, and almost invisible at larger oblique angles (> 60°) (Stavenga et al., 2016). The setae that have both the structural red and pigmentary orange elements appear to be red at normal viewing direction because structural colours are much brighter than pigmentary colours. Hence, structural red overshadows pigmentary orange and determines the final visual appearance. At mid-range viewing directions (e.g. 30°), the reflectance spectrum is a mixture in between structural red and pigmentary orange. However, this slight swift in hue is almost inconspicuous. At larger oblique angles (> 60°), the intensity of the structural red is too weak to be seen, consequently the pigmentary orange becomes the dominant visual factor. Hence, setae with pigments appear less iridescent than that without.

Table S1. Raw Raman dataset for unpigmented spider silks.

Click here to Download Table S1

Table S2. Raw Raman dataset for yellow.

Click here to Download Table S2

Table S3. Raw Raman dataset for chemical and feather standards.

Click here to Download Table S3

Table S4. Raw Raman dataset for orange/red.

Click here to Download Table S4

Table S5. Raw Raman dataset for black.

Click here to Download Table S5