Refractive index and dispersion of butterfly chitin and bird keratin measured by polarizing interference microscopy

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Abstract: Using Jamin-Lebedeff interference microscopy, we measured the wavelength dependence of the refractive index of butterfly wing scales and bird feathers. The refractive index values of the glass scales of the butterfly *Graphium sarpedon* are, at wavelengths 400, 500 and 600 nm, 1.572, 1.552 and 1.541, and those of the feather barbules of the white goose *Anas anas domestica* are 1.569, 1.556 and 1.548, respectively. The dispersion spectra of the chitin in the butterfly scales and the keratin in the bird barbules are well described by the Cauchy equation $n(\lambda) = A + B/\lambda^2$, with $A = 1.517$ and $B = 8.80 \cdot 10^3$ nm^2 for the butterfly chitin and $A = 1.532$ and $B = 5.89 \cdot 10^3$ nm^2 for the bird keratin.

OCIS codes: (180.0180) Microscopy; (160.1435) Biomaterials; (160.5320) Photorefractive materials; (170.1420) Biology.

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1. Introduction

Structural coloration is a widespread, well-recognized phenomenon in the animal kingdom [1–5]. Notably many butterfly species feature beautifully colored wings due to a cover of nanostructured scales [6,7]. Many bird species are also strikingly colored due to feathers with shiny barbs or barbules [8–10]. For a detailed understanding of the reflection properties of the scales and feathers their three-dimensional structure as well as the refractive index of the composing material has to be known. For butterfly wing scales the material in question is the biopolymer chitin and for bird feathers this is keratin [11,12].

Mason [13] extensively investigated the structural colors of insects by using immersion fluids. He thus estimated the refractive index of *Morpho* butterfly scales at about 1.55. The intense blue reflection of the *Morpho* scales when in air was fully extinguished by a well-matching immersion fluid, but a light brown color remained, indicating that the studied scales contained an absorbing pigment. Indeed, Vukusic et al. [14] found for *Morpho* scales a complex refractive index, with real part 1.56 ± 0.01 and imaginary part 0.06 ± 0.01, via measurements of the scale reflectance and transmittance in various immersion fluids.

The refractive index of optical materials invariably depends on the wavelength, i.e. optical materials are dispersive. So far the dispersion of butterfly wing scales has not been studied. We encountered a highly favorable scale type to tackle that question, namely the glass scales of the swordtail *Graphium sarpedon*, the Common Bluebottle [15,16]. These scales are attractive because they are unpigmented and thus the imaginary component of the refractive index is negligible. Furthermore, the glass scales are approximately ideal plane-parallel plates, making them very suitable for quantitative analysis. Applying Jamin-Lebedeff interference microscopy, we were able to measure the wavelength dependence of the scale refractive index, i.e. the dispersion. For comparison we applied the same approach to the so-called white scales of *G. sarpedon* [15].

Mason also investigated bird feathers and estimated the refractive index at 1.54 (blue spongy feathers [17]) and 1.55-1.60 (iridescent peacock barbules [18]). Schmidt [19] also reported a refractive index value of ~1.55 (hummingbird). Since then several studies have produced similar values [1,20]. The dispersion of bird feathers has also remained unstudied, and we therefore have extended our interference microscopy measurements to the unpigmented feathers of the white goose, *Anas anas domesticus*.

2. Materials and methods

Butterfly wing scales were taken from the swordtail *Graphium sarpedon*, specifically the glass and white scales [15,16]. The scales were isolated by gently pressing wing pieces on to a microscope slide. The isolated scales were covered by a cover slip after immersion of the scales in a fluid with refractive index between 1.46 and 1.64 (series A of Cargille Labs, Cedar Grove, NJ, US). The wavelength dependence of the refractive index of each immersion fluid was derived by using the Cauchy equation $n(\lambda) = A + B/\lambda^2$ and calculating the parameters A and B from the fluid's refractive index value, given for the wavelength 589 nm, and its Abbe number.

The microscope slides with scales were mounted on the stage of a Zeiss Universal Microscope, which was set up for Jamin-Lebedeff interference microscopy [21] (see also http://www.accessengineeringlibrary.com/mghpdf/0071738207_ar028.pdf). As light sources we used a tungsten lamp or a high-pressure mercury lamp, and specific wavelengths were

selected by inserting narrowband interference filters (bandwith 12-16 nm) below the condenser. The microscope objective was a Zeiss Pol-Int I 10x/0.22. Photographs were taken with a Kappa DX40 color camera (Kappa Optronics, Gleichen, Germany) or a Coolsnap ES monochrome camera (Photometrics, Tucson, AZ).

The Jamin-Lebedeff interference microscope has a Jamin interferometer positioned between the condenser and the microscope objective, and a fixed Sénarmont compensator and a rotatable analyzer between the objective and the eyepiece. The compensator was designed for the mercury green line, λ = 546 nm, but reliable results could also be obtained at other wavelengths. The Jamin interferometer relies on the fact that an object (o) with thickness d and refractive index n_o in a reference medium (r) with refractive index n_r creates an optical path length difference of $G = (n_r - n_o)d$. This can be compensated by rotating the analyzer by an angle $\Delta\alpha$ so that $G = (\Delta\alpha/180)\lambda$, where the angle is given in degrees. The analyzer angle difference thus depends on the refractive index of the reference medium by

$$\Delta\alpha = 180(n_r - n_o)d / \lambda. \tag{1}$$
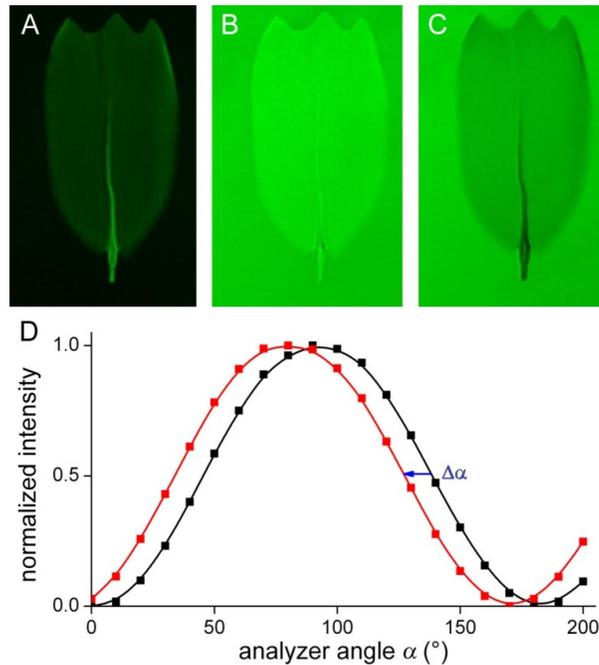
Fig. 1. Jamin-Lebedeff interference microscopy of an isolated glass scale of *Graphium sarpedon*. **A-C** Images for three angular positions of the analyzer differing by 60° of a scale immersed in a fluid with refractive index 1.46 at 546 nm. (**D**) Normalized brightness as a function of the analyzer angle, measured in two regions of interest, one region in the scale (red curve) and the other adjacent to it (black curve). The two curves are shifted along the abscissa axis by a negative analyzer angle difference, $\Delta\alpha$, indicating that the refractive index of the scale is >1.46.

In principal the value of the scale refractive index can be obtained by finding the value of n_r where $\Delta\alpha = 0$. In practice, however, it is extremely difficult to find a precisely matching immersion fluid. Therefore a more reliable (and realizable) procedure is to determine $\Delta\alpha$ for a number of n_r values around the expected n_o value. This was achieved as follows. Interference images of the scales were photographed for 20 angular analyzer positions (α), in steps of 10° (Fig. 1). The images were evaluated by taking two regions of interest (ROI), one on the object and another adjacent to the scale, where the surrounding reference medium occupied the space. The brightness (I) of the two ROI was determined with ImageJ (rsbweb.nih.gov/ij). The

20 images thus yielded two sinusoidal brightness curves, $I_o(\alpha)$ and $I_r(\alpha)$, that were shifted along the α-axis; that is $I_o(\alpha) = I_r(\alpha + \Delta\alpha)$. For a fixed wavelength λ, $\Delta\alpha(n_r)$ is a linear function (Eq. (1), and thus, by applying several immersion media with different refractive indices, the value of n_o follows from the line's zero-crossing, $\Delta\alpha(n_r) = 0$, where $n_o = n_r$ (Fig. 2). The scale's dispersion, or, the wavelength dependence of the scale's refractive index, $n_o(\lambda)$, was obtained by determining the zero-crossing, $\Delta\alpha(n_r) = 0$, for a number of wavelengths. Actually, the experimental practice was that we determined $\Delta\alpha(n_r,\lambda)$ for a certain scale, when immersed in a fluid with refractive index n_r, for a number of chosen wavelengths (we used $\lambda = 390, 451, 494, 546, 588, 650$ and 715 nm). This procedure was then repeated for another scale in another medium. Finally, the Cauchy equation $n(\lambda) = A + B/\lambda^2$ was fitted to the measured dispersion spectra, $n_o(\lambda)$, yielding the scales' Cauchy coefficients A and B.

The procedure described above for butterfly scales was applied in an identical fashion to barbules of white goose feathers (*Anas anas domesticus*). Barbs with barbules were isolated from the feathers with fine scissors.

3. Results

Isolated glass scales of the swordtail *Graphium sarpedon*, immersed with a fluid with refractive index in the range $n_r = 1.46\text{-}1.62$, were observed with a Jamin-Lebedeff interference microscope. Figure 1(A)–1(C) shows a scale in a reference medium with $n_r = 1.46$ for three angular positions of the analyzer; the light wavelength was $\lambda = 546$ nm. The differences in brightness of the scale and the reference medium demonstrate that the scale refractive index deviates from that of the medium. The brightness of two areas, one in the scale and another in the adjacent medium, was evaluated as a function of the analyzer angle. The two identical shaped brightness curves are shifted with respect to each other along the abscissa, yielding the analyzer angle difference, $\Delta\alpha$ (Fig. 1(D)).
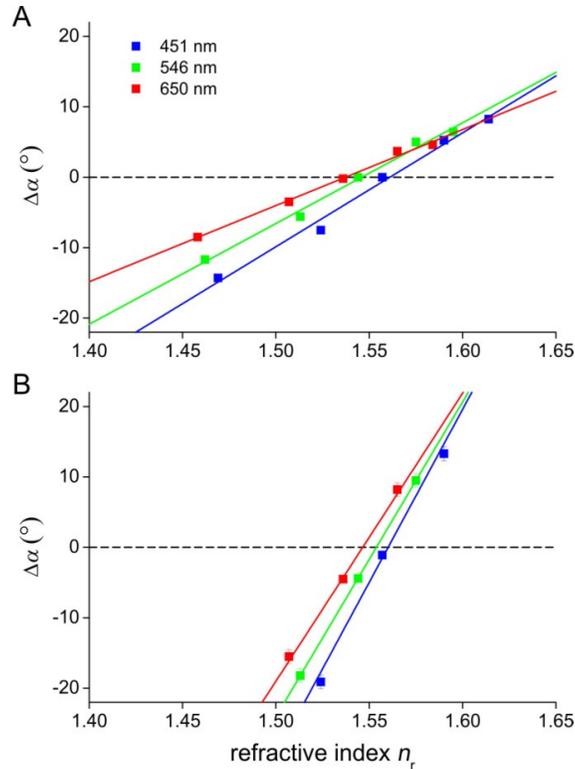
Fig. 2. The analyzer angle difference, Δα, as a function of the refractive index of the reference medium, n_r. (**A**) Δα values obtained for three wavelengths, 451, 546 and 650 nm from *G. sarpedon* glass scales fitted with linear functions. (**B**) Δα values obtained from white goose feather barbules with linear fits.

By applying different immersion fluids, we determined the value of Δα for a range of refractive indices, n_r, and light wavelengths, λ. Figure 2(A) presents Δα(n_r) for three wavelengths, λ = 451, 546, and 650 nm. Figure 2(B) shows, for the same wavelengths, Δα(n_r) obtained with the same procedure from the barbules of white goose feathers, *Anas anas domesticus*. For each wavelength, the measured values of Δα of both the scales and barbules are well fitted by a linear function, Δα(n_r) = an_r + b. With Eq. (1) this yielded the refractive index, n_o = -b/a, and the thickness, d = aλ/180.

Figure 3 presents the refractive index values thus derived for a number of wavelengths. The data could be well described by the Cauchy dispersion equation, $n_o(\lambda) = A + B/\lambda^2$, yielding for the butterfly glass scales A = 1.517 ± 0.001 and B = 8.80·10^3 ± 0.15·10^3 nm^2 and for the feather barbules A = 1.532 ± 0.001 and B = 5.89·10^3 ± 0.21·10^3 nm^2. Their refractive index at 586 nm is 1.542 and 1.549, respectively, and the Abbe numbers are 19.0 and 32.3, respectively. Each of the data points in Fig. 3 was obtained from a different scale or feather barbule, immersed in a different reference medium. The derived thickness values thus slightly varied; for the glass scales the linear fits yielded d = 404 ± 28 nm, in excellent agreement with both anatomical results and thin film modeling of reflectance and transmittance measurements [16], and for the feather barbules d = 1.37 ± 0.18 μm.

We similarly studied the white scales of *G. sarpedon*, but in less detail, because measurements at λ = 546 nm yielded a refractive index value identical to that of the glass scales: n_o = 1.546. Because the white and glass scales are made of the same material, chitin, we thus assume that the dispersion characteristic of the white scales is identical to that of the glass scales (Fig. 3) The thickness of the white scales following from a linear fit to the Δα(n_r) data was 410 ± 32 nm.
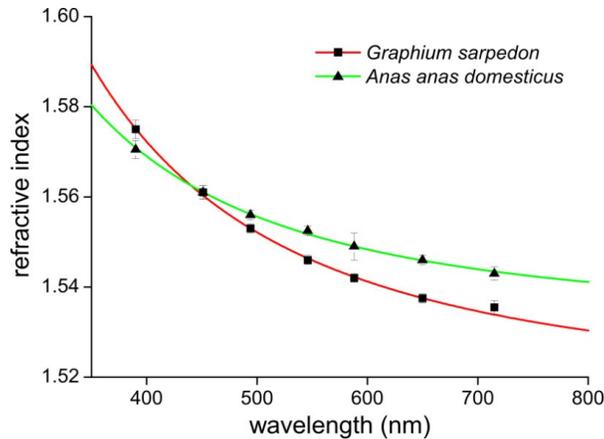
Fig. 3. Refractive index values derived from the zero-crossings of the linear fits of the butterfly glass scales (Fig. 2(A)) and the bird feather barbules (Fig. 2(B)), fitted with the Cauchy equation.

4. Discussion

Using classical interference microscopy, we have measured dispersion spectra for butterfly wing scales and bird feather barbules. Previous studies on *Morpho* scales, using refractive index matching fluids, yielded a value around 1.55 [13]. Those scales are however pigmented and thus have a complex refractive index [14]. To avoid that complexity, we selected the glass scales of *G. sarpedon*, because these scales are unpigmented and thus have a real refractive index. Furthermore, they approximate plane-parallel plates [16], making them ideal for interferometric investigation. Similarly, the barbules of white goose feathers are unpigmented and platelike [22] and thus could be analyzed with the same method. We found that Jamin-Lebedeff interferometry is a reliable and robust method to determine the refractive index of both butterfly scale chitin and feather keratin with unprecedented detail for the whole (human) visible wavelength range. Previous estimates using optical modeling of iridescence phenomena must be considered as less accurate, especially when an absorbing pigment is present [14,20]. The present study shows that both chitin and keratin have substantial dispersion. A similar finding has recently been reported for the chitin in the multilayers coloring the elytra of the Jewel Beetle *Chrysochroa fulgidissima* [23]. The Cauchy parameters for the unpigmented beetle layers were found to be $A = 1.51$ and $B = 1.53 \cdot 10^4$ nm^2, slightly different from the values found for the *Graphium* glass scales.

The wavelength dependence of the refractive index has remained neglected in the numerous quantitative studies of butterfly wing scale and feather optics. Fortunately, the small variation of the refractive index of no more than a few percent in the visible wavelength range (Fig. 3) will not have a major impact on the results of optical models. Nevertheless, the obtained parameters of the Cauchy equation will allow ready implementation in future optical modeling studies.

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