

Structural ultraviolet ornamentation in the butterfly *Hypolimnna bolina* L. (Nymphalidae): visual, morphological and ecological properties

Darrell J. Kemp^{A,B,C} and Joseph M. Macedonia^B

^ASchool of Tropical Biology, James Cook University, PO Box 6811, Cairns, Qld 4870, Australia.

^BSchool of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA.

^CCorresponding author. Email: darrell.kemp@jcu.edu.au

Abstract. Many butterflies exhibit structurally coloured wing patches that are stunningly bright and iridescent in their appearance, yet functionally obscure. These colours are often exaggerated in males, which suggests a sexually selected origin. We studied the visual properties, morphological basis, and interindividual variation of structural wing colouration in the common eggfly, *Hypolimnna bolina* L. (Nymphalidae). Males of this territorial species possess highly directional UV/violet colouration that fully overlaps smaller white patches on their dorsal wing surfaces. We sampled 56 males, including territorial residents and non-resident ‘floaters’ and assessed the properties of their structural colour using reflectance spectrometry and scanning electron microscopy. The patches reflect strongly in the UV range (300–400 nm), with a peak of ~360 nm, and the wing scales in these regions exhibit a ridge-lamellar surface architecture that has known function in other species as a multiple thin-film interference mirror. Peak UV brightness was variable, and both brightness and peak hue varied systematically across age classes. UV brightness was also related to hue independently of the age-related variation. Territorial residents possessed duller UV markings than their non-resident contemporaries, which is not consistent with exaggeration due to male–male competition. The high phenotypic variance is, however, consistent with a putative role for this male-limited trait as a sexual ornament.

Introduction

With the exception of bioluminescence, animal colour patterns result from the selective absorption of light from pigments and the selective reflectance of light from surface structures. Both of these mechanisms usually apply in concert (Rutowski *et al.* 2005; Shawkey and Hill 2005), but there are colours of which variation is almost exclusively influenced by the presence of pigments or the action of specific surface structures. For example, whereas variation in the ‘redness’ of finch plumage is primarily determined by the presence of carotenoid pigments (Hill 1996), variation in the blue and green iridescent colouration of male damselflies is determined by variation in surface nanostructures (cuticular multilayers: Fitzstephens and Getty 2000). These two classes of colours, respectively termed ‘pigmentary’ and ‘structural’ colours, often distinguish themselves by the nature of their visual effects, with many structural colours shifting in hue or brightness with their angle of view (Vukusic *et al.* 2001; Osorio and Ham 2002). Colours of different provenance may be differentially costly or difficult to produce, thus influencing their utility as signals of individual condition or quality (Fitzstephens 1998; McGraw *et al.* 2002).

Relative to colours influenced primarily by pigments, structural colours have received less attention in studies of

sexual selection and signal design/content. This is true even in cases where both colour mechanisms coexist in a single ornamental colour pattern. In male guppies, for example, pigment-based orange markings co-occur with structural blue/violet and green, and research has almost exclusively focused on the orange markings (which result from both carotenoid and pteridine pigments: Grether *et al.* 2005). The reduced interest in structural colour is puzzling on two counts: first, because structural colour features prominently in sexually dimorphic ornamentation (Brunton 1998; Eaton and Lanyon 2003); and, second, because some structural colours may also indicate aspects of mate quality, such as developmental stability (Fitzpatrick 1998). Part of the reason may be that many structural signals peak in the ultraviolet (UV) waveband, and thus lie outside the range of human visibility (Silberglied 1979; Hausmann *et al.* 2003). In any event, the research that has been conducted into structurally coloured ornaments suggests that they do serve as sexual signals (Silberglied and Taylor 1978; Johnsen *et al.* 1998; Sweeney *et al.* 2003) and that they do carry potentially useful information on phenotypic quality (Fitzstephens and Getty 2000; McGraw *et al.* 2002; D. J. Kemp and R. L. Rutowski, unpublished data).

Day-flying lepidopterans (butterflies and moths) provide an excellent system for investigating the cause and function of structural colour (Ghiradella 1998; Vukusic and Sambles 2003). This form of colouration is extremely widespread within the group and often contributes to male-limited ornamentation (thus, sexual dichromatism). Structural colour mechanisms in butterflies represent some of the most complex and novel optical mechanisms in nature (Vukusic *et al.* 1999, 2000, 2001). Studies have also revealed this ornamentation to be highly phenotypically variable (Brunton and Majerus 1995; Kemp 2006), heritable, and condition-dependent (D. J. Kemp and R. L. Rutowski, unpublished data). However, whereas signalling function has been determined in a few cases (Silberglied and Taylor 1978; Sweeney *et al.* 2003), the overall number of studied species is limited, and the full potential complement of signalling functions has not been investigated. In the species for which most is known, *Colias eurytheme*, male-limited structural UV is used in mate recognition by both sexes (Silberglied and Taylor 1978), but it is still not fully understood whether variation in signal characteristics (brightness, hue, etc.) is used in intraspecific female mate choice, or what information may be ultimately conveyed.

In this study we address the nature and function of male-limited structural colouration in the nymphalid butterfly *Hypolimnas bolina* (L). Males of this species possess ovoid regions of blue/UV (hereafter, UV) on their dorsal fore- and hindwings (see Results) that are extremely bright, iridescent, and visible from a limited range of viewing angles. The UV fully overlaps several smaller white markings that are set against a black background; thus, each marking appears (to human viewers) as an inner circle of UV/white surrounded by a halo of UV/black. This colour scheme is also present in males of the congeneric *H. misippus* (females of both species lack these UV markings, although in *H. bolina* they do possess a dull bluish refulgence on their dorsal wings). The iridescent UV is thought to arise from dedicated surface structures, although the mechanism responsible has not been documented. Male *H. bolina* (and *H. misippus*) are also strongly territorial, and compete for the ownership of mating territories via conspicuous aerial displays (see the description below). Importantly, the male's dorsal wing surface is displayed via several ritualised flight manoeuvres during both courtship and male–male contests. This, coupled with the male-limited nature of the iridescent markings, makes this species an excellent model for addressing sexual selection-based hypotheses regarding the potential signalling function of structural colouration.

Our aims were three-fold. First, we set out to identify the optical mechanism responsible for the production of UV in male *H. bolina*. The brightness and angular-dependence of this colour has the hallmark of a multilayer thin-film mechanism, and we mount a preliminary investigation of this hypothesis using spectrometry and scanning electron

microscopy. Second, we investigate whether (and how) the reflectance characteristics of the UV patches vary with age (wing-wear). Female butterflies will be generally benefited by choosing younger mates; hence, one hypothesis for the use of structural (rather than pigmentary) ornamentation in sexual signalling is that it better magnifies interindividual differences in age (Fitzpatrick 1998; Kemp 2006). The UV patches would then act as an indicator of mate quality. Last, we investigate whether UV reflectance characteristics vary among territorial residents and non-residents in a manner supportive of its use as a signal of aggression and/or dominance. This question has been partially addressed in *H. bolina* by Rutowski (1992), who demonstrated that colour manipulation had no effect upon the subsequent territorial success of existing residents. However, similar work (in the territorial swallowtail *Papilio polyxenes*: Lederhouse and Scriber 1996) revealed effects when butterflies were manipulated before gaining residency, which suggests that the timing of the manipulation may have a large bearing on the effect. Both studies used gross manipulations of colour, and neither investigated the existence of natural colour variation among residents and non-residents, which should offer a reasonable starting point for addressing this hypothesis.

Materials and Methods

Study species and behaviour

Hypolimnas bolina (the 'varied eggfly' (Braby 2000) or 'blue-moon butterfly' (Ramsay and Ordish 1966)) is a generally common butterfly with a widespread distribution throughout southern Asia and the Pacific and Indian oceanic regions. In tropical north Queensland, male *H. bolina* are active almost throughout the entire year (Kemp 2001a) and defend perching territories for long periods (7–8 h) each day (Rutowski 1992). Favoured perching locations often occur at distinctive locations such as sunlit forest edges and along corridors through rainforest vegetation, or in rainforest clearings (see Kemp and Rutowski 2001 for further information). These sites are not centred upon adult or larval food resources (although they may contain both) and are believed to offer the best locations by which to detect and intercept passing receptive females (Kemp and Rutowski 2001). Males perch on the outermost foliage of bordering trees and shrubs, either in the sun or the shade, and with wing postures ranging from fully open to completely closed, depending upon their body temperature (Kemp and Krockenberger 2002).

Courtship has been described for both this species and *H. misippus* (Stride 1956, 1957, 1958; Rutowski 1992), and appears similar in both cases. Generally, upon having detected a flying female, a male will assume a position directly below her and adopt a 'quivering', shallow-wing-beat flying action. Males sometimes sweep from side to side below the female in a horizontal arc (of roughly 0.5–1.0 m), which is reminiscent of a pendulum swing, all the time maintaining the opening position such that their dorsal wings are displayed to the female. A successful courtship is terminated when the female lands (usually on vegetation) and copulation ensues. Unsuccessful courtships can last several minutes, and may be terminated by the female embarking upon an ascending flight, a 'zig-zag' flight through foliage, or a sudden dive into the undergrowth (Stride 1958). Male–male contests, which generally ensue when one male is detected by a territory resident, consist of the two males circling around or hovering near each other for a varying length of time until one male departs the area. Regardless of the dura-

tion of the contest, the departing male always retreats with a stereotyped 'flap-glide' style of flight, which is not seen at any other time (Rutowski 1992; Kemp 2000) and in which his wings are held fully open for short gliding stretches (thus exposing the iridescent dorsal colouration). Departing males rarely return to the contested site, but if they do they are swiftly escorted out again by the prior 'winner' (almost always the resident: Kemp 2000).

Male dorsal colouration is therefore displayed both during courtship and during the course of male–male contests, and may have been (and/or presently be) subject to sexual selection acting in one or both of these contexts.

Butterfly sampling

One of us (DJK) sampled butterflies for this study from a series of perching territories near Cairns in north Queensland (16°53'S, 145°45'E), from 14 December 2002 to 31 January 2003, and between 0900 and 1200 hours. These territories are highly sought-after locations (as adjudged by the frequency of occupation by territory residents and rates of intrusion by non-residents), and have featured in several previous investigations into this species' behavioural ecology (refer to Kemp 2000, 2005; Kemp and Rutowski 2001). The sampling methodology is outlined in more detail in Kemp (2005). Briefly, territorial residents were captured, marked and released in the area, with males marked on their ventral hind-wings with a green ultra-fine permanent marker (Sanford Sharpie brand). Males that were seen in sole attendance at the site of capture on any subsequent day were classified as *bona fide* residents. Once a resident (thus defined) was identified he was caught, along with at least one other male that flew in to perch at that site on that day. These 'replacement residents' are thought to represent the population of non-resident 'floaters'. Throughout the study, unmarked males that perched near the study area were also caught, marked and released.

Age estimation

The age of free-flying adult butterflies is extremely difficult to measure accurately. Because the wings of butterflies and other insects degrade irreversibly with use, estimates of wing wear have often been used as surrogate measure of age (e.g. butterflies: Rutowski 1985; Karlsson 1994; Kemp 2000; other insects: Plaistow and Siva-Jothy 1996; Plaistow and Tsubaki 2000). In this study we placed butterflies in five age classes, using standard classification criteria based upon the extent of tattering of the wing margins:

- (1) intact margins, no visible damage;
- (2) very few (<3) visible chips along margins;
- (3) multiple (3–5) chips along margins, some larger areas of lost wing;
- (4) many chips, slight 'tattered' appearance along margins, areas of lost wing; and
- (5) wing margin almost entirely chipped and 'tattered', large areas of wing membrane missing.

In reality, wing wear is a measure of adult activity, which will by necessity covary with age. However, although the relationship between age and wing wear (as presently assessed) must be positive, the exact nature of this relationship has not been determined for this species. Reproductively active adult male *H. bolina* live up to several months in the field (Kemp 2001b).

Captured butterflies were killed, their forewings removed, mounted on black card with spray adhesive (3M, Pentaluma, CA, USA; applied to the ventral surfaces), then pressed flat for 24 h. The use of glue has been previously determined not to affect the dorsal colouration of this species; it does not penetrate through to the dorsal side.

Reflectance spectrometry

We measured wing colour using the 'beam' method of reflectance spectrometry (Endler 1990). We used an Ocean Optics USB-2000 spectrophotometer (25 averaged spectra, 125-ms integration time), with

the probe at 45° (to the horizontal) and pulsed illumination provided at 90° by a PX-2 xenon light source. Fig. 1 illustrates the measurement set-up. As in many butterfly species that possess structural colouration, the UV portion of the *Hypolimnias* reflectance curve (see Fig. 2) is strongly dependent upon the orientation of the wing relative to the light source and receiver. Angle-dependence of reflectance arises as a consequence of the optical mechanisms at play, including constructive interference and diffraction (Vukusic *et al.* 2001). In *H. bolina*, the viewing/illumination angle at which the UV reflectance is brightest varies among individuals (D. J. Kemp, unpublished data). We therefore rotated each sample wing surface within a 5–6° range (indicated in Fig. 1 by the symbol γ) to maximise the height of the UV reflectance curve, and thus exclude variation due to the peak reflectance angle. This ensured that we assessed each specimen's peak UV brightness. Reflectance in all cases was expressed as a proportion of that obtained from a magnesium oxide white standard, which provides relatively high and equal reflectance across the entire 300–700-nm range.

In three specimens we also investigated the directionality of reflectance from the UV patches by first finding the orientation of maximum brightness (using the method above), then rotating the wing $\pm 5^\circ$ (i.e. $\gamma + 5^\circ$ and $\gamma - 5^\circ$) (Fig. 1) and capturing the spectra at each orientation. This was done for both forewings of each specimen.

Electron microscopy

We obtained scanning electron micrographs using a Leica-Cambridge Stereoscan 360 FE scanning electron microscope set to a 15.0 kV accelerating voltage and zero stub tilt, with samples cold-sputter coated prior with gold. Using several fresh specimens, we sampled pieces of wing from the largest forewing UV/white patch, its UV/black halo, and from the immediately adjacent black forewing area (see Fig. 2a). We placed scale bars on the images that were verified previously against optical diffraction gratings.

Statistical analyses

We summarised the spectral data to give measures of UV brightness (mean percentage reflectance amplitude from 300 to 450 nm) and hue

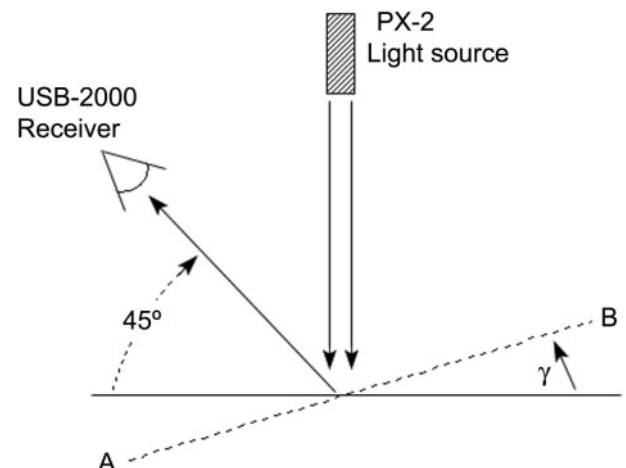


Fig. 1. Diagrammatic representation of the spectrometry set-up used to measure the colour of the dorsal surface of the forewing of male *H. bolina*. The receiver was angled at 45° relative to the horizontal, and focused to capture light from an approximately circular 2-mm area. The section AB indicates the wing surface, which was rotated a fraction from the horizontal position (represented as the angle γ) in order to locate each specimen's angle of maximum reflectance, the so-called 'best angle' (see text).

(the wavelength corresponding to the peak UV reflectance amplitude). We also calculated long-wave brightness as the sum of percentage reflectance amplitudes from 500 to 700 nm. These parameters were normally distributed (see Results), and we use standard parametric statistics throughout.

In multivariable analyses of UV parameters, we found the best-fitting model using a best-subsets approach based upon minimisation of Akaike's information criterion (AIC). This criterion is an information-theoretic derivative of the log-likelihood function that, in the case of observational data, facilitates direct comparison between candidate models containing varying numbers of parameters (Burnham and Anderson 2002). We investigated the significance of individual variables contained in the best-fitting models using the Wald statistic, and investigated the direction and size of the relationship using semi-partial correlations. Model fitting was conducted using the generalised linear/non-linear modelling function of STATISTICA version 7.0 software. Means are given with 95% confidence intervals, unless otherwise stated.

We conducted statistics on UV parameters of the inner UV/white circle, which we assume is also representative of variation occurring in the UV/black halo. This assumption seems realistic because, among other things, each colour pattern element is seen to fade equally with age (D. J. Kemp, unpublished data).

Results

Spectral properties of the dorsal UV

Fig. 2*b, c* illustrates spectral scans of the larger forewing UV/white patch and the UV/black halo. Scans taken when the wing was oriented at the best angle for viewing the iridescent UV (the solid lines) feature a Gaussian-shaped reflectance peak centred around 350–370 nm and extending from below 300 nm to ~450–470 nm. The fact that this

reflectance extends above 400 nm explains the violet appearance of this wing region to human observers. The limited-view nature of UV reflectance is indicated by the scans of each region taken with the wing subsequently rotated 180° in the horizontal plane (the dashed lines), in which the short wavelength peak is almost entirely absent. These scans present flat or gently sloping lines around 30–40% (UV/white patch) and 3–5% (UV/black halo), which are consistent with the whitish and black appearance of these regions, respectively (to the human observer). The 'ground' colour of the dorsal surface is similar to that of the UV halo in the 'UV off' position: black (i.e. the dashed line in Fig. 2*c*).

We captured reflectance spectra from the centre of each specimen's white forewing patch with the wing oriented for maximum UV reflectance (as described in Materials and Methods). Left and right values for UV brightness (mean percentage reflectance from 300–450 nm) were highly correlated ($r = 0.928$, $N = 56$, $P < 0.0001$), as were the values for UV hue ($r = 0.929$, $N = 56$, $P < 0.0001$), and we averaged them. Repeat measurements on 18 randomly chosen samples indicated high repeatability in both spectral characteristics (brightness: $r = 0.927$, $P < 0.0001$; hue: $r = 0.939$, $P < 0.001$), thus indicating that these spectral characteristics can be reliably quantified using reflectance spectrometry. UV brightness varied among individuals from 23.4% to 94.7% ($N = 56$; mean = $55.1 \pm 3.8\%$), and peak hue varied from 325 to 381 nm ($N = 56$, mean = 355 ± 3.6 nm). The distribution of neither parameter deviated from normality

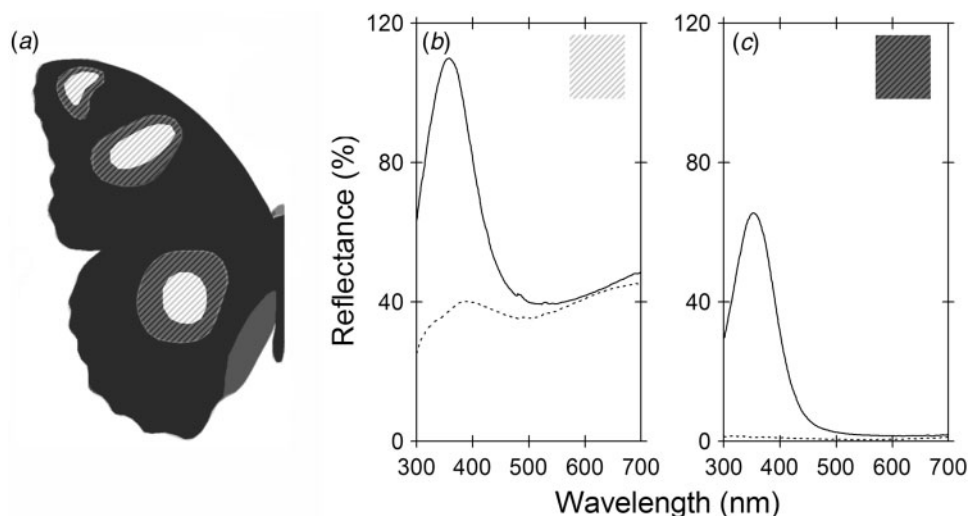


Fig. 2. (a) Stylised illustration of the dorsal wing surface of a male *Hypolimnas bolina*. The hatched regions indicate the coverage of iridescent UV. Note that it overlaps the ovoid-shaped white patches and extends onto the black ground colour, thus giving rise to an ovoid 'UV on white' patch surrounded by a 'UV on black' halo. Panels (b) and (c) show reflectance scans taken from the forewing white patch and its UV halo, respectively, with the wing situated at the best angle for viewing UV (solid line) and then rotated 180° (dashed line) in the horizontal plane. Reflectance of the wing ground colour is identical to that of the dashed line in (c). The scans represent averages of those obtained for three specimens. Reflectance can exceed 100% because it is measured relative to that provided by a white reflectance standard (see text).

(Kolmogorov–Smirnov $d = 0.060–0.062$, $P > 0.20$). Even among the 21 individuals without visible wing fading (i.e. those of wing wear Classes 1 and 2) there was considerable variance, with UV brightness ranging from 49.4 to 94.7% and peak hue ranging from 342 to 381 nm. UV brightness was positively correlated with peak hue (Pearson's $r = 0.65$, $N = 56$, $P < 0.001$), a phenomenon that we explore further below.

Rotation of sample wings $\pm 5^\circ$ from the optimum viewing angle (i.e. angle γ in Fig. 1) further indicated that the UV has a strong directional component not seen in the coincident long-wave reflectance (Table 1). For example, rotation of the wing a further 5° towards the probe resulted in a 40–50% reduction in mean UV (300–450 nm) brightness, whereas there was only a 0.7% reduction in mean long-wave (500–700 nm) brightness. There were also changes in peak UV hue with orientation, but these were less systematic than those seen in brightness (i.e. peak hue both increases and decreases across samples and orientations) (Table 1).

Wing scale morphology

Scanning electron micrographs of the UV/white, UV/black and black regions of the forewing indicated characteristic differences in wing-scale arrays and scale surface morphologies. In the UV/white and UV/black regions (Fig. 3b), scales are situated in discrete rows, and there are two distinct scale types: 'cover scales', which appear semi-transparent and have intact terminal margins, and the underlying 'ground scales', which have serrated terminal margins. By contrast, in the black (non-UV) region, scales are situated more haphazardly, and there appears to be just one type of scale with a serrated terminal margin (Fig. 3a). Higher-magnification imaging (Fig. 3c, d) revealed that both the cover and ground scales in the UV/white region, and the cover scales in the UV/black halo, exhibit a surface architecture characteristic of the iridescence-producing wing scales of other butterflies (e.g. *Eurema* (Ghiradella *et al.* 1972), *Colias* (Ghiradella 1974) and *Morpho* (Vukusic *et al.* 1999)). The key feature of this architecture (Fig. 3d) is that the longitudinal ridges, which occur on all butterfly wing

scales (Ghiradella 1998), bear a series of cuticular outgrowths running along their lateral face. These outgrowths – known as 'lamellae' (Ghiradella 1998) or 'flanges' (Lawrence *et al.* 2002) – present (to incident light striking the wing's surface) a series of alternating air–cuticle layers, which in other butterflies has been shown to function as a quarter-wave interference device (Ghiradella *et al.* 1972). Scales in the black, non-UV region, and the serrated 'ground' scales in the UV/black halo (Fig. 3c) do not exhibit this 'ridge-lamellar' architecture; instead, the lateral ridge face exhibits fine-scale near-vertical striations (termed 'microribs' by Ghiradella 1998).

Ecology of the UV patches

UV reflectance characteristics of the UV/white patch varied systematically with age (Figs 4 and 5), with decreases across age classes in both UV brightness (ANOVA of regression, $F_{1,54} = 34.1$, $P < 0.0001$, $R^2 = 0.39$), and peak hue (ANOVA of regression, $F_{1,54} = 36.9$, $P < 0.0001$, $R^2 = 0.40$). This supports the hypothesis that the structural UV offers a signal of male age. Forewing length (a measure of body size) was negatively related to UV hue ($r = -0.28$, $N = 56$, $P < 0.05$) but not brightness ($r = -0.15$, $N = 56$, $P < 0.05$). The UV characters also varied between territorial residents and non-residents: residents possessed UV markings that were less bright (residents ($n = 21$): $48.0 \pm 7.3\%$; non-residents ($n = 35$): $59.4 \pm 3.9\%$; $t_{54} = 3.12$, $P < 0.005$) and peaked at a shorter wavelength (residents: 349 ± 7 nm; non-residents: 358 ± 4 nm; $t_{54} = 2.39$, $P < 0.05$). However, residents were also older than non-residents (as reported for this dataset by Kemp 2005), which could explain the difference in UV parameters, which covary with themselves and with age.

To further assess the covariance between age, body size (forewing length), territorial residency and male colouration, we constructed the most parsimonious multivariable model of each UV characteristic. In each case we included residency status (0 = resident, 1 = non-resident), forewing length, age (wing-wear class) and the other UV parameter (brightness or hue), plus all interactions with residency status. The best-fitting model of UV brightness (AIC =

Table 1. Spectral properties (UV brightness and peak hue) of the white forewing patch with the wing situated at three different orientations to the spectrometer probe: the angle conducive to maximum UV brightness (the 'best angle'), rotated 5° anticlockwise from this angle, and rotated 5° clockwise

UV brightness is mean percentage reflectance from 300 to 450 nm, and hue is the wavelength corresponding to the UV peak. Differences from values obtained at the best angle are given in parentheses. For comparison, the average percentage long-wave (500–700 nm) brightness for these samples and orientations is given in the bottom row. All values are averages obtained from three different wing samples

Specimen	Best angle		Best angle -5°		Best angle $+5^\circ$	
	Brightness (%)	Peak hue (nm)	Brightness (%)	Peak hue (nm)	Brightness (%)	Peak hue (nm)
A	78.1	372	63.4 (–14.7)	370 (–2)	44.3 (–33.8)	361 (–11)
B	85.8	369	73.2 (12.6)	373 (+4)	48.4 (–37.4)	382 (+13)
C	95.7	366	72.6 (–23.1)	373 (+7)	48.9 (–46.8)	344 (–22)
Mean UV (300–450 nm)	86.5	369	69.7 (–16.8)	372 (+3)	47.2 (–39.3)	362 (–7)
Mean long-wave (500–700 nm)	32.9		30.5 (–2.4)		32.2 (–0.7)	

422.12, $G_3 = 422.12$, $N = 56$, $P < 0.000001$) included UV hue (Wald = 15.30, $P < 0.0001$, semi-partial $r = 0.321$), wing-wear class (Wald = 9.56, $P < 0.005$, semi-partial $r = -0.306$), and forewing length (Wald = 2.06, $P = 0.151$, semi-partial $r = 0.139$). Thus, there were significant ‘effects’ of both hue and age upon brightness, but no effects relating to territorial residency. Although body size was included in the most informative model, this effect was non-significant. In the case of UV hue, the best-fitting model (AIC = 413.73, $G_2 = 40.14$, $N = 56$, $P < 0.000001$) included UV brightness (Wald = 12.27, $P < 0.0005$, semi-partial $r = 0.318$) and wing-wear class (Wald = 10.30, $P < 0.005$, semi-partial $r =$

-0.301). Hence, similar to the analysis of brightness, UV hue was related most clearly to the other UV trait (brightness) and age. The lack of an effect of territorial residency indicates that there were no differences between residents and non-residents in each UV parameter once the other existing covariances were taken into account.

These results are suggestive of two independent relationships: (1) a relationship between UV brightness and hue, and (2) an effect of age upon both parameters. We further assessed the independent nature of these two relationships by looking at the covariance between hue and brightness in ‘fresh’ samples (Wing-wear Classes 1 and 2, which we

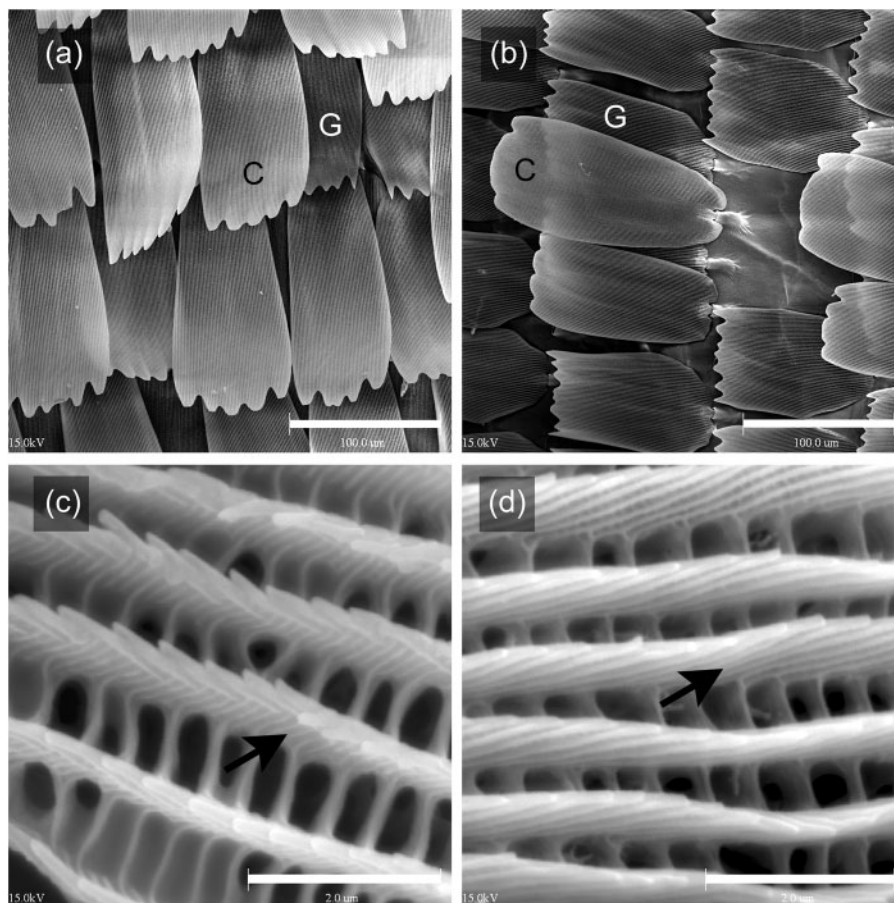


Fig. 3. Scanning electron micrographs of different regions and individual scales from the dorsal forewing. (a) Scales in the area of non UV-reflective black region. Cover scales (denoted by ‘C’) and ground scales (denoted by ‘G’) appear morphologically similar. Magnification: $\times 400$, scale bar = 100 μm . (b) Scales in the large, ovoid UV/white patch. Here the cover scales are semi-transparent and have largely intact margins, whereas the underlying ground scales are strongly serrated. Magnification: $\times 400$, scale bar = 100 μm . (c) Close-up of a cover scale from the black wing region. The arrow indicates the lateral face of a ridge, where microribs can be seen running nearly vertically, and short, angled lamellae can be seen terminating along the ridge top. Cover and ground scales in the black region, and ground scales in the UV/black halo, have this surface morphology. Magnification: $\times 21\,400$, scale bar = 2.0 μm . (d) Close-up of a single ‘cover’ scale from the UV/white region. The arrow indicates the stacks of lamellae that extend down the face of each ridge. This is the ridge-lamellar architecture characteristic of a multi-layer thin-film device. Ground scales in this region and cover scales in the UV/black halo exhibit this surface architecture. Magnification: $\times 21\,400$, scale bar = 2.0 μm .

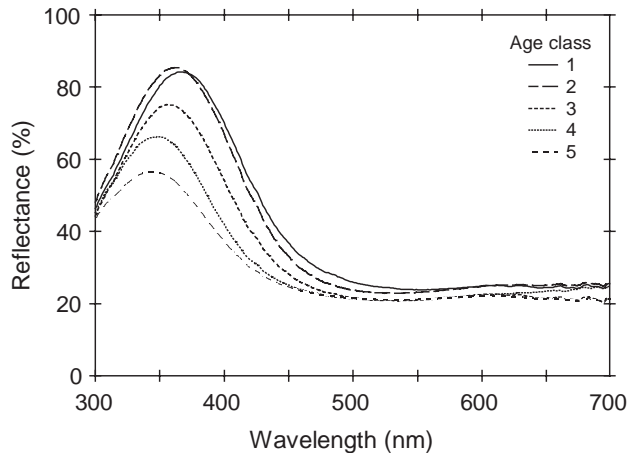


Fig. 4. Average reflectance spectra of the white forewing patch of males grouped in five age classes (based on wing-wear; 1 = youngest class). Sample sizes are given in Fig. 5.

grouped together because these two classes did not vary in brightness) (Fig. 5). If the positive covariance between UV brightness and hue was purely a consequence of an age effect upon each parameter, then no such covariance should exist among these fresh samples. However, the two UV parameters are more closely related in fresh samples ($r = 0.77$, $N = 21$, $P < 0.001$) than in worn samples ($r = 0.36$, $N = 35$, $P < 0.05$) (Fig. 6), which strengthens the assertion that the observed covariance between UV brightness and hue represents a real and ‘fundamental’ relationship.

Discussion

Previous workers have noted that species of *Hypolimnas* possess limited-view dorsal violet or UV markings (Stride 1958; Rutowski 1992; Braby 2000); however, the mechanism responsible for this colouration has not been investigated, and its adaptive significance is almost entirely unknown. In this study we set out to investigate how this colour is produced in *H. bolina*, and whether it has potential as a signal of male age and/or aggression (or fighting ability). The salient features of our results are as follows:

- (1) The colour patch, which has been variously referred to as purplish-blue (Common and Waterhouse 1981; Braby 2000) or bluish (McCubbin 1971), reflects light strongly from just above 400 nm to below 300 nm, with a peak around 350–370 nm. The patch therefore presents a true UV colour.
- (2) Wing scales in this colour patch (but not the adjacent ‘black’ area) exhibit a surface architecture characteristic of the multi-thin-film morphology known to generate iridescent UV reflectance in butterflies (Ghiradella *et al.* 1972; Ghiradella 1974, 1998; Vukusic *et al.* 1999). This, coupled with the marked angle-dependence of *Hypolimnas* UV (Table 1, Fig. 2), suggests that this colour results primarily from thin-film interference. Scales of the non-UV wing regions exhibit a morphology characteristic of an unspecialised or generic lepidopteran scale (see, for example, fig. 12 of Ghiradella 1998).
- (3) Both UV hue and brightness vary systematically with wing-wear, which supports the hypothesis that this

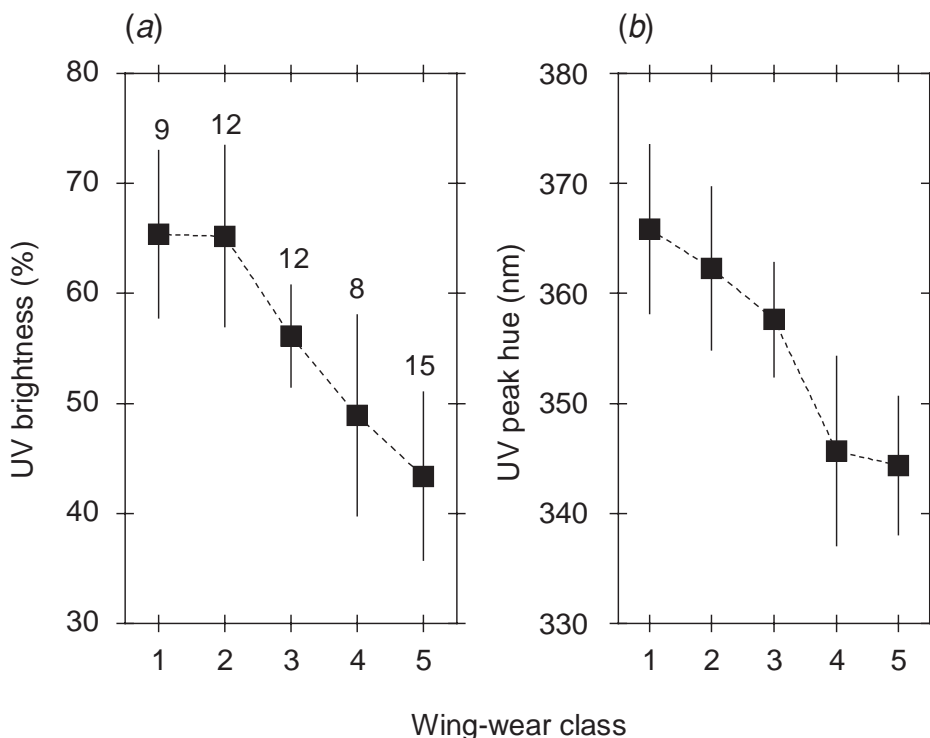


Fig. 5. UV characteristics of the white forewing patch of males in five age classes (1 = youngest class). (a) Brightness represents average percentage 300–450-nm reflectance, and (b) hue is the wavelength corresponding to the UV peak. Sample sizes for each class are given in the brightness plot, and the spreads indicate the 95% confidence interval.

colour could signal male age (or prior adult activity). There is also covariance between the two visual UV characteristics (i.e. hue and brightness) that occurs independently of their relationship with age.

- (4) UV characteristics vary between territorial residents and non-residents, but this seems most likely to stem from wing-wear differences between these two groups (as indicated by the multivariate analyses). In any event, residents possessed less bright UV than non-residents, which is opposite of that expected if this trait evolved as a signal of dominance/aggression.

We discuss these findings in light of previous work on butterfly structural colouration and the potential signal function of these UV patches in *Hypolimnias*.

Proximate mechanisms of UV signal generation and variation

Our finding that *H. bolina* generate their dorsal UV using the ridge-lamellar thin-film morphology is perhaps not surprising, given that this surface architecture is widely distributed among iridescent nymphalids, pierids, rhodinids and other families (Ghiradella 1998; Vukusic *et al.* 1999, 2001). Of the different specialised ridge morphologies in iridescent butterfly scales, this architecture is closest to the generic lepidopteran scale, and may represent the most primitive photonic elaboration (Ghiradella 1998). Indeed, comparison of the non-iridescent (Fig. 3c) and iridescent (Fig. 3d) scales in *H. bolina* indicates a broad similarity except that the iridescent scales have taller ridges adorned with more lamellae. Both scale types have lamellae terminating along the ridgetops, thus contributing to a sawtooth profile. However, the ridges of iridescent scales appear more tightly spaced, which is again consistent with the situation in other butterflies (Ghiradella 1998, and references therein). In *Colias eurytheme*, a species for which these wing structures have been extensively studied (Ghiradella 1974; Kemp 2006; Kemp *et al.* 2006), interindividual variation in peak UV brightness is related to the spacing of these ridges (Kemp *et al.* 2006). Phenotypic variation in UV is similarly high for both species

(see Kemp 2006 for *C. eurytheme*); hence, it will be interesting to see whether scale ridge density is also an important determinant of this variation in *H. bolina*.

An interesting feature of the present findings concerns the covariance between UV brightness and hue (Fig. 6), and the variation observed in both these parameters across age classes (Fig. 5). Because wing scales are lost over time, a reduction in UV brightness is expected, and is also seen in *C. eurytheme* (Kemp 2006). However, hue does not vary with age in this species (Kemp 2006), nor is there any relationship between hue and brightness (D. J. Kemp, unpublished data). One potentially important difference is that in *Colias* the UV-producing structures occur exclusively on the cover scales (Ghiradella 1974), whereas photonic structures occur on both ground and cover scales in the *Hypolimnias* UV/white patch. This implies a candidate explanation for the hue-related variances and covariances in *H. bolina*. Given that cover scales are more likely to be lost over time, peak UV hue could reduce with age if the ground scales reflect shorter-wavelength UV than do the cover scales. Age-related losses of cover scales would subsequently reduce both UV brightness and peak hue (as shown in Fig. 5). Extending this possibility further, it could also explain an 'intrinsic' relationship between UV brightness and hue provided there is variation in the relative coverage of cover versus ground scales. An individual with larger or more densely packed cover scales (which are semi-transparent), may exhibit both a brighter and longer-wave UV peak because of (a) the greater spatial coverage and overlap of UV-reflective structures, and (b) the relatively greater contribution of the long-wave-reflecting cover scales. The key prediction of this scenario, that cover scales reflect longer-wave UV, therefore stands as an interesting prediction for future work.

Potential UV signalling functions

Regardless of why these UV parameters vary with wing wear, it is clear that this component of the male dorsal visage could indicate age. This possibility has also been suggested for the structural colours of many birds (Fitzpatrick 1998)

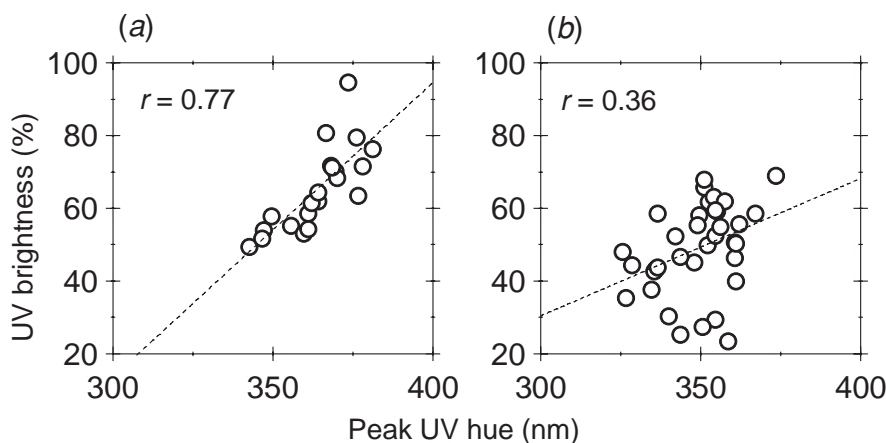


Fig. 6. Linear relationships between peak UV hue and UV brightness in (a) 'fresh' males (Age Classes 1 and 2) and (b) 'worn' males (Age Classes 3–5). UV brightness and hue are defined as in Fig. 5. Pearson's *r*-values are given in each plot as a measure of the effect size.

and butterflies (Kemp 2006). In insects, the ability of females to choose younger mates may be important if this affects their likelihood of receiving a high-quality male ejaculate. Specific data on this point are presently lacking for *H. bolina*, but declines in sperm quality are known for insects (Siva-Jothy 2000), and prior mating history is known to affect the nutritional quality of future nuptial donations in other butterflies (Rutowski *et al.* 1987). Male *H. bolina* invest, on average, ~3.0–3.5% of their body mass in the ejaculate (Rutowski 1992), but this could still constitute an important investment for females, most of whom mate only once in their lifetime (Kemp 2001c). Young males, who are more likely to be virgins, will also subject a female to a shorter copulation (Rutowski *et al.* 1987; Kemp 2001c), which would subsequently reduce the risk of predation during copula. Hence, there are ways in which male age could be a relevant concern to female *H. bolina*, and the UV varies in a way that would see it as a good age indicator. The UV could also indicate old age and thus viability, but because UV brightness decreases with age, selection for such an honest signal of age could not be responsible for the exaggeration of this signal. Further research will be required to assess the legitimacy of these arguments.

Finally, along with the findings of Rutowski (1992), this research indicates that the male UV is unlikely to serve as a badge of territorial aggression and/or dominance. Wallace (1889) and then Silberglied (1984) promoted the hypothesis that bright male butterfly colouration evolved in the context of aggressive competition among males. Efforts to evaluate this hypothesis have been restricted to two studies (Rutowski 1992; Lederhouse and Scriber 1996), each of which relied on a colour manipulation of existing territorial residents, but which produced directly conflicting results. We found that resident *H. bolina* actually possess duller dorsal UV markings than their contemporaries, which suggests that this trait did not become exaggerated in the context of male–male competition for territorial ownership. This does not mean that the UV serves absolutely no role in male–male communication, as it may function in species and/or sex recognition. However, these findings do suggest that we must look elsewhere to understand why males possess these bright, highly saturated and directional wing patterns. The signal is likely to be costly, both because of the costs of development (D. J. Kemp and R. L. Rutowski, unpublished data) and the potential cost of increased predation (Lyytinen *et al.* 2005), and may henceforth provide an honest signal of male quality. Future work may therefore profitably focus on the significance of variation in this trait to mating decisions of conspecific females.

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References

- Braby, M. F. (2000). 'Butterflies of Australia: their Identification, Biology and Distribution.' (CSIRO: Canberra.)
- Brunton, C. F. A. (1998). The evolution of ultraviolet patterns in European *Colias* butterflies (Lepidoptera: Pieridae): a phylogeny using mitochondrial DNA. *Heredity* **80**, 611–616. doi:10.1046/j.1365-2540.1998.00336.x
- Brunton, C. F. A., and Majerus, M. E. N. (1995). Ultraviolet colours in butterflies: intra- or inter-specific communication? *Proceedings of the Royal Society of London. Series B. Biological Sciences* **260**, 199–204.
- Burnham, K. P., and Anderson, D. R. (2002). 'Model Selection and Multimodel Inference: a Practical Information-Theoretic Approach.' (Springer: New York.)
- Common, I. F. B., and Waterhouse, D. F. (1981). 'Butterflies of Australia.' (CSIRO: Melbourne.)
- Eaton, M. D., and Lanyon, S. M. (2003). The ubiquity of avian ultraviolet plumage reflectance. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **270**, 1721–1726. doi:10.1098/rspb.2003.2431
- Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society* **41**, 315–352.
- Fitzpatrick, S. (1998). Colour schemes for birds: structural coloration and signals of quality in feathers. *Annales Zoologici Fennici* **35**, 67–77.
- Fitzstephens, D. M., and Getty, T. (2000). Colour, fat and social status in male damselflies, *Calopteryx maculata*. *Animal Behaviour* **60**, 851–855. doi:10.1006/anbe.2000.1548
- Ghiradella, H. (1974). Development of ultraviolet-reflecting butterfly scales: how to make an interference filter. *Journal of Morphology* **142**, 395–410. doi:10.1002/jmor.1051420404
- Ghiradella, H. (1998). Hairs, bristles and scales. In 'Microscopic Anatomy of Invertebrates'. (Ed. M. Locke.) pp. 257–287. (Wiley-Liss: New York.)
- Ghiradella, H., Aneshansley, D., Eisner, T., Silberglied, R. E., and Hinton, H. E. (1972). Ultraviolet reflection of a male butterfly: interference color caused by thin-layer elaboration of wing scales. *Science* **178**, 1214–1217.
- Grether, G. F., Cummings, M. E., and Hudon, J. (2005). Countergradient variation in the sexual coloration of guppies (*Poecilia reticulata*): drosopterin synthesis balances carotenoid availability. *Evolution* **59**, 175–188.
- Hausmann, F., Arnold, K. E., Marshall, N. J., and Owens, I. P. F. (2003). Ultraviolet signals in birds are special. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **270**, 61–67. doi:10.1098/rspb.2002.2200
- Hill, G. E. (1996). Redness as a measure of the production cost of ornamental coloration. *Ethology, Ecology and Evolution* **8**, 157–175.
- Johnsen, A., Andersson, S., Örnborg, J., and Lifjeld, J. T. (1998). Ultraviolet plumage ornamentation affects social mate choice and sperm competition in bluethroats (Aves: *Luscinia s. svecica*): a field experiment. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **265**, 1313–1318. doi:10.1098/rspb.1998.0435
- Karlsson, B. (1994). Feeding habits and change of body composition with age in three nymphalid butterfly species. *Oikos* **69**, 224–230.
- Kemp, D. J. (2000). Contest behavior in male butterflies: does size matter? *Behavioral Ecology* **11**, 591–596. doi:10.1093/beheco/11.6.591

- Kemp, D. J. (2001a). Reproductive seasonality in the tropical butterfly *Hypolimnas bolina* (Lepidoptera: Nymphalidae) in northern Australia. *Journal of Tropical Biology* **17**, 1–12. doi:10.1017/S0266467401001018
- Kemp, D. J. (2001b). Age-related site fidelity in the territorial butterfly *Hypolimnas bolina* (L.) (Lepidoptera: Nymphalidae). *Australian Journal of Entomology* **40**, 65–68. doi:10.1046/j.1440-6055.2001.00199.x
- Kemp, D. J. (2001c). The ecology of female receptivity in the territorial butterfly *Hypolimnas bolina* (L.) (Nymphalidae): implications for mate location by males. *Australian Journal of Zoology* **49**, 203–211. doi:10.1071/ZO01027
- Kemp, D. J. (2005). Contrasting lifetime patterns of territorial success in the nymphalid butterflies *Hypolimnas bolina* and *Melanitis leda*: a question of flight physiology? *Australian Journal of Zoology* **53**, 361–367. doi:10.1071/ZO05036
- Kemp, D. J. (2006). Heightened phenotypic variation and age-based fading of a nano-structural ultraviolet wing ornament. *Evolutionary Ecology Research* **8**, 515–527.
- Kemp, D. J., and Krockenberger, A. K. (2002). A novel method of behavioural thermoregulation in butterflies. *Journal of Evolutionary Biology* **15**, 922–929. doi:10.1046/j.1420-9101.2002.00470.x
- Kemp, D. J., and Rutowski, R. L. (2001). Spatial and temporal patterns of territorial mate locating behaviour in *Hypolimnas bolina* (L.) (Lepidoptera: Nymphalidae). *Journal of Natural History* **35**, 1399–1411. doi:10.1080/002229301750384329
- Kemp, D. J., Vukusic, P., and Rutowski, R. L. (2006). Stress mediated covariance between nanostructural architecture and ultraviolet butterfly colouration. *Functional Ecology* **20**, 282–289. doi:10.1111/j.1365-2435.2006.01100.x
- Lawrence, C., Vukusic, P., and Sambles, R. (2002). Grazing-incidence iridescence from a butterfly wing. *Applied Optics* **41**, 437–441.
- Lederhouse, R. C., and Scriber, J. M. (1996). Intrasexual selection constrains the evolution of the dorsal color pattern of male black swallowtail butterflies, *Papilio polyxenes*. *Evolution* **50**, 717–722. doi:10.2307/2410844
- Lyytinen, A., Lindström, L., and Mappes, J. (2005). Ultraviolet reflection and predation risk in diurnal and nocturnal Lepidoptera. *Behavioral Ecology* **15**, 982–987. doi:10.1093/beheco/arl102
- McCubbin, C. (1971). 'Australian Butterflies.' (Nelson: Melbourne.)
- McGraw, K. J., MacKillop, E. A., Dale, J., and Hauber, M. E. (2002). Different colors reveal different information: how nutritional stress affects the expression of melanin- and structurally based ornamental plumage. *Journal of Experimental Biology* **205**, 2343–2347.
- Osorio, D., and Ham, A. D. (2002). Spectral reflectance and directional properties of structural coloration in bird plumage. *Journal of Experimental Biology* **205**, 2017–2027.
- Plaistow, S. J., and Siva-Jothy, M. T. (1996). Energetic constraints and male mate-securing tactics in the damselfly *Calopteryx splendens xanthostoma* (Charpentier). *Proceedings of the Royal Society of London. Series B. Biological Sciences* **263**, 1233–1238.
- Plaistow, S. J., and Tsubaki, Y. (2000). A selective trade-off for territoriality and non-territoriality in the polymorphic damselfly *Mnais costalis*. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **267**, 969–975. doi:10.1098/rspb.2000.1098
- Ramsay, G. W., and Ordish, R. G. (1966). Australian blue moon butterfly *Hypolimnas bolina nerina* in New Zealand. *New Zealand Journal of Science* **9**, 719.
- Rutowski, R. L. (1985). Evidence for mate choice in a sulphur butterfly (*Colias eurytheme*). *Zeitschrift für Tierpsychologie* **70**, 103–114.
- Rutowski, R. L. (1992). Male mate-locating behavior in the common eggfly, *Hypolimnas bolina* (Nymphalidae). *Journal of the Lepidopterists' Society* **46**, 24–38.
- Rutowski, R. L., Gilchrist, G. W., and Terkanian, B. (1987). Female butterflies mated with recently mated males show reduced reproductive output. *Behavioral Ecology and Sociobiology* **20**, 319–322. doi:10.1007/BF00300677
- Rutowski, R. L., Macedonia, J. M., Morehouse, N., and Taylor-Taft, L. (2005). Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **272**, 2329–2335.
- Shawkey, M. D., and Hill, G. E. (2005). Carotenoids need structural colours to shine. *Biology Letters* **1**, 121–124. doi:10.1098/rsbl.2004.0289
- Silberglied, R. E. (1979). Communication in the ultraviolet. *Annual Review of Ecology and Systematics* **10**, 373–398. doi:10.1146/annurev.es.10.110179.002105
- Silberglied, R. E. (1984). Visual communication and sexual selection among butterflies. In 'The Biology of Butterflies'. (Eds R. I. Vane-Wright and P. R. Ackery.) pp. 207–223. (Academic Press: London.)
- Silberglied, R. E., and Taylor, O. R. (1978). Ultraviolet reflection and its behavioral role in the courtship of the sulphur butterflies *Colias eurytheme* and *C. philodice* (Lepidoptera, Pieridae). *Behavioral Ecology and Sociobiology* **3**, 203–243. doi:10.1007/BF00296311
- Siva-Jothy, M. T. (2000). The young sperm gambit. *Ecology Letters* **3**, 172–174. doi:10.1046/j.1461-0248.2000.00146.x
- Stride, G. O. (1956). On the courtship behaviour of *Hypolimnas misippus* L. (Lepidoptera, Nymphalidae), with notes on the mimetic association with *Danaus chrysippus* L. (Lepidoptera, Danaidae). *British Journal of Animal Behaviour* **4**, 52–68.
- Stride, G. O. (1957). Investigations into the courtship behaviour of the male of *Hypolimnas misippus* L., (Lepidoptera, Nymphalidae), with special reference to the role of visual stimuli. *Animal Behaviour* **5**, 153–167.
- Stride, G. O. (1958). Further studies on the courtship behaviour of African mimetic butterflies. *Animal Behaviour* **6**, 224–230. doi:10.1016/0003-3472(58)90055-1
- Sweeney, A., Jiggins, C., and Johnsen, S. (2003). Polarized light as a butterfly mating signal. *Nature* **423**, 31–32. doi:10.1038/423031a
- Vukusic, P., and Sambles, J. R. (2003). Photonic structures in biology. *Nature* **424**, 852–855. doi:10.1038/nature01941
- Vukusic, P., Sambles, J. R., Lawrence, C. R., and Wootton, R. J. (1999). Quantified interference and diffraction in single *Morpho* butterfly scales. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **266**, 1403–1411. doi:10.1098/rspb.1999.0794
- Vukusic, P., Sambles, J. R., and Lawrence, C. R. (2000). Structural colour: colour mixing in wing scales of a butterfly. *Nature* **404**, 457. doi:10.1038/35006561
- Vukusic, P., Sambles, J. R., Lawrence, C. R., and Wootton, R. J. (2001). Structural colour: now you see it – now you don't. *Nature* **410**, 36. doi:10.1038/35065161
- Wallace, A. R. (1889). 'Darwinism: an Exposition of the Theory of Natural Selection, with some of its Applications.' (Macmillan & Co.: London.)

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