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RESEARCH ARTICLE

Spiders do have melanin after all

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ABSTRACT

Melanin pigments are broadly distributed in nature – from bacteria to fungi to plants and animals. However, many previous attempts to identify melanins in spiders were unsuccessful, suggesting that these otherwise ubiquitous pigments were lost during spider evolution. Yet, spiders exhibit many dark colours similar to those produced by melanins in other organisms, and the low solubility of melanins makes isolation and characterization difficult. Therefore, whether melanins are truly absent or have simply not yet been detected is an open question. Raman spectroscopy provides a reliable way to detect melanins *in situ*, without the need for isolation. In this study, we document the presence of eumelanin in diverse species of spiders using confocal Raman microspectroscopy. Comparisons of spectra with theoretically calculated data falsify the previous hypothesis that dark colours are produced solely by ommochromes in spiders. Our data indicate that melanins are present in spiders and further supporting that they are present in most living organisms.

KEY WORDS: Pigment, Araneae, Eumelanin, Raman spectroscopy

INTRODUCTION

Melanins are important biomolecules that perform diverse functions and are produced endogenously by almost all living organisms. Melanins operate in the innate immune defense mechanisms of insects and crustaceans (Cerenius and Soderhall, 2004; Nappi and Christensen, 2005). Their anti-oxidation properties protect organisms from thermal and chemical stresses (Rózanowska et al., 1999), as well as from radiation exposure (Tugay et al., 2011). Melanins may also modify the properties of materials by acting as stiffening agents, thereby increasing mechanical strength and abrasion resistance of bird feathers (Butler and Johnson, 2004), plant seeds and insect cuticle (Riley, 1997). However, the most widespread function of melanins is colour production. Because melanins strongly absorb light across all visible wavelengths, eumelanins produce many of the dark brown to black colours (Riddle, 1909) and provide UV protection for many organisms (Brenner and Hearing, 2008; Gao and Garcia-Pichel, 2011). Pheomelanins are reddish in isolation and, in combination with eumelanin, produce the vast diversity of melanin-based black to brown colouration seen in animals (Simon and Peles, 2010). Counter-intuitively, the high refractive indices of melanins also enable them to efficiently scatter light and thereby produce vibrant structural colours. For example, in birds (Eliason et al., 2013) and insects (Berthier, 2007), melanin granules can be deposited in sub-micrometre periodic structures that scatter and reflect specific wavelengths of light, producing a variety of hues across the whole rainbow of colours.

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Given these diverse functions, it is not surprising that melanins have been described in virtually all major lineages of living organisms, including protists (Gao and Garcia-Pichel, 2011), bacteria (Nosanchuk and Casadevall, 2006), fungi (Tugay et al., 2011), plants (Riley, 1997) and animals (Eliason et al., 2013). This includes eumelanins in many arthropods, such as crustaceans and insects (Cerenius and Soderhall, 2004). However, pheomelanins have only been very recently reported in invertebrates (Galván et al., 2015; Speiser et al., 2014) and Raman spectrometry may facilitate the discovery of pheomelanins across major lineages of organisms in the future. Despite the ubiquity of melanin in nature, all studies to date have failed to identify melanins in spiders, and a recent genomic study suggested that spiders lack the metabolic pathway known to synthesize melanins endogenously in other organisms (Croucher et al., 2013). Hence, spiders are argued to lack melanins (Foelix, 2011; Holl, 1987; Oxford and Gillespie, 1998; Seligy, 1972) and some chemical data suggest that ommochromes, especially ommins, instead produce their dark colours (Seligy, 1972). This hypothesis also suggests that spiders may have evolved novel mechanisms to replace the diverse functions of melanins in other organisms.

The low solubility of melanins (Gonçalves et al., 2012) makes them difficult to detect and analyze chemically. Raman spectrometry is a useful tool to detect and distinguish between different types of melanin in both living (Galván et al., 2013) and fossilized (Beimforde et al., 2011) specimens *in situ*, without the need for pigment extraction and purification. Raman spectrometry measures the energy change resulting from the excitation laser inelastically interacting with different modes of molecular vibrations (Raman scattering). The resulting spectrum is commonly used as a fingerprint to identify molecules (Cheng et al., 1995). Using qualitative spectrometry (such as Raman), rather than chemical analysis, makes the identification of melanins in biological systems easier. Here, we use confocal Raman microspectroscopy data to test for the presence of eumelanin in a diverse group of spiders.

MATERIALS AND METHODS

Spiders

We tested 14 species of spiders with black or brown body patches from six different families (see Table 1). These included species near the base of the spider phylogeny (tarantulas, Therophosidae) as well as species from five distantly related families distributed across the Entelgynae (orb spiders, Araneidae; wolf spiders, Lycosidae; golden silk spiders, Nephilidae; jumping spiders, Salticidae; and cobweb spiders, Theridiidae). Most were collected live in the local area (Akron, OH), but the tarantulas and black widows were purchased from the pet trade. Three taxa were previously collected and stored in ethanol: *Caerostris* (Andasibe-Mantadia National Park, Madagascar), *Gasteracantha* (Florida, USA), and *Maratus* (Queensland, Australia).

Confocal Raman microspectroscopy

Raman spectra of pigments and spiders 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

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Table 1. Eumelanin is detected in all 14 species of spiders across six families investigated

Family	Species	Source	Specimen condition	Eumelanin signal intensity
Theraphosidae (tarantulas)	*Brachypelma smithi* (Mexican redknee tarantula)	Black hair	Molt	Strong
	Grammostola rosea (Chilean rose tarantula)	Black and non-black hair	Ethanol preserved	Moderate
	Lampropelma violaceopes (Singapore blue tarantula)	Black hair	Molt	Moderate
Theridiidae (cobweb weavers)	*Latrodectus hesperus* (western black widow)	Black hair	Freshly dead	Strong
Salticidae (jumping spiders)	*Phidippus johnsoni* (Johnson jumper)	Black and white hair	Freshly dead	Strong
	Maratus chrysomelas	Black hair	Ethanol preserved	Moderate
	Maratus speciosus (coastal peacock spider)	Black hair	Ethanol preserved	Moderate
	Maratus robinsoni (rainbow jumping spider)	Black hair	Ethanol preserved	Weak
Araneidae (orbweavers)	*Gasteracantha cancriformis* (spinybacked orbweaver)	Black hair	Ethanol preserved	Strong
	Caerostris darwini (Darwin's bark spider)	Black and white hair	Ethanol preserved	Strong
	Argiope aurantia (yellow garden spider)	Black hair	Frozen	Strong
	Argiope trifasciata (banded garden spider)	Black hair and non-black cuticle	Frozen	Strong
Nephilidae (golden silk orbweavers)	*Nephila clavipes*	Black hair	Frozen	Strong
Lycosidae (wolf spiders)	*Schizocosa ocreata*	Black hair and non-black cuticle	Ethanol preserved	Moderate

Signal intensity is qualitatively estimated based on the ratio of the height of the peaks at 1380 and 1580 cm^{-1} to the background baseline. Raw spectral data can be downloaded from Tables S1–S3.

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 300 to 2500 cm^{-1}. The laser beam cross-section diameter is around 40 µm. The system was operated with LabSpec 5 software with an integration time of 5 s ×2 accumulations. For each spider, both light and dark colours were examined when possible, because eumelanin signatures should only occur in the dark regions. Spectra were collected from three different locations for both colour types on each particular spider. We collected spectra from black body patches of all 14 spider species and non-black body patches of five spider species. We used *Sepia officinalis* (cuttlefish) eumelanin (M2649, Sigma-Aldrich) as a standard and collected Raman spectra under the same conditions as reported above. Replicate spectra were smoothed, normalized, averaged and plotted using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA) without baseline correction.

Theoretical Raman spectrum simulation

Previously, only two categories of pigment were identified in spiders: (1) ommochromes and (2) bilins (Oxford and Gillespie, 1998). Ommins, a subcategory of ommochromes, were suggested to produce the black (dark) colouration in spiders (Seligy, 1972), so we tested for their presence. However, ommin standards are not commercially available. We therefore used a standard method to calculate the theoretical Raman spectrum of ommin A, based on the molecular structure of the pigment (Holl, 1987). We used Spartan 14 Quantum Mechanics Program (Wavefunction, Inc., Irvine, CA, USA) with HF/6-31G* [Hartree-Fock method with 6-31G(d) basis set], assuming no solvent effect (vacuum), at 298.15 K and 532 nm excitation wavelength. To verify the method's accuracy, synthetic β-carotene (Sigma-Aldrich C9750) served as a control for the theoretical calculated spectrum of ommin A. We calculated the theoretical spectrum of β-carotene and compared this to its empirically collected spectrum (Fig. 1). The theoretical Raman spectrum of eumelanin was not calculated because eumelanins are complex polymers whose chemical structures are not fully understood and greatly vary between different organisms.

RESULTS

The Raman spectra for *S. officinalis* melanin showed two broad peaks (Fig. 2B,D, grey dotted line): one lower intensity around wavenumber 1380 and the other higher intensity around wavenumber 1580. The peak positions and the shape of the spectrum matched eumelanin signatures and were consistent with previous reports (Huang et al., 2004; Perna et al., 2013).

Spectra collected from all black spider body patches (Fig. 2A,C, blue circle) also showed signatures for eumelanin (Fig. 2B,D, blue line), whereas spectra collected from non-black spider body patches (Fig. 2A,C, red circle) did not (Fig. 2B,D, red dashed line). Instead, spectra from non-black spider body patches showed highly reproducible and steadily increasing curves, with positive correlations between Raman signal intensity and wavenumber as the basal spectra (Fig. 2B,D, red dashed line). Spectra are summarized in Table 1 (see also Figs S1 and S2).

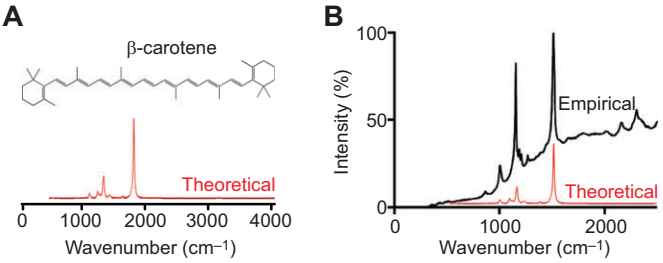

Fig. 1. Theoretical and empirical Raman spectra of β-carotene. (A) Theoretical Raman spectrum based on the structure of β-carotene. (B) Empirical Raman spectrum measured from commercially available synthetic β-carotene, overlaid with the theoretical spectrum (after fitting correction, to adjust the wavenumbers). Smoothened spectra without baseline correction are shown.

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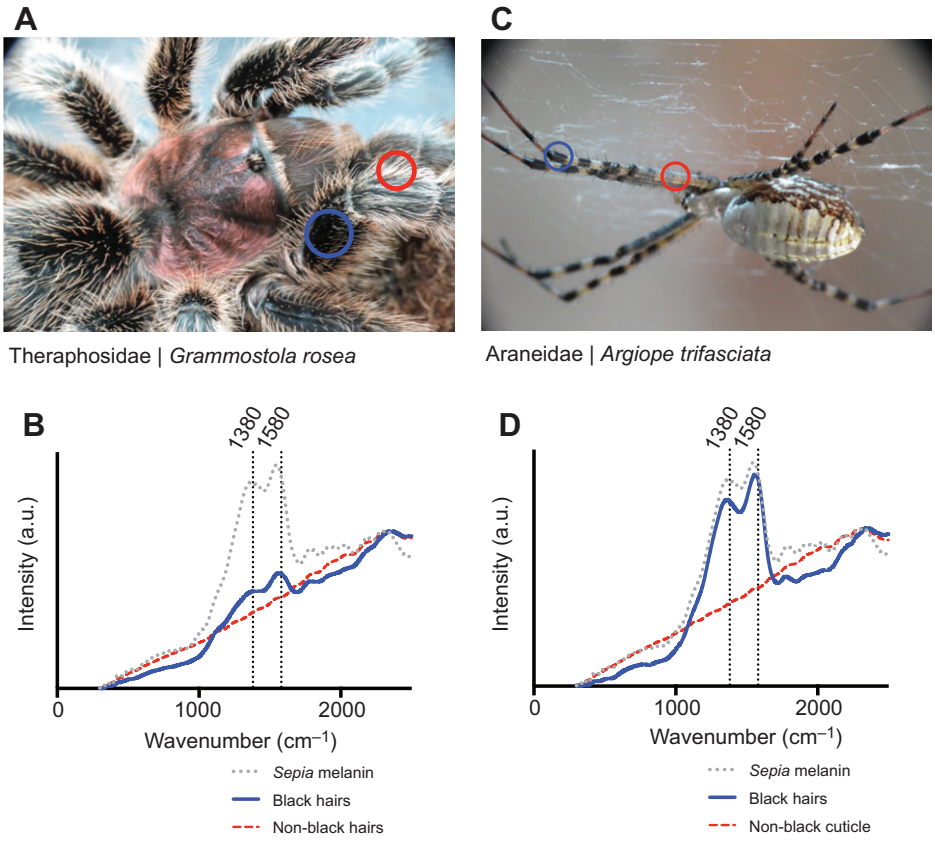

Fig. 2. Representative Raman spectra and their source organisms. (A) Adult female Chilean rose tarantula *Grammostola rosea*. (B) Raman spectra of black (blue solid line) and non-black (red dashed line) hairs, measured from areas circled in blue and red in A. (C) Adult female banded garden spider *Argiope trifasciata*. (D) Raman spectra of black hairs (blue solid line) and non-black cuticle (red dashed line), measured from areas circled in blue and red in C. Raman spectrum of *Sepia officinalis* melanin (grey dotted line; positive control) is overlaid in B and D. Smoothed spectra without baseline correction are shown. Raw spectral data can be downloaded from Tables S1–S3.

In theory, Raman spectrum can be predicted based on the molecular structure of a pigment. Hence, although no ommin pigment standard is commercially available, the spectrum of ommin A can be predicted based on its known chemical structure (Holl, 1987). We used β-carotene as an initial test of whether Raman spectra can be faithfully predicted based on the molecular structure of a pigment. β-carotene is a common pigment that belongs to the carotenoid family and it has a relatively simple chemical structure compared with ommins. The shape of the predicted Raman spectrum for β-carotene based on theoretical quantum mechanics calculations (Fig. 1A) matched well with its empirical spectrum (Fig. 1B). However, the position (wavenumber) of the peaks is systematically overestimated. This is a bias introduced by the chosen calculation method. In general, the Hartree–Fock (HF) method overemphasizes the occupation of bonding orbitals and the bonds are systematically calculated as too short. Therefore, the vibrational frequencies (wavenumbers) are generally predicted to be too high. However, this kind of overestimation can be easily fixed by application of a systematic scaling factor to the calculated spectrum (Fig. 1A). This procedure yields an almost perfect match between the theoretical and the empirical spectra (Fig. 1B).

Using a theoretical calculation, we showed that the predicted (uncorrected) Raman spectrum of ommin A (Fig. 3, red line) is dissimilar to the eumelanin signature spectrum (Fig. 2B,D, gray dotted line) or the spectra from black body patches of spiders (Fig. 2B,D, blue line).

DISCUSSION

Our Raman data suggest that the black colour of spiders is due to eumelanin, rather than the previously suggested ommin A. We detected eumelanin signatures from the black body patches of 14 different species of spiders across six families (Fig. 2B,D, blue line; Table S2, Fig. S1). Neither the Raman spectra from non-black body patches of the same spiders (Fig. 2B,D, red dashed line; Table S3, Fig. S2), nor the calculated Raman spectrum of ommin A (Fig. 3, red line) showed those signatures. Because the spiders we sampled are broadly distributed across the Araneae, our data suggest that eumelanin is widespread in spiders, contrary to previous conclusions (Oxford and Gillespie, 1998).

Why have melanins not previously been detected in spiders? Prior attempts used relatively simple techniques (e.g. thin-layer

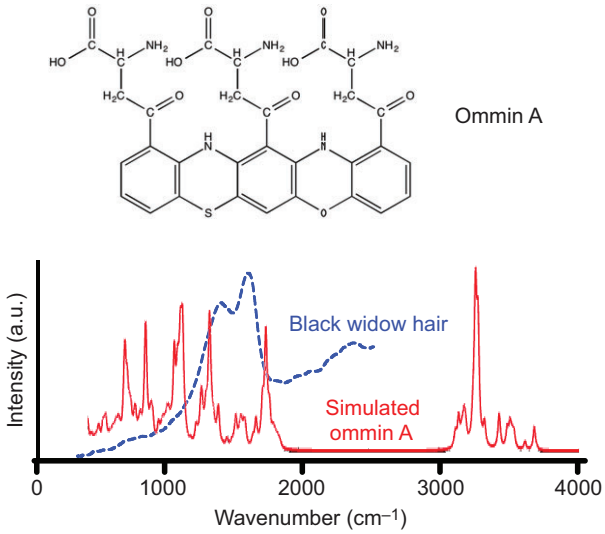

Fig. 3. Simulated Raman spectrum of ommin A based on its molecular structure. A spectrum from black widow (*Latrodectus hesperus*) hair is overlaid for comparison. Smoothened spectra without baseline correction are shown.

chromatography) that are inefficient for detecting melanins. Moreover, these techniques required extraction and purification of melanins, which may have been hindered by their low levels of solubility and low volumes per spider (Seligy, 1972). That the biosynthetic pathway of melanin in spiders was not detected in a recent genomic study may simply illustrate a limitation of homologous sequence comparison to identify evolutionary shifts in enzymes. Genes associated with melanin pigmentation that were not found in spiders include: *ebony*, *eiger*, *grim*, *Gustatory receptor 28b*, *Melanization protein 1*, *Peptidoglycan recognition protein LC*, *Serine protease 7*, *Serpin 27A*, *Serpin 77Ba*, *yellow*, *yellow-f* and *yellow-f2* (Croucher et al., 2013). However, seven genes associated with melanin synthesis were found in the same study (Croucher et al., 2013): *basket*, *dorsal*, *hemipterous*, *Hemolectin*, *Neuroglian*, *Rho1* and *Toll*. Spiders may use different enzymes or substrates to synthesize melanins, as is true in insects that produce pheomelanin (Galván et al., 2015). In a similar case, the pigment gadusol was known to be present in fish, even though fish lacked the genes known to produce it. A recent study found a new gadusol synthetic pathway in fish and other vertebrates that differs from the previously known pathway in invertebrates (Osborn et al., 2015). Therefore, documenting the presence of melanin in spiders should spur investigation of its biosynthetic pathways and numerous other questions. For instance, are other types of melanin, such as pheomelanin, present in spiders? Are melanins involved in the spider's immune system, cuticle sclerotization and/or structural colour production? The courtship displays of many adult male wolf spiders involves using conspicuous brushes of black hairs (tufts) on their forelegs (Hebets and Uetz, 2000). Could melanins act as honest signals in spiders as they appear to function in some birds (D'Alba et al., 2014; Jawor and Breitwisch, 2003)? Regardless, our results may close the door on the mystery of why one group of organisms would lack an otherwise widespread pigment – it was there all along.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.A.B., M.D.S. and B.-K.H. conceived research. B.-K.H. designed, performed research and analyzed data. T.A.B., M.D.S. and B.-K.H. wrote the manuscript. T.A.B. and M.D.S. provided scientific leadership to B.-K.H.

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Supplementary information
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