Functional optics of glossy buttercup flowers
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Buttercup (Ranunculus spp.) flowers are exceptional because they feature a distinct gloss (mirror-like reflection) in addition to their matte-yellow coloration. We investigated the optical properties of yellow petals of several Ranunculus and related species using (micro)spectrophotometry and anatomical methods. The contribution of different petal structures to the overall visual signal was quantified using a recently developed optical model. We show that the coloration of glossy buttercup flowers is due to a rare combination of structural and pigmentary coloration. A very flat, pigment-filled upper epidermis acts as a thin-film reflector yielding the gloss, and additionally serves as a filter for light backscattered by the strongly scattering starch and mesophyll layers, which yields the matte-yellow colour. We discuss the evolution of the gloss and its two likely functions: it provides a strong visual signal to insect pollinators and increases the reflection of sunlight to the centre of the flower in order to heat the reproductive organs.

1. Introduction

Many flowering plants display brightly coloured flowers to distinguish themselves from their environment and to attract pollinators [1–4]. In virtually all plant species floral coloration is pigmentary, i.e. the visual signal is the result of light scattering by cells and organelles containing pigments that absorb light in a wavelength-specific way [5–7]. Diffusely reflected light with wavelengths outside the absorption range of the pigment thus determines the colour of the petals. The absorption range depends on the nature of the pigment, e.g. blue-absorbing carotenoids yield yellow colours (reviewed by [6]).

Structural coloration is exceedingly rare among flowers, because the interiors and surfaces of flowers are very irregularly shaped and ordered [19,20]. In those plant species with flowers that do have structures with periodicity of the order of the wavelength of light, structural coloration and iridescence can only be observed by applying very local and directional

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illuminated [19]. Consequently, under natural viewing and illumination conditions the coloration of flowers is generally pigmented and not structural [19,21].

The flowers of many buttercups (Ranunculus spp.) are a clear exception to this rule, as they feature a distinct gloss (i.e. a mirror-like reflection) in addition to their overall yellow coloration [19,22,23]. Previous studies suggested that the gloss is due to the very flat and smooth epidermal layer that acts as a mirror [19,22], possibly enhanced by an air layer immediately below the epidermis [24]. Vignolini et al. [24] applied optical multilayer theory to model the reflectance spectra, but stated that an optical model of the whole complex petal structure was missing. A comprehensive overview that quantifies the contribution of different petal components to the visual signal of Ranunculus flowers has not yet been developed, leaving the complex nature of the flowers’ coloration unknown.

Inspired by the likely structural origin of the buttercup’s glossiness [19,23,24], we present here an in-depth study of the reflection characteristics of Ranunculus flowers. The genus Ranunculus comprises more than 500 species with a virtually cosmopolitan distribution [25]. We investigated the detailed spectral characteristics of three annual, herbaceous meadow buttercups, i.e. Ranunculus repens L., R. acris L. and R. lingua L. as well as the glossy flowers of the related celandine Ficaria verna Huds. (formerly known as R. ficaria) and the non-glossy flowers of the kingcup (also called marsh marigold) Caltha palustris L. (all Ranunculaceae). We applied various spectrophotometric methods, anatomical studies and a recently developed optical model [26] to assess the contributions of different petal components to the buttercup flowers’ bright-yellow coloration. We conclude that the coloration of buttercup flowers is due to a very rare combination of structural and pigmented coloration, and we discuss the functional significance of the gloss.

2. Material and methods

2.1. Plant material, photography and spectrophotometry

Flower samples were obtained from meadows around Groningen, The Netherlands. Flowers were photographed with a Nikon D70 digital camera having an F Micro-Nikkor (60 mm, f/2.8) macro-objective (Nikon, Tokyo, Japan). Petal details were photographed with an Olympus SZX16 stereomicroscope equipped with an Olympus DP70 digital camera (Olympus, Tokyo, Japan) and a Zeiss Universal Microscope (Zeiss, Oberkochen, Germany) with a Mueller DCMS510 camera (Mueller Optronic, Erfurt, Germany). To visualize the contribution of the gloss, photographs of pieces of petal were made by using white light with both a parallel and a crossed analyser on the microscope.

The overall reflectance and transmittance spectra of the dominant-coloured (central) area of the petals of at least five different plants were measured with an integrating sphere. For reflectance measurements, the flower was directionally illuminated from within the integrated sphere; the illuminated area had a diameter of about 5 mm. For transmittance measurements, the petal was illuminated from outside the sphere with an optical fibre at an area with diameter approximately 1 mm (for equipment details see [26]). The spectral characteristics of very small petal areas (5–10 μm diameter) were measured with a microspectrophotometer (MSP), following [26]. For measurements of the absorbance spectrum of the buttercup petal’s pigment, small pieces of petal were immersed in water. Transmittance spectra were measured on the isolated tissues with the MSP, from which absorbance spectra were calculated. For details about imaging scatterometry, see the electronic supplementary material (see also [19]).

2.2. Cryo-electron microscopy

Cryo-electron microscopy (cryoSEM) was applied to study the anatomy of R. acris petals. Petals were glued in a special copper holder and quickly frozen in nitrogen slush. The samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryoSEM. Electron micrographs were acquired from uncoated frozen samples.

2.3. Thin-film optics

We calculated the reflectance of a thin film with thickness d and refractive index n in air using the classical Airy formulae. With normal illumination the reflectance spectrum has extrema at wavelengths \( \lambda_n = 4nd/u \), with u an integer; the reflectance is maximal when u is odd and minimal when u is even [14,27]. The wavenumbers of the extrema (\( k_n = 2\pi/\lambda_n \)) hence are a linear function of n: \( k_n = ku \), with slope \( s = \pi/(2nd) \). We assumed an upper epidermis with a refractive index of \( n = 1.4 \) [24], which faced air on both sides and contained \( \beta \)-carotene with peak absorbance \( A = 1.4 \) (measured at 448 nm; see below). In our modelling, we considered a number of cases where the thin film had a Gaussian-distributed varying thickness with mean 2.7 μm and standard deviation \( \sigma = 0, 25, 50, 75, 100 \) and 125 nm.

2.4. Modelling petal reflectance and transmittance

The diffuse coloration of flowers is due to the random pathways of light propagating in the petal interior [26,28]. We modelled petal reflectance and transmittance by applying Kubelka & Munk [29] theory—which is often used to model the optical properties of plant leaves [30–35]—combined with a calculation procedure for a stack of absorbing and scattering layers (electronic supplementary material). By implementing model parameters found in real flowers, the relative contribution of different floral elements to the overall visual signal can be quantified [7,26,28].

The parameters of the model were based on our anatomical findings. We assumed for the upper epidermis a Gaussian-distributed varying thickness with mean \( d = 2.7 \) μm and standard deviation 125 nm, for the stach layer a thickness of 38 μm [36], and for the mesophyll and lower epidermis thicknesses of 100 and 40 μm, respectively. For the pigment of the upper epidermis, we estimated a peak absorption coefficient \( \kappa = A/(\log_{10} e) = 1.4/(0.4433 \times 2.7) = 1.2 \text{ μm}^{-1} \); for the mesophyll and lower epidermal layers we assumed peak absorption coefficients of 0.02 and 0.04 μm\(^{-1}\), respectively. By comparing the experimental and simulated data, the scattering coefficients of the stach layer, mesophyll and lower epidermis were estimated to be 10, 5 and 2 mm\(^{-1}\), respectively. Finally, the surface reflectance of the lower epidermis (which is solely due to differences in refractive indices between the very last layer and air) was assumed to be 0.1 (and hence the transmittance was 0.9).

3. Results

3.1. Spectral measurements and anatomy

For all investigated buttercup flowers, the reflectance and transmittance was low in the blue wavelength range and high above 500 nm (figure 1). The long-wavelength reflectance was markedly higher than the transmittance. This is distinctly different
from the majority of flowers, where the (long-wavelength) reflectance is lower than the transmittance [7]. The transmittance spectra showed a slight depression at approximately 680 nm, which suggests the presence of α-chlorophyll. The flowers of *C. palustris* exhibited a very low reflectance in the ultraviolet (UV) compared with the *Ranunculus* species. Whereas for the kingcup the reflectance and transmittance spectra were rather similar, for the buttercups these spectra differed from each other in the UV, suggesting that their petals are asymmetric in pigmentation and/or anatomy.

The buttercups are glossy, as is clearly visible in the main petal area (figures 1a–c and 2a,c). When observed with a low-power microscope, applying oblique illumination, the gloss was avoided and neat rows of effectively scattering granules were then discernible in the main petal area (figure 2b). While applying epi-illumination with polarized light, the gloss was prominently visible with a parallel polarizer and analyser (figure 2c), but it was fully extinguished by a crossed analyser; then only the depolarized backscattering from the inner petal structures remained (figure 2d). The starch granules below the epidermis are white (figure 2c), indicating that the yellow colour of the petals is due to a short-wavelength absorbing pigment concentrated in the upper epidermis. Indeed, the isolated upper epidermis, obtained by gently peeling, shows a bright-yellow colour, clearly due to a homogeneously distributed blue-absorbing pigment (figure 2f).

The mesophyll and lower epidermal layers markedly contribute to the light scattering. Focusing at the lower epidermal layer reveals yellow reflections with bright contours shining through. The cell contours became distinctly visible when focusing at the mesophyll layer (figure 3a,b), showing that the boundaries of the mesophyll cells are strongly scattering. When the air gaps are filled with water, the reflectance is about twofold smaller (electronic supplementary material, figure S2), because the interior of the flower is then more homogeneous and the refractive index contrast between the normally air-filled gaps and the surrounding cells is diminished.

We further investigated the pigmentation of the epidermal layers by measuring the reflectance and transmittance spectra of very small petal areas with an MSP. The shapes of the reflectance spectra measured abaxially from the bright contours and in between the contours were different (compare figure 3c, mesophyll and lower epidermis), which is likely to be due to wavelength-dependent scattering of the mesophyll cells. Both spectra showed a distinctly depressed reflectance at approximately 680 nm, characteristic for α-chlorophyll, which is absent in the spectra measured from non-glossy, diffusely reflecting areas at the adaxial side (figure 3c, upper epidermis), indicating an asymmetrical

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**Figure 1.** Reflectance and transmittance spectra of petals of four Ranunculaceae species. (a) *Ranunculus repens*, (b) *R. acris*, (c) *R. lingua* and (d) *Caltha palustris*. Green curves: reflectance, R; red curves: transmittance, T.
pigment deposition in the petal. Transmittance measurements on the isolated upper and lower epidermal layers of *R. acris* yielded the absorbance spectrum of a blue-absorbing pigment (figure 3d) very similar to the spectrum reported for β-carotene dissolved in hexane; yet, the peaks in the measured buttercup spectra (at 425, 448 and 478 nm) were more pronounced (figure 3d). For the upper epidermis, the estimated absorbance value at the peak wavelength 448 nm was 1.4 ± 0.3, whereas for the lower epidermis the absorbance was more variable: 0.8 ± 0.5. The distinct peak at approximately 680 nm in the absorbance spectrum of the lower epidermis corroborated our previous finding of the presence of a minor amount of α-chlorophyll (figure 3d; see also the electronic supplementary material, figure S2a, dry).

Cryo-electron microscopy (CryoSEM) on *R. acris* petals showed that petals consist of differently structured layers. The upper epidermis has a very constant thickness of approximately 2.7 μm and a very smooth surface (see also [19]). Air spaces separated the plate-like upper epidermis and the underlying starch granule layer; locally the upper epidermis touched the starch granules, providing mechanical support. The size of the air spaces is variable, which is mostly due to the irregularly shaped starch cells (figure 4b). We combined these new anatomical data with previous findings [36–38] to conceive a diagram of the inner structure of the *Ranunculus* petals (figure 4b). The very thin upper epidermis (which contains highly concentrated carotenoid pigment) faces slanted palisade parenchymal cells [36,37] that are filled with grainy starch. The mesophyll and lower epidermis form additional layers; both contain carotenoid pigment and α-chlorophyll, mostly in the boundary areas. In between the cells variably sized air spaces exist that contribute to the overall scattering.

The electron micrographs demonstrated that the upper epidermis is essentially a thin plate separated from the starch layer by (mostly) air space, and it may thus act as an optical thin film in air. Indeed, the adaxial reflectance spectra
of very small petal areas feature characteristic thin-film oscillations (figure 5a and electronic supplementary material, figure S3a,c). The oscillations were absent in the blue wavelength range, where the carotenoid pigment strongly suppressed the reflectance (figure 5a and electronic supplementary material, figure S3a,c). However, in the partially unpigmented glossy petals of *Ficaria verna* oscillations are also visible in the blue wavelength range (electronic supplementary material, figure S4; white glossy *Ficaria* and *Ranunculus* morphs are found occasionally [22]), corroborating our findings of an optical thin film. By fitting a linear function to the wavelengths of the reflectance extrema converted into wavenumbers, we derived from *R. repens*, *R. acris* and *R. lingua* average thickness values of $2.3 \pm 0.3\, \mu m$, $2.7 \pm 0.4\, \mu m$ and $3.1 \pm 0.5\, \mu m$, respectively, which is in good agreement with the anatomy (figure 4a).

### 3.2 Optical modelling

We performed optical modelling to gain further understanding of the upper epidermis acting as a thin film. In the UV and long-wavelength range the reflectance featured strong oscillations, depending on the variation in the thickness, but in the blue wavelength range all calculated reflectance spectra exhibited a distinct trough, due to the absorbing $\beta$-carotene (figure 5c). The oscillations vanished when the standard deviation of the thickness exceeded approximately 5% (figure 6c, $\sigma \geq 100\, \text{nm}$; see also electronic supplementary material, figure S5), explaining why clear oscillations are only measurable from very small areas. To investigate the possible contributions of additional petal layers, we also considered an optical model incorporating a narrow air layer in between the epidermis and the starch layer, thus causing a multilayer (sensu [24]). This yielded oscillating spectra with a similar periodicity to the single thin film but with slightly higher peak values (electronic supplementary material, figure S5). However, because the air space size is highly variable (figure 4a) and the starch layer will act as a wide-field scatterer rather than as a plane reflector, we conclude that multilayer interference is unrealistic.

Quantitative insight into flower coloration can be gained by treating the petals as a stack of layers, where each layer is characterized by its specific reflectance and transmittance spectra [26]. Our anatomical studies showed that the...
buttercup’s upper epidermis can be considered as a homogeneous medium with negligible scattering, and that the starch, mesophyll and lower epidermal layers strongly scatter. We applied a Kubelka–Munk-layer-stack model (Material and methods, electronic supplementary material, [26]) to an R. acris petal treated as a four-layer stack. This yielded distinctly different adaxial and abaxial reflectance spectra (figure 5d). The adaxial reflectance (R_{ad}) had a deeper trough in the blue wavelength range than the abaxial reflectance (R_{ab}), due to the higher concentration of carotenoid pigment in the upper epidermis compared with the lower cell layers, whereas the abaxial reflectance spectrum had a pronounced dip at approximately 680 nm, due to the exclusive abaxial presence of chlorophyll (figure 5d).

4. Discussion
4.1. The coloration toolkit: a pigmented thin film and underlying backscattering layers
This study describes the special combination of buttercup flowers’ pigmented thin-film reflector and strongly scattering underlying starch and mesophyll layers. Pigmentary filtering of structural coloration is common in various animal taxa, such as butterflies (e.g. [14,39]) and birds (e.g. [40]), but is very rare among flowers. In flowers of the vast majority of plant species, specular reflections are absent, because of the presence of conical epidermal cells and/or epidermal microstructures [5,20] that scatter light into a wide angular space [19]. When directionally illuminated, buttercup flowers display a strong gloss in addition to an overall matte-yellow colour (figures 1 and 2).

The glossy petals of Ranunculus have fascinated scientists for more than a century [5,22,23,38]. Pioneering work by Parkin [36,37] showed that the starch cells are arranged in a slanted manner and occur in many Ranunculus species. More recently, Vignolini et al. [24] discovered that the epidermal and starch layers are separated by an air layer, a feature that had not been described in any plant system before. By combining previous and new anatomical findings, spectroscopy and optical models, we show that buttercup flowers feature a special coloration system.

The clearly oscillating reflectance spectra measured from very small petal areas of Ranunculus and Ficaria species demonstrated that the epidermal layer acts as a thin film. The upper epidermis locally has a constant thickness, and optical modelling showed that this thickness—independent of the air space and starch layer—determines the periodicity of the oscillations. The thickness of the upper epidermis can thus be directly derived from measurements of the petal reflectance (figure 5 and electronic supplementary material, figures S3 and S4). Reflectance measurements from various areas of the same petal yielded thicknesses varying by more than 10%, resulting in smooth reflectance spectra when measured from larger areas (cf. figures 1 and 5c). The upper epidermis is only locally flat; over larger areas the surface is wrinkled, so that the gloss is restricted to those areas
that have the right mirror position with respect to the light source and observer (figure 2a,c).

The upper epidermis contains a high concentration of carotenoid pigment and therefore it serves as an effective spectral filter for the light backscattered by the underlying starch and mesophyll layers. Mesophyll cells commonly occur in flowers [7], but starch cells are almost exclusively found in buttercups [5,22,25], where they act as strong, diffuse reflectors, enhancing the buttercup’s brilliance (figures 2b,c, 5d and electronic supplementary material, figure S2).

An intriguing question concerns the radiation of petal glossiness. Phylogenetic reconstruction using recently published phylogenies [25] shows that glossiness is predominant among Ranunculus species and also occurs in related genera (electronic supplementary material, figure S6). The most conservative interpretation would be that glossiness—due to a thin-film epidermis—is the ancestral state for Ranunculus, and also for Ficaria, Oxygraphis and Halerpestes (electronic supplementary material, figure S6), which are more distantly related genera [25,41]. In several species glossiness has been lost over the course of evolution (electronic supplementary material, figure S6). The starch layer is also found in most Ranunculus species [25]. Large-scale sampling of Ranunculaceae species will elucidate the ancestral origin and the anatomical variation between species.

4.2. Functionally glossy?

The glossiness presumably has two non-mutually exclusive functions: enhancing the visual signal to pollinators and increasing the light reflection to the reproductive organs. For nearly normally incident light, the reflectance amplitude of the gloss is no more than approximately 5% (figure 6a), but the reflectance amplitude considerably increases with an increasing angle of incidence, i.e. away from normally incident light (figure 6a). Therefore, pollinators that approach the flower under a large mirror angle will perceive the gloss as a flash. The spectral modulation of the gloss signal is nonetheless rather low (i.e. a glare without a distinct hue), especially under a large incident angle (figures 5a and 6a); hence the colour contrast, which is considered important for pollinators to detect flowers [1,42–44], is limited. We conclude that the bright flash may provide a long-distance signal to pollinators, whereas at short distances the diffuse UV-yellow coloration will constitute the flower’s visual signal.

Buttercup flowers are heliotropic [45–49], and when ambient temperatures are low they have approximately the shape of a paraboloid (figure 6b). Under these circumstances, incident sunlight that reaches the petal surface under a large angle will not be reflected to the outside but towards the central flower area where the reproductive structures are located (see also [50]). This will cause increased floral temperatures, which enhances seed and pollen maturation and is preferred by pollinators [45–53]. Thus, due to the combined effect of near-closure and heliotropism of the flowers, the petals’ thin film will enhance light reflection to the reproductive organs. Indeed, under natural conditions, the centre of the paraboloid-shaped glossy flowers of the arctic buttercup, R. adoneus, were found to be several degrees warmer than the ambient air [54].

Abiotic factors, including sunlight, have been previously documented to impose selective pressures on flower anatomy and coloration [53,55,56]. For example, Koski & Ashman [56] recently reported that exhaustive amounts of internal UV reflections can be deleterious for plant reproduction. Based on a large geographical sample, they showed that the size of the UV-absorbing area of a (quasi)paraboloid-shaped flower depends on the amount of sunlight. Experiments confirmed that high levels of solar irradiance (as in regions close to the Equator) favour phenotypes with larger UV-absorbing petal areas, so as to reduce (harmful) UV reflectance to the pollen and prevent DNA damage [56]. Future experiments, e.g. manipulation of the internal light reflections, will illuminate the functional significance of the buttercup’s remarkable anatomy.

**Authors’ contributions.** All authors performed measurements; C.J.v.d.K. and D.G.S. analysed results; C.J.v.d.K. and D.G.S. drafted the manuscript, which was approved by all authors.

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