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Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*

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Animal colouration is typically the product of nanostructures that reflect or scatter light and pigments that absorb it. The interplay between these colour-producing mechanisms may influence the efficacy and potential information content of colour signals, but this notion has received little empirical attention. Wing scales in the male orange sulphur butterfly (*Colias eurytheme*) possess ridges with lamellae that produce a brilliant iridescent ultraviolet (UV) reflectance via thin-film interference. Curiously, these same scales contain pterin pigments that strongly absorb wavelengths below 550 nm. Given that male UV reflectance functions as a sexual signal in *C. eurytheme*, it is paradoxical that pigments in the wing scales are highly UV absorbing. We present spectrophotometric analyses of the wings before and after pterin removal that show that pterins both depress the amplitude of UV iridescence and suppress a diffuse UV reflectance that emanates from the scales. This latter effect enhances the directionality and spectral purity of the iridescence, and increases the signal's chromaticity and potential signal content. Our findings also suggest that pterins amplify the contrast between iridescent UV reflectance and scale background colour as a male's wings move during flight.

Keywords: pterin pigments; structural colour; ultraviolet; thin-film iridescence; *Colias*; colour signal

1. INTRODUCTION

With the exception of bioluminescence, animal colouration is generated through complex interactions between incident light and animal integument. Within the integument (e.g. skin, scales, feathers) colour can be produced either by pigments or physical structures that interact with light (Land 1972; Fox 1976; Herring 1994). Pigments do not produce colours *per se*, but act as filters that selectively absorb some wavelengths while permitting the reflection of others (Bradbury & Vehrencamp 1998). Pigment-based colours tend to be non-directional or diffuse, changing little in appearance with the orientation of incident light or viewer.

In contrast, structural colours arise from light that is reflected from nanoscale structures and can range from diffuse, broadband reflections produced by incoherent scattering (e.g. the blue sky) to narrow bandwidth reflection of high purity and intensity produced by constructive interference. The orderly structures that produce such effects include, for example, thin-film multilayer reflectors (Greenewalt *et al.* 1960; Ghiradella 1989, 1991; Parker *et al.* 1998), surface gratings (Parker 1998; Parker *et al.* 1998), collagen arrays (Prum & Torres 2003, 2004), and nanosphere arrays (Prum *et al.* 2004). Butterflies possess a particularly broad diversity of nanostructural colour production mechanisms (e.g. Ghiradella 1991), with some of the more striking examples including structural 'colour mixing' (Vukusic *et al.* 2000) and three-dimensional photonic crystals (Vukusic & Sambles 2003).

Investigations of colouration as honest signals of quality have focused primarily on pigment-based colouration (Hamilton & Zuk 1982; Lozano 1994; Olsen & Owens 1998; Hill 1999; McGraw & Hill 2000; Grether *et al.* 2001; Blount *et al.* 2003), but structural colours are being studied with increasing frequency from this perspective (Johnsen *et al.* 1998, 2003; Keyser & Hill 1999; McGraw *et al.* 2002; Doucet & Montgomerie 2003; Siefferman & Hill 2003). Most animal colours, however, are generated through a combined effects of these mechanisms. Here we suggest that the combination of pigmentary and structural mechanisms on incident light reflections warrant close attention, because this interplay may be crucially important for the spectral composition of a signal and the information it may convey. Despite being mentioned recently in the literature (Grether *et al.* 2004; Vukusic *et al.* 2004) this idea has only just begun to be addressed empirically (Shawkey & Hill 2005).

The orange sulphur butterfly, *Colias eurytheme*, presents an ideal system for studying the relationship between the optical properties of pigments and physical structures during colour signal production. The dorsal surfaces of this butterfly's wings exhibit a predominantly yellow-orange colour that results from the absorbance properties of pterin pigments deposited in the wing scales during pupation (Watt 1964). Additionally, in males the dorsal yellow-orange area displays a brilliant, iridescent ultraviolet (UV) colouration. This directional UV reflectance is produced by nanostructural lamellae that are arranged like shingles along parallel ridges of specialized scales (Ghiradella 1991). This yellow-orange and UV reflectant area is bordered by a dark brown band of melanin-containing scales.

Past research on *C. eurytheme* and other sulphurs suggests that male UV colouration is important for species

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recognition and sexual identification (Silberglied & Taylor 1978; Rutowski 1977; Rutowski 1985). However, no investigation has evaluated exactly how the pterin pigments shape the spectral properties of the UV reflection. Having pterins in scales that produce an iridescent UV reflectance is especially curious, because several of the pterins found there absorb maximally in the UV (Watt 1964). Thus, the pterins absorb the very UV wavelengths that the specialized nanonstructures reflect. In this study we sought to determine the effects of pterins on the UV reflectance of the wing scales of male *C. eurytheme*, and further, to understand the implications of these effects for the evolutionary design of this colour signal.

2. MATERIAL AND METHODS

(a) *Terminology*

We define 'intensity' (or 'brightness') as the sum of all individual wavelength amplitudes under a spectral curve over a given wavelength distribution. 'Hue' (e.g. red, green) is defined as the most intensely reflected wavelength. 'Chroma', or colour saturation, is a property of the steepness of the slope between the strongest and weakest portions of the spectrum, as well as the width of the peak formed by the strongest wavelengths. 'Reflectance' is the ratio of incident to reflected light and is calculated relative to a near-perfect diffuse reflector (a white standard).

(b) *Specimens*

The butterflies used in this study were reared in the lab from eggs laid by females captured in alfalfa fields in and around Chandler, Arizona in the summer of 2004.

(c) *Pterin extractions*

The effects of pterins on colour production in *C. eurytheme* were evaluated by comparing the spectral properties of wings before and after the extraction of these pigments. Wings from dead males were removed and wetted in 70% isopropyl alcohol until visibly saturated, and then transferred for two minutes into a 1% NH_4OH solution made with reagent grade water. This extraction treatment rendered the wings white in appearance except for their dark melanic markings. Wings were then placed on paper towels to dry and held down by glass microscope slides to keep them flat. Reflectance spectra of the same wings were recorded before and after pterin extraction (see below). To ensure that our alcohol pre-treatment did not affect the reflectance properties of the wings independently of extraction with NH_4OH, reflectance spectra of wings were compared before alcohol immersion, following immersion and drying, and two days following immersion and drying. Comparisons were made using a two-way ANOVA with factors for treatment and spectral segment (see 'statistical comparisons'). As anticipated, a significant effect of spectral segment was detected, but no treatment effect was found for the UV+ or UV− wing orientations (see 'reflectance spectrophotometry'): $F_{3,108}=0.0$, $p=1.0$.

Our extraction procedure was designed to remove all pterins while leaving the UV iridescent structural mechanism unaltered. Capillary electrophoresis was used to confirm that all pterins had been removed by the NH_4OH extraction process. Each of five matched pairs of unextracted and extracted *C. eurytheme* male forewings were placed in 70% isopropyl alcohol until visibly saturated, wicked with paper towel to remove excess alcohol, and then placed in an Eppendorf vial with 200 µl of 0.1 M NaOH under dim light. After 3 min the wings were removed and the vials were kept in a dark ice chest until centrifuged at 12 000 RPM for three minutes to remove scales and other debris from the supernatant. We then loaded 30 µl of supernatant from each sample into a Beckman P/ACE 2050 capillary electrophoresis system that was interfaced with a PC running system GOLD SPECTRUM ANALYSIS software (v.7). A neutrally charged marker (0.1% dimethylformamide) was run as an internal calibration standard along with the wing samples. The identity and relative amounts of pterins in wing extracts were assessed by comparing their retention times and peak size to those of known quantities of purified pterin standards.

(d) *Reflectance spectrophotometry*

To measure reflectance from the orange area of male dorsal wing surfaces, forewings were cut from bodies and mounted on black matte card stock. Mounted male wings were then placed on a universal stage and illuminated in a darkened room by a xenon light source (Ocean Optics PX-2). The illuminant was conducted through a fiber optic cable and presented perpendicular to the wing surface. Wing reflectance was captured with a collimating lens (Ocean Optics 74-UV) having a 1° acceptance angle that was positioned 45° relative to the plane of the wing. Light captured by this lens was transmitted through another fibre optic cable to an Ocean Optics USB2000 spectrometer connected to a laptop computer running OOIBASE32 software. The software was set to obtain spectra that were an average of 20 successive scans at an integration time of 75 ms. A glass slide with a matte white coating of magnesium oxide was used as the reflectance standard. The reflectance spectrum of this standard is flat and nearly 100% across the wavelengths of interest (300–700 nm).

Spectral measurements were obtained from near the centre of the orange area of each dorsal forewing in the UV+ orientation, in which iridescent UV reflectance was most intense, and in a UV− orientation, in which UV reflectance was least intense. To find the precise location of maximum UV reflectance, we placed the wing on a flat universal stage (i.e., pitch, yaw, and roll at 0°) with the wing base pointed toward the azimuth of the collimating lens. We then varied the roll of the universal stage until the intensity of the UV signal was maximized and recorded this spectrum. For the UV− orientation the forewing was turned 180° around the axis perpendicular to the wing surface so the wing base was pointing away from the azimuth of the collector and a spectrum was collected from the same location on the wing.

(e) *Graphical comparison of wing spectra*

Each reflectance spectrum was reduced to a pair of values plotted as a single point in two-dimensional 'colour space' using Endler's (1990) segment classification method. Spectra were partitioned into four, 100 nm-wide colour segments corresponding roughly to UV to violet (300–400 nm; 'UV' wavelengths segment), violet to green (400–500 nm; 'S' or short wavelengths segment), green to orange (500–600; 'M' or medium wavelengths segment), and orange to red (600–700 nm; 'L' or long wavelengths segment). The intensity of each spectral segment was calculated by summing the individual wavelength intensities within each segment (Q_{UV}, Q_S, Q_M, Q_L). The relative intensity of each segment then was calculated by dividing each segment's intensity by

the entire spectrum's intensity ($Q_T = Q_{UV} + Q_S + Q_M + Q_L$). Hence, $UV = Q_{UV}/Q_T$, $S = Q_S/Q_T$, $M = Q_M/Q_T$, $L = Q_L/Q_T$. Subtraction of UV from M and S from L produces two values, X and Y, respectively, that are plotted as a single point in two-dimensional colour space.

Chroma (C) is measured as the distance of the point for a specific colour from the origin of the colour space graph (i.e. $X = 0.0$, $Y = 0.0$), and was calculated as (after Endler 1990)

$$C = \sqrt{X^2 + Y^2}. \quad (2.1)$$

Hue (H) is the angle of a colour score relative to the graph origin. For example, 'red' is located at the top of the graph (see inset in figure 2) and has the coordinates $X = 0.0$, $Y = 1.0$; yellow is 90° clockwise to red ($X = 1.0$, $Y = 0.0$), and so on (after Endler 1990)

$$H = \mathrm{ArcSin}(X)/C. \quad (2.2)$$

Differences in colour contrast (CC) between the UV+ and UV− wing positions before and after pterin extraction were calculated as the Euclidian distance between pairs of colour scores using this equation

$$CC = \sqrt{(X_1 - X_2)^2 + (Y_1 - Y_2)^2}. \quad (2.3)$$

(f) *Statistical comparisons of wing spectra*

Reflectance intensity of the male dorsal forewing was compared separately for UV+ and UV− wing orientations using a two-way ANOVA for treatment (before versus after extraction) and spectrum segments (four segments; see above). *Post hoc* pairwise comparisons were made between treatments (before vs after extraction) within each spectral segment using paired-sample t-tests with sequential Bonferroni correction (Rice 1989). Differences in hue angle and chroma before and after pterin extraction also were examined for the UV+ and UV− wing orientations using a protected paired t-test design.

Intensity contrast between the two wing orientations was compared for the UV wavelengths before and after pterin extraction. Individual wavelength amplitudes first were summed from 300–400 nm independently for the two wing orientations prior to pterin extraction ($n = 20$). The UV− sums then were subtracted from the UV+ sums, leaving 20 before extraction contrast values. The same process was carried out on the extracted specimens, yielding 20 after extraction contrast values. These before and after extraction contrast values were compared with a paired-samples t-test.

Prior to running ANOVAs and t-tests (all tests two-tailed with $\alpha = 0.05$), we confirmed that no data distributions differed significantly from a normal distribution (Komogorov–Smirnov one-sample tests). All statistical procedures were conducted with SPSS (MACINTOSH v.10.0 and WINDOWS v.10.1).

3. RESULTS

(a) *Pterin pigments*

Capillary electrophoresis showed that the NaOH extract from intact *C. eurytheme* male forewings contained the pterins described by Watt (1964) for this species. By comparison, NaOH extracts from forewings previously extracted using NH_4OH lacked any detectable amount of pterins when analysed using capillary electrophoresis. We thus are confident that our NH_4OH-extraction method successfully removed all pterins from the wings.

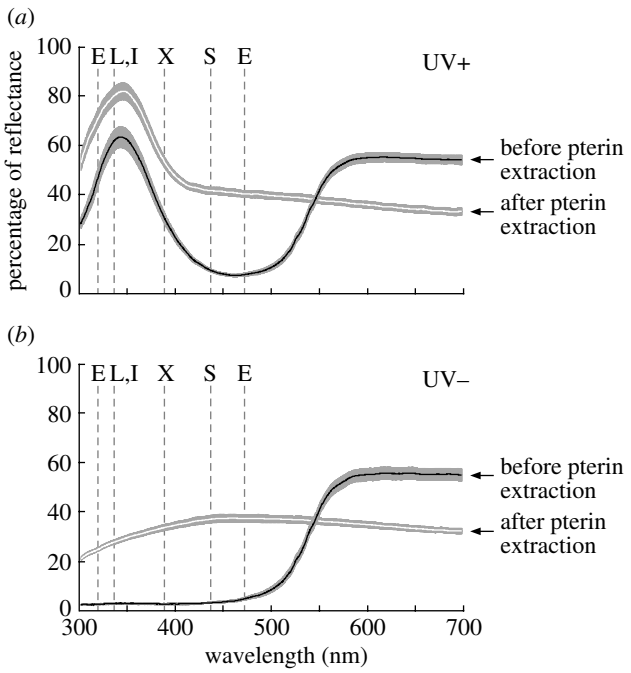

Figure 1. Reflectance spectra of male *Colias eurytheme* dorsal forewing. Illumination is normal (perpendicular) to wing surface in both panels. (*a*) UV+ orientation: receptor angle at 45° to normal in the direction of the wing base. (*b*) UV- orientation: receptor angle at 45° to normal in the direction of the wing tip. Black lines are reflectance means prior to pterin extraction; white lines are reflectance means following pterin extraction, grey surrounds are standard errors. Letters and the vertical dashed grey lines below them identify spectral locations of absorbance peaks for erythropterin (E; two peaks above 300 nm), leucopterin (L), isoxanthopterin (I), xanthopterin (X) and sepiapterin (S). Pterin absorbance peak data are from Watt (1964).

(b) *Reflectance spectra*

Unextracted forewings in the UV+ orientation exhibit two reflectance peaks: a UV peak of ≈63% at $\lambda_{max} = 340$ nm and an 'orange' peak of ≈56% at $\lambda_{max} = 620$ nm (figure 1*a*, black line). The characteristics of this orange colour are typical of oranges and reds in nature, with reflectance rising swiftly around 520 nm and remaining high and essentially flat from 585–700 nm. The spectra of unextracted forewings in the UV− orientation closely resemble the UV+ spectra, except that they reflect little light in the UV wavelengths (≈3% across all UV wavelengths, see figure 1*b*, black line).

Pterin extraction significantly altered the reflectance spectrum across all measured wavelengths. First, overall reflectance was increased in the UV, short and middle wavelengths in both UV+ and UV− orientations (figure 1*a*,*b*, white lines). This increase in reflectance from roughly 300–550 nm is expected, as the pterins that were extracted absorb most strongly in that wavelength region. However, the change in UV reflectance was not equal for both UV+ and UV− orientations. In the UV+ orientation, UV reflectance increased 20% (from ≈62 to 82% at $\lambda_{max} = 345$ nm), whereas in the UV− orientation, UV reflectance increased ≈27% (figure 1*b*, white line). The increase in percent UV reflectance in the UV+ orientation is smaller than would be predicted by simple addition of the reflectance due to scattering alone and due to thin-film interference alone. The underlying cause of this apparent non-additive effect is unclear at this time.

Table 1. Intensity, hue and chroma differences of male *Colias eurytheme* ($n=20$) for unaltered and pterin-extracted wings. (A two-way ANOVA with factors for spectral segment (four, 100 nm wide segments) and treatment (pre- and post-extraction) was conducted for each wing orientation, and subsequent paired-sample *t*-tests were adjusted for multiple comparisons using the sequential Bonferroni method (Rice 1989).)

(*a*) *intensity: UV+*			
factor	$F_{3,152}$	p	
segment	248.620	<0.001	
treatment	68.348	<0.001	
segment X treatment	210.018	<0.001	
segment	paired t (d.f.=19)	p	significant difference
300–400 nm	−9.156	<0.001	brighter after extraction
400–500 nm	−34.020	<0.001	brighter after extraction
500–600 nm	1.533	0.142	none
600–700 nm	16.020	<0.001	brighter before extraction
(*b*) *intensity: UV−*			
factor	$F_{3,152}$	p	
segment	1224.278	<0.001	
treatment	420.163	<0.001	
segment X treatment	869.397	<0.001	
segment	paired t (d.f.=19)	p	significant difference
300–400 nm	−53.235	<0.001	brighter after extraction
400–500 nm	−33.863	<0.001	brighter after extraction
500–600 nm	0.239	0.814	none
600–700 nm	−13.229	<0.001	brighter before extraction
(*c*) *hue and chroma*			
parameter	paired t (d.f.=19)	p	significant difference after extraction
hue angle: UV+	−36.753	<.001	increase (bluer)
chroma: UV+	14.823	<.001	decrease (paler)
hue angle: UV−	−22.234	<.001	increase
chroma: UV−	111.117	<.001	decrease

Pterin extraction also resulted in a significant reduction in reflectance of long wavelengths in both wing orientations (from ≈55 to ≈33%, figure 1*a*,*b*). This change in reflectance is not due to the extraction of the pterins *per se* because they do not absorb in this wavelength region, but may instead be due to the loss of the scattering properties associated with the granules in which pterins are deposited (see §4). In accordance with the spectral changes described above, changes in hue and chroma followed the same pattern for both wing orientations after pterin extraction: hue angle increased significantly (i.e., reflection became 'bluer') and chroma decreased significantly (i.e., reflection became paler; table 1).

Converting spectra to colour scores revealed that the Euclidian distance between UV+ and UV− wing orientations was significantly greater before than after pterin extraction ($t=12.20$, $p<0.001$, d.f.=19; figure 2). Similarly, UV intensity contrast between the two wing orientations was significantly greater before than after pterin extraction ($t=3.122$, $p<0.01$, d.f.=19).

4. DISCUSSION

Studies of behavioural interactions mediated by colour signals in animals have often focused on pigmentary and structural colouration as separate components of an animal's appearance (Bradbury & Vehrencamp 1998). Indeed, past behavioural work on *C. eurytheme* attempted to manipulate the yellow-orange and UV colouration separately, treating them as distinct features of the appearance of male butterflies (Silberglied & Taylor 1978). However, our results suggest ways in which pigments enhance the colouration produced via structural mechanisms.

(a) *The effects of pterins on wing colouration and iridescence*

Pterin pigments have several specific effects on the spectral characteristics of the colour arising from the dorsal wing surface in male *C. eurytheme*. First, pterins absorb a diffuse reflectance in wavelengths below about 550 nm that is revealed when the pigments are removed. This diffuse reflectance is structural in origin, because it can be eliminated and the wing rendered transparent by immersing an extracted wing in a fluid with a refractive index (e.g. 1.5 for xylene) very close to that of insect cuticle (1.6; Ghiradella *et al.* 1972). We suspect that this diffuse white reflection is produced by scattering from non-lamellar nanoscale structures on the wing surface (e.g. Stavenga *et al.* 2004).

Second, the removal of pterins appears linked to an unexpected *decrease* in reflectance in the middle and long wavelengths (yellow–red; 550–700 nm). This decrease cannot be due to absence of pigment molecules, which only absorb light, but must be related to absence of the small ellipsoid beads or granules which contain the pterins (Yagi 1954; Ghiradella *et al.* 1972). These beads are suspended beneath the scale surface latticework in pierid butterflies, and have been suggested to increase wing reflectance by scattering light (Stavenga *et al.* 2004). The pterin extraction procedure removes these beads (e.g. J. M. Kolyer & A. Reimschuessel 1970, personal observations), which in turn may decrease the amount of light scattered.

Third, and perhaps most importantly, the presence of pterin pigments amplifies the iridescent properties of the UV signal. Spectral differences between UV+ and UV− wing positions show heightened colour contrast in the

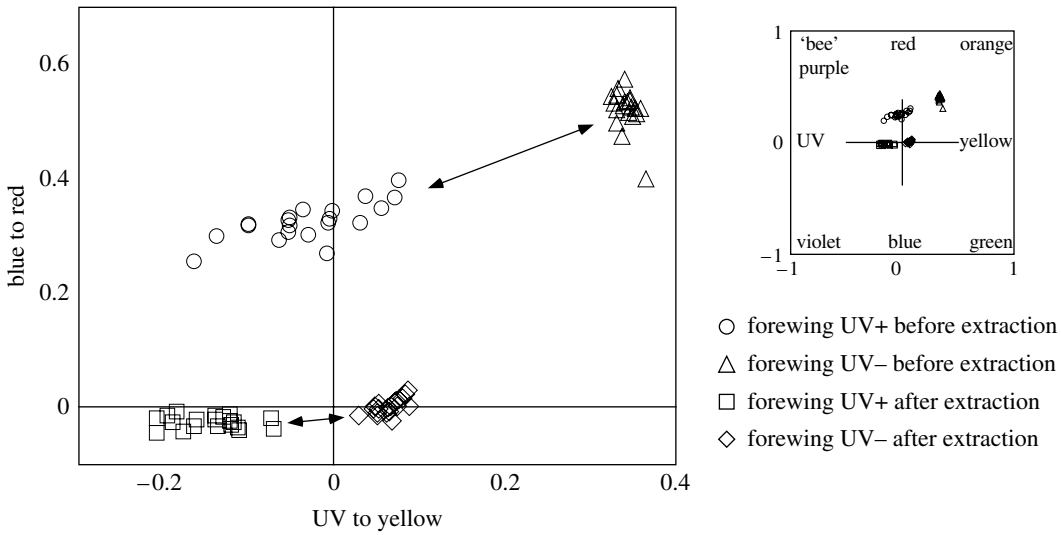

Figure 2. Male *C. eurytheme* forewing colour scores before and after pterin extraction plotted in colour space of Endler (1990). Chroma increases with distance from the origin (i.e. $X=0$, $Y=0$) in any direction. Hue is the angle of a colour score relative to the *y*-axis above the origin. 'Red' is located at the top of the graph (see inset) and has the coordinates $X=0$, $Y=1$; yellow is 90° clockwise to red ($X=1$, $Y=0$), etc. For example, the forewing in the UV− orientation before pterin extraction (triangles) possess greater chroma but a lower hue angle, than the forewing in the same orientation after pterin extraction (diamonds). 'Bee purple' in the small colour space graph (upper right), refers to the human perceptual equivalent of purple in animals with a UV-sensitive visual photopigment.

presence of pterins. Colour space analysis, which evaluates hue and chroma independent of intensity, reveals that the pterins increase colour contrast as the UV flashes off and on during wing movement (figure 2). With small changes in wing position, the reflectance spectra of pterin-containing wings shift from vividly bimodal to unimodal. Wings with pterins removed are largely achromatic with a broadband white reflectance, and any colour shift due to wing position occurs as a slight increase in the UV reflectance.

Intensity contrast also is augmented by the pterin absorption in those areas of the wings that reflect UV from overlying lamellar thin-films. Wings containing pterins should change during a wing beat cycle from weakly UV reflecting (≈3%) to highly UV reflecting (≈63%), about a 20-fold increase in UV brightness. In contrast, the UV reflectance of wings lacking pigments would be expected to shift from ≈30 to 82%, less than a threefold change in UV intensity.

In sum, multilayer thin-film nanostructures, broadband scattering features, and selectively absorbing pigments all contribute to a signal that is brilliantly iridescent and strongly chromatic, and comprised of complementary colours (UV and yellow-orange). For visual systems with an array of both long and short wavelength photoreceptors, as in pierid butterflies (Shimohigashi & Tominaga 1991), this colour combination is thought to contrast strongly with the background against which these butterflies are most likely to be viewed, which is mostly green, UV absorbing foliage.

(b) *Iridescent UV reflectance and honest signalling*

What are the potential functional consequences of this combination of pigments and structures on the use of colour in signalling? We propose four that may also apply to other species that use iridescent colouration in their signals. First, our results suggest that, in *C. eurytheme*, pterin pigments in the wings amplify (*sensu* Hasson 1989) the changes in the colour and intensity of the iridescent UV reflections that will be visible to receivers as a male moves his wings during flight. Strongly flashing visual signals elicit strong behavioural responses in other butterflies (Magnus 1958). We expect also that the frequency of UV flashes produced by *C. eurytheme* males during flight should fall within the female's ability to discriminate the pulses of UV light individually (Nakagawa & Eguchi 1994; Rutowski *et al.* unpublished data), making this a conspicuous and highly salient signal for conspecifics.

Second, pterins may facilitate assessment of the male's iridescent UV signal. The ability of males to produce a bright iridescent UV signal appears to be costly and condition dependent (Kemp & Rutowski, unpublished data), due to the additional cuticular material and the developmental precision required for the construction of highly reflective lamellar arrays. The absorption of diffuse UV by pterins ensures that the UV wavelengths reflected from the wings contain information only about the quality of the lamellar nanostructures the male has been able to produce. In this way, the pterins are potentially contributing to signal honesty (Hasson 1990, 1991).

Third, the cost of pterins must be factored into the total cost of producing an effective and informative UV signal. As Zahavi (1975) suggested, the costs of sexual advertisement signals may be disproportionately higher for low quality individuals than for individuals of higher quality. These costs may be exacerbated when the signal arises as a product of several components because selection requires individuals to garner the resources needed to construct all components. Here we have described a signal whose design involves combining precisely constructed nanostructures with pigments that are highly selective in the wavelengths they absorb (such as pterins but including carotenoids and other pigments). Males that are unable to deposit adequate amounts of these pigments may be disadvantaged when competing for mates.

Last, because of their role in colour signal production in male *C. eurytheme*, pterins may themselves provide

important information for females. Different signals may be correlated with different aspects of a bearer's condition or quality (Fitzpatrick 1998; Brooks & Couldridge 1999; McGraw *et al.* 2002; Shawkey & Hill 2005), but even within a colour patch that involves multiple colour producing mechanisms, the concerted actions of these mechanisms may encode multiple types of information about the signaller (Grether *et al.* 2004). As suggested, the lamellar arrays that produce UV colouration in *C. euryheme* males may be costly and thereby honestly signal the bearer's condition. In addition, we speculate that pterin pigments in this butterfly may signal condition by being correlated with larval adaptation to variation in host-plant quality (e.g. nitrogen availability, Slansky & Feeny 1977). Females, therefore, are potentially able to gain information about several aspects of a prospective mate from the spectral characteristics of one colour patch (e.g. dorsal wing surface). Work is ongoing in our laboratory to explore these possibilities.

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