

Blue integumentary structural colours in dragonflies (Odonata) are not produced by incoherent Tyndall scattering

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Summary

For nearly 80 years, the non-iridescent, blue, integumentary structural colours of dragonflies and damselflies (Odonata) have been attributed to incoherent Tyndall or Rayleigh scattering. We investigated the production of the integumentary structural colours of a damselfly – the familiar bluet, *Enallagma civile* (Coenagrionidae) – and a dragonfly – the common green darner, *Anax junius* (Aeshnidae) – using fibre optic spectrophotometry and transmission electron microscopy (TEM). The reflectance spectra of both species showed discrete reflectance peaks of ~30% reflectance at 475 and 460 nm, respectively. These structural colours are produced by light scattering from closely packed arrays of spheres in the endoplasmic reticulum of box-shaped epidermal pigment cells underlying the cuticle. The observed reflectance spectra do not conform to the inverse fourth power relationship predicted for Tyndall/Rayleigh scattering. Two-dimensional (2-D) Fourier analysis of the TEM images of the colour-producing arrays reveals ring-shaped distributions of Fourier power at intermediate spatial frequencies, documenting a quasiordered nanostructure. The nanostructured Fourier power spectra

falsify the assumption of spatial independence of scatterers that is required for incoherent scattering. Radial averages of the Fourier power spectrum indicate that the spheres are substantially nanostructured at the appropriate spatial scale to produce visible colours by coherent scattering. However, the spatial periodicity of the arrays is apparently too large to produce the observed colour by coherent scattering. The nanospheres could have expanded substantially (~50%) during preparation for TEM. Alternatively, coherent light scattering could be occurring *both* from the surfaces and from structures at the centre of the spheres. These arrays of colour-producing spheres within pigment cells have convergently evolved at least 11–14 times independently within the Odonata. Structural colouration from arrays in living cells has also fostered the convergent evolution of temperature-dependent colour change in numerous odonate lineages.

Key words: structural colour, dragonfly, damselfly, coherent scattering, Tyndall scattering, Rayleigh scattering, pigment cells, *Enallagma civile*, *Anax junius*.

Introduction

The colours of organisms are produced by molecular pigments and by interactions of light with nanostructures in the integument. The latter form of colouration, referred to as structural colour, is an important component of the phenotype of a diversity of animals (Fox, 1976; Parker, 1999; Srinivasarao, 1999; Prum and Torres, 2003a) and even some plants (Lee, 1997).

Structural colours are common and broadly distributed in insects (Fox, 1976; Parker, 1999). Most insect structural colours are produced by periodic nanostructures in the cuticle (Parker, 1999) or the scales of the wings (Ghiradella, 1991). Exceptionally, the blue integumentary structural colours of dragonflies and damselflies (Odonata) are produced by spherical nanostructures within living epidermal cells that lie below the cuticle (Vernon et al., 1974; Charles and Robinson, 1981).

For nearly 80 years, since Mason (1926), the non-iridescent structural blue colours of dragonflies and damselflies (Odonata) have been universally hypothesized to be produced by incoherent Tyndall or Rayleigh scattering (Vernon et al., 1974; Fox, 1976; Charles and Robinson, 1981; Sternberg, 1996; Corbet, 1999; Parker, 1999; Srinivasarao, 1999). Here, we report an investigation of the anatomy and physics of structural colour production in two distantly related odonates, in which we test whether these colours are produced by incoherent or coherent scattering.

Incoherent and coherent scattering

Although mechanisms of structural colour production are often described as quite diverse (Fox, 1976; Parker, 1999; Srinivasarao, 1999), almost all of them can be productively

understood as aspects of light scattering (Prum and Torres, 2003a,b). As light propagates through a medium, substantial scattering of light waves occurs at the interfaces of materials with different refractive indices. Light scattering from multiple objects or interfaces can be classified as either incoherent or coherent (Prum and Torres, 2003a,b).

Tyndall and Rayleigh scattering refer to incoherent light scattering by particles smaller than the wavelengths of visible light. Light scattering is incoherent when the objects scattering the light are randomly distributed over the spatial scale of visible light wavelengths. As a consequence of the spatial independence of scatterers, scattered light waves will be random in phase, and the phase relationships among scattered light waves can be ignored. Thus, incoherent scattering can be described by the properties of the individual scatterers alone – size, refractive index of the scatterer and refractive index of the surrounding medium. Traditionally, Tyndall scattering has been used to refer to incoherent scattering by small particles near the size of visible wavelengths, whereas Rayleigh scattering is used to refer to incoherent scattering by all small particles down to the size of a molecule (Young, 1982). Thus, Rayleigh scattering includes Tyndall scattering. Tyndall scattering has been more frequently used to refer to incoherent scattering in organisms because the objects in organisms that are hypothesized to scatter light incoherently are similar in size to wavelengths of visible light and are much larger than individual molecules (e.g. Huxley, 1975; Fox, 1976). However, we prefer to use Rayleigh scattering for all incoherent scattering in organisms because most of the testable predictions about incoherent small particle scattering are derived from Lord Rayleigh's work (Young, 1982). For example, Rayleigh predicted that the magnitude of light scattering is inversely proportional to the fourth power of the wavelength, producing blue colours, or ultraviolet hues (Bohren, 1987). Because the phases of each of the incoherently scattered light waves are independent of one another, incoherently scattered colours do not exhibit iridescence, or strong changes in hue, with angle of observation or illumination.

Coherent light scattering occurs when spatial periodicity in the distribution of the scattering objects results in nonrandom phase relationships among scattered waves (Prum and Torres, 2003a). Physical models of coherent scattering describe the colour produced in terms of the differential reinforcement or interference among the scattered light waves from multiple scattering interfaces. Coherent scattering from different structural classes of nanostructures has been described as a variety of different mechanisms including constructive interference, reinforcement, diffraction and thin-film interference, but all these phenomena share the common physical mechanism of coherent scattering.

Coherently scattering nanostructures with laminar or crystal-like structures frequently exhibit iridescence. Recently, however, we have identified a new class of coherently scattering nanostructures that do not produce prominent iridescence, which we have termed quasiordered arrays (Prum

et al., 1998, 1999a,b, 2003; Prum and Torres, 2003a,b, 2004). Quasiordered arrays have periodic spatial distribution of light-scattering interfaces at the spatial scale of nearest neighbours, but they lack laminar or crystal-like periodicity at larger spatial scales. Recent photonic analyses of two-dimensional (2-D) quasiordered (= amorphous or disordered) photonic crystals of parallel fibres demonstrate that quasiordered arrays can exhibit a complete photonic band gap – a range of light frequencies that cannot be transmitted in any direction through the material (Jin et al., 2001). Since photonic band gaps in light transmission are a consequence of coherent back scattering of these untransmissible frequencies (Joannopoulos et al., 1995), these analyses demonstrate independently that quasiordered arrays are capable of coherently scattering light.

Mason (1923, 1926) established the first set of criteria for identifying incoherent Tyndall scattering in biological tissues: (1) variation in refractive index, (2) particles smaller than 600 nm, (3) scattered light blue and transmitted light red, (4) depth or shade of blue dependent on particle size (larger produces whiter), (5) scattered light polarized in plane normal to plane of incidence (but dependent on particle size) and (6) intensity inversely proportional to the fourth power of the wavelength. Mason's traditional criteria for identifying incoherent Tyndall scattering do not include an examination of the critical assumption of spatial independence of scatterers. Furthermore, Mason (1923, 1926) did not appreciate the potential of quasiordered arrays to produce non-iridescent structural colours by coherent scattering. Consequently, since Mason (1923, 1926), iridescence has frequently been cited as a defining feature of coherent scattering (interference, etc.), and many non-iridescent blue structural colours have been indiscriminately attributed to incoherent Tyndall, Rayleigh scattering (Fox, 1976; Herring, 1994; Parker, 1999; Srinivasarao, 1999).

In previous research, we have tested and rejected the traditional incoherent scattering explanations for the production of non-iridescent structural colours in avian feather barbs (Prum et al., 1998, 1999b, 2003), avian skin (Prum et al., 1999a; Prum and Torres, 2003b) and mammalian skin (Prum and Torres, 2004). We know of no instances in which a biological structural colour attributed to incoherent Rayleigh or Tyndall scattering has been critically tested by showing both the spatial independence of the scatterers and the congruence between the reflectance spectrum and the inverse fourth power prediction of Rayleigh.

Odonate structural colour

Odonates exhibit a variety of red, yellow, brown and black pigmentary colours and a variety of structural colours, including pruinescence (e.g. many libellulids), iridescence (e.g. *Calopteryx maculata*) and common non-iridescent blue colours (Fig. 1; Gorb, 1995; Corbet, 1999; Silsby, 2001). The blue integumentary structural colours of odonates were attributed to incoherent Tyndall scattering by Mason (1926) after examination of an *Enallagma* damselfly (Coenagrionidae). Mason (1926) did not measure whether the

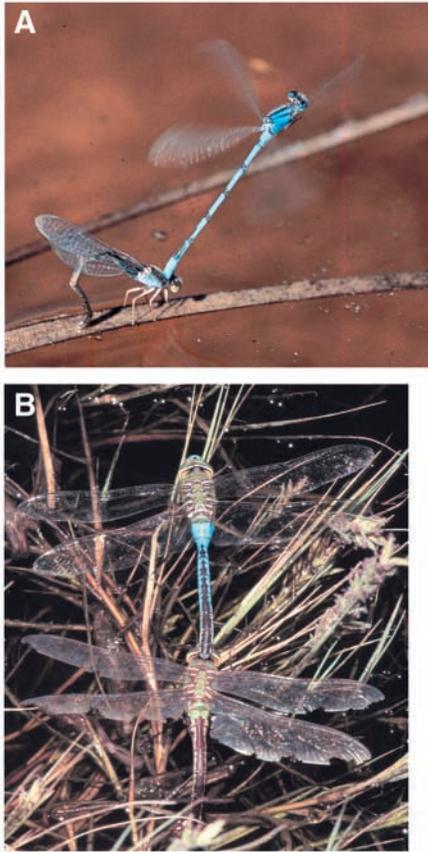


Fig. 1. Mating pairs of two odonates with non-iridescent, blue, integumentary structural colouration. (A) The familiar bluet, *Enallagma civile* (Coenagrionidae). The male displays structural blue colour on the abdominal and thoracic segments. In this photo, the female integument is covered with sediment from muddy water where eggs are being laid. (B) The common green darner, *Anax junius* (Aeshnidae). The male shows blue structural colour on abdominal segments, but the female is entirely green. Photos by Roy J. Beckemeyer.

reflectance spectra was congruent with the inverse fourth power prediction of Rayleigh but he did note that the blue structural colour of *Enallagma* appeared to lack the polarization predicted by Tyndall scattering. Despite the lack of critical support, Mason (1926) concluded that the blue colour was produced by Tyndall scattering by “fine particles enclosed in a chitinous protein”. The Tyndall scattering hypothesis has been cited without question, without exception and without testing for the past seven decades (Vernon et al., 1974; Fox, 1976; Charles and Robinson, 1981; Sternberg, 1996; Corbet, 1999; Parker, 1999; Srinivasarao, 1999).

The integumentary nanostructures that produce these structural colours were first described by Vernon et al. (1974) in a phylogenetically broad sample of blue Australian odonates including aeshnids, amphipterygids and coenagrionids. Vernon et al. (1974) documented that the structural colours are produced by light scattering from spherical objects that are closely but irregularly packed within integumentary pigment

cells immediately below the cuticle. Apparently, the spatial distribution of the light-scattering spheres met with the general expectations for an incoherently scattering array, because the description of the anatomy of the structurally coloured cells did not lead to a reevaluation of the Tyndall scattering hypothesis. Subsequently, Charles and Robinson (1981) used scanning electron microscopy to describe the same anatomical structures in a North American damselfly, the familiar bluet, *Enallagma civile* (Coenagrionidae). Neither Vernon et al. (1974) nor Charles and Robinson (1981) identified the material within the nanospheres. Even though the colour-producing nanostructures are produced by pigment cells and may be composed of pigment molecules, the colours produced are still structural. Because many pigments also have high refractive indices, nanostructured arrays of pigment granules are often involved in structural colour production: e.g. pterines and purines in vertebrate iridophores, and melanosomes in avian feather barbules. Vernon et al. (1974) referred to the colour-producing cells as chromatophores, a term that is usually reserved for pigment cells involved in physiological colour changes. Here, we refer to these cells more generally as pigment cells, since many species with this form of structural colouration do not exhibit physiological colour change.

Here, we examine the anatomy and physics of the non-iridescent blue structural colours of two distantly related North American odonates, a damselfly and a dragonfly: the familiar bluet, *Enallagma civile* (Coenagrionidae, Zygoptera; Fig. 1A), and the common green darner, *Anax junius* (Aeshnidae, Anisoptera; Fig. 1B). We used spectrophotometry to measure the reflectance spectra of the integument of these odonates and transmission electron microscopy (TEM) to document the anatomy of the cuticle and structural colour-producing cells. We then used 2-D Fourier analysis of the TEM micrographs to characterize the spatial periodicity of the colour-producing nanostructures, to test the spatial independence of scatterers and to predict the reflectance spectrum produced by coherent scattering from these nanostructures (Prum and Torres, 2003a). Our results falsify the spatial independence of the light scatterers that is assumed by incoherent scattering mechanisms and provide some support for the conclusion that the array of light-scattering spheres is appropriately nanostructured to produce visible colours by coherent scattering.

Materials and methods

Species sampled and microscopy

Adult (post-teneral) specimens of *Enallagma civile* (Hagen) (Coenagrionidae) and male *Anax junius* (Drury) (Aeshnidae) were collected in Lawrence, KS, USA in August 2002 and June 2003 by R.O.P. Specimens of *Enallagma* and *Anax* were fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde) for 12 h, stored in cacodylate buffer and prepared for TEM by a standard protocol (Prum and Torres, 2003b). These specimens were sufficiently preserved to reveal the general anatomy of the cuticle and epidermal pigment cells, but they showed extensive degradation of the colour-producing

nanospheres within the pigment cells. To improve preservation of the nanostructure of the light-scattering spheres, a subsequent sample of four *Enallagma civile* were fixed and embedded following the rapid method of Hayat and Giaquinta (1970). Specimens were sectioned (~100 nm thick) with a diamond knife and viewed with a JEOL EXII transmission electron microscope. Digital micrographs were taken with a Soft-Imaging Megaview II CCD camera (1024×1200 pixels).

Reflectance spectra

Reflectance spectra of living *Enallagma* and *Anax* were measured with an Ocean Optics USB2000 fibre optic spectrophotometer and Dell laptop computer. Reflectance was measured with normal incident light at 6 mm distance from a 3 mm² patch of the integument. The colour of preserved specimens changed rapidly to a deep brown or black with no measurable hue.

2-D Fourier analysis

Coherent scattering of visible wavelengths is a consequence of nanoscale spatial periodicity in refractive index of a tissue. Following a theory of corneal transparency by Benedek (1971), we have developed a method of using the discrete 2-D Fourier transform to analyze the periodicity and optical properties of structural coloured tissue and to predict its reflectance spectrum due to coherent scattering (Prum et al., 1998, 1999a,b, 2003; Prum and Torres, 2003a,b).

The digital TEM micrographs of the rapidly fixed specimens of *Enallagma civile* were analyzed using the matrix algebra program MATLAB (version 6.2; www.mathworks.com) on a Macintosh G4 computer. The scale of each image (nm pixel⁻¹) was calculated from the number of pixels in the scale bar of the micrograph. A 1024 pixels² portion of each array was selected from each image for analysis. Because the molecular composition of the colour-producing nanospheres is unknown, we could not calculate an average refractive index of the nanostructure based on the frequency distribution of its components as in our previous applications of the method. However, we estimate the average refractive index of the material within the spheres necessary to produce congruence with the observed reflectance spectrum.

The Fourier transform was calculated with the 2-D fast Fourier transform (FFT2) algorithm (Briggs and Henson, 1995). We then calculated the 2-D Fourier power spectrum, or the distribution of the squares of the Fourier coefficients. The 2-D Fourier power spectrum resolves the spatial variation in refractive index in the tissue into its periodic components in any direction from a given point. The 2-D Fourier power spectrum was expressed in spatial frequency (nm⁻¹) by dividing the initial spatial frequency values by the length of the matrix (pixels in the matrix × nm pixel⁻¹).

We calculated radial averages of the power spectra using 100 spatial frequency bins, or annuli, between 0 and 0.02 nm⁻¹ and expressed them in terms of % total Fourier power. Composite radial averages were calculated from a sample of power spectra from five TEM images of the best preserved

Enallagma sections to provide an indication of the predominant spatial frequency of variation in refractive index in the tissue over all directions.

We produced predicted reflectance spectra for *Enallagma civile* based on the 2-D Fourier power spectra of the TEM micrographs, the image scales, estimated values of the average refractive index of the material and estimating the expansion of the arrays during preservation. First, a radial average of the % power was calculated for concentric radial bins, or annuli, of the power spectrum corresponding to fifty 10 nm-wide wavelength intervals between 300 and 800 nm (covering the light spectrum visible to insects). The radial average power values were expressed in % visible Fourier power by normalizing the total power values across all potentially visible spatial frequencies (i.e. potentially scattering light between 300 and 800 nm) to 1. The inverse of the spatial frequency averages for each wavelength were then multiplied by twice the estimated average refractive index of the medium and expressed in terms of wavelength (nm). A composite predicted reflectance spectrum was produced by averaging the normalized predicted spectra from a sample of five TEM images of *Enallagma civile*. Values of the average refractive index and % expansion during tissue preparation were estimated by producing a reflectance spectrum congruent with the observed reflectance peaks.

Phylogenetic analysis

The distribution of non-iridescent blue integumentary structural colour was estimated from a review of odonate diversity (by J.A.C.) and standard references (Corbet, 1999; Silsby, 2001). The phylogenetic pattern in the evolution of integumentary blue was estimated using a recent and comprehensive phylogeny of the odonates (Rehn, 2003). The estimated number of evolutionary events to describe that diversity was calculated using MacClade 4 (Maddison and Maddison, 2000).

Results

Colour

In *Enallagma civile*, a non-iridescent, blue structural colour is distributed broadly over the dorsal and lateral surfaces of the thoracic and abdominal segments. The dorsal surface of the thorax and the anteriodorsal surfaces of the abdominal segments are covered by black patches or stripes (Fig. 1A). The blue integument patches are slightly larger in males than females. In male *Anax junius*, structural blue integumentary colour is restricted to the six anterior abdominal segments. The two most anterior segments are entirely blue on their dorsal and lateral surfaces, but the next four have prominent dorsal and lateral black stripes (Fig. 1B). All other portions of the cuticle are green (Fig. 1B). Most female *Anax junius* are entirely green.

The reflectance spectra of the blue portions of the integument of both *Enallagma civile* and male *Anax junius* revealed discrete peaks of 475 nm and 460 nm, respectively

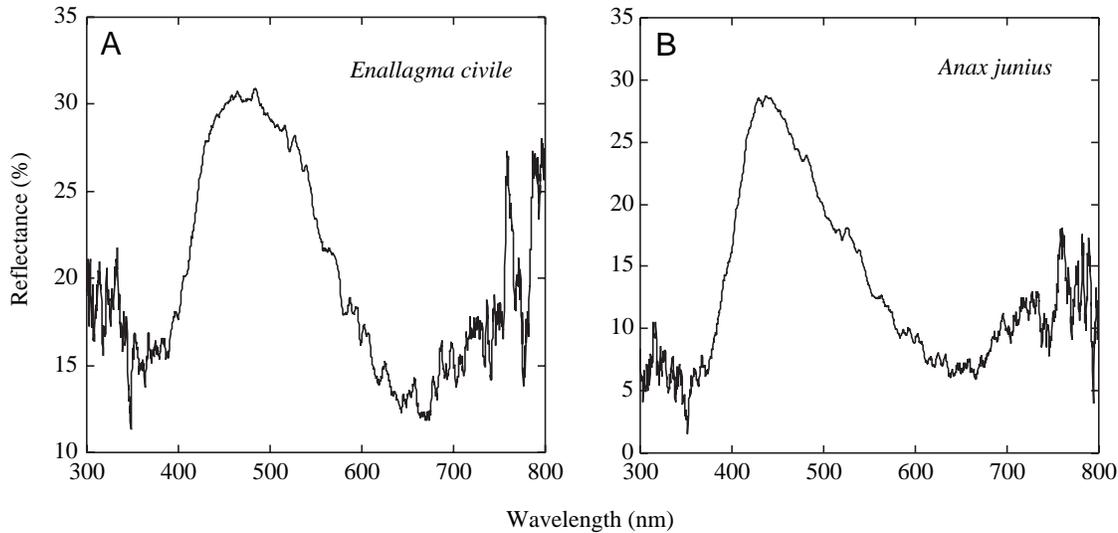


Fig. 2. Reflectance spectra of blue integument of (A) the familiar bluett, *Enallagma civile* (Coenagrionidae), and (B) the common green darner, *Anax junius* (Aeshnidae).

(Fig. 2). The reflectance spectra of both species lacked the inverse fourth power relationship predicted for incoherent Rayleigh scattering.

Anatomy

Transmission electron micrographs of the integument of *Enallagma civile* and *Anax junius* were entirely consistent with previous anatomical descriptions of other blue odonates (Vernon et al., 1974; Charles and Robinson, 1981). In both

Enallagma and *Anax*, the cuticle consists of chitin $\sim 5\text{--}8\ \mu\text{m}$ thick (Fig. 3A). Immediately below the cuticle is a layer of box-shaped pigment cells (Fig. 3A,B). In *Enallagma*, the light-scattering nanospheres filled the distal two-thirds of the pigment cells, creating a solid layer $\sim 5\text{--}10\ \mu\text{m}$ thick, and the ommochrome spheres were restricted to the basal third of the cells (Fig. 3A,B). Vernon et al. (1974) identified these ommochrome pigments as xanthommatin and dihydroxanthommatin in the pigment cells of a broad diversity of blue odonates. Occasional cells showed cell nuclei and other cellular organelles restricted to the basal third of the cell volume (Fig. 3A). In all blue *Anax junius* specimens observed, the ommochrome pigment granules were restricted to the basal portions of the integumentary pigment cells, in the state

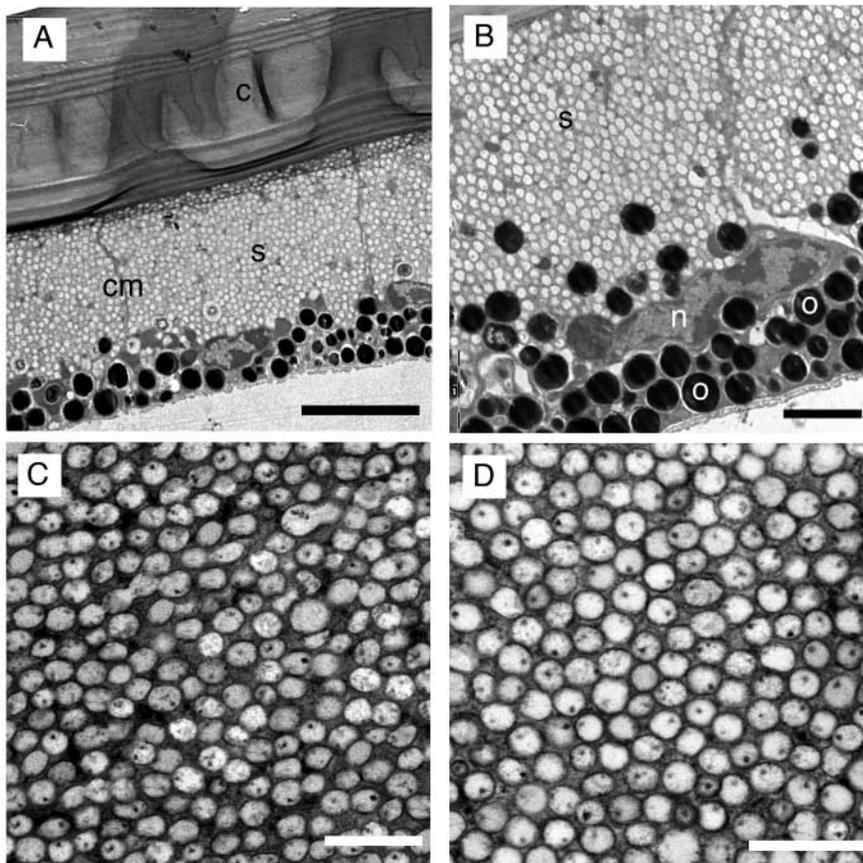


Fig. 3. Transmission electron micrographs of the integument of the familiar bluett, *Enallagma civile* (Coenagrionidae), prepared by rapid fixation (Hayat and Giaquinta, 1970). (A) Cuticle and epidermal pigment cells at $6000\times$ magnification. Scale bar, $5\ \mu\text{m}$. (B) Epidermal pigment cell showing the light-scattering nanospheres and ommochrome pigment at $10\ 000\times$ magnification. Scale bar, $2\ \mu\text{m}$. (C) The light-scattering nanospheres at $50\ 000\times$ magnification. Scale bar, $500\ \text{nm}$. Some neighbouring spheres appear to be 'budding' off each other within the same pocket of endoplasmic reticulum. (D) Light-scattering nanospheres at $50\ 000\times$ magnification, showing the dark internal spot within many spheres. Scale bar, $500\ \text{nm}$. Abbreviations: c, cuticle; cm, cell membranes of neighbouring pigment cells; n, nucleus; o, ommochrome granules; s, light-scattering spheres.

that produces structural blue colour and identical to *Enallagma*. (During temperature-dependent colour change in *Anax*, these ommochrome granules are capable of migrating upwards amongst the light-scattering nanospheres; see Discussion.)

The light-scattering nanospheres in the epidermal pigment cells were ~200–300 nm in diameter and packed immediately adjacent to one another. As previously reported (Vernon et al., 1974; Charles and Robinson, 1981), the light-scattering spheres were enclosed within pockets of the endoplasmic reticulum. Like Vernon et al. (1974), we observed occasional 'budding' of these spherical bodies from one another within a single pocket of the endoplasmic reticulum (Fig. 3C). Many spheres showed a darkly staining central spot (Fig. 3C,D). In the less well preserved sections, spheres showed dark lines radiating from the centre of the sphere (Fig. 4B,C; Vernon et al., 1974). The variation in position of the dark spots within the spheres indicates that each sphere may have a linear structure, perhaps a fold of endoplasmic reticulum, that runs through the centre of the sphere from pole to pole (Fig. 3C,D). Vernon et al. (1974) did not identify the material within these spheres but suggested that the spheres could be either crystal or liquid filled. The radiating structures within the degrading spheres could indicate a composition of biocrystalline or proteinaceous materials. The nanospheres differ from the ommochrome granules in being exclusively spherical and highly consistent in diameter. Further research is required to

describe the composition, structure and development of these nanospheres.

The green portions of the integument in *Anax junius* entirely lack underlying epidermal pigment cells. Rather, the green hue appears to be produced by a green pigment within the cuticle itself.

Fourier analysis

Two-dimensional Fourier analysis of TEM micrographs of the light-scattering nanospheres produced power spectra with a ring-shaped distribution of peak Fourier power at intermediate spatial frequencies (Fig. 5A). The ring-shaped Fourier power distribution shows that the arrays are highly nanostructured at intermediate spatial frequencies and equivalently periodic in all directions. The composite radial average power spectra from a sample of five micrographs of *Enallagma civile* shows a peak spatial frequency of 0.00307 nm^{-1} (Fig. 5B). This peak spatial frequency is of the same order of magnitude as the wavelengths of visible light waves, which indicates substantial nanostructure at the appropriate spatial scale to produce visible colours (Fig. 5B). This spatial frequency corresponds to an average centre-to-centre distance between neighbouring spheres of 322 nm (Prum et al., 1998, 1999a; Prum and Torres, 2003a,b). This substantial nanostructure (Fig. 5A,B) falsifies a fundamental and critical assumption of all incoherent scattering models, including Tyndall, Rayleigh and Mie scattering.

Although the arrays are appropriately nanostructured to produce visible light by coherent scattering, predicting the peak hue due to coherent scattering from the arrays of nanospheres in *Enallagma* is subject to substantial error. Assuming a minimal average refractive index in the cells of 1.35 (typical of cellular cytoplasm), the 2-D Fourier power spectra of *Enallagma civile* predict a peak reflectance of 796 nm, which is at the long wavelength extreme of

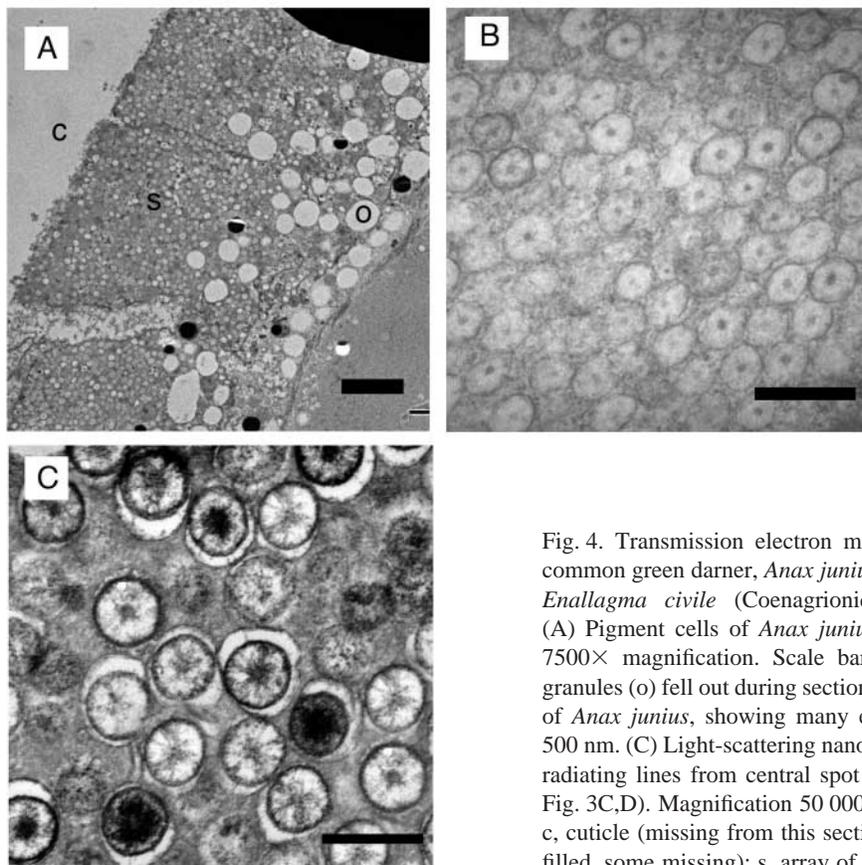


Fig. 4. Transmission electron micrographs of the integument of the common green darner, *Anax junius* (Aeshnidae), and the familiar bluett, *Enallagma civile* (Coenagrionidae), prepared with 12 h fixation. (A) Pigment cells of *Anax junius* with superficial cuticle missing at 7500 \times magnification. Scale bar, 2 μm . Most of the ommochrome granules (o) fell out during sectioning. (B) Light-scattering nanospheres of *Anax junius*, showing many entirely degraded spheres. Scale bar, 500 nm. (C) Light-scattering nanospheres of *Enallagma civile*, showing radiating lines from central spot (compare with rapidly fixed cells in Fig. 3C,D). Magnification 50 000 \times , scale bar, 500 nm. Abbreviations: c, cuticle (missing from this section); o, ommochrome granules (some filled, some missing); s, array of light-scattering spheres.

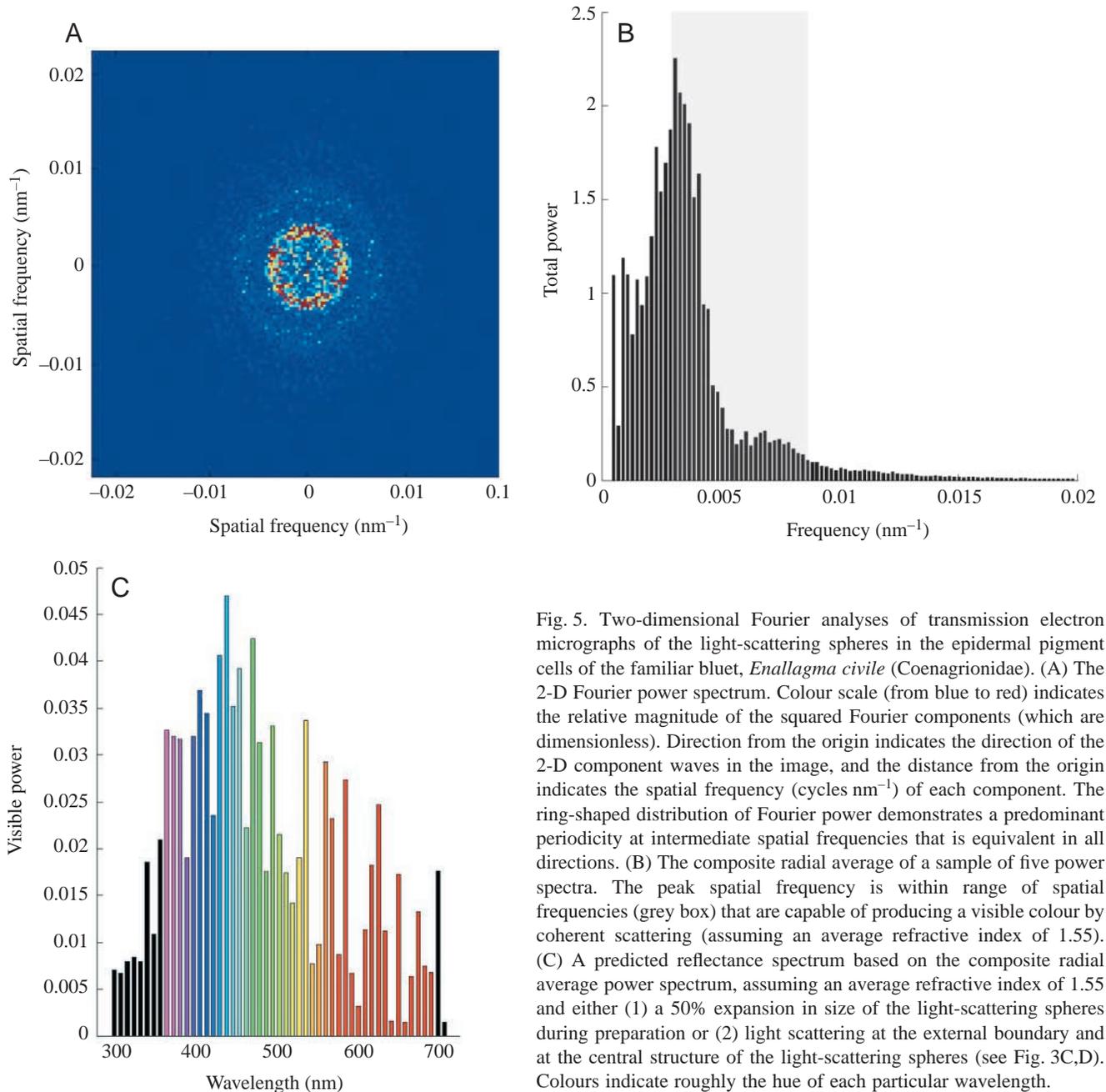


Fig. 5. Two-dimensional Fourier analyses of transmission electron micrographs of the light-scattering spheres in the epidermal pigment cells of the familiar bluett, *Enallagma civile* (Coenagrionidae). (A) The 2-D Fourier power spectrum. Colour scale (from blue to red) indicates the relative magnitude of the squared Fourier components (which are dimensionless). Direction from the origin indicates the direction of the 2-D component waves in the image, and the distance from the origin indicates the spatial frequency (cycles nm⁻¹) of each component. The ring-shaped distribution of Fourier power demonstrates a predominant periodicity at intermediate spatial frequencies that is equivalent in all directions. (B) The composite radial average of a sample of five power spectra. The peak spatial frequency is within range of spatial frequencies (grey box) that are capable of producing a visible colour by coherent scattering (assuming an average refractive index of 1.55). (C) A predicted reflectance spectrum based on the composite radial average power spectrum, assuming an average refractive index of 1.55 and either (1) a 50% expansion in size of the light-scattering spheres during preparation or (2) light scattering at the external boundary and at the central structure of the light-scattering spheres (see Fig. 3C,D). Colours indicate roughly the hue of each particular wavelength.

the visible spectrum and ~40% longer than the wavelength of the observed reflectance peak. With a more realistic average refractive index of 1.55 (a value typical of protein), the nanostructure would have had to expand by ~50% during preparation for TEM and imaging to produce the observed peak hue of 475 nm (Fig. 5C). Consequently, the nanospheres in life could have been distributed with an average centre-to-centre distance of 153 nm (see Discussion for problems with this explanation).

An alternative explanation may be that substantial light scattering occurs at both the periphery of the spherical nanostructures and at the dark (i.e. electron-dense) structures

within the spheres. Many of the light-scattering spheres show a conspicuous dark spot (Figs 3, 4). This spot could be an indication of a molecular structure that organizes the material within the spheres and perhaps contributes to their size. If this internal structure has a distinct refractive index (e.g. a fold of endoplasmic reticular membrane that runs from pole to pole within the spheres), then light scattering could occur at both the peripheral boundaries and the centre of the spheres. This would essentially double the fundamental spatial frequency of variation in refractive index and produce exact congruence with the observed reflectance spectrum, assuming an average refractive index of 1.55 (Fig. 5C).

Table 1. Provisional list of odonate clades with noniridescent, blue, integumentary structural colour (Corbet, 1999; Silsby, 2001)

Suborder	Family	Subfamily	Example	
Anisoptera	Aeshnidae	Aeshinae	<i>Aeshna</i> , Anax	
		Brachytroninae	<i>Brachytron praetense</i>	
Zygoptera	Libellulidae		<i>Acisoma panorpoides</i>	
	Amphipterygidae	Philoganginae	<i>Philoganga</i>	
		Diphlebiinae	Diphlebia	
		Calopterygidae	Caliphaeinae	<i>Caliphaea thailandica</i>
		Chlorocyphidae		<i>Platycypha</i>
		Dicteriidae		<i>Heliocharis amazona</i>
		Polythoridae		<i>Cora marina</i>
		Lestidae	Lestinae	<i>Orolestes octomaculatus</i>
	Megapodagrionidae	Sympecmatinae	Austrolestes	
		Argiolestinae	<i>Argiolestes ochraceus</i>	
		Megapodagrioninae	<i>Megapodagrion</i>	
	Perilestidae		Various Old World genera	
	Coenagrionidae	Argiinae	<i>Argia vivida</i>	
		Coenagrioninae	<i>Coenagrion</i> , Enallagma	
Ischnurinae		Ishnura		
Pseudagrioninae		<i>Pseudagrion</i>		
Platycnemeidae		Platycnemidinae	<i>Platycnemis pennipes</i>	
Platystictidae	Calicnemidinae	<i>Risioicnemis serrata</i>		
	Sinostictinae	<i>Sinosticta ogatai</i>		
	Protoneuridae	<i>Ellatoneura glauca</i>		

Some families and subfamilies have been separated into component genera based on the phylogeny of Rehn (2003). Genera in bold type indicate that the anatomy of the light-scattering pigment cells from some species has been described by Vernon et al. (1974), Charles and Robinson (1981) or in the present study.

Phylogenetic analysis

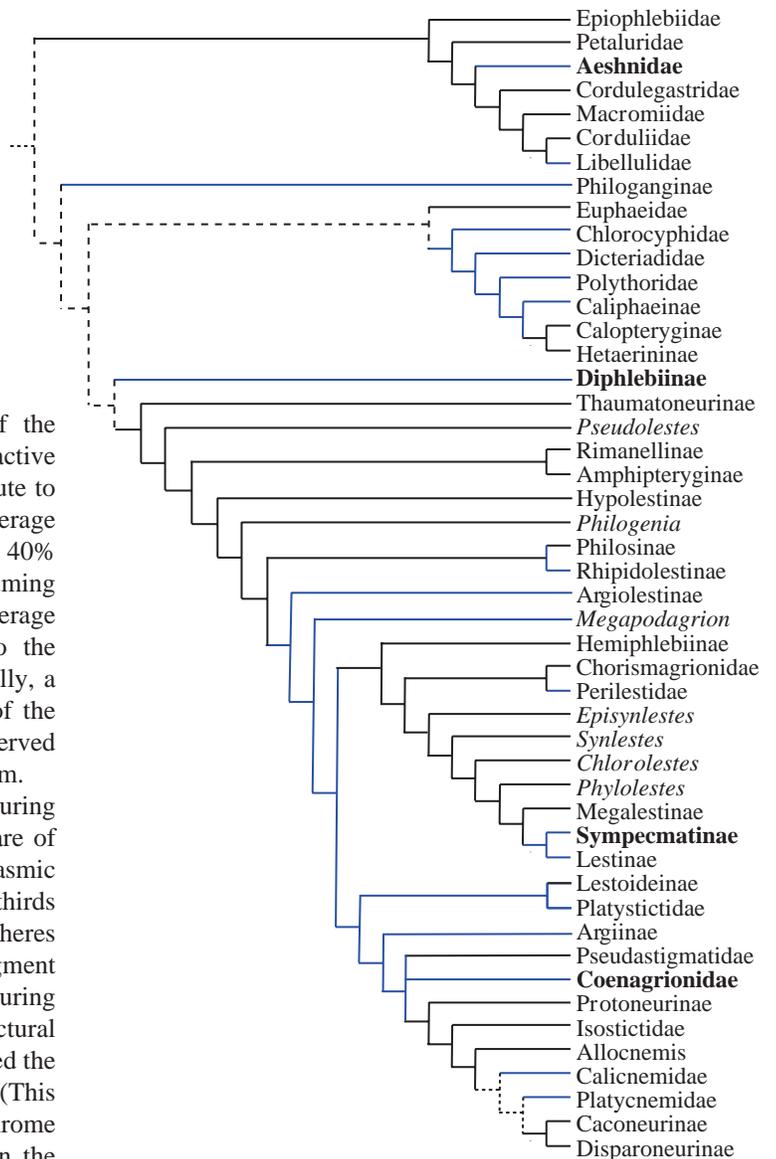
Non-iridescent, blue, integumentary structural colours are known in at least 14 families within all major clades of odonates (Table 1). Based on the most recent and comprehensive phylogenetic hypothesis for the odonates (Rehn, 2003), a parsimony analysis of the phylogenetic distribution of non-iridescent integumentary blue colour indicates that at least 11 historically independent evolutionary derivations (with up to three losses) of this mode of structural colouration have occurred within the odonates (Fig. 6). Given the lack of resolved phylogenies within most odonate families, there may have been additional independent origins and losses within large clades that have a diversity of species with and without structural colour (e.g. Aeshnidae, Chlorocyphidae, Coenagrionidae). Structural colouration from quasiordered nanostructures within pigment cells has evolved numerous times within odonates. Including the results from the present study and those of Vernon et al. (1974) and Charles and Robinson (1981), the anatomy of the structural colour-producing pigment cells of odonates has been described from Aeshnidae, Diphlebiinae, Sympecmatinae and Coenagrionidae. According to this hypothesis of character evolution, each of these four clades represents an evolutionarily independent origin of this form of structural colouration (Fig. 6, boldface). These evolutionarily independent instances show remarkable parallelism in anatomy and structure.

Discussion

The discrete, saturated blue peaks in the reflectance spectra of blue, structurally coloured *Enallagma civile* and *Anax junius* (Fig. 2) falsify a fundamental prediction of the incoherent scattering hypothesis. The intensity of light scattering is not inversely proportional to the fourth power of the wavelength, as predicted by incoherent Tyndall/Rayleigh scattering. The ring-shaped distribution of 2-D Fourier power at intermediate spatial frequencies (Fig. 4) also falsifies a critical assumption of the incoherent scattering models, including Tyndall, Rayleigh and Mie scattering. Because the phases of scattered waves from neighbouring spheres are not random at the spatial scale of wavelengths of visible light, incoherent scattering mechanisms cannot appropriately describe the colour production by these structures. The ring-shaped distributions of Fourier power spectrum document that the colour-producing arrays of light-scattering spheres are equivalently nanostructured in all directions and can be considered quasiordered in spatial organization (Prum et al., 1999a, 2003; Prum and Torres, 2003a,b, 2004). Like other quasiordered arrays, the structural colours produced by these odonate pigment cells are not iridescent.

Although the arrays of light-scattering spheres are appropriately nanostructured to produce visible colours by coherent scattering, there are still substantial uncertainties about the precise mechanism of colour production in these arrays. The predicted reflectance spectrum based on the Fourier

Fig. 6. Phylogenetic reconstruction of the evolution of non-iridescent, blue, integumentary structural colouration in Odonata based on a phylogeny by Rehn (2003). Lineages in blue have evolved this form of structural colour, and lineages in black lack it. Lineages with broken lines have ambiguous character states (either present or absent). Parsimonious reconstruction of the character indicates that blue structural colour evolved at least 11 independent times within Odonata (with 14 total changes). The taxa in bold type include species whose pigment cell structures have been described in the present study, in Vernon et al. (1974) or in Charles and Robinson (1981).



power spectra produces a substantial overestimate of the wavelength of peak reflectance. Uncertainty about refractive indices of the composite materials is unlikely to contribute to this error because even assuming the lowest reasonable average refractive index (1.35 for cytoplasm) produces a 40% overestimate in the peak wavelength. Alternatively, assuming a 50% expansion in the size of the spheres and an average refractive index of 1.55 produces an excellent fit to the observed reflectance spectrum (Fig. 4C). More specifically, a doubling of the peak spatial frequency, or a division of the array into more spheres with half the diameter of the observed spheres, would produce the observed reflectance spectrum.

The 50% expansion in the size of the spheres during preparation is not reasonable. The spherical structures are of unknown composition within pockets of the endoplasmic reticulum of the pigment cells. Because more than two-thirds of the 'volume' of these pigment cells consists of nanospheres that are outside the cellular cytoplasm, these unusual pigment cells may be particularly susceptible to perturbation during fixation. During fixation, all specimens rapidly lost structural colour and turned black, indicating that whatever produced the structural colour was perturbed by fixation itself. (This perturbation was not caused by migration of ommochrome granules, which remained in their basal position within the pigment cells after fixation; Figs 3A,B, 4A.) However, the size of the nanospheres in the fixed pigment cells of *Enallagma*, *Anax* and the other species described by Vernon et al. (1974) did not vary extensively. Furthermore, if the nanospheres expanded ~50% during fixation, then the array would have required twice the number of spheres before preservation to retain the same volume and would double the predominant spatial frequency. Such a big change in nanostructure during fixation would probably create more massive perturbation of pigment cell structure than is documented in these images (Figs 3, 4).

Alternatively, light scattering may be occurring at *both* the periphery of the spheres *and* the centres of the spheres. If the spot within many of the light-scattering spheres is an indication of an internal structure of a different refractive index (e.g. a central fold of endoplasmic reticulum) running through the centre of the spheres, then this structure could also cause light scattering. This would exactly double the peak spatial frequency of variation in refractive index and would produce

a reflectance spectrum that is exactly congruent with the observed reflectance spectrum (Fig. 5C). The variable position of the spot indicates that it is produced by a linear structure running from pole to pole through the nanospheres (e.g. imagine a jar of pitted olives; Fig. 3C,D). Because these polar structures are not oriented in a single direction within the cells (Fig. 3C,D), their orientation is not periodic in any particular planes, and the coherently scattered reflections would lack iridescence or polarization.

The chemical composition, refractive index and structure of the material within the light-scattering spheres in odonate pigment cells needs to be investigated further. Understanding the composition of the spheres would provide a further test of the coherent scattering hypothesis by establishing whether the refractive index of the spheres is high enough to produce the observed magnitude of reflectance. Our investigation gives only indirect evidence on the identity of these spheres. In the

more degraded preparations, the spheres also showed a series of electron-dense lines radiating from the central spot (Fig. 4B,C; also reported by Vernon et al., 1974), but these lines are absent in the better preserved, quickly fixed samples of *Enallagma* (Fig. 3C,D). Vernon et al. (1974) suggested that these radiating lines may indicate a crystalline structure within the spheres, perhaps like spherical versions of the purine crystals of vertebrate iridophores. However, these lines may also be an indication of how material degrades rather than representing its intact structure. Vernon et al. (1974) have further shown that the development of the light-scattering nanospheres in teneral *Austrolestes* dragonflies (Lestidae) is similar to the ommochrome pigment granules. Both are produced by deposition of materials into pockets of the endoplasmic reticulum. Vernon et al. (1974) documented occasional anomalous or chimeric structures that were developmentally and anatomically intermediate between typical ommochrome granules and the light-scattering nanospheres. One section of *Enallagma civile* observed here documents the interconnections between neighbouring light-scattering spheres, indicating that these aggregations of spheres are complexly organized like pearls on a string (Fig. 3C).

Additional research is required to determine the mechanism of colour production in these odonate pigment cells. Most importantly, the biochemical composition and structure of the light-scattering spheres within these odonate pigment cells needs to be documented and it needs to be determined whether they change substantially in size or have an internal light-scattering structure within them.

We have now tested and rejected the traditional hypothesis of incoherent Rayleigh or Tyndall scattering for structural colours from avian feather barbs (Prum et al., 1998, 1999b, 2003), avian skin (Prum et al., 1999a, 2003b), mammalian skin (Prum and Torres, 2004) and the odonate integument (present study). We know of no hypothesized instance of biological colour production by incoherent scattering that has been verified by examining whether the scattering objects are spatially independent and whether the reflectance spectrum conforms to Rayleigh's inverse fourth power law. The historical lack of appreciation of coherent scattering by quasicrystalline arrays has led to the erroneous association of non-iridescent blue colours with incoherent scattering. Additional putative examples of incoherent scattering in various vertebrates and invertebrates need to be tested critically to identify the actual anatomical and physical conditions under which it may occur in organisms.

Evolution of nanostructured arrays

Here, we document that two of the most distantly related odonate clades – aeshnid dragonflies and coenagrionid damselflies – have essentially identical structural colour-producing pigment cells. Previous work by Vernon et al. (1974) further documents that two additional clades of structurally coloured odonates – lestid and amphipterygid damselflies – have identical colour-producing pigment cell anatomy.

A comparative phylogenetic analysis of the distribution of non-iridescent, blue, integumentary structural colour in odonates indicates that this anatomy has evolved 11–14 times independently. This estimate is quite conservative, since the available phylogenies do not yet resolve the genera and species within the several diverse clades that include large numbers of structurally coloured and nonstructurally coloured taxa (e.g. Aeshnidae, Chlorocyphidae, Coenagrionidae). This feature could have evolved convergently additional times within several of these groups.

Like many odonates with non-iridescent, blue, integumentary structural colours (Sternberg, 1996; Corbet, 1999), *Anax junius* is capable of temperature-dependent colour change from blackish, at low body temperatures, to blue at higher body temperatures (Corbet, 1999). Vernon et al. (1974) have shown that temperature-dependent colour change in other odonates occurs by vertical migration of the ommochrome spheres from the basal third of pigment cells during the high-temperature blue condition to become uniformly distributed among the light-scattering spheres of the entire cell area. There, the ommochrome granules absorb sufficient incident light to prevent the creation of the blue structural colour. Congruent with Vernon et al. (1974), our observations indicate that there are no major anatomical differences between pigment cells of odonates that have or lack the capacity for temperature-dependent colour change. Taxa with temperature-dependent colour change appear to differ only in the capacity of the ommochrome pigment vesicles to migrate among the light-scattering nanospheres in physiological response to basal body temperature (Sternberg, 1996). Nothing is known about the mechanism of ommochrome granule motility during physiological colour change but it may be facilitated by cytoskeletal filaments.

Most insects' colours are restricted to the cuticle. The evolution of structural colour production by living pigment cells in the epidermis below the cuticle in numerous independent lineages of odonates has also fostered the convergent evolution of temperature-dependent colour change in many odonate clades (e.g. Aeshnidae, Lestidae, Megapodagrionidae, Coenagrionidae; Sternberg, 1996; Corbet, 1999).

It has been hypothesized that green odonate colours are produced by a combination of structural Tyndall blue and a cuticular pigmentary yellow. However, the green integument of *Anax junius* completely lacks the subcuticular pigment cells that produce the structural colour. This green colour appears to be produced exclusively by a green cuticular pigment.

Odonates are a highly visual order of insects, and the colour of the integument is known to function in mate choice and intraspecific communication in many species (reviewed in Corbet, 1999). It appears likely that the combination of advanced visual perception and epidermal, or subcuticular, pigmentary cells has fostered the independent evolution of structural colouration in many lineages of odonates. The sexual dimorphism of structural colouration in many lineages suggests that sexual selection and mate choice may have

played an important role in the evolution and diversification of these colours. More detailed work is needed on the physical mechanisms of colour signalling, development and heritability of structural colouration in odonates in order to understand whether these colours may function as honest indicators of condition. The temperature dependency of structural colour in many lineages would provide a mechanism for such signalling to evolve, but it is uncertain whether information on such an ephemeral aspect of condition as body temperature would be of utility in mate choice. Future studies on odonate behaviour should incorporate understanding of the physics of structural colouration in investigations of its function and evolution.

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