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# Study of melanin localization in the mature male *Calopteryx haemorrhoidalis* damselfly wings

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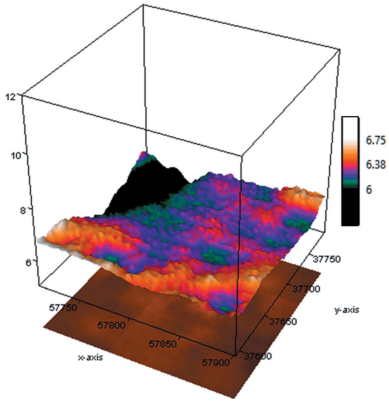
Damselflies *Calopteryx haemorrhoidalis* exhibiting black wings are found in the western Mediterranean, Algeria, France, Italy, Spain and Monaco. Wing pigmentation is caused by the presence of melanin, which is involved in physiological processes including defence reactions, wound healing and sclerotization of the insect. Despite the important physiological roles of melanin, the presence and colour variation among males and females of the *C. haemorrhoidalis* species and the localization of the pigment within the wing membrane remain poorly understood. In this study, infrared (IR) microspectroscopy, coupled with the highly collimated synchrotron IR beam, was employed in order to identify the distribution of the pigments in the wings at a high spatial resolution. It was found that the melanin is localized in the procuticle of the *C. haemorrhoidalis* damselfly wings, distributed homogeneously within this layer, and not associated with the lipids of the epicuticle.

## 1. Introduction

*Calopterygidae*, a family of damselflies also known as demoiselles, are notable for their pigmented wings and striking body colours (Cordoba-Aguilar & Cordero-Rivera, 2005). The *Calopteryx haemorrhoidalis* species with black wings only inhabit the western Mediterranean, Algeria, France, Italy, Spain and Monaco (Clausnitzer, 2009). The wings of the mature male damselfly of this species have dark-brown pigmentation, whereas the female damselfly wings are light brown (Stavenga *et al.*, 2012). Several hypotheses regarding the role of wing colouration in insects have been proposed (Sugumaran, 2002; Stoehr, 2006). Melanins are high-molecular-weight polymeric phenolic and/or indolic compounds which possess negatively charged and hydrophobic characteristics (Bridelli *et al.*, 1999; Eisenman & Casadevall, 2012; Nappi & Vass, 1993; Dubovskiy *et al.*, 2013). It is believed that wing pigmentation is caused by the presence of melanin, which has been considered as an indicator for male recognition and quality (Svensson *et al.*, 2007). Aside from providing pigmentation, melanin is involved in physiological processes including defence reaction, wound healing and sclerotization (Sugumaran, 2002; Stoehr, 2006). It is believed that the pigmentation in some damselfly species including *C. haemorrhoidalis* is related to an immune response (Contreras-Garduño *et al.*, 2006; Freitak *et al.*, 2005). This mechanism



involves the recognition of pathogens by various receptors that trigger the metabolic reactions that lead to the production of chemical signals (de Oliveira & De Marco Júnior, 2009). It was also reported that melanin plays a role in the encapsulation of parasites (Fabricant *et al.*, 2013; Sugumaran, 2002). Despite the important physiological roles of melanin, the presence and colour variation among males and females of the *C. haemorrhoidalis* species, as well as the localization of the pigment within the wing membrane, *i.e.* whether melanin is associated with procuticle or nanostructured epicuticle, remain poorly understood. Hence, the aim of this work was to identify and investigate the localization of melanin in *C. haemorrhoidalis* damselfly wings. This has been achieved using transmission-mode infrared (IR) and microspectroscopy at the Infrared Microspectroscopy (IRM) beamline at the Australian Synchrotron, in order to spatially map the distribution of the pigment in the wing membranes.

## 2. Materials and methods

### 2.1. Damselfly wing preparation

Five *C. haemorrhoidalis* damselfly specimens (mature male damselflies) collected from Els Ports, situated at the borders of Catalonia, Valencia and Aragon (Fig. S1 in the supporting information), were used in this study. The damselfly wing membranes were dissected into square sections of approximately 5 mm × 5 mm on the dorsal side of the forewing using a surgical blade. The wing samples were then briefly rinsed with MilliQ $H_2O$ (resistivity of 18.2 MΩ $cm^{-1}$, Millipore, USA) and finally blow-dried using 99.99% purity nitrogen gas as described elsewhere (Hasan *et al.*, 2012).

### 2.2. Extraction of lipids

Chloroform (CHROMOSLV for HPLC ≥ 99.8%) from Sigma Aldrich was used to remove lipids from the wing membranes. The sections of the mature male damselfly wing were placed in 1 mL of chloroform for 1 h in the dark. After lipid extraction, the wing samples were washed with chloroform to remove any possible contamination and then retained for the subsequent surface characterization and chemical composition analysis. Synthetic melanin was purchased from Sigma Aldrich.

### 2.3. Synchrotron Fourier transform infrared microspectroscopy

The synchrotron Fourier transform infrared microspectroscopy (FTIR) measurement was performed at the IRM beamline using a Bruker Vertex 80v spectrometer coupled with a Hyperion 2000 FTIR microscope (Bruker Optik GmbH, Ettlingen, Germany) and a liquid-nitrogen-cooled narrow-band mercury–cadmium telluride (MCT) detector.

The spatially resolved distribution of the chemical functional groups presented by the damselfly wings (before and after chloroform treatment) was mapped and characterized in transmission mode at a high resolution, which was critical in the surface characterization of the wing samples (Hasan *et al.*, 2012; Ivanova *et al.*, 2013). In this study, the synchrotron transmission measurement was performed using a 36× IR objective (NA = 0.50; Bruker Optik GmbH, Ettlingen, Germany) with the aperture size adjusted to 4 µm × 4 µm for every pixel; the spectra were acquired at 4 µm step intervals between pixels.

In practice, the wing section was held on a gap between aluminium support frames using polyimide (Kapton) tape to fix both sides of the wing section (see Fig. S1). Synchrotron FTIR chemical maps were then acquired to cover an area of 160 µm × 160 µm on the wing membrane. For each pixel, the synchrotron FTIR spectrum was recorded within a spectral range of 3800–700 $cm^{-1}$ using 4 $cm^{-1}$ spectral resolution and 16 co-added scans. The Blackman–Harris three-term apodization, power-spectrum phase correction and a zero-filling factor of 2 were set as default acquisition parameters using the *OPUS* 7.2 software suite (Bruker).

### 2.4. Spectral pre-processing and hierarchical cluster analysis

Hierarchical cluster analysis (HCA) was performed on the transmission maps using *CytoSpec*, Verison 1.4.02 (Cytospec Inc., Boston, MA, USA) (Vongsvivut *et al.*, 2015). Prior to HCA, the spectra in each SFTIR map were converted to the second derivative using (i) a nine-point Savitzky–Golay algorithm to remove broad-baseline offset and curvature, and (ii) vector normalization to account for the differences in path length. HCA analysis was then carried out within the spectral ranges 3040–2810 and 1800–930 $cm^{-1}$ using Ward's algorithm. These specific spectral regions were chosen in order to obtain optimal information for the major biomolecules, including lipids, proteins and nucleic acids, without the contribution of noise and atmospheric interference (*i.e.* carbon dioxide and water vapour) present in the middle range (2810–1800 $cm^{-1}$).

## 3. Results and discussion

The insect-wing cuticle is composed of the epicuticle and the procuticle, both of which can be further divided into two distinct sub-layers (Lockey, 1988; Ivanova *et al.*, 2013). In the case of the epicuticle, these two sub-layers are referred to as the outer and inner epicuticle. Chloroform treatment for 1 h was found to be sufficient to remove the entire epicuticle. Examination of the scanning electron micrographs confirmed that the outer epicuticle of the wing membrane had been removed (Figs. 1*a* and 1*b*). The wing cuticle with the *C. haemorrhoidalis* was measured to be 1.8–2.0 µm thick and the epicuticle layer was estimated to be approximately 433.4 nm (Truong *et al.*, 2017). In order to reconfirm the chemical nature of the dark-brown colour of the damselfly wing, the spectra collected from both natural and dewaxed *C. haemorrhoidalis* damselfly wings using FTIR were compared (Fig. 1*c*).

Despite the major bands observed for the mature male wing, which can be attributed to the wax component of the wing, notable characteristic peaks corresponding to melanin were observed (Figs. 1*c* and 1*d*). In the mature male wing, the

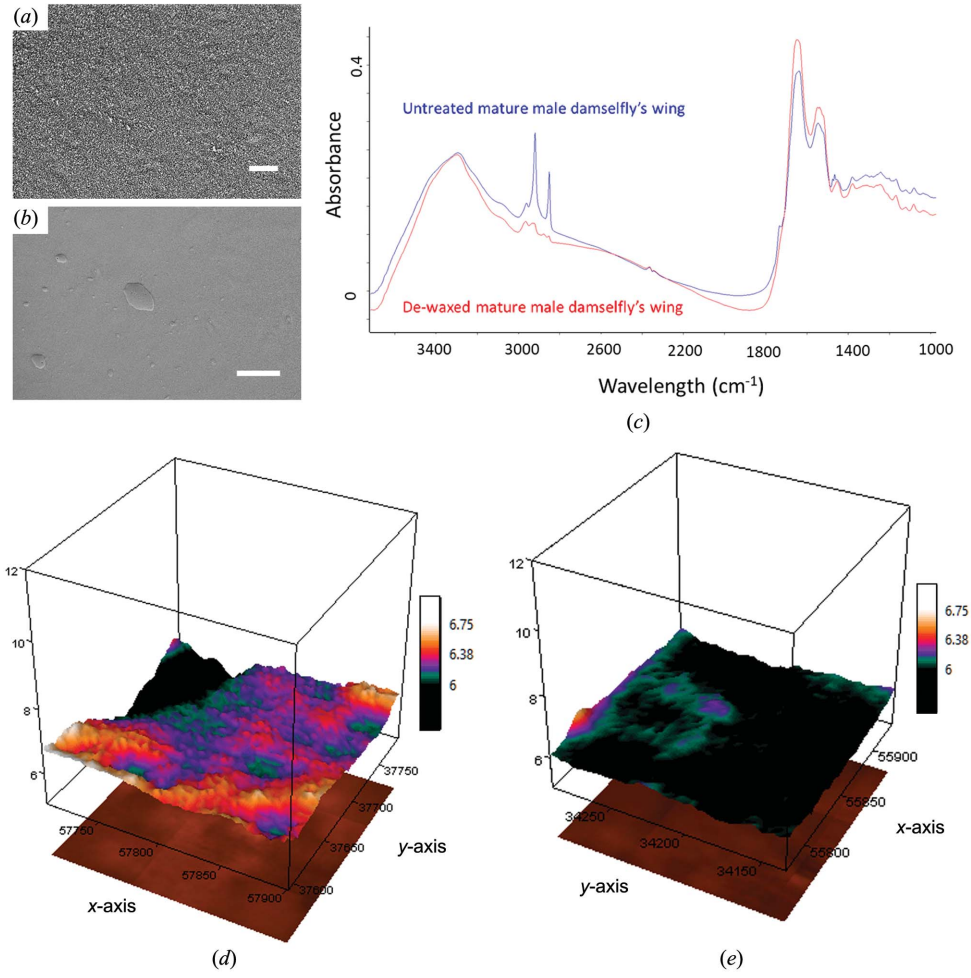



**Figure 1**
SEM micrographs of (*a*) an untreated mature male damselfly and (*b*) a dewaxed mature male damselfly wing with removed epicuticle (scale bar 4 µm). (*c*) Average IR spectra of untreated and dewaxed wing surfaces. Their corresponding transmission IR maps observed for (*d*) an untreated mature male damselfly wing, showing its natural surface nanostructures, and (*e*) a dewaxed mature male damselfly wing with substantially smoother morphology, indicating the loss of surface nanostructures, respectively (*x* and *y* axis units are given in µm). Note that the transmission IR maps were obtained based on integrated band areas (amide I) in the range 1680–1610 $cm^{-1}$ and were set to display the same scale in order to obtain a fair comparison.

bands at 1464, 1447, 1410 and 1240 $cm^{-1}$ (Fig. 1*c*) were similarly present in the spectra of synthetic melanin analogues shown in Fig. S2 and reported in previous studies (Kimura *et al.*, 2015; El-Batal & Al Tamie, 2016). The band at 1464 $cm^{-1}$ can be attributed to the aliphatic C—H groups and the band at 1447 $cm^{-1}$ represents symmetrical stretching of C═O (Bridelli *et al.*, 1999; Mbonyiryivuze *et al.*, 2015). The band present at 1410 $cm^{-1}$ is representative of the OH groups of phenolic compounds and the weak band at 1240 $cm^{-1}$ can be assigned to the C—OH stretching of the phenolic groups (Turick *et al.*, 2002; Bridelli *et al.*, 1999). It has been concluded that the dark-brown pigment detected in the damselfly wings is melanin. While these bands were present within the mature male wing membrane, only three bands at 1448, 1369 and 1236 $cm^{-1}$ were prominent in the spectra obtained for the dewaxed wing (Figs. 1*c* and 1*e*). These bands represent C═O symmetrical stretching, C═N stretching in the indole ring and C—OH stretching of the phenolic groups, respectively (Bridelli *et al.*, 1999). The presence of these bands clearly indicates that melanin is detectable in the wing membrane after the dewaxing treatment using chloroform. It was previously found that melanin was associated with chitin in the veins of damselfly *Calopteryx japonica* wings and fungal membrane (Stavenga *et al.*, 2012; Eisenman & Casadevall, 2012). The removal of the lipids from the outer epicuticle of the wing is evident from the analysis of the FTIR spectra (Fig. 1*c*). This is also in agreement with our previous work (Ivanova *et al.*, 2013), in which it was demonstrated that the outer-epicuticle of the *Hemianax papuensis* dragonfly wings was removed after a 1 h treatment with chloroform.

To confirm the uniformity of melanin distribution across intact and dewaxed wing membranes, the synchrotron-IR map was further processed using a pattern-recognition approach, a so-called hierarchical cluster analysis, in order to access spectral similarity within the two main spectral regions (3020–2800 and 1830–1000 $cm^{-1}$) (Fig. 2). The HCA maps revealed that both intact (Fig. 2*a*) and dewaxed (Fig. 2*b*) wings exhibited the majority of the spectral information shown in Fig. 1(*c*). This suggests that there is chemical homogeneity across both wings.

To summarise, the location of the melanin pigment is most likely to be in the procuticle of the *C. haemorrhoidalis* damselfly wings and not associated with lipids of the epicuticle. Using synchrotron-sourced IR microspectroscopy, melanin appeared to be homogenously distributed inside the wing membrane. Confirmation of the presence of melanin in the procuticle layer of the wings contributes to an understanding of how pigment has formed during different stages of insect development.

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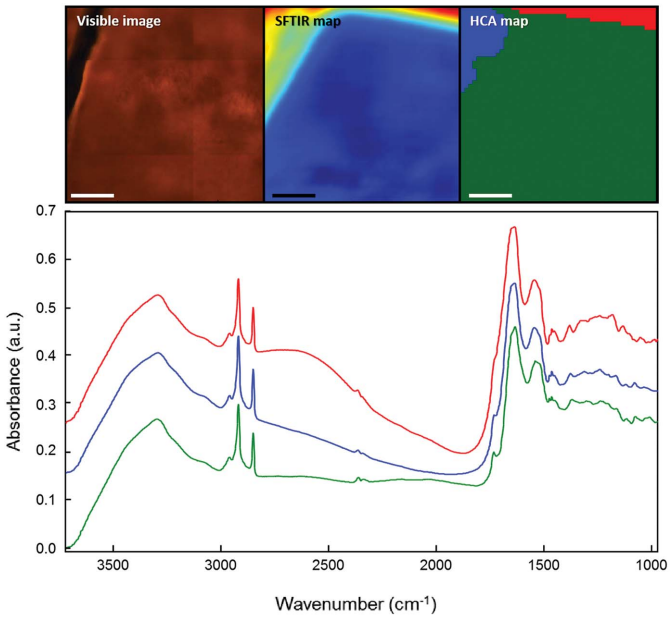
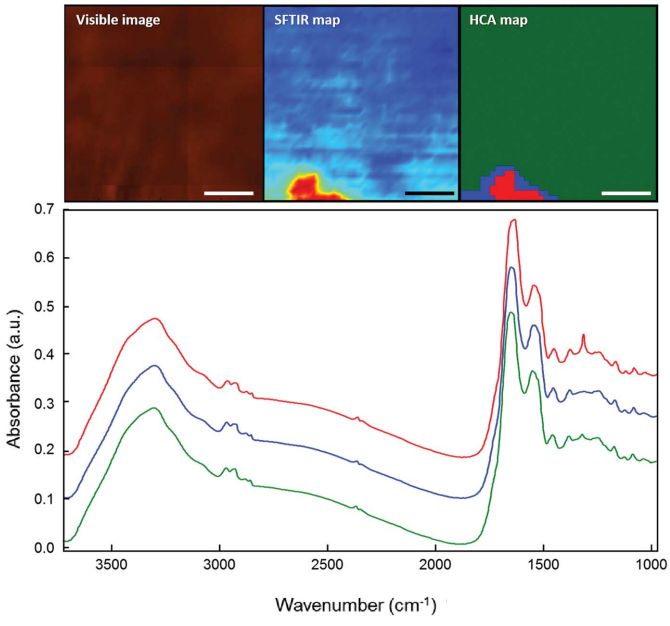



(*a*)




(*b*)

**Figure 2**
Hierarchical cluster analysis (HCA) of (*a*) an untreated mature male damselfly wing, in comparison with (*b*) a dewaxed mature male damselfly wing. The HCA results in each case were obtained using spectral information in the ranges 3020–2800 and 1830–1000 $cm^{-1}$ with three clusters for intact and dewaxed wings (scale bar 40 µm).

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