

The nanoanatomical basis of sexual dimorphism in iridescent butterfly colouration

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Abstract. Structurally generated colours are at least as commonplace and varied components of animal signals as pigment colours, yet we know far less about the former, both in terms of the patterns and phenotypic variation and of their underlying correlates and causes. Many butterflies exhibit bright and iridescent colour signals that arise from a characteristic ‘ridge-lamellar’ scale surface nanoarchitecture. Although there are multiple axes of functional variation in these traits, few have been investigated. Here we present evidence that sexual dimorphism in the expression of a sexually homologous ridge-lamellar trait (iridescent ultraviolet) is mediated by sex differences in the density of lamellar-bearing scale ridges. This trait – ridge density – has also been causally related to iridescent signal variation in other coliadines (e.g. *C. eurytheme*), which suggests that it may offer a common basis to both intra- and intersexual differences in ultraviolet wing reflectance among these butterflies.

Additional keywords: *Eurema hecabe*, Lepidoptera, pierid, ultraviolet (UV).

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Introduction

The striking diversity of animal colouration has long served as a platform for the study of evolution, genetics and optics (Newton 1704; Ford 1964; Prum and Brush 2002). Studies using colour as a dependent variable have, in turn, revealed much about how animals produce, develop and express this trait (Kemp and Rutowski 2011). In the field of behavioural ecology, however, the past several decades have heralded a focus on the biochemistry of pigmentary colour production as a major determinant of signalling costs, and thereby colour signal evolution. This viewpoint has been criticised (e.g. Griffith *et al.* 2006) in representing a narrow view of colour signal evolution, and is also one that largely ignores the visual effects and potential honest signalling role of structural colouration (Kemp and Rutowski 2011).

In contrast to ‘pigment’ colour (which arises through selective light absorption), structural colours arise due to the select reflection of narrow wavebands from optically functional surface architectures, and are often very bright, highly saturated and iridescent (Land 1972; Kinoshita *et al.* 2008). The colour-generating surface structures themselves are constructed during ontogeny using semitransparent materials such as cuticle (as in insects) or keratin (as in birds) (Ghiradella 1994; Prum *et al.* 1999). There are cogent arguments for how the costs/difficulties of building these arrays, including the challenges of achieving

the nanoscale regularity necessary for optimal photonic function, could render structural colours as honest signals of individual quality (Fitzpatrick 1998). Indeed, evidence is rapidly mounting for the condition-dependence of structurally coloured ornamentation across such disparate taxa as birds (Keyser and Hill 1999; Johnsen *et al.* 2003), butterflies (Kemp and Rutowski 2007; Kemp 2008a), spiders (Lim and Li 2007) and damselflies (Fitzstephens and Getty 2000).

The potential communicatory role of structural colouration was recognised at least over a century ago (e.g. Darwin 1871; Wallace 1889), but researchers are only beginning to fully probe the ways in which these colours may function as adaptive signals. Much of what is known at present stems from model vertebrate systems such as guppies (e.g. Kodric-Brown 1985, 1989) and blue tits (e.g. Andersson *et al.* 1998; Hunt *et al.* 1999). However, an incredible diversity of structural colour-producing mechanisms also exists in the invertebrates, particularly among showcase visual signal groups such as butterflies and beetles (e.g. Ghiradella 1991, 1994; Srinivasarao 1999; Seago *et al.* 2009; Vukusic 2006). Indeed, butterflies exhibit a greater diversity than most groups (Vukusic *et al.* 2001; Prum *et al.* 2006; Saranathan *et al.* 2010). This rich source of variation is being increasingly examined, both from the perspective of understanding how biological surfaces may be fashioned to generate colour (Ghiradella 1991, 1994; Vukusic and Sambles

2003), and to understand why and how selection operates to produce such signals (Kemp *et al.* 2006; Kemp and Rutowski 2007; Kemp 2008a, 2008b).

In terms of understanding the causes and consequences of variation in structural colouration, one potentially insightful yet underutilised contrast is between the males and females of the same species. Many butterflies are sexually dimorphic for wing colouration, with the females showing reduced or vestigial expression of homologous wing colour traits that are more highly exaggerated in males. This exaggeration is thought to be due primarily to sexual selection for mate attractiveness, which is adjudged (at least partially) on the basis of visual signals delivered during courtship (Wiklund 2003; Robertson and Monteiro 2005; Costanzo and Monteiro 2007; Kemp 2007, 2008b). Sexual dimorphism in the expression of structural colouration is commonplace, mostly with respect to the presence or size of such markings, but sometimes involving differences in total reflectance and/or hue. In the latter case, there is an opportunity to examine how variation in underlying nanostructure gives rise to intraspecific variability in signal expression (Fitzstephens and Getty 2000; Kemp *et al.* 2006). Insight into the proximate basis of signal variation is essential for a complete appraisal of signal evolution, particularly in relation to sexual selection-based hypotheses.

The large grass yellow butterfly (*Eurema hecabe*) offers excellent empirical opportunities for the study of structural colouration. Both sexes of this species exhibit homologous structurally generated ultraviolet (UV) on their dorsal wings (Rutowski *et al.* 2007). Males express these markings across most of their dorsal fore- and hind-wing surfaces, whereas females display less bright markings on their proximal forewings (Fig. 1a–b). UV reflectance in both sexes depends on phenotypic condition, which is mediated primarily by success in acquiring larval resources (Kemp 2008a), and females prefer males with brighter UV (Kemp 2008a, 2008b). The UV is reflected from a Type I ridge-lamellar system (Vukusic *et al.* 2000), the physics of which is well understood (Kinoshita and Yoshioka 2005). Briefly, an air–chitin multilayer is built up on longitudinal ridges that run at regular intervals along the uppermost (dorsal) surface of specialised wing scales (Fig. 1c). The chitinous layers, known as ‘lamellae’, constitute the optically dense multilayers, which function along with their alternating air layers to constructively interfere UV. The entire ridge-lamellar framework is underlain by the body of the scale, which is rich in the pteridine pigments responsible for the wing’s ‘human’ yellow appearance. Because these pteridines are situated beneath the ridge-lamellar multilayers, and absorb strongly below 400 nm, they are thought to amplify the ‘limited-view’ nature of the wing’s UV signal by ensuring that only iridescent (coherently scattered) UV is reflected from its surface (Rutowski *et al.* 2005).

Our goal in this study was to explore the nanoarchitectural basis of sexual dimorphism in the expression of UV wing reflectance in *E. hecabe*. We focus on total reflectance because this characteristic is known to be highly phenotypically variable (Kemp 2006, 2008a), strikingly dimorphic (Rutowski *et al.* 2007), and of verified salience to mate choice (Kemp 2008b). The wing nanostructures are best approximated as thin-film multilayer reflectors, and although there are many

potential axes of nanoarchitectural variation, theory suggests that overall reflectance in such a system is primarily a function of the total number of layers and the density of reflectors (Land 1972; Kinoshita *et al.* 2008). On the basis of prior work in the related coliadinine *Colias eurytheme* (Kemp *et al.* 2006), we predicted that whole-wing UV reflectance would be determined by the density of lamellae-bearing ridges. Moreover, we predicted that if there is a common basis to both intra- and intersexual differences then males should show a greater density of UV-bearing scale ridges than females. We used reflectance spectrometry and electron microscopy to search for covariance between nanostructural variables and UV wing reflectance in both males and females.

Materials and methods

Provenance of specimens

We used 45 male and 45 female butterflies, which we chose haphazardly (essentially at random with respect to UV reflectance) from among the F1 offspring of 90–100 dams sampled from around Cairns, north Queensland, Australia. All butterflies were reared in the laboratory at James Cook University using full-spectrum greenhouse-cultivated *Sesbania cannabina* as a larval host, and under standard conditions of 26–28°C and 14:10 L:D.

Reflectance spectrometry

The techniques for measuring iridescent butterfly wing colouration are well developed and documented elsewhere (e.g. Kemp 2006). Briefly, we collected reflectance spectra from a central 5-mm region of each individual’s right dorsal forewing using an Ocean Optics USB-4000 spectrophotometer (10 averaged scans, 100 ms integration time). Illumination was provided normal to the wing surface by an Ocean Optics PX-2 pulsed xenon light source, with the USB-4000 collector probe set at 45°. Owing to variability among individuals in their viewing/illumination angle conducive to maximal UV reflectance, the wing was set on a glass slide and placed on a universal stage that was rotated 3–7° (around the wing’s proximal–distal axis) to find the angle of maximal UV reflection. Wings were always arranged horizontally, so that their base pointed towards the collector. We initially summarised UV reflectance as both the (a) mean and (b) maximum reflection amplitude in the 300–400-nm range, which was assessed relative to a magnesium oxide white standard. All measurements were highly repeatable (left- to right-wing correlations for each sex: $r > 0.95$, $n = 45$, $P < 0.0001$), and averaged for subsequent analyses. Our measures of mean and maximum reflectance were themselves strongly correlated ($r > 0.903$, $n = 45$, $P < 0.0001$), and yielded identical primary conclusions (at $\alpha = 0.05$). In keeping with prior work on the system (e.g. Kemp 2008a, 2008b), here we report only the results for mean reflectance.

Electron microscopy

For each individual we excised three replicate sections from the approximate area of wing for which reflectance spectra were collected, before sputter coating them with 5 nm of gold (2 min at 35 mA). We obtained the SEMs using a JEOL JSM-6480 LA scanning electron microscope set to backscattered electron

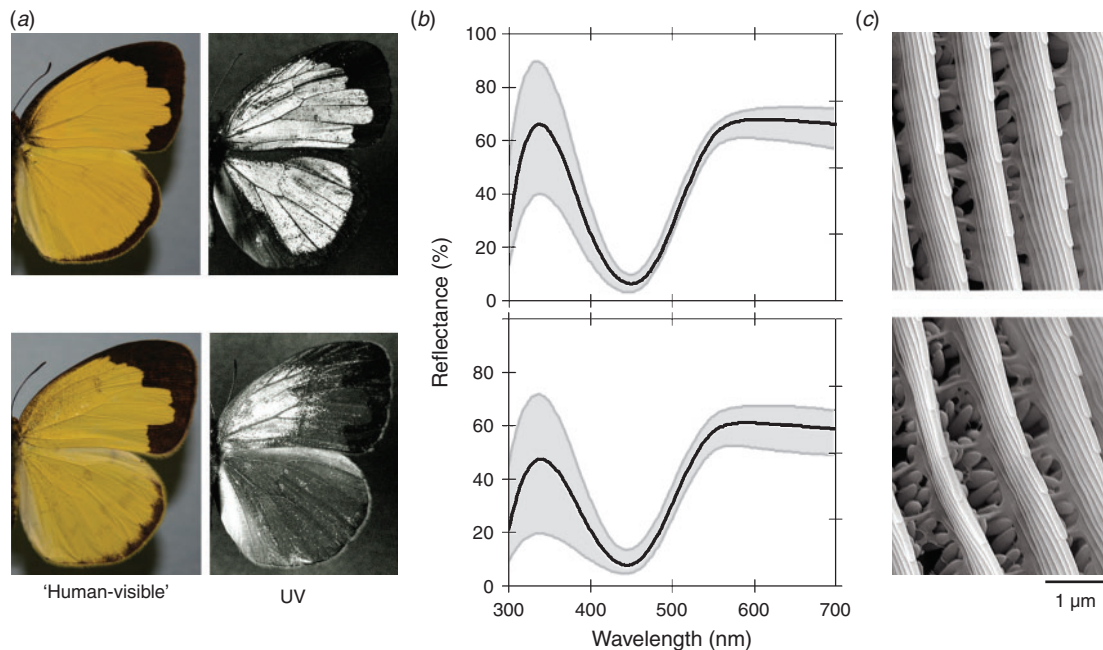


Fig. 1. (a) Dorsal wing colouration of male (above) and female (below) *Eurema hecabe*, as photographed under 'human visible' (400–700 nm) and UV (300–400 nm) light; (b) reflectance spectra showing the average (solid line) and minimum–maximum range (shaded areas) for males and females ($n = 20$ for each sex); (c) scanning electron micrographs (SEMs) of male and female wing scales. (Modified from Kemp 2008b.)

imaging with an accelerating voltage of 15 kV, spot size of 49, zero stub tilt and 12 mm working distance.

Morphometrics

We focussed on the density of longitudinal ridges and the number of lamellae per ridge as key nanostructural predictors of UV reflectance (based on Kemp *et al.* 2006). Using 2000 \times magnification SEMs, we counted the number of ridges that spanned a 30- μ m scale bar centred upon a single wing scale, perpendicular to the ridge axis. Measurements were repeated for three haphazardly selected wing scales per section of wing. We also used the 20000 \times magnification SEMs to count the number of lamellae per ridge for one wing scale per section of wing. Within-sample variation was low for both parameters; thus we averaged the replicate measures for each individual.

While this study focuses on two nanostructural parameters, we fully expect that signal properties will be determined by a potentially broad spread of additional nanoscale features, such as lamellar cross-sectional width and/or spacing (which together determine the 'optical distance' of the interference layers). Similarly, signal hue is theoretically anticipated to be influenced by various axes of nanostructural variation (as above) and by variation in materials refractive index. Accurate assessment of these covariances is beyond the reach of this study, and to be presented elsewhere.

Statistical analysis

We explored the relationship between wing scale nanostructure and UV reflectance using a REML-based information-theoretic approach (Burnham and Anderson 2002). REML-based

parameter estimation is more flexible, requires fewer assumptions, and is preferable over least-squares when there are multiple random covariates. Least-squares estimation of slope is also known to be biased in situations where both variables (predictor and response) are subject to error. The information-theoretic approach involves calculating all possible linear combinations of the predictor variables and interactions, then ranking these candidate models in terms of parsimony according to Akaike's Information Criterion (AIC). The AIC is a derivative of the log-likelihood function, which is adjusted for the increase in model parsimony arising as a consequence of increasing predictors. Specific models are outlined in the results when presented. Aside from the AIC ranking, the only other 'rule' specified during best subsets computations was that interaction terms could only be present along with both relevant main effects. We investigated the significance of individual terms in most-parsimonious models using the Wald statistic.

Given that both independent and dependent variables were measured with random error components, we used Model II regression (i.e. Reduced Major Axis) techniques to estimate bivariate slopes and confidence regions. All analyses were conducted according to the procedures of Sokal and Rohlf (1995) and using Statistica ver. 7.0.

Results

Morphometric and spectrometric measures

As would be expected from a random sample, male wings reflected more UV than female wings ($t_{88} = 9.68$, $P < 0.0001$). Males also exhibited a greater average density of ridges on their wing scales ($t_{88} = 4.98$, $P < 0.0001$), but there was no sex

difference in the number of lamellae per ridge ($t_{88}=1.53$, $P=0.129$) (Table 1).

Nanostructural basis of UV reflectance

We initially ran a best-subsets GLM analysis of UV reflectance, in which we specified sex (fixed factor), ridge density, lamellar frequency, and first-order interactions as predictive factors (Table 2). The most parsimonious model was significant overall ($G_3=75.15$, $P<0.0001$), and included three individually significant effects: sex (Wald=6.30, d.f.=1, $P<0.05$), ridge density (Wald=5.11, d.f.=1, $P<0.05$) and the sex*ridge density interaction (Wald=4.07, d.f.=1, $P<0.05$). As identified previously, male wings reflected more UV, as did individuals with more dense scale ridges (Fig. 2a–b). The significant sex*ridge density interaction indicated that UV reflectance increased with scale ridge density in females but not in males.

On the basis of this interaction, we then constructed models individually for each sex, which included the variables ridge density, lamellar number and the ridge*lamellae interaction

(included to explore any potentially more subtle interactive effects among morphological variables). As anticipated, nanostructure successfully predicted UV reflectance in females but not males (Table 3). In females, the most parsimonious model was, again, one including only ridge density (higher density = greater UV reflectance) (Fig. 2a), with no additive or interactive effect of lamellae number. In males, no linear combination of variables explained the observed variation in UV reflectance ($P>0.80$). Although the (Reduced Major Axis) slope estimate for the relationship between ridge density and UV reflectance in males was positive (Fig. 2b), the relationship itself was deemed non-significant.

Morphometric sex differences

The most parsimonious predictive model of sex (based on morphometrics) again only included ridge density (AIC=106.6, $G_1=22.1$, $P<0.0001$) (Table 4), which underscores the denser packing of ridges on male wing scales (Fig. 2). That

Table 1. Descriptive morphometric and spectrometric measures obtained from the wings of 45 male and 45 female *Eurema hecabe*

UV reflectance is the wing's mean % reflectance over the wavelength interval 300–400 nm. The variable 'Ridge density' refers to the density of longitudinal scale ridges that bear the stacks of UV-reflective 'lamellae'

Trait	Sex	Mean	95% confidence limits	Minimum–Maximum (Range)
UV reflectance (%)	F	56.15	53.93–58.38	37.21–75.77 (38.56)
	M	70.36	68.41–72.31	57.16–86.23 (29.07)
Ridge density (per 1 μm)	F	0.926	0.907–0.945	0.767–1.083 (0.316)
	M	0.984	0.970–0.997	0.907–1.083 (0.176)
Lamellar number (per ridge)	F	6.496	6.32–6.66	5.33–7.33 (2.00)
	M	6.674	6.51–6.84	6.00–7.67 (1.67)

Table 2. A summary of best-subsets predictive modelling of wing UV reflectance in *Eurema hecabe* on the basis of sex and wing-scale ultrastructure

The five most parsimonious predictive linear models are shown, in order of their Akaike's Information Criterion (AIC) score. Smaller values of the AIC indicate improved fit. The variable 'Ridges' refers to the density of longitudinal scale ridges that bear the stacks of UV-reflective 'lamellae'

Model (combination of predictor variables)	d.f.	LL-ratio	P	AIC
1. Sex + Ridges + S*R	3	75.1	<0.0001	600.8
2. Sex + Ridges	2	71.2	<0.0001	602.7
3. Sex + Ridges + Lamellae + S*R	4	75.1	<0.0001	602.8
4. Sex + Ridges + Lamellae	3	71.2	<0.0001	604.7
5. Sex + Ridges + Lamellae + S*R + S*L	5	75.2	<0.0001	604.7

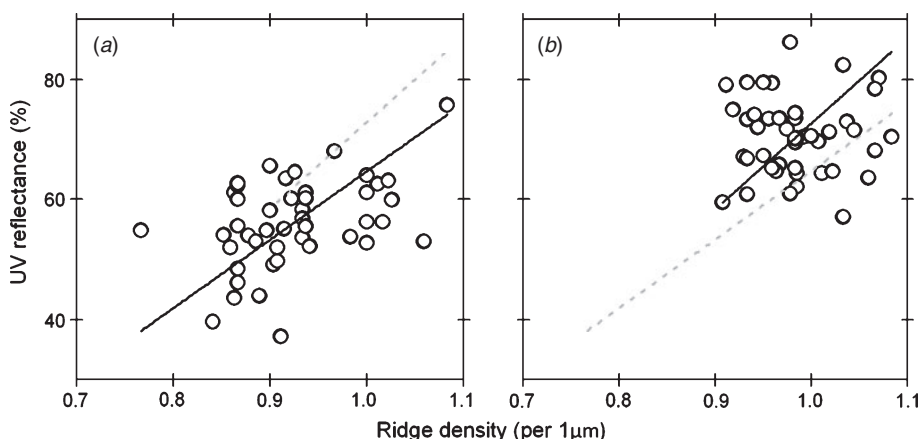


Fig. 2. Bivariate scatterplots expressing wing UV reflectance (y-axis) as a function of wing scale ridge density (x-axis) for (a) female and (b) male *E. hecabe*. The wing scale ridges bear the lamellar thin-films (Fig. 1c) that reflect UV light (Fig. 1a). Reduced Major Axis (RMA) slopes are represented as solid lines for each sex, and are given as half-tone dashed lines in each opposite-sex plot for comparison. Pearson's R correlation estimates are also given along with significance estimates. RMA slopes are approximated by the equations: UV reflectance = $-49.33 + 114.039 \times$ ridge density (females) and UV reflectance = $-70.37 + 143.07 \times$ ridge density (males). Note that only the female relationship attained statistical significance.

Table 3. A summary of best-subsets modelling of UV reflectance for male and female *Eurema hecabe*

Model (combination of predictors)	d.f.	Model statistics		
		LL-ratio	<i>P</i>	AIC
Males				
Lamellae	1	0.0531	0.818	298.9
Ridges	1	0.0262	0.871	298.9
Ridges + Lamellae	2	0.0753	0.963	300.9
Ridges + Lamellae + R*L	3	0.464	0.927	302.5
Females				
Ridges	1	9.10	<0.005	301.8
Ridges + Lamellae	2	9.13	<0.05	303.8
Ridges + Lamellae + R*L	3	10.54	<0.05	304.4
Lamellae	1	0.0318	0.858	310.9

Table 4. A summary of best-subsets modelling of sex in *Eurema hecabe* on the basis of wing ultrastructure (as explained in Table 2)

Model (combination of predictor variables)	d.f.	Model statistics		
		LL-ratio	<i>P</i>	AIC
1. Ridges	1	22.1	<0.0001	106.6
2. Ridges + Lamellae	2	23.2	<0.0001	107.6
3. Ridges + Lamellae + R*L	3	23.2	<0.0001	109.5
4. Lamellae	1	2.36	0.125	126.4

‘lamellae number’ did not feature in the most parsimonious model also indicates that males and females do not differ in this feature.

Discussion

Researchers have known for centuries that the iridescent colours of butterfly wings result from a form of structural colouration (Kinoshita 2008). It was not until work led by Ghiradella in the early 1970s (Ghiradella *et al.* 1972; Ghiradella 1974) that the causative multilayer ‘ridge/lamellar’ architecture (perhaps the most widespread agent of iridescent signal production in the Lepidoptera) was convincingly documented. Since Ghiradella’s work, multiple investigators have explored multiple axes of phenotypic variation in this trait (Kemp 2006, 2008a; Kemp and Rutowski 2007; Rutowski *et al.* 2007). However, there has been only one attempt to uncover the nanostructural basis of such variation, that by Kemp *et al.* (2006) in male *Colias eurytheme*. Because female *C. eurytheme* do not express sexually homologous UV, this investigation could not yield insights into the proximate basis of sexual differences in the expression of ridge-lamellar UV.

Eurema hecabe is somewhat of an exception among coliadines in that females exhibit a homologous yet less-bright version of ridge-lamellar UV, a typically male-limited trait (Rutowski *et al.* 2007; Kemp 2008b). This species allows examination of the sexual basis to nanostructural colour variation, and we provide first insights into the quantitative proximate basis of sexual dimorphism. Two salient findings are: (1) that male and female *E. hecabe* vary in the density of lamellar-bearing ridges on their wing scales, and (2) that this trait covaries with UV reflectance across the sexes (and in females) in the manner

predicted if it were causal (Fig. 2). Aside from the lack of such a relationship in males, our data are consistent with the simplest hypothesis, that sexual dimorphism in UV is driven – at least to some extent – by variation in the same factor (ridge density) that underlies intrasexual UV variation (and which is also seen in male *C. eurytheme*: Kemp *et al.* 2006).

To unequivocally demonstrate a causal role for scale ridge density would, of course, require direct manipulation of this parameter; a feat not easily achieved. The study of *C. eurytheme* by Kemp *et al.* (2006) perhaps comes closest, in that subjects were reared under stressful conditions that would have affected the development of scale nanostructure. The fact that ridge density also covaried in a similar way with UV reflectance in *C. eurytheme* supports (to some extent) the putative causality of the relationship we report for female *E. hecabe*. It remains possible, however, that ridge density covaries with some other unmeasured aspect of wing scale nanostructure, which is instead the true determinant of UV reflectance. As in *C. eurytheme*, the absolute explanatory power of ridge density for UV reflectance in *E. hecabe* is rather weak ($r^2 \sim 0.25$ across all individuals, irrespective of sex), which suggests, at the very least, that a role exists for other, unstudied nano-, micro- or macroscale structural determinants of UV (e.g. Stavenga *et al.* 2006, 2010).

It is notable that ridge density did not covary significantly with UV reflectance in males. We suggest that this most likely stems from the relatively reduced variance in UV reflectance among males (Kemp 2008b). Given that we sampled individuals essentially at random (within the sexes), it follows that our male sample captured less absolute phenotypic variance in this trait than our female sample. In fact, the absolute range seen in female ridge density was nearly twice that seen among males (Table 1; Fig. 2a–b). Kemp *et al.* (2006) reported significant covariance between ridge density and UV reflectance among two samples of male *C. eurytheme* comparable in size to that studied here, but an important difference was that their samples were chosen to maximise variation in the UV. Here our primary goal was to identify sexual differences, which prescribed that we sampled individuals randomly, and which may, in turn, have restricted our ability to detect covariances between nanostructure and reflectance. We consider this especially likely for our restricted male sample, whose range of ridge density (0.176) was also considerably lower than the range of values (0.324) in the sample of *C. eurytheme* studied by Kemp *et al.* (2006).

Lack of strength in relationships between nanostructure and reflectance may also exemplify a broader issue in these studies (*sensu* Luke *et al.* 2009): the problem of relating intrascale structure to a macro- or even supermacroscale trait (whole- or part-wing UV reflectance). Whole-wing reflectance is what signal receivers would appraise, and is therefore most directly related to selection, but the relationship between this trait and intrascale architecture will also be determined by intermediate (interscale) factors such as scale layering and stacking effects (Stavenga *et al.* 2006, 2010). The strongest proximate relationships between nanostructure and colour should reside in the links between intrascale nanostructure and single-scale reflectance (e.g. Vukusic *et al.* 1999). Our ability to model this system in a ‘bottom-up’ sense will therefore rely on such evidence,

which emphasises the importance of incorporating single-scale reflectance measurements into future studies of this nature.

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