

The mirror crack'd: both pigment and structure contribute to the glossy blue appearance of the mirror orchid, *Ophrys speculum*

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Summary

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- The Mediterranean orchid genus *Ophrys* is remarkable for its pseudocopulatory pollination mechanism; naïve male pollinators are attracted to the flowers by olfactory, visual and tactile cues. The most striking visual cue is a highly reflective, blue speculum region at the centre of the labellum, which mimics the corresponding female insect and reaches its strongest development in the mirror orchid, *O. speculum*.
- We explored the structure and properties of the much-discussed speculum by scanning and transmission electron microscopic examination of its ultrastructure, visible and ultraviolet (UV) angle-resolved spectrophotometry of the intact tissue, and mass spectrometry of extracted pigments.
- The speculum contrasts with the surrounding labellar epidermis in being flat-celled with a thick, smooth cuticle. The speculum is extremely glossy, reflecting intense white light in a specular direction, but at more oblique angles it predominantly reflects blue and UV light. Pigments in the speculum, dominantly the cyanidin 3-(3''-malonylglucoside), are less diverse than in the surrounding regions of the labellar epidermis and lack quercetin copigments.
- Several physical and biochemical processes interact to produce the striking and much-discussed optical effects in these flowers, but the blue colour is not produced by structural means and is not iridescent.

Introduction

The mirror orchid

The morphological distinctiveness, complexity and commercial importance of orchid flowers have promoted them to popular models for studies of floral development, functional morphology, reproductive biology and plant–pollinator interactions. The genus *Ophrys* is well suited for study in each of these disciplines, and particularly for analysis of the interface between floral morphology and pollinator attraction.

The floral bauplan of *Ophrys* is typical of most species of the subfamily Orchidoideae, which includes most of the European terrestrial orchids (Rudall & Bateman, 2002). Stamen and pistils are congenitally fused into a gynostemium wherein the single fertile stamen bears two anther locules, each containing a club-shaped pollinarium with an adhesive viscid disc at the proximal end linked to a pollen mass (pollinium) towards the distal end. The stigmatic surface is located immediately below the viscid discs. The inferior ovary is rich in minute ovules. The perianth consists of two closely spaced whorls, each composed of three organs, the three petals being located immediately distal to the three sepals. The lower median petal, termed the labellum, is often larger and usually more complex than the lateral petals. The resulting floral morphology, showing unusually strong bilateral symmetry, was recognized by

Darwin (1862) as strongly encouraging transfer of pollinaria between inflorescences to facilitate cross-pollination.

The bilateral symmetry of the orchid flower, and morphological complexity of the labellum, are especially strongly expressed in the genus *Ophrys* (Fig. 1a). A comparative overview of the genus by Bradshaw *et al.* (2010) demonstrated that this complexity extends to the micromorphological scale, revealing a wide range of epidermal cell types located in specific regions of the labellum and presumably reflecting its unusual pseudocopulatory mode of pollination. Most *Ophrys* species attract a limited range of species of flying insect (typically hymenoptera), relying on naïve males to attempt to mate with the female-mimicking flowers on at least two successive orchid inflorescences (Cozzolino & Widmer, 2005; Jersáková *et al.*, 2006; Ayasse *et al.*, 2010).

Ophrys flowers use three successive cues to attract insects (Cozzolino & Widmer, 2005; Vereecken *et al.*, 2007; Schiestl & Cozzolino, 2008; Schlüter & Schiestl, 2008), each emphasizing the labellum. First to impact upon the insect's senses is the complex cocktail of volatile pseudophomones (Borg-Karlson, 1990; Schiestl *et al.*, 2003; Mant *et al.*, 2005; Vereecken & Schiestl, 2009). Next come the visual cues of the flower; initially focusing on the various shapes and colours of the perianth segments in aggregate, the visual focus switches to the labellum as the insect approaches the flower. Once the insect has landed on the labellum,

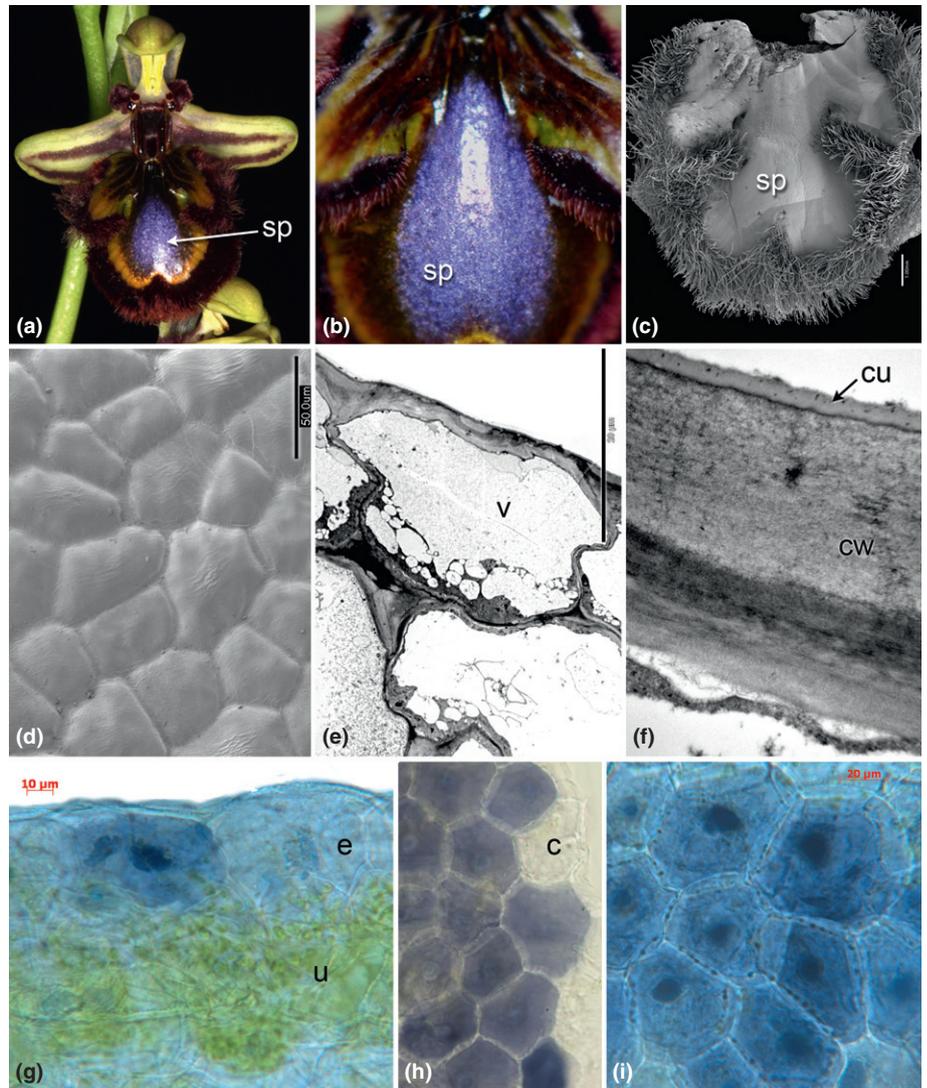


Fig. 1 Flower of *Ophrys speculum*; imaged using: (a, b) a standard digital camera; (c, d) scanning electron microscopy; (e, f) transmission electron microscopy; and (g–i) light microscopy of unstained material. (a) Entire flower from above. (b) Close-up view of the blue speculum region of the labellum. (c) Entire dissected labellum, showing smooth central speculum (sp) and peripheral trichomes. (d) Detail of the smooth speculum surface. (e) Transverse section of adaxial epidermis of speculum. (f) Detail of outer wall (cw) and cuticle (cu) of speculum epidermis. (g) Transverse section of unstained dissected speculum, showing adaxial epidermis containing blue pigment and underlying layers containing green chloroplasts. (h, i) Surface views of blue adaxial epidermis; the blue colour has leached out of cut epidermal cells in (h). c, cut cell; cu, cuticle; cw, cutinized cell wall; e, adaxial epidermis; sp, speculum; u, underlying layers; v, vacuole.

the shape and micromorphological textures of the adaxial epidermis maintain the illusion of a female insect by providing tactile cues that increase the ardour of the male insect and encourage the vertical orientation needed for successful acquisition or deposition of the pollinaria (Kullenberg, 1961).

Thus far, the olfactory cues of *Ophrys* have received more scientific attention than the visual and tactile cues. Yet the functional morphology of the later-stage cues is equally remarkable; we presume that these contrasting cues are mutually reinforcing (Giurfa *et al.*, 1994; Kulachi *et al.*, 2008). Many insects use multiple cues to reinforce their search image of a flower, enhancing recognition of target flowers and thus optimizing their foraging efficiency (Whitney *et al.*, 2009a; Leonard *et al.*, 2011).

The present study focuses on the impressive visual cues provided by the *Ophrys* labellum, paying particular attention to the speculum, which is a comparatively reflective blue region, varying in complexity of outline, located at or near the centre of the labellum of most *Ophrys* spp. (Fig. 1a). In many species this remarkable feature is generally accepted as mimicking the glossy wings and/or body of the pollinating species, and thus plays a key role in pollination within the genus.

We selected for study the widespread Mediterranean mirror orchid, *O. speculum*, because (as its name suggests) its remarkable speculum is exceptionally large, simple in outline and highly reflective, being perceived by the human eye as a brilliant blue (Fig. 1b). The flower of *O. speculum* is known to contain several anthocyanin pigments (Strack *et al.*, 1989), but many authors (including Bradshaw *et al.*, 2010) have speculated that the labellum owes its remarkable lustre more to physically induced structural colour than to biochemically induced pigmentation colour.

Structural components of colour

Table 1 provides a series of definitions for optics-based terms used throughout this manuscript. ‘Colour’ is the appearance resulting from the relative amount of light of each wavelength across the human visible wavelength range emanating from an object. This definition can be adapted to take into account the visual capabilities of different animals. Structural colour is the term given to an apparent colour produced by periodically arranged materials that do not necessarily contain pigment. If the scale in which the periodicity occurs is of the same order of

magnitude as that of the wavelengths of light striking the object in question, light reflected from the interfaces between the materials interferes constructively for certain wavelengths. This results in reflection and/or transmission of light of different wavelengths in different directions (Kinoshita, 2008). Although several structural mechanisms can generate different colour effects, a defining feature of structural colour is that it is iridescent, or angle-dependent – the colour changes as the angle of observation is altered. Pigment-based colours never show this property. However, a structural component can also optically modify a pigment-based colour: in such a case, the appearance of the colour is not only determined by pigments, but also depends on the anatomy of the surrounding structures. For example, the highly reflective yellow colour of the buttercup, *Ranunculus acris*, is caused by structural enhancement of a yellow pigment (Galsterer *et al.*, 1999; Vignolini *et al.*, 2012).

Structural colour has been well studied in animals, but its presence in the plant kingdom has only recently begun to be analysed in detail (Glover & Whitney, 2010). Blue-green iridescence in the leaves of tropical understorey plants has been attributed to multilayered structures (Graham *et al.*, 1993; Gould & Lee, 1996). Similarly, there have been a few reports of iridescent blue fruits (Lee, 1991; Lee *et al.*, 2000). We recently described the presence of diffraction gratings on the petals of several angiosperm species, confirming that cuticular striations can generate iridescent colours that are superimposed on the underlying pigment colour (Whitney *et al.*, 2009b).

In this interdisciplinary study, we apply several analytical techniques with the aim of determining both the causes and relative importance of biochemical and structural effects in producing the much-discussed *Ophrys* ‘mirror’. We conclude that the visual effect is the product of a combination of factors. The colour is the product of pigmentation, but the final appearance of the labellum is modified considerably by the combination of this pigment with specular reflection arising from the ultrastructure of the cell wall

and cuticle. The labellum does not exhibit iridescence, but its colour does appear angle-dependent, as a result of the strong reflection of white light from the glossy cuticle at certain angles.

Materials and Methods

Plant material

Several plants of *Ophrys speculum* Link were provided by S.M. from his personal collection. Plants in the early stages of flowering were shipped to the Department of Plant Sciences, University of Cambridge, and maintained on a south-facing windowsill with light watering until all flowers had been exploited.

Optical analysis

To determine the colour response of the flower, images were obtained with a standard digital camera and compared with images obtained using a UV-sensitive camera (Fuji Finepix camera equipped with a quartz objective and a Baader U-filter 2" HWB 325-369).

Reflection measurements were taken from the central blue region of the labellum (speculum) using a commercial reflection/backscattering probe (Ocean Optics, Dunedin, Florida, USA). One end of the probe was directly coupled onto a spectrometer (QE65000 Ocean Optics, 200–950 nm) while the other end was linked to a light source (DH-2000 Deuterium Tungsten Halogen Light Sources) providing illumination with a fixed numerical aperture of 0.22.

In order to better characterize the optical response of the flower, angular resolved spectra were collected using a goniometer. The illumination arm can be held in a determined fixed position while the sample and the collection arm are rotated independently.

Microscopy

For scanning electron microscope (SEM) examination (Fig. 1c,d), flowers were fixed in formalin acetic alcohol (FAA) and stored in 70% ethanol. Specimens were passed through an ethanol series up to 100% ethanol and critical-point dried using a Tousimis Autosamdri 815B (Tousimis, Rockville, Maryland, USA). Specimens were then mounted on aluminium stubs, coated in platinum using a sputter coater (Emitech K550, EMITech, Fall River, Massachusetts, USA), and examined under a Hitachi S-4700 SEM at 2 kV.

For transmission electron microscope (TEM) examination (Fig. 1e,f), 2 mm squares were dissected from the labellum using a mounted needle, fixed in 2.5% glutaraldehyde in phosphate buffer at pH 7.4, and stored in 70% ethanol until needed. Samples were then stained in 2% osmium tetroxide solution and passed through an ethanol and resin series before being polymerized for 18 h under vacuum. Semithin (0.5–2 µm) and ultrathin sections (14 nm) were cut using an ultramicrotome (Reichert-Jung Ultracut, Reichert Microscope, Depew, New York, USA). The semithin sections were mounted on glass slides and stained with toluidine blue in phosphate buffer, before being examined under a light microscope. The ultrathin sections were placed on Formvar-coated

Table 1 Glossary of terms from the field of optics used within this manuscript

Term	Definition
Colour	Appearance resulting from the relative amount of light emanating from an object at each wavelength. The perceived colour depends on the receptivity of the photoreceptors in the eye of the observer
Structural colour	Colour produced by light interference rather than pigmentation. Reflection of particular wavelengths of light by periodically arranged materials causes colour, irrespective of the chemical characteristics of the material (including whether or not it contains pigments)
Iridescence	An optical effect where the apparent colour of an object changes as the angle of observation is altered, as a consequence of different wavelengths of light being reflected at different angles
Specular reflection	Reflection of light at the same angle as it arrives at a surface, as seen most strongly in a mirror
Salience	Conspicuousness against the background – a red button is more salient on a blue coat than a blue button

grids and stained automatically with uranyl acetate and lead citrate using an Ultrastainer (Leica EM Stain, Leica Mikrosysteme GmbH, Vienna, Austria) before being examined under the TEM.

For light microscope (LM) examination (Fig. 1g–i), fresh, unstained labella were hand-sectioned using a single-edged razor blade and mounted in a drop of water on a microscope slide, covered with a glass cover slip, and imaged using a Leitz Diaplan photomicroscope fitted with a Leica DC500 digital camera.

Metabolite analysis

Labella of *O. speculum* were excised from flowers using a razor blade. The inner blue section and the outer brown section were separated under a dissecting microscope, flash-frozen in liquid nitrogen and placed in tubes containing 1 ml cold methanol containing 1% hydrochloric acid. Pigments were extracted by shaking gently overnight at room temperature in the dark and subsequently stored at -80°C .

Absorbance spectra of the crude extracts containing pigments from either the blue or brown regions of the labellum were obtained between 300 and 700 nm on a Jasco V-550 UV-VIS spectrophotometer (Jasco, Essex, UK). As per Davey *et al.* (2004), flavonoids from the crude methanolic extracts were analysed by high-performance liquid chromatography (HPLC; Surveyor system, Thermo Scientific, San Jose, CA, USA), the eluant being analysed by both photodiode array (PDA) spectrometry and time-of-flight mass spectrometry using electrospray ionization (Finnigan LCQ DECA XP, Thermo Scientific). Data were analysed using Xcalibur software (Thermo Fisher Scientific). Samples (injection volume, 20 μl) were resolved on a Luna C18 column (250 \times 2.0 mm; Phenomenex, Macclesfield, UK) using 0.5% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.2 ml min^{-1} , with a gradient of increasing B as follows: initial A : B, 95 : 5 v/v; A : B at 2 min, 95 : 5; A : B at 42 min, 0 : 100; A : B at 47 min, 0 : 100; A : B at 48 min, 95 : 5; A : B at 53 min, 95 : 5. The eluant was monitored for absorbance between 200 and 800 nm with the MS operating in positive ion mode (settings: capillary temperature 230 $^{\circ}\text{C}$; capillary voltage 27 V; spray voltage 3.5 kV; sheath gas flow rate 9.28 (arb.)); centroid data collection). Mass ions were detected between 100 and 1200 m/z , using quercetin (Sigma) to tune and calibrate the MS. Metabolite fragmentation (ms/ms) on selected masses was carried out under the following settings: isolation width 1.0 m/z , 50% normalized collision energy, activation $Q=0.250$, activation time 30 ms. The identification of metabolites was based on their absorbance spectra and mass spectral data as compared with published data (the online flavonoid database at <http://metabolomics.jp/wiki/Index:FL>) and with a reference flavonol, quercetin-3- β -D-glucoside, 20 μM and anthocyanin, cyanidin 3-O-glucoside chloride, 200 μM (Sigma).

Results

The speculum has a smooth, flat surface with layered cell wall

The labellum of *O. speculum* (Fig. 1a,b) has a complex adaxial epidermal surface (see also Bradshaw *et al.*, 2010). Its epidermal

cells are smooth, nonstriated and nonpapillate, or consist of long, spirally twisted trichomes, the latter concentrated along the periphery of the labellum (Fig. 1c).

The adaxial epidermis of the speculum region is composed entirely of smooth, flat-topped cells (Fig. 1d) that show little or no doming in transverse section (Fig. 1e,g). Each epidermal cell contains a large vacuole, most of the cytoplasm and organelles lying close to the inner cell wall (Fig. 1e). The epidermis of the speculum incorporates the bulk of the blue pigment (Fig. 1g,i), as does the epidermis of heavily pigmented regions located elsewhere in the flower. When the fresh tissue of the speculum is cut, the blue colour leaches out rapidly (Fig. 1h). The epidermal cell wall is thickest on the outer surface, where it is overlain by a thick (*c.* 0.5 μm) cuticle that covers the entire surface. Although the cutinized cell wall displays some layering (Fig. 1f), our TEM images do not indicate an ordered multilayered structure of sufficient regularity and dimensions to generate structural colour of the kind responsible for the blue scales of *Morpho* butterfly wings (Vukusic *et al.*, 1999).

The speculum is highly UV-reflective

Although the blue colour of the speculum is exceptionally striking to the human eye, many insects perceive colours differently from human vision. In particular, it is common for insects to perceive light in the UV range of the spectrum (Briscoe & Chittka, 2001). To assess whether the *Ophrys* labellum is UV-reflective, we compared a photograph of the flower taken using a standard camera with one taken with maximal sensitivity in the 325–369 nm range (Fig. 2a,b). It is clear from these images that the blue speculum region of the labellum is highly UV-reflective. The reflectivity in this range has two components – it is partly the result of the specular reflected signal from the cuticle and partly the result of a more diffuse signal caused by light that has entered the cells but not been absorbed by the pigment within.

To investigate this response more fully, we compared the reflection of the labellum of mature and senescent flowers using a commercial reflection/backscattering probe (Ocean Optics). A peak of reflection was observed between 350 and 400 nm (Fig. 2c) in both flowers, but was more evident in the mature flower, confirming that a strong UV signal is detectable from the labellum of a receptive flower. This UV signal is likely to enhance the salience of the speculum, potentially facilitating pollinator landing on the labellum.

The speculum reflects white light strongly in the specular direction, but blue light at other angles

In order to better characterize the optical response of the speculum, angular resolved spectra were collected using a goniometer (a schematic diagram of the experimental setup is presented in Fig. 3a). Fig. 3(b) shows the scattering behaviour of the speculum in colour scale blue to green to yellow (the yellow colour in the chart shows a greater proportion of reflected light compared with blue). The collection angle is plotted on the y -axis and the wavelength on the x -axis. At the specular reflection direction (indicated by the white dotted line in the

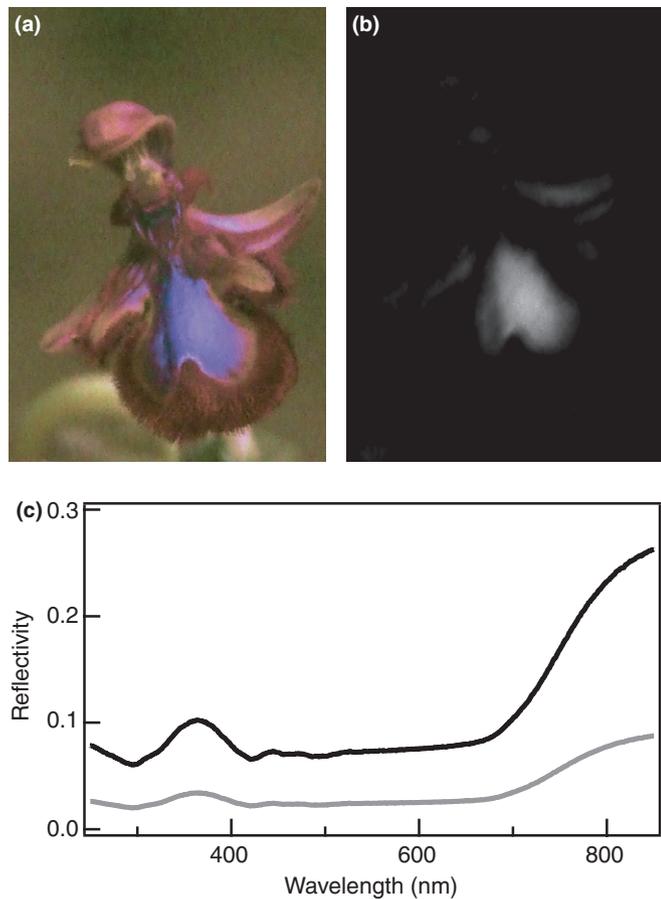


Fig. 2 Reflectance from the *Ophrys speculum* flower. (a) Flower photographed in daylight. (b) The same flower photographed with a UV-sensitive camera. (c) Reflectance spectra of mature (black line) and senescent (grey line) flowers.

image), the absorption from the pigment is less significant than with the other scattering angles. Specular reflection is a reflection of white light at the same angle as it arrives at a surface, as seen most strongly in a mirror. Across the horizontal band between 20° and 40°, light of all wavelengths is reflected equally across the spectrum, constituting an angularly broadened mirror-like response. This behaviour is in contrast to a perfectly planar surface where specular reflection occurs only for a collection angle of 30° matching the illumination angle. However, as the reflective surface of the speculum is convex and the diameter of the illumination spot on the sample is *c.* 2 mm, the light is reflected not only at one specific angle but in the angular range between 20° and 40°.

Above *c.* 40°, light reflection is limited predominantly to the UV-blue and the infrared. This analysis suggests that the speculum contains a pigment that absorbs in the wavelength window between 420 and 650 nm. The combination of such a pigment with the specular reflection from a mirror-like surface results in a speculum that appears blue at high observation angles but whiter in a specular observation direction.

The contribution of specular reflection to the total reflectivity of the speculum is analysed further in Fig. 3(c), where the integrated intensity as a function of the collection angle is

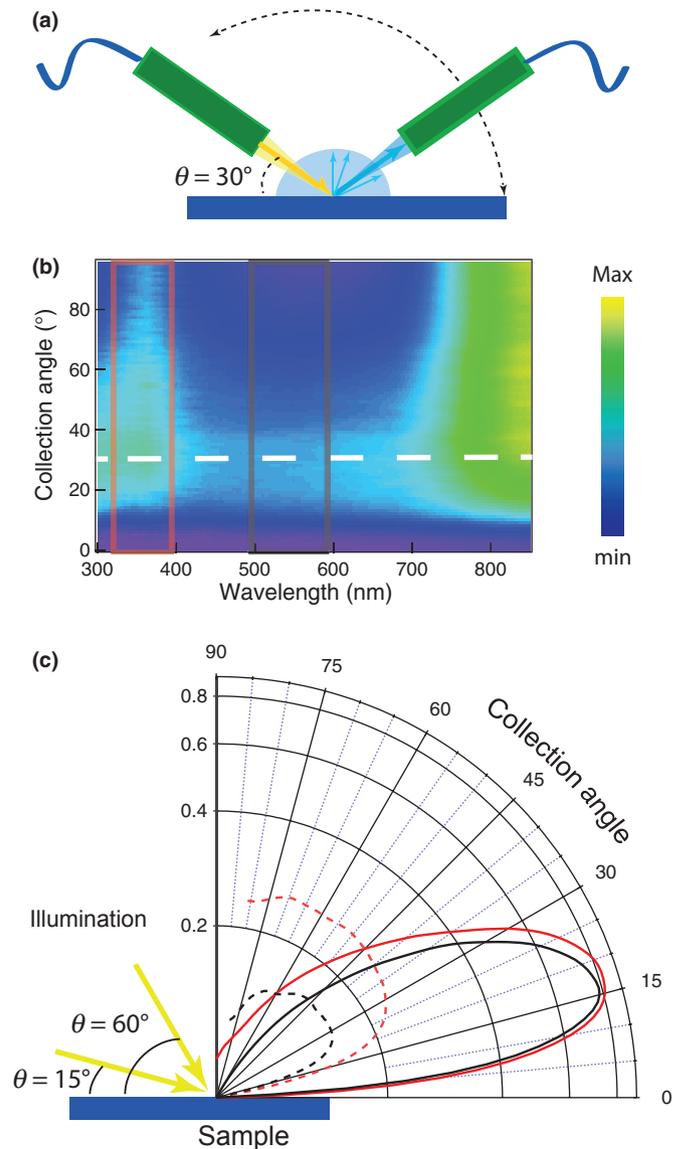


Fig. 3 Optical characterization of the *Ophrys speculum* flower. (a) Diagram of the goniometer assembly. During measurements, the illumination arm can be fixed at a definite angle (30° in the diagram) and the collection arm is varied to collect the scattered light in the plane perpendicular to the sample, as shown by the dotted black double-headed curve. (b) Scattering measurements from the speculum obtained with the configuration shown in (a). The collection angle is plotted on the y-axis and the wavelength on the x-axis. The dotted white line corresponds with the specular reflection direction, while the two coloured rectangles indicate the regions in which we integrated the spectra for the analysis reported in (c). (c) Polar scattering intensity distribution. The graph shows the integrated intensity as a function of the illumination angle. To obtain the curves, we integrated the intensity of the reflected light in two wavelength intervals: 300–400 nm (red lines) and 500–600 nm (black lines), for two angles of collection for two contrasting incidence angles (15° incidence, shown as solid lines, and 60° incidence, shown as dashed lines).

recorded for two contrasting incidence angles (15° and 60°) and two wavelength regions. In the polar graph, the integrated intensity is shown for the two incidence angles of 15° (solid lines) and 60° (dashed lines) and for the two wavelength regions of 300–400 nm, where the pigment does not absorb (red), and

500–600 nm, where the pigment does absorb (black). For an angle of incidence of 15°, glossiness is dominant and the light is almost entirely reflected in the specular reflection direction across the entire spectrum. This result suggests that the optical appearance of the speculum results from the interplay of the glossy cuticle and the diffuse blue and UV light filtered by the pigment. At low illumination and observation angles, the signal from the air–cuticle interface predominates, resulting in a broad-band (white) gloss. At larger angles of incidence (dashed lines), the contribution of the specular reflection is much smaller, making the spectral response of pigment scattering across a wide angular range more clearly visible. This observation is explained by blue (and UV) diffuse isotropic scattering from the pigment-bearing tissue and specular reflection arising from the smooth surface. Finally, the red dashed line spans a greater angular range than the black dashed line, presumably because the pigment within the tissue absorbs some light in the 300–400 nm range.

The speculum contains only cyanidin pigments, whereas the rest of the labellum also contains delphinidin and quercetin

Absorption spectra of the crude extracts were obtained from the blue speculum, brown labellum fringe and the yellow lateral petals (Fig. 4). Peak absorption in the 500–560 nm regions indicated the presence of anthocyanins with λ_{max} at *c.* 529 nm (Harborne, 1984: 64–65). Absorption between 350 and 380 nm in the brown labellar margin suggested the possible presence of flavonols acting as copigments (Shoji *et al.*, 2007). Absorption peaks at 419 and 653 nm indicated the presence of Chl*a*, especially in the yellow lateral petals.

The speculum

Only one pigment from the blue speculum was resolved at 17.97 min by HPLC (Figs 5, 6). The absorption spectrum and the λ_{max} of 516 and 280 nm of the metabolite at this time indicated a structure resembling anthocyanins. This spectrum matched published spectra of cyanidin-3-glucoside or cyanidin-3-sophoroside (Zhang *et al.*, 2008), malonyl ester of cyanidin-3-glucoside (Lee, 2002) and cyanidin-3-glucoside or peonidin-3-glucoside (Hong & Wrolstad, 1990). The molecular mass ions of the metabolite eluting at 17.97 min were determined in the positive ionization mode. The total mass scan of the peak detected the molecular mass ions *m/z* 535, 593, 611 and 758 (Table 2). These masses were searched against the online flavonoid database at <http://metabolomics.jp/wiki/Index:FL> and in published manuscripts. The parent anthocyanin aglycone proved to be a cyanidin (Giusti *et al.*, 1999a,b; Zhang *et al.*, 2008; Mullen *et al.*, 2010) with the following putative identifications for *m/z*: 535 cyanidin 3-(3"-malonylglucoside), 593 cyanidin 3-(6"-dioxalylglucoside), 611 cyanin or cyanidin 3,5-diglucoside, and 758 genticyanin C or cyanidin 3-glucoside-5-(6-p-coumaroylglucoside). Fragmentation spectra were obtained for the parent mass ion of 534, which produced daughter ions of 448.9 and 287.04 *m/z*. According to Giusti *et al.* (1999a,b) and Mullen *et al.* (2010), the

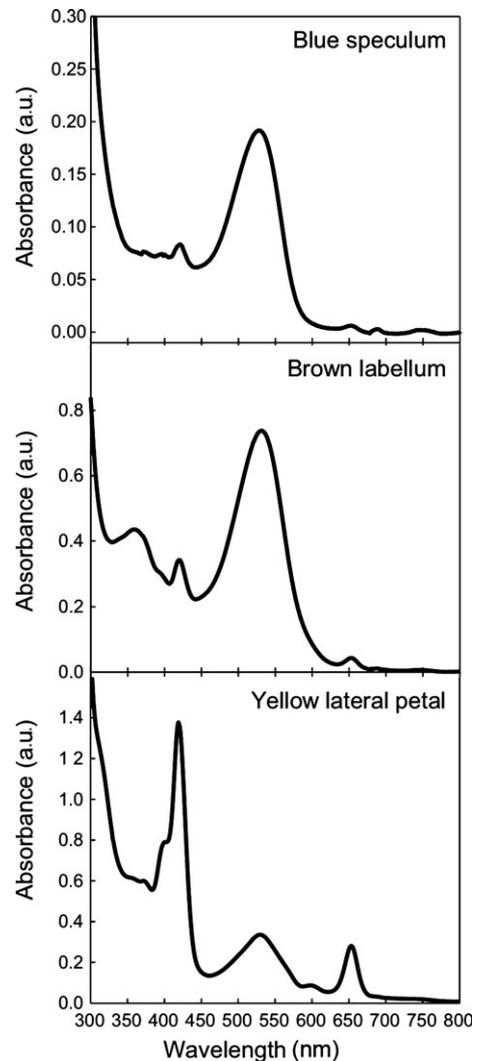


Fig. 4 Absorbance spectra from crude solvent extracts (methanol with 1% HCl) of the blue speculum, brown labellar margins and the yellow lateral petals of *Ophrys speculum* flowers.

mass 535.3 is the cyanidin 3-(3-malonylglucoside), which is the mass of cyanidin (287) + hexose minus H₂O (162.2) + malonic acid minus H₂O (86.1). When cyanidin 3-(3-malonylglucoside) is fragmented, it also produces the mass 449.1 (535–86.1), corresponding with cyanidin 3-(3-malonylglucoside) minus the malonyl group. The mass ion 287 is also produced by cyanidin 3-(3-malonylglucoside; 449.1) minus the mass of hexose (162.2), thus forming the cyanidin aglycone. It was not possible to obtain an authentic standard for cyanidin 3-(3-malonylglucoside); instead cyanidin 3-O-glucoside chloride was used to confirm the absorption spectrum (λ_{max} of 514 and 280 nm), parent mass (449.01, minus chloride ion) and fragmentation pattern (forms the mass of cyanidin (287) minus the glucoside) of a cyanidin-glycoside (Table 2, Fig. 6h).

Brown labellum fringe

Six peaks were resolved in the brown section chromatogram (Figs 5, 6). The absorption spectra of two peaks (retention times

17.9 and 18.6 min) were characteristic of an anthocyanin, and four peaks (retention times 18.4, 19.3, 20.3 and 21.1 min) were characteristic of flavonols (Mabry *et al.*, 1970). The peak eluting at 17.92 min had the same absorption spectrum, parent ion and fragment ion mass as that found at a similar time in the blue section, and hence was identified as cyanidin 3-(3''-malonylglucoside). The peak at retention time 18.6 min, slightly co-eluted with another peak at 18.4 min and had a λ_{\max} of 522 nm, indicating the presence of delphinidin-3-rutinoside or cyanidin-3-rutinoside (Toki *et al.*, 1996; Vera De Rosso & Mercadante, 2007). The remaining peaks at 18.4, 19.3, 20.3 and 21.1 min all had absorption spectra with a

λ_{\max} of 356 nm that are characteristic of a flavonol such as isorhamnetin-rutinoside ($\lambda_{\max} = 356$ nm) or a glycoside of quercetin such as quercetin-glucoside, -rutinoside or -rhamnoside ($\lambda_{\max} = 358$ nm; Mabry *et al.*, 1970). The mass spectra of these four flavonol peaks all revealed mass ions of the same molecular weight as glycosylated or malonylated quercetin (Table 2). Variation in the conjugate species explains the differences in retention time for the same quercetin compound. The masses of other compounds, especially the flavonols luteolin and kaempferol, were also present in the online mass searches. However, the *Ophrys* labellum compound is unlikely to have these chemistries, as the UV traces for these compounds are closer to 330 and 370 nm, respectively. The reference compound, quercetin-3- β -D-glucoside, had a similar retention time to the four flavonols in the extract and had a λ_{\max} of 356 nm. Therefore, the main compound present in the labellar fringe is likely to be a mix of cyanidin and delphinidin with a copigment of quercetin.

Discussion

Analysis of the optical properties of the intensely blue-coloured speculum of *O. speculum* indicates that the visual effects are achieved by multiple factors. A pigment located in the adaxial epidermis, absorbing in the green–red region of the spectrum and diffusely reflecting blue and UV light, operates in combination with a highly reflective mirror-like surface that provides intense specular reflection and causes the blue colour to be somewhat angle-dependent. UV photography and simple reflectance spectrometry indicate that the speculum is highly reflective in the UV, a part of the spectrum known to be visible to many insects, including the hymenopteran pollinators characteristic of *Ophrys* flowers (Briscoe & Chittka, 2001). The high degree of salience (conspicuousness) that this UV signal provides to the *O. speculum* flower is likely to enhance pollinator handling of the flower, perhaps facilitating landing in the optimal position for pseudocopulation and eventual pollen transfer.

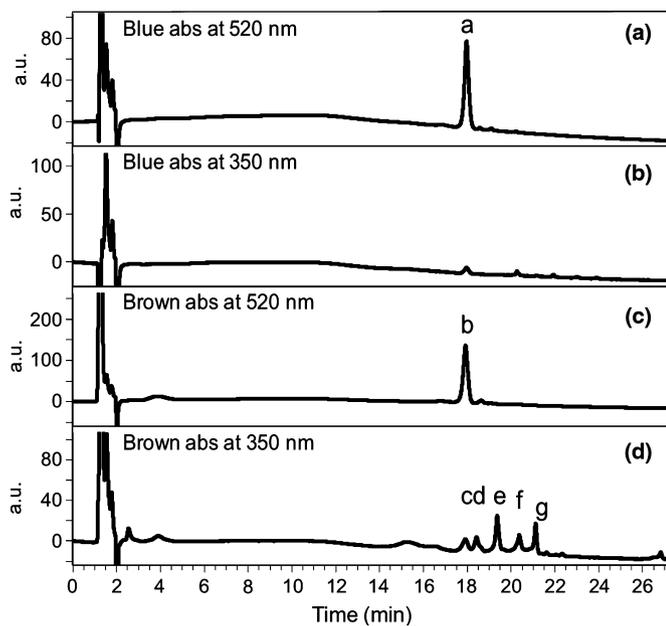


Fig. 5 High-performance liquid chromatography (HPLC) chromatograms (absorbance (abs) at 520 and 350 nm) of the crude extract from the blue speculum and brown sections of the *Ophrys speculum* labellum. a, b, cyanidin 3-(3''-malonylglucoside); d, delphinidin; c, e–g, flavonols.

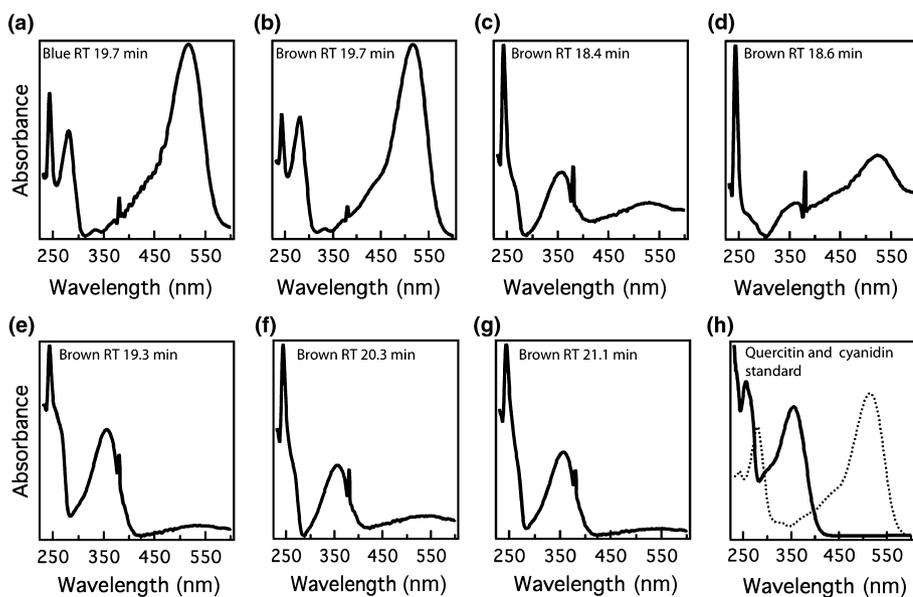


Fig. 6 Analysis of the labellum pigments of *Ophrys speculum*. (a) Absorption spectrum of the metabolite from the blue speculum of *O. speculum* eluting at 17.97 min. (b–g) Absorbance spectra of the metabolites from the peripheral brown section eluting at 17.97, 18.4, 18.6, 19.3, 20.3 and 21.1 min, respectively. (h) Absorption spectrum of quercetin-3- β -D-glucoside (solid line) and cyanidin-3-O-glucoside (dashed line) standards. RT, retention time.

Table 2 High-performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis of extract from the blue speculum and brown labellum fringe sections of *Ophrys speculum*

	Peak RT (min)	λ_{\max}	Parent ions <i>m/z</i>	Fragment ions <i>m/z</i>	Putative metabolite identification
Blue	17.97	516, 280	534.90 592.78 611.16 757.86	448.9, 287.04	Cyanidin 3-(3''-malonylglucoside), Cyanidin 3-(6''-dioxalylglucoside), Cyanin or cyanidin 3,5-diglucoside Cyanidin 3-glucoside-5-(6-p-coumaroylglucoside)
Brown	17.97	515, 280	534.90 592.78 611.16 757.86	448.9, 287.04	Cyanidin 3-(3''-malonylglucoside), Cyanidin 3-(6''-dioxalylglucoside), Cyanin or Cyanidin 3,5-diglucoside Cyanidin 3-glucoside-5-(6-p-coumaroylglucoside)
	18.42	356	534.90	448.9, 287.04	Cyanidin 3-(3''-malonylglucoside),
	18.64	522, 356	460.7 550.9 609.9 684.9		Apigenin 7-(6''-methylglucuronide) Quercetin 3-(6''-malonylgalactoside) Quercetin 3-galactoside-7-rhamnoside Delphinidin 3-(6''-malonylsambubioside)
	19.36	355	609.9 685.9 712.7 765.05	658.69, 496.83	Quercetin 3-glucoside-7-rhamnoside Delphinidin 3-(6''-malonylsambubioside) Quercetin 3-(6''-malonylglucoside)-7-glucoside Myricetin 3-O-(4''-O-acetyl-2''-O-galloyl)-alpha-L-rhamnopyranoside (for 658 fragment)
			804.78 927.2	658.93, 496.87	Gossypetin 3-sophoroside-8-glucoside Myricetin 3-O-(4''-O-acetyl-2''-O-galloyl)-alpha-L-rhamnopyranoside (for 658 fragment)
	20.37	356	590.3 610.49 683.96 759.9 764.99	658.85, 496.74	Quercetin 3-(2'',3'',4''-triacylgalactoside) Quercetin 3-glucoside-7-rhamnoside Delphinidin 3-(6''-malonylsambubioside) Delphinidin 3-sambubioside-5-glucoside Myricetin 3-O-(4''-O-acetyl-2''-O-galloyl)-alpha-L-rhamnopyranoside (for 658)
			927.12	658.97, 552.57	Myricetin 3-O-(4''-O-acetyl-2''-O-galloyl)-alpha-L-rhamnopyranoside (for 658)
			1021 1181		Cyanidin 3-(6-malonylglucoside)-7-(6-caffeoylglucoside)-3'-glucoside Cyanidin 3-(6''-p-coumaryl-2''-sinapylsambubioside)- 5-(6-malonylglucoside)
	21.11	356	425.7 494.1 550.7 684.83 759.7		Quercetin 7,3',4'-trimethyl ether 3-sulfate Quercetagenin 3'-methyl ether 3-glucoside Quercetin 3-(6''-malonylgalactoside) Delphinidin 3-(6''-malonylsambubioside) Quercetin 3-sambubioside-7-glucoside
	Quercetin	20.17	356	464.09	396.08

RT, retention time (min); λ_{\max} , maximum absorption between 200 and 800 nm on the photodiode array detector; MS, main parent monoisotopic mass ions (positive ionization) of each peak and the fragment ions where detected. Metabolite identification was based on reference to published data and by searching the monoisotopic mass ions on the flavonoid database at <http://metabolomics.jp/wiki/Index:FL>. For each mass, > one metabolite is usually identified on the database, therefore only one example for each mass is provided here.

The intense specular reflection from the labellum provides the characteristically extreme glossiness, a feature that has been hypothesized to improve the sexual mimicry of the flower by resembling the sheen on the folded wings of an insect at rest. A similar glossiness has been reported for some other flowers. For example, the dark petal spots of *Gorteria diffusa*, a South African daisy, achieves pollination by mimicking female bombyliid flies; here too, glossiness has been hypothesized to mimic the visual appearance of folded insect wings (Ellis & Johnson, 2010). Our micrographs indicate that the glossiness arises from a thick (c. 0.5 μm) and extremely smooth, ridgeless layer of cuticle deposited on top of unusually flat epidermal cells. The cuticle layer extends between cells, reducing the visibility of individual cell boundaries.

The overall effect produced by this cuticular layer is of a thin mirror coating the flower surface. The highly reflective yellow colour of the buttercup, *Ranunculus acris*, is also the result of a mirror-like cuticle (Galsterer *et al.*, 1999; Vignolini *et al.*, 2012). The *O. speculum* mirror layer is made more effective by the flatness of the adaxial epidermal cells. Petal epidermal cells are frequently conical-papillate (Kay *et al.*, 1981; Whitney *et al.*, 2011) and, failing that, they are usually lenticular or gently domed. The extreme flatness of the epidermal cells in the speculum region provides a better backdrop to the mirror than a biological surface can usually achieve.

Analysis of the reflection of different wavelengths of light from the speculum at different angles confirms that there is a structural component to the appearance of the speculum. At the specular

angle (set at 30° in our analysis), light of all wavelengths is reflected with equal efficiency. Similar results are obtained with different angles of incidence. However, at increased angles of collection relative to the sample, a strong blue and UV reflection is observed; little, if any, reflection of other wavelengths is evident until the far-red region of the spectrum is reached. This analysis explains why the colour of the speculum appears to shift as the flower is reoriented. At angles where the specular reflection is strong, a mirror-like effect dilutes the apparent blueness of the tissue. However, when the flower is shifted to angles other than the specular, the intense blue reflection again dominates the signal. In addition, the gently convex shape conferred on the speculum by curvature of the labellum contributes to the apparent shift of colour with angle of observation, by modifying the contribution of the gloss. These effects are evident in Fig. 1(b), where some apparent blue-and-white speckling observed under strong specular illumination is the result of slight variations in angle and cuticle thickness across the surface of the speculum. This angular dependence is a defining feature of a structural contribution to colour, but in this case there is no evidence that the blue hue itself is produced through structural means. If the blue colour was achieved by a multilayered structure, as is the case for the *Morpho* butterfly, the hue of the colour (i.e. the relative amounts of blue, green, yellow and/or red light) would change at different observation angles (Vukusic *et al.*, 1999). Instead, it is only the relative contributions of blue and broad-band reflected (white) light, not blue and other narrow bandwidths of light, that alter as angle varies. Accordingly, we cannot define the speculum as truly iridescent. In response to the description by Bradshaw *et al.* (2010) of epidermal morphology of the labellum of a range of *Ophrys* spp., Vereecken *et al.* (2011) also reported that unpublished data suggested that the *O. speculum* labellum was not truly iridescent. In further support of this observation, we note that, although the cell walls of the speculum epidermal cells appear to contain layers of cellulose in our TEM analysis, those layers are irregular in depth and shape. Only regular structures can generate colour by interference, whereas the disordered layering evident in Fig. 1(f) is unable to generate a colour signal.

Our optical analysis indicated the likely presence of a blue pigment in the speculum, absorbing light between 420 and 650 nm. Biochemical analysis confirmed the presence of an anthocyanin, cyanidin 3-(3''-malonylglucoside). This was the only pigment detected in the speculum tissue, using an analytical approach competent to reveal any flavonoids present. Anthocyanins are commonly found in flowers, generating the red–blue range of colours. Cyanidins produce a bluer colour than some other anthocyanins, such as pelargonidins, although they are usually more magenta than purple/blue. The particular hue produced by the cyanidin is determined by several other factors, including the nature of any side groups on the molecule, their interactions with metal ions, the pH of the vacuolar liquid and the co-occurrence of any other pigments. It is likely that an alkaline vacuolar pH or formation of a complex with iron or magnesium ions is responsible for the blue hue of this cyanidin, although further analyses of cellular ion content would be necessary to define how the particular shade is produced. Although we detected Chl in the biochemical analysis, it was primarily found in the brown fringes of the

labellum, so it is unlikely that this pigment is contributing greatly to the blue colour of the speculum. George *et al.* (1973) also found cyanidins in the intense blue-purple flowers of the Australian enamel orchids (*Elythranthera* spp.), although it remains to be determined whether there is a structural component to the appearance of these highly glossy flowers.

We detected the presence of the same cyanidin and possibly also delphinidin-3-rutinoside in the region of the labellum surrounding the speculum. This tissue also contains four copigments of a flavonol, which are most likely glycosylated or malonylated quercetins. The flavonols appear to modify the absorption range of the anthocyanins to produce a brown coloration in the tissue. It is the exclusion of the flavonoid copigments from the speculum that permits the striking purity of the blue colour of the cyanidin in the labellum of *O. speculum*.

The pure glossy blue of the *O. speculum* flower has fascinated scientists and naturalists for many years. The intensity of the colour, and its apparent angular dependence, led to speculation that it is produced using structural rather than pigment-based mechanisms (cf. Bradshaw *et al.*, 2010). From our analysis, we conclude that the visual effect is the product of a combination of factors – the colour is the result of pigmentation, but the final appearance of the labellum is modified by the combination of this pigment with the specular reflection arising from the ultrastructure of the cell wall and cuticle. A single pure cyanidin produces the basic blue colour, most likely as a result of an alkaline vacuolar pH or formation of a complex with metal ions. The spectral purity of the pigment colour is enhanced by backscattering from a disordered multilayer structure in the lower wall of the epidermal cells. The flat surface of the epidermal cells is enhanced by an exceptionally smooth mirror composed of cuticle, providing both glossiness and a strong specular reflection, which is angle-dependent even though the colour itself is not iridescent. In combination, these features produce a striking optical effect that presumably contributes to the pollination efficiency and thus potentially to the reproductive success of the species.

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