

Remarkable iridescence in the hindwings of the damselfly *Neurobasis chinensis chinensis* (Linnaeus) (Zygoptera: Calopterygidae)

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The bright green dorsal iridescence of the hindwings of *Neurobasis chinensis chinensis* males, very rare in Odonata, is known to play a significant role in their courtship behaviour. The mechanism responsible for such high contrast and spectrally pure colour has been investigated and found to be optical interference, producing structural colour from distinct laminations in the wing membrane cuticle. The ventral sides of these iridescent wings are dark brown in colour. In a single continuous membrane of wing cuticle, this is an effect that requires a specialized structure. It is accomplished through the presence of high optical absorption ($k = 0.13$) within two thick layers near the ventral surface of the wing, which leads to superior dorsal colour characteristics. By simultaneously fitting five sets of optical reflectivity and transmissivity spectra to theory, we were able to extract very accurate values of the complex refractive index for all three layer types present in the wing. The real parts of these are $n = 1.47, 1.68$ and 1.74 . Although there is often similarly significant dorsal and ventral colour contrast in other structurally coloured natural systems, very few system designs comprise only a single continuous membrane.

Keywords: structural colour; iridescence; interference; multilayer; damselfly

1. INTRODUCTION

The wings of Odonata consist of a framework of narrow veins, interconnected by thin membranes. They are composed of cuticle, a structurally complex and variable material, which can contribute to the mechanical properties of insect wings under flight-related stress (Neville 1975, 1993; Vincent 1980; Newman & Wootton 1986; Smith *et al.* 2000). In some insects, the cuticle shows refractive-index periodicity, thereby creating strong optical effects through interference or Bragg scattering. These structural optical effects can combine with the effects of additional brightly coloured pigmentation (Fox 1976; Farrant 1997) to result in colour addition or a range of more complex optical effects. In many of these cases, the optical properties associated with the pigmentary and structural colour components are rather different. Whereas a lack of directionality, mediocre intensity and sometimes fluorescent properties (Fox 1976; Lawrence *et al.* 2002; P. Vukusic, unpublished data) are associated with pigmentation, structural colour is generally marked by properties such as angle dependence or specific limited directionality (Vukusic *et al.* 2002), high brightness (Ghiradella 1991; Vukusic *et al.* 1999), colour reflectivity that may be beyond the accessible wavelength range of pigmentation (Ghiradella *et al.* 1972) and even polarization effects (Neville & Caveney 1969; Vukusic *et al.* 2000b). The evolutionary selection pressures associated with the development of individual and often complex optical systems are inherently dependent upon species' interaction with conspecifics and predators and are the

subject of many elegant reviews (Fox & Vevers 1960; Fox 1976; Parker 2003).

Many adult Odonata exhibit complex behaviour, variously associated with the establishment and defence of territories and with courtship, mating, mate defence and oviposition (Corbet 1999). Courtship behaviour is principally found in calopterygoid Zygoptera: in Calopterygidae, Chlorocyphidae and Euphaeidae. An important variable underpinning such behaviour in male Odonata is their body colour and patterning. Conspicuously bright body coloration is common and is involved in both intersexual and intrasexual behaviour. Odonate wings, however, are generally transparent or banded with dull brown or light brown pigmentation. It is in this respect that the damselfly *Neurobasis chinensis* is unusual: although its forewings are completely transparent, its hindwings are opaque with a dorsal surface that conspicuously reflects green at normal incidence and a ventral surface that appears dull brown. The green dorsal colour, changing hue with the angle of observation under diffuse illumination, has been identified as structural in origin. Furthermore, the brown ventral side, wholly lacking the dorsal green iridescence, implies a more complex ultrastructure than might be expected in a thin wing membrane of the kind found in damselflies. This study was therefore undertaken to characterize one of the few species of Odonata that exhibits conspicuous wing iridescence and to answer the question: how does the structure of an *N. chinensis* wing allow selective reflection of bright green dorsally and dull brown ventrally?

2. MATERIAL AND METHODS

The green iridescent hindwings of a preserved male damselfly *N. chinensis* were examined using scanning and transmission electron microscopy (SEM and TEM) and reflection and

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transmission spectroscopy. A Hitachi S-3200N electron microscope was used for SEM; the samples were cold sputtered with 4 nm of gold. TEM analysis was undertaken after fixing samples in 3% glutaraldehyde at 21 °C for 2 h followed by rinsing in sodium cacodylate buffer. Subsequent fixing in 1% osmic acid in buffer for 1 h was followed by block staining in 2% aqueous uranyl acetate for 1 h, dehydration through an acetone series (ending with 100% acetone) and embedding in Spurr resin (Spurr 1969). After microtoming, sample sections were stained with lead citrate and examined using a JEOL 100S TEM instrument. Reflection and transmission spectra were collected using an optical fibre connected to a computer-driven ARC Spectro-Pro 3001 spectrometer. A Wotan Halostar KLR35 50 W bulb was used to illuminate the sample. Absolute values of transmissivity through the wing were determined by successively illuminating a single transparent membrane window with collimated laser light. An argon-ion and four helium-neon lasers provided nine accessible wavelengths.

In addition to the two reflection spectra taken from each side of the iridescent wing, a transmission spectrum was recorded with the wing orientated normal to the incident beam. A long-focal-length convex achromatic lens in front of the wing ensured that the beam spot specifically passed through a region of iridescent wing membrane without overlying any wing venation. To verify absolute values of reflectivity (R) and transmissivity (T), a task that is generally not possible with natural samples and materials using spectrometers, even when they are equipped with integrating spheres, the R and T values associated with the wing surfaces were measured using single-wavelength laser lines. In this way, it was possible to measure the intensity of all reflected or transmitted light. When this was ratioed with the total intensity of incident light, it yielded absolute values of R and T at several wavelengths (Vukusic *et al.* 1999). Modifications to the intensity scaling of the five sets of R and T spectra recorded for the wing were made in light of this. Consequently, the experimental intensities shown are believed to be accurate to $\pm 3\%$.

With good knowledge of the intensity magnitudes of the four reflected spectra and one transmitted spectrum, it was possible to apply an optical multilayer theory (Kaiser & Pulker 2003) to derive values for their complex refractive indices. Multilayer thicknesses, measured during TEM analysis, were used as a basis on which to model theory for all five datasets simultaneously. This has a distinct advantage over fitting datasets individually: it removes the degeneracy associated with fitting unknown layers and structures for which many different models may give identical optical responses.

The optical theory was applied using the well-documented method by which a characteristic matrix for the multilayer system is employed to calculate R and T . The optical parameters that were allowed to vary to obtain the best fit of theory to experimental data were the complex refractive indices of the materials comprising the multilayers. Additionally, to allow for variation in the layer thicknesses of the samples, associated with random fluctuations produced naturally or during the preparation process, all layer thicknesses were allowed to vary by between 4% and 8%. This figure was judged acceptable after quantitative comparison of 12 different sections of the same region of wing using TEM and SEM.

3. RESULTS

The dorsal side of the hindwing of *N. chinensis* is green at normal incidence (figure 1*a*), becoming distinctly blue

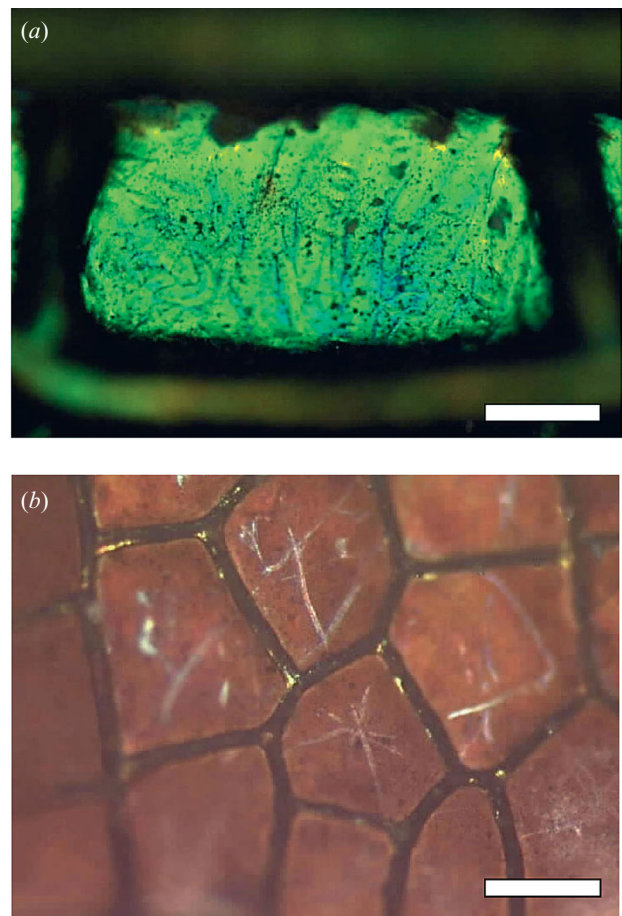


Figure 1. (a) Optical image showing the iridescence observed from the dorsal side of an *N. chinensis* hindwing. The bright region is a single membranous window surrounded on all four sides by wing venation. (b) The ventral side of the same region of wing is brown in colour. The bright diagonal lines are the result of scratch damage. Scale bars: (a) 150 μm and (b) 750 μm .

towards grazing (incidence parallel to the surface). Optically, this indicates both that the colour is derived structurally and that this structure is multilayered. The ventral sides of the hindwings are deep brown in colour (figure 1*b*), with no observable green iridescence. This colour matches that of the small, *ca.* 1 cm^2 , tip of the dorsal wing surface that does not exhibit green iridescence.

Electron microscopy, both SEM and TEM, shows the arrangement of the expected multilayering (figure 2). The wing membrane close to the dorsal surface comprises up to 12 periodic dark- and light-contrasted layers, while the membrane close to the ventral surface is far less periodic, comprising only a few layers of varying thickness and of less distinct contrast (figure 2*c*). There appears to be no discernible helicoidal arrangement of chitin fibrillae in any of the layers; accordingly, initial experiments searching for any circularly polarized reflection from the wings recorded no signal. Both the dorsal and ventral surfaces of these hindwings, and those of the forewings, exhibit the familiar fine wax microsculpture (WM in figure 2*b* and faintly in figure 2*d*) associated with the wings of some other Odonata (Gorb *et al.* 2000). While it has been suggested that this structure affects UV reflectivity in certain Odonata (Gorb *et al.* 2000), recent optical experiments with the

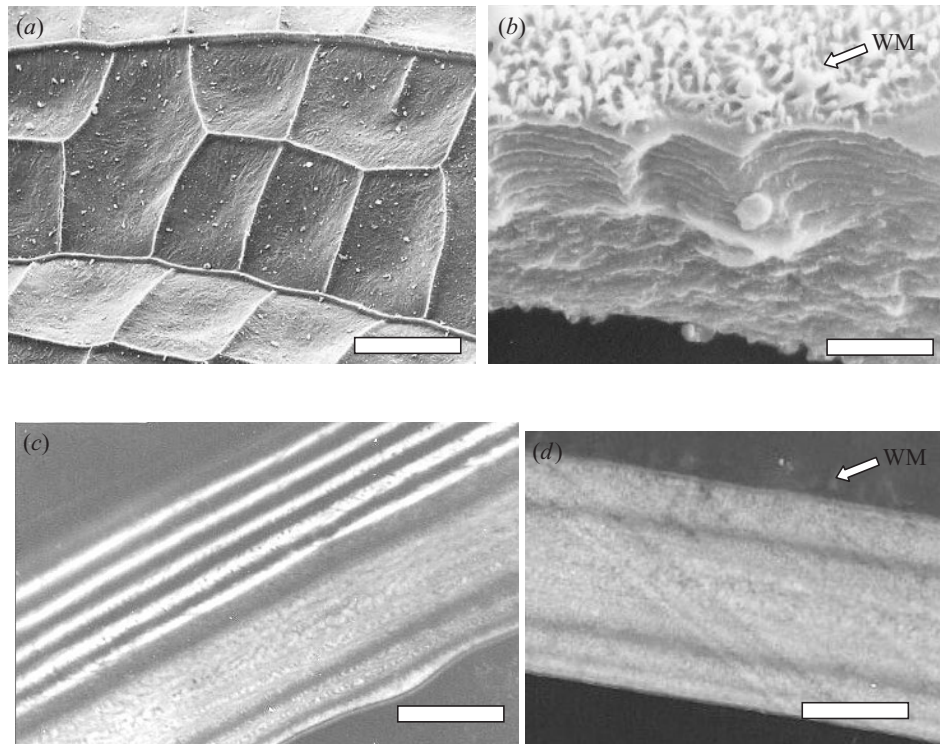


Figure 2. (a,b) SEM images showing (a) the dorsal surface and (b) the fractured edges of an *N. chinensis* iridescent hindwing membrane (dorsal surface uppermost); with (c,d) TEM images showing cross-sections through (c) an iridescent hindwing membrane (dorsal surface uppermost) and (d) the non-iridescent membrane from the tip of a hindwing. Scale bars: (a) 500 μm ; (b) 1 μm ; (c) 850 μm and (d) 850 μm . WM: wax microsculpture.

transparent wings of *Aeshna cyanea* have indicated that such surface microstructure has negligible optical effect at near-UV and visible wavelengths (P. Vukusic, unpublished data).

Reflection spectra taken from the green iridescent wing surface at several angles of incidence reveal a curious wavelength dependence. At angles around normal incidence (5° in figure 3a), there is a strong reflectivity maximum at 510 nm, a feature responsible for the overtly green iridescence. Additionally, distinct but subsidiary maxima centred at ca. 600 and 660 nm comprise a low-intensity tail. At wider angles of incidence (50° in figure 3b), these maxima are observed to shift to shorter wavelengths in a manner generally commensurate with the response of multilayer systems; their relative intensities also change in a predictable way.

Reflection spectra taken at different angles from the brown-coloured ventral wing surfaces exhibit what appear to be maxima associated with multilayering (15° in figure 3c and 60° in figure 3d). However, their overall distribution and superposition with the absorption associated with brown pigmentation, assumed to be melanin, appears brown to human visual systems.

Figure 3 shows the comparison between the resulting theoretical models and the corresponding experimental data. Despite some divergence at either end of the recorded wavelength band, the consistency of this simultaneous fitting of all five datasets is good, especially given the uncertainty in the extent of the dispersive nature of the material. This is a recognized difficulty encountered when modelling natural systems (Vukusic 2003). The fitting process yielded three different layer refractive indices

(table 1); the identities of these layers closely corresponded to those of the three differentially contrasted layers observed in the TEM images (figure 2c; i.e. the three layers referred to are contrasted white and black (principally on the dorsal side) and grey (limited to the ventral side)).

4. DISCUSSION

Optically absorbing substrates enhance the saturation of structurally coloured systems. While the physics of this have been described in many texts (e.g. Fox 1976), none has quantified the extent of such absorption in natural systems. With this investigation, we have both broadly characterized the iridescence associated with *N. chinensis* and modelled the optical absorption responsible for the contrast in colour and intensity between the dorsal and ventral sides of the wing. This is principally present in the form of highly absorbing layers in the ventral part of the wing, apparently coincident with the ventral component of the membrane, which is of course a double structure. The two principal grey-contrast layers, situated nearer the ventral side of the wing, have absorption coefficients of $k = 0.13 \pm 0.02$. This is significantly higher than the absorptions found in a broad range of other coloured species, and is, above all, responsible for the difference in colour between the dorsal and ventral wing surfaces. If these k -values were significantly lower, the green iridescence would be visible from both sides of the wing. This is confirmed by theoretically modelling the system without the optical absorption associated with the ventral layers.

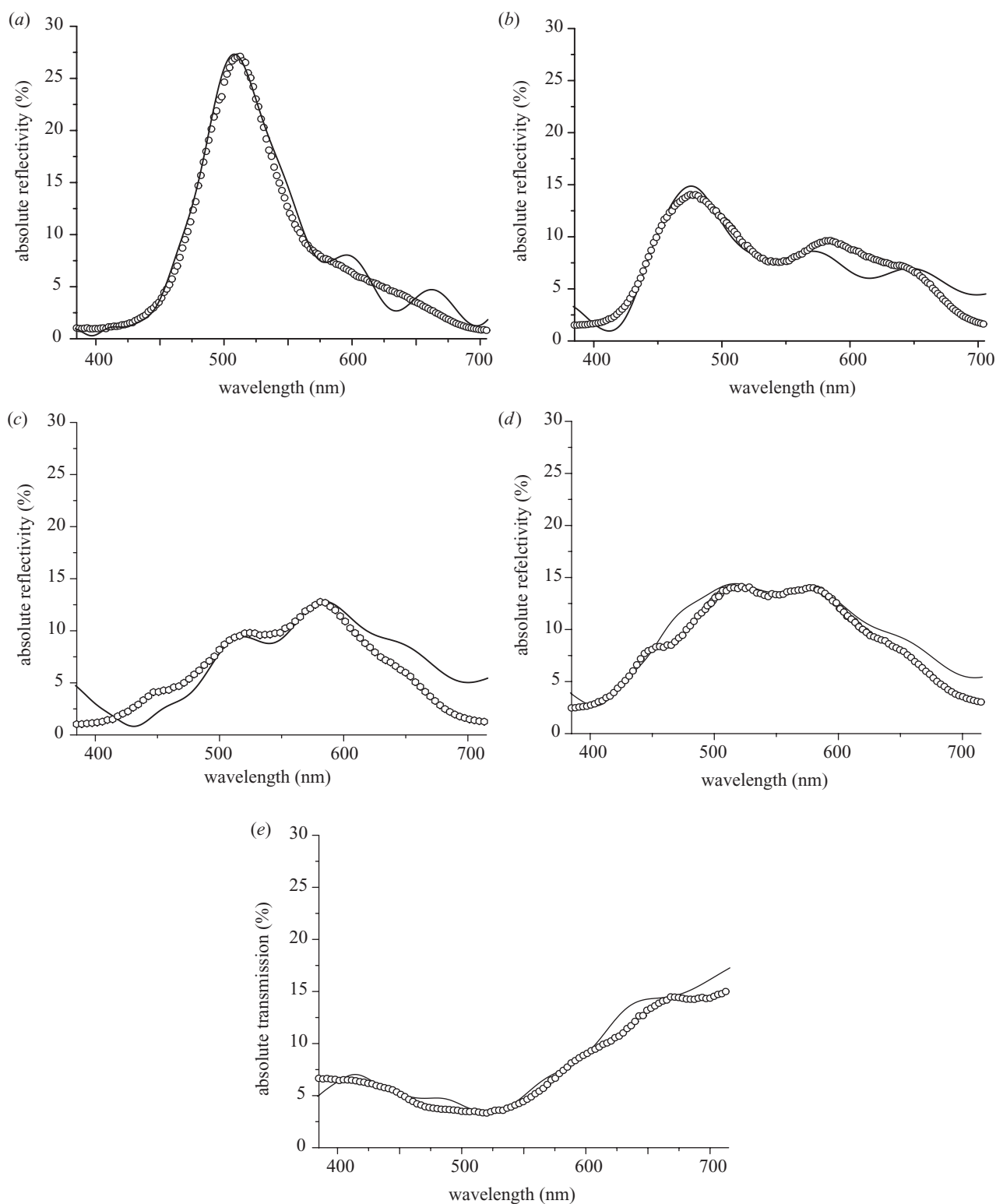


Figure 3. Comparison of theoretical models (solid line) and experimental data (circles) for (a,b) reflectivity from the iridescent dorsal side of a hindwing at 5° and 50° respectively, (c,d) reflectivity from the non-iridescent ventral side of a hindwing at 15° and 60°, respectively, and (e) transmission through the iridescent wing of light incident normally on the dorsal side.

The refractive index contrast between neighbouring layers in the *N. chinensis* system is less than that generally found in many other structurally coloured terrestrial insects: it is 0.18 for the layers close to the dorsal surface and as low as 0.06 for those close to the ventral surface. Furthermore, theoretical modelling indicates that the dark-contrast layers in the system are themselves optically absorbing, with $k = 0.06 \pm 0.03$. As a result of this and of

the low refractive index contrast, the intensity of the peak wavelength reflected from the dorsal side at or near normal incidence is lower than would otherwise be expected for an equivalent number of layers in, say, a lepidopteran structurally coloured system (Vukusic *et al.* 2000a).

We believe that the optical absorption in *N. chinensis* is caused by the diffuse presence of melanin throughout many of the layers in the damselfly wing. Several attempts

Table 1. The complex refractive indices of the three regions of different contrast (white, grey and black) shown in figure 2c.

	contrast in TEM image	n	k
layer type 1	white	1.68 ± 0.01	0.00
layer type 2	black	1.47 ± 0.02	0.06 ± 0.03
layer type 3	grey	1.74 ± 0.02	0.13 ± 0.02

were made to remove melanin from the wing with a range of solvents and reducing agents, in order to measure the resulting change in optical properties, but these were unsuccessful. This is generally found to be a difficult procedure owing to the robust nature of certain types of melanin (Prota *et al.* 1988), but the process was especially difficult in this case because of the fragile nature of the specimen. However, a similar optical comparison has been completed for the structurally coloured elytra of two species of beetle (Fox 1976). When the left elytra from one *Chrysochus cobaltinus* Leconte and one *Lytta vesicatoria* Linnaeus were bleached, washed and then dried, their iridescence was observed to deteriorate markedly. Both were then painted ventrally with Indian ink of high optical absorbance. Compared with the untreated right elytra, those backed by Indian ink were deeper and brighter in colour.

Although many Zygoptera have abdomens with structural colour, relatively few have structurally coloured wings. Most are found in the superfamily Calopterygoidea, to which *Neurobasis* belongs. Courtship and agonistic behaviour are widespread in this group (Corbet 1999), and there is good evidence that wing colour and pattern contribute significantly in some representatives of most calopterygoid families: *Calopteryx* spp. (Calopterygidae) (Heymer 1972, 1973; Waage 1973; Kumar & Prasad 1977; Ruppell 1989), many Chlorocyphidae and *Euphaea* spp. (Euphaeidae) (Ueda 1992; Orr 1996, 2003; A. G. Orr, personal communication) as well as *Neurobasis*.

Kumar & Prasad (1977) have described the courtship of *N. c. chinensis* in detail. During the reproductive period, a male tries to 'court' females passing through his territory using a non-flickering display of the strikingly coloured hindwings and the coloured abdomen. Males are observed alighting briefly on, or flying close to, water surfaces beneath the overflying females. During this process, only the forewings are used for flying; the hindwings are held motionless and obliquely spread. Together with the coloured abdomen and white terminal abdominal segments, their high-intensity structural colour is not only significantly conspicuous against an insipid brown background of stream or pond water but it is also prominent in front of the multitude of other backgrounds that water and vegetation might provide. Immediately after such a performance the male moves off, flies above and attaches to the female and then attempts coitus. It seems clear that the remarkable green wing iridescence, in the hindwings only, of *N. c. chinensis* is particularly adapted to this kind of courtship display in flight, with the hindwings held still while the forewings deliver the flight strokes.

It is interesting to compare in more detail the structural colours associated with lepidopteran wings and the iridescence described for *N. chinensis*. The optical systems differ in several fundamental ways. The elongate independently stroking wings of Odonata, with their network of veins and many small usually transparent cells, contrast with the more sparsely veined, simultaneously stroking lepidopteran wings, dorsally and ventrally covered with flattened scales, each with a short stalk fitting into its own socket on the wing membrane. A major function of these scales is to provide the insect's colour and patterning, through pigmentation (Nijhout 1991) or structural periodicity (Vukusic *et al.* 2000a). Structurally coloured lepidopteran scales show multilayering of several characteristic designs (Vukusic *et al.* 2000a). In some species, this multilayering is incorporated into discrete ridges that extend the full length of each iridescent scale. In other species, continuous multilayering extends completely across the scale, in some cases further modulated to produce complex optical effects involving colour stimulus synthesis and even polarization rotation (Vukusic *et al.* 2000b). Comparing the multilayering of *N. chinensis* and the continuously multilayering lepidopteran systems highlights two key points. First, lepidopteran systems comprise layers of cuticle and air while *N. chinensis* multilayers consist of layers of cuticle alone. Based on the modelling completed in this work, we see that the refractive index contrast between neighbouring layers is much lower in *N. chinensis* than in lepidopterans, being *ca.* 0.56 for the Lepidoptera and *ca.* 0.20 for *N. chinensis*. Optically, a higher refractive index contrast between neighbouring layers of a system gives higher reflectivity (Land 1972) and a modified spectral character. For example, the near-normal-incidence optical response of the *N. chinensis* system is illustrated in figure 3a. If the refractive index contrast between layers was increased to 0.56, to match that of most lepidopteran systems, not only would the peak reflected wavelength be reduced to 420 nm, but also the intensity of this reflectivity would be increased from 30% to *ca.* 80%. This would occur even under circumstances of unchanged layer optical absorption and thickness. Second, iridescent lepidopteran scales lie on a wing membrane beneath which lies an additional double layer of usually brown or dark scales (R. I. Vane-Wright, personal communication). In *N. chinensis* several optically absorbing layers close to the ventral wing surface form a highly absorbing background; in the Lepidoptera the iridescent scales generally show little, if any, optical absorption. In a few cases, such scale absorption is limited to the lower part of the iridescent scale (Vukusic *et al.* 2001); in several others, it is believed to be distributed throughout the scale (Vukusic *et al.* 1999). Substantial optical absorption in Lepidoptera is created by diffuse pigmentation throughout the wing membrane itself and by the brown or dark scales on the reverse side of the wing (Nijhout 1991). Clearly, both kinds of system incorporate a strongly absorbing background, but implemented in different ways. Its presence increases the saturation of the reflected structural colour and augments the quality of the displayed iridescence.

It is also interesting to compare the optical systems of iridescent Coleoptera with that of *N. chinensis*. As with the Lepidoptera, coleopteran optical systems are diverse

(Parker 2000). As an example, we consider the type of multilayer associated with many green and blue iridescent buprestid beetles, such as *Chrysochroa vittata* Fabricius (Mossakowski 1980). This beetle exhibits up to 10 double layers, each of high- and low-refractive-index cuticle, forming a continuous series in the outer part of the exocuticle of body and elytra. There is no evidence of a helicoidal arrangement of cuticle fibrils within these layers and it is not a system that reflects circularly polarized light. Most beetles exhibiting these properties are scarabs (Neville & Caveney 1969).

TEM preparation of a section of one of these elytra produces strong differential image contrast between the layers, similar to that on the dorsal side of the *N. chinensis* wing. The refractive indices of cuticle layers in this beetle, and indeed in the majority of others studied, are believed to lie in the range of 1.40–1.73 (Neville & Caveney 1969; Mossakowski 1980; Parker *et al.* 1998). However, these values have not been definitively verified. Several basic models for beetles' multilayer systems suggest that a refractive index contrast of 0.05–0.30 between neighbouring layers appears to fit some experimental data. None of these models, however, incorporates optical absorption, and such absorption is present, caused by a dark underlayer, generally found in the inner part of the exocuticle.

Multilayer systems are not found on the membranous hindwings of beetles. However, those on the elytra and body are clearly much more similar to those of *N. chinensis* hindwings than are lepidopteran systems. In these beetles and in *N. chinensis*, the optical properties of the cuticle have been controlled in such a way that conspicuous iridescent reflection is achieved with relatively low refractive index contrast and probably relatively little structural differentiation between layers. Visibility is further enhanced by underlying absorption. While the physiological and ecological pressures on beetles and Odonata are significantly different, they appear to have reached a marked convergence in the physical structures underpinning their iridescence.

Finally, the ultrastructure of the zygopteran wing membrane cuticle would repay further study. With a typical thickness of *ca.* 1.7 μm for the double layer, the *Neurobasis* hindwing membrane is close to the value usually quoted for the thickness of the epicuticular layer alone. Smith *et al.* (2000) found similar membrane thicknesses in the hindwings of the desert locust, *Schistocerca gregaria* Forskål. Since electron microscopy revealed no structural differentiation and no chitin residues were detected in pyrolysis followed by spectrophotometry, they concluded that the locust membrane probably consists of two layers of epicuticle (which is not known to contain chitin) back to back, and that the considerable differences in mechanical properties that they found within the wing had a chemical rather than an ultrastructural basis. However, the layered appearance of the dorsal membrane component of *Neurobasis* wings suggests the presence of procuticle, which is characteristically layered and contains optically evident chitin fibrillae. This discovery and that of Gorb *et al.* (2000) show that considerable structural complexity can exist in very thin cuticles. Indeed, since the entire body cuticle of the tiniest insects may be within

this thickness range, the structure of very thin insect cuticles needs far more investigation.

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