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Characterization of the green iridescence on the chelicerae of the tube web spider, *Segestria florentina* (Rossi 1790) (Araneae, Segestriidae)

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Abstract. *Segestria florentina* (Rossi 1790) (Segestriidae) displays iridescent green coloration on the paturons of the chelicerae. This was confirmed by reflectance measurements, which gave a spectral peak at 505 nm. Scanning electron microscopy did not identify cuticular surface scales or sculpturing, suggesting that the cause of the iridescence was subsurface. Transmission electron microscopy revealed 86 alternate dark and light layers in the exocuticle, the mean dimensions of which were 126 nm ± 28 nm and 88 nm ± 55 nm respectively. The identity of each layer was initially unclear. However, by using a combination of materials with different refractive indices in calculations of theoretical reflectance spectra, we concluded that they were most likely to be composed of chitin and air, since a peak of 480 nm was obtained, which most closely matched that which was recorded. The function of the green color is not clear, since *S. florentina* has relatively poor vision and relies predominantly on vibratory and acoustic signals. The study provides useful information relevant to research into the evolution of structural colors in spiders and, more generally, in nature.

Keywords: Structural color, photonic, multilayer reflector

Structural colors are the result of the interaction of light with physical structures, termed photonic crystals, which are in or on the surface of a substratum. They have been identified from a diverse range of taxa, most notably butterflies (Ghiradella 1991; Kinoshita et al. 2002; Biró et al. 2003; Vukusic et al. 2004; Ingram 2008) and birds (Prum et al. 1999; Zi et al. 2003; Li et al. 2005; Vigneron et al. 2006), which have long been the focus of photonics research since their structural colors are very obvious. There are, however, many more instances of structural coloration, which are yet to be described – for example, in spiders. Of the few studies conducted, almost all have concentrated on jumping spiders (Salticidae), as structural colors occur mainly in this family. Multilayer reflectors have been the most commonly occurring photonic crystal, identified from modified setae or cuticular scales (Cutler & Richards 1972; Hill 1979; Holl 1987; Land et al. 2007) and epicuticular surface sculpturing (Parker & Hegedus 2003) on the abdominal and cephalothoracic regions, on which these colors are typically located. Diffraction gratings have also been discovered, individually or in combination with a multilayer reflector (Kochalka 1980; Parker & Hegedus 2003). Here, we extend the literature on spider photonics by describing the structural origin of the green iridescence on the chelicerae of the tube-web spider, *Segestria florentina* (Rossi 1790), using a combination of electron microscopy, spectroscopy and optical modelling. Structural colors have not previously been examined from this haplogyne family. It is therefore hoped that the results will provide useful information, which when considered with those describing iridescence in the highly-derived Salticidae, will contribute to our understanding of the evolution of structural color in spiders.

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Spectral measurements were taken from a specimen of *S. florentina*, which was provided in 70% ethanol by The Natural History Museum (London). A chelicera was dissected from the specimen and illuminated with a halogen source incident at 0° to the surface (''normal incidence''). Reflected light returning along the same illumination pathway (''backscatter'') was measured using an Avantes Avaspec 2048/2 spectrometer. The reflection was normalized using a white diffusive standard. Due to the difficulty of measuring spectra from such a small specimen and also the curvature of the sample, a second measurement was taken from the second chelicera of the same individual, for confirmation. A 2 mm^2 section of the dorsal paturon was dissected from one sample and glued with Araldite™ to a glass slide. This provided a sufficiently flat surface from which to measure reflectance spectra with a Roper Spectral-DV™ spectrometer mounted on an Olympus microscope, equipped with a rotating slide holder. This experimental set-up enabled spectra to be recorded from multiple points on the specimen. Data were then collated using Mélange image processing software. The specimen was illuminated, as before, with a halogen source at close to normal incidence and backscattered light (375–700 nm) was collected from a narrow cone around the same angle. Spectra were acquired at 20× magnification.

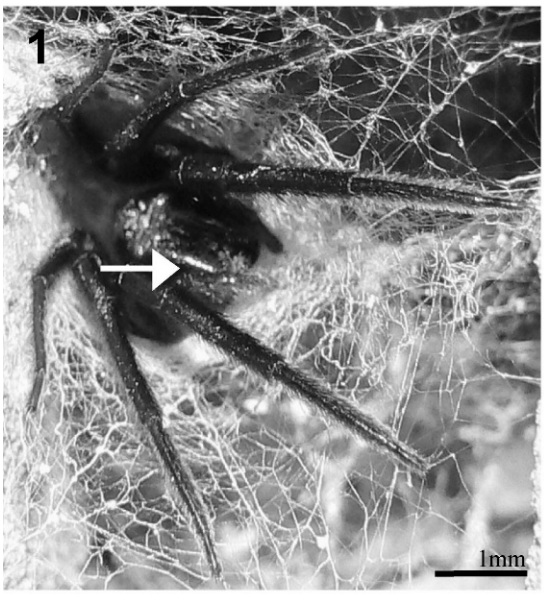
Previous studies have identified cuticular surface and subsurface structures as the cause of interference colors in spiders. A sample was therefore prepared for scanning and transmission electron microscopy (SEM and TEM respectively). For the former, a chelicera was transferred from 70% ethanol to 70% acetone and then dehydrated through a graded acetone series before being critical point dried. It was then coated with gold, mounted on a metal stub using Araldite™ and viewed with a Philips XL30 Field Emission SEM. Images were recorded digitally. A further sample was prepared for TEM, which was immersed in 70% acetone and then

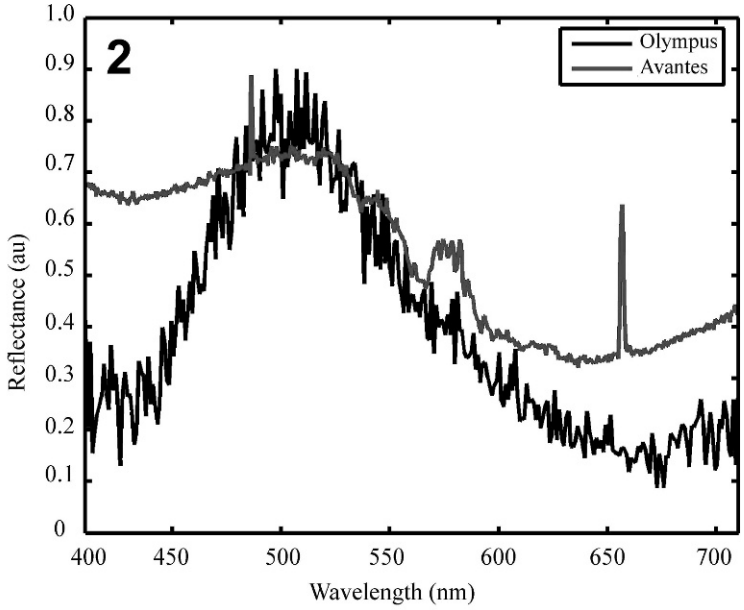
progressively dehydrated through increasingly concentrated acetone to 100% dried acetone. Samples remained here for 48 h before beginning the resin infiltration process using TAAB medium grade resin. Previous experience (Kennaway et al. 2004) has shown that the most important factor in successfully infiltrating terrestrial arthropods is first to ensure that there is no residual water in the specimens (through extended dehydration times) and secondly to ensure that the resin has penetrated the specimens properly (by use of thinned, or low viscosity resin). The resin infiltration process consisted of immersing the sample in a mixture of resin and acetone and progressively increasing the concentration of resin in the mixture. This was achieved over four days starting with a mixture of approximately 20% resin, 80% dried absolute acetone. Each mixture was changed after about 12 h and each step was carried out for 24 h. Finally, the infiltrated sample was placed in a resin-filled, flat-ended BEEM capsule and polymerized for 8 h at 70° C. Sections for TEM were cut at 70–90 nm thickness, collected onto grids and counterstained using alcoholic uranyl acetate and Reynold's lead citrate, prior to examination in a Hitachi H7100 TEM. Images were recorded onto film and then scanned from prints.

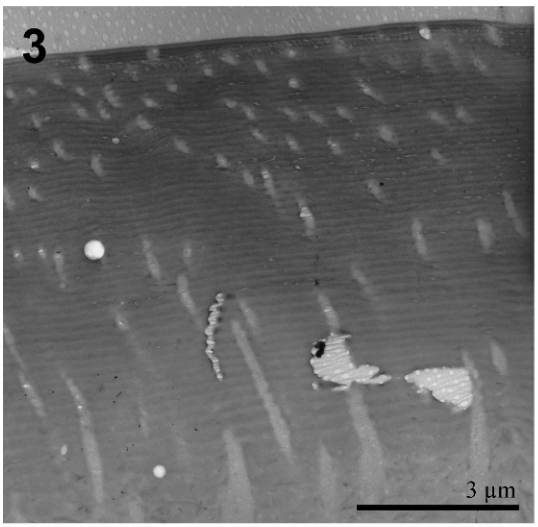
Theoretical reflectance spectra were calculated by the continued-fraction method, which relies on exactly solving Maxwell's equations for an arbitrarily stratified medium (Dereux et al. 1988). In this method, the reflection coefficients for transverse-electric and transverse-magnetic polarized light are expressed in terms of the surface impedances, which take the form of continued fractions. These continued fractions terminate for a finite number of layers deposited onto an infinitely thick substrate (of known refractive index). Their values are determined as soon as the thickness and the refractive index of all the layers are known and the incidence conditions are given. The incidence angle was set to 0° (incidence medium: air) so that the specular reflection occurred exactly in the backward direction (''backscatter''). Layer thickness values were determined from TEM cross-sectional images and used for calculation. The stratified medium was assumed to be formed by the alternation of low and high density materials (the reason why we chose to model the stack of layers by a periodic multilayer will be explained later on). The precise identity of the two constituent materials was, however, unclear in the absence of data concerning the refractive indices of materials comprising spider reflectors. Since these values are hard to determine experimentally, we used the results from a number of different approaches: 1) a basic infiltration test (using acetone) for detecting the presence of empty (air-filled) layers (see Parker 2000); 2) previous studies, which have reported spider reflectors from the abdominal and cephalothoracic regions as being comprised of air and chitin layers (Parker & Hegedus 2003; Land et al. 2007); 3) using the aforementioned information, we alternated the refractive indices of possible constituent materials to obtain the best fit between the empirical and theoretical spectra. The refractive index of the organic material was taken to be constant over the whole spectral range. The refractive index of the substrate (beneath the stratified medium) was taken to be equal to that of the bulk organic material. Although the continued-fraction method allowed us to calculate the reflectance for all layers appearing on the TEM images, such a calculation did not lead to a clear peak in the spectrum because variations of the layer thickness across the stack tended to destroy light interference. For this reason, we chose to model the actual structure by a periodic multilayer stack with the same number of layers but constant thicknesses.

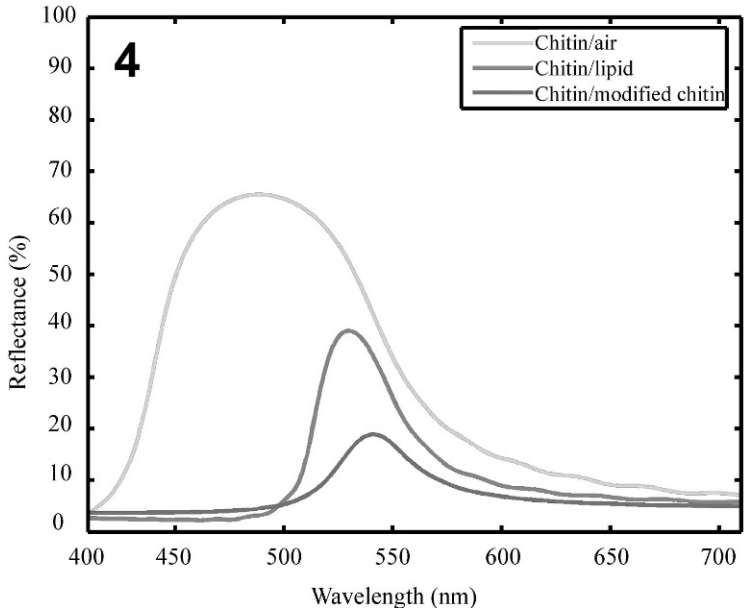
Initial observations of the chelicerae showed that under most angles of illumination and observation, the bright green iridescence originated from the dorsal surface of the paturon (Fig. 1). This was confirmed by the two measured spectra taken from this region, which indicated two similar reflectance peaks around 505 nm (green) (Fig. 2). Scanning electron microscopy revealed that the dorsal surface of the paturon was predominantly smooth, devoid of scales or surface sculpting, suggesting that the origin of the iridescence lay below the cuticular surface. Sections of the paturon were, therefore, examined with the TEM and showed that the exocuticle is composed of an outer region extending 7–8 μm towards the interior, in which around 86 alternate dark and light layers were observed (Fig. 3). Measurements of the thickness of each layer showed that they were highly variable: 126 nm ± 28 nm and 88 nm ± 55 nm, respectively. For this reason, the mean values were used for the theoretical reflectance spectra calculation.

The material of the high density layers was assumed to be chitin, based on previous literature (Richards 1951; Parker & Hegedus 2003). The results of the acetone test showed no color change, suggesting that the less dense layers were not composed of air. Based on this, we modeled spectra using a combination of chitin and a lipid with a refractive index (RI) of 1.46 (Bausch & Lomb), since cuticle contains lipid (Richards 1951). Also chitin and a low RI chitin-based material ($n = 1.40$) (Bernard & Miller 1968), and finally chitin and air, to give weight to the acetone test. Assuming a refractive index of chitin equal to 1.56 (real part) (Land 1972), the optical path length across a chitin/low density bi-layer at normal incidence is equal to $\delta = (d_{chitin} \times n_{chitin}) + (d_{low} \times n_{low})$, where d_{chitin} and d_{low} are the average actual thicknesses of the chitin layer (126 nm) and low density layer (88 nm), $n_{chitin} = 1.56$ (chitin) and $n_{low} = 1.0$, 1.40 or 1.46 (air, lipid, or low RI chitin-based material) are the refractive indices respectively. The condition of constructive interference upon reflection at normal incidence in a quarter wavelength multilayer stack, $\delta = m \times \lambda/2$, predicts that the Bragg wavelength ($m = 1$) is located in the visible range: 567 nm (air), 637 nm (lipid) and 648 nm (low RI chitin-based material) from the green end of the spectrum to the red (Fig. 4), suggesting that the low density layers are most likely to be composed of air. The failure of the acetone test to identify air as a constituent is likely due to the fact that the structure is sealed. The reflectance of a stack made of 50 alternating layers of chitin and 1) air 2) modified chitin and 3) lipid was calculated at normal incidence. We chose to consider the first 50 alternating layers (over 86 in total) due to the large fluctuations observed in layer thicknesses. The averaged thickness values of these layers, $d_{chitin} = 115$ nm and $d_{low} = 62$, were found to be slightly lower than the ones cited above, leading to a shift of the Bragg wavelengths to shorter wavelengths: 481 nm (air), 530 nm (lipid), and 538 nm (low RI chitin-based material). An imaginary part (i) was added to the refractive index of chitin and modified chitin to take into









Figures 1–4.—*Segestria florentina* (Rossi 1790) (Segestriidae). 1. Frontal view of an adult female, indicating green iridescent chelicera. 2. Reflectance spectra (au - arbitrary units) recorded from the paturon using two types of spectrometer (Avantes / Olympus microscope-mounted). Both illumination and measurement angles were set to 0° (normal incidence) in each case. 3. Scanning electron micrograph of a transverse section through a paturon. 4. Theoretical reflectance spectra (%) from the paturon, by the continued-fraction technique. Incidence and reflection angles set to 0°. Three combinations of materials were used: Chitin/air, chitin/lipid and chitin/modified chitin with the refractive indices set to 1.0 (air), 1.56 (chitin), 1.46 (modified chitin), and 1.40 (lipid).

account optical absorption in the chitin material (n_{chitin} = 1.56 + i × 0.05, n_{low} =1.40 + i × 0.05). This complex refractive index was assumed to be constant across the whole wavelength range (350–710 nm), in a first approximation. The results showed that the best agreement with the measured reflectance spectra was obtained using chitin and air (Fig. 4).

The chelicerae display some of the most striking structural colors found in spiders (Jackson 1982). They are probably most well known from the Salticidae, however, they also occur in the Segestriidae. In both cases, naturalists and scientists have been aware of their existence for some time and yet the cause of the color has remained unexplained. In the current study we chose to examine the iridescent green chelicerae of *S. florentina*, from which the cause of the color was identified as a constructively-interfering multilayer reflector. Studies have identified this type of reflector elsewhere in spiders (Cutler & Richards 1972; Hill 1979; Holl 1987; Parker & Hegedus 2003; Land et al. 2007) and it is emerging as the most common type of photonic crystal in this group, as it is in butterflies (Ingram 2008) and structurally colored animals in general (Parker

2006). This convergently evolved optical structure typically provides an effective method of displaying bright color to conspecifics without attracting the unwanted attention of predators (Parker 1998). However, it is unclear if this is the intended function of the color in *S. florentina*. Unconfirmed reports suggest that the similarly green iridescent chelicerae of the unrelated genus, *Phidippus* (Salticidae), are employed in conspecific (mate) recognition (Irene Lindsey, pers. comm.). Additional evidence for this originates from studies of vision in the related species, *P. regius* Koch 1846, which showed that the eyes have a corresponding peak spectral sensitivity in the green (de Voe 1975). It is unlikely that the chelicerae of *S. florentina* function in mate recognition, since the family is known to have relatively poor vision, relying predominantly on vibratory (Barth 1982) and acoustic signals (Gertsch 1979). To determine the role of the color, behavioral and visual data are required to determine if the green has some adaptive function in signaling.

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