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Female mating preferences and male signal variation in iridescent *Hypolimnas* butterflies

Darrell J. Kemp [a,b,*], David Jones [a], Joseph M. Macedonia [c], Andrew K. Krockenberger [a,d]

[a] School of Marine and Tropical Biology, James Cook University, Queensland, Australia
[b] Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia
[c] Department of Biology, Florida Southern College, Lakeland, FL, U.S.A.
[d] Centre for Tropical Biodiversity and Climate Change, James Cook University, Queensland, Australia

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Nature's most striking, complex and innovative colour signals are generated via selective reflectance from optical nanostructures (rather than selective absorbance by pigments), a phenomenon known as structural coloration. These colours reach their height of visual brilliance as sexual signals, a context in which they also express both great functional innovation and high evolutionary lability. However, owing to a historical preoccupation with pigments, we know relatively little about the evolutionary causes and consequences of innovation in structurally coloured sexual signals, especially in exemplar arthropod taxa. In this study we addressed the possibility that species differences in intraspecific mating preferences may contribute to visual and functional variation in structural coloration. We contrasted mate preferences and signal properties between two closely related butterfly species (*Hypolimnas alimena* and *Hypolimnas bolina*) that possess male-elaborated structural coloration. *Hypolimnas bolina* offers a valuable comparative basis because females are known to prefer highly bright and limited-view ultraviolet markings, which males generate via complex nanoscale surface multilayer arrays. Male *H. alimena*, by contrast, display less bright and weakly iridescent dorsal blue, arising from a simpler surface microarchitecture. In two separate experiments, we found that female *H. alimena* did not distinguish between males spanning a graded range of 0.25–1.4× natural peak brightness. Only once the dorsal blue was completely obscured did male mating success suffer. Furthermore, a sample of wild phenotypes indicated greater variance for signal brightness in male *H. bolina* than *H. alimena*, but no difference in peak hue (i.e. signal colour). These results supported a priori predictions, and are consistent with a scenario whereby directional female preference has driven male signal exaggeration in *H. bolina*, but not its less ornamented close congener.
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Coloration is an important and intriguing component of phenotypic diversity. Animal colour traits function adaptively as visual signals in contexts such as crypsis, aposematism and mimicry, and in social and sexual signalling (Osorio & Vorobyev, 2008). As sexual signals, coloration may facilitate interspecific communication (e.g. mate recognition) and/or intraspecific communication (e.g. mate or rival assessment). In the former case, selection is expected to maximize interspecific differences, and therefore to act in a stabilizing manner within a species to produce discrete badges of species identity. In the latter case, strong intraspecific vectors of directional selection can reinforce runaways in colour traits, leading to phenotypic exaggeration in the selected sex (usually males; Darwin, 1874). Exaggeration in this context refers to the extent the trait removes the phenotype from its putative optimum for viability (Bonduriansky, 2007; Bonduriansky & Rowe, 2005). Phenotypically, this often equates to a colour signal of high visual conspicuousness, that is, one with strongly contrasting within-pattern elements and/or that contrasts highly with its viewing background (Endler, 2012; Endler & Mielke, 2005). Selective trait exaggeration of this manner mediates against viability by demanding important resources, increasing the risk of predation and/or reducing the opportunity for resource acquisition (Rowe & Houle, 1996). An intriguing outcome of directional sexual selection is that, aside from directing overall greater levels of exaggeration, target traits tend to evolve greater phenotypic variation (Kirkpatrick & Ryan, 1991; Pomiankowski & Møller, 1995). This is understood to occur because individuals of varying phenotypic and/or genetic quality are differentially able to afford the marginal viability costs associated with increasing trait expression (Grafen, 1990; Rowe & Houle, 1996; Zahavi, 1975).

* Correspondence: D. J. Kemp, Department of Biological Sciences, Macquarie University, Sydney (North Ryde), New South Wales 2109, Australia.
E-mail address: darrell.kemp@mq.edu.au (D. J. Kemp).

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Mechanistically, an exaggerated visual signal can be generated by the sequestration in surface tissues of pigments that produce conspicuous colours (Grether, Kolluru, & Nersissian, 2004; Hill, 1991). Alternatively, conspicuous colour signals can also be generated by arrays of nanoscale surface structures that interact with light in highly specific ways (Land, 1972). Reflectance from nanostructural arrays can be extremely high, and confined to narrow spectral bandwidths, hence structural colours constitute nature's brightest and most deeply saturated visual signals (Srinivasarao, 1999; Vukusic & Sambles, 2003). They also exhibit unique visual properties, such as plane polarization and iridescence, and can be highly restricted in their range of viewing angles (Vukusic, Sambles, Lawrence, & Wootton, 2001). In the context of sexual signalling, these properties have the potential to increase signal conspicuousness (e.g. by creating strobe effects) or reduce signalling costs (e.g. by restricting the angular emission function of the signal; see Whitney et al., 2009 for an interesting example in regard to iridescent flower coloration). The structural provenance of these visual effects also means that, through examination of the causative nanoarchitectures, empiricists can gain insights into functional signal innovation (Maia, Rubenstein, & Shawkey, 2013; Wickham, Large, Poladian, & Jermiin, 2006). However, owing to a historical preoccupation with pigment-based indicator signals, we know relatively little about how selection has shaped the manifold visual and functional features of structural coloration (Kemp, Herberstein, & Grether, 2012).

Butterflies are an excellent group for investigating the causes and consequences of variation in colour signals. Their wing colours showcase some of the most visually exaggerated and functionally innovative signals in nature (Prum, Quinn, & Torres, 2006; Shawkey, Morehouse, & Vukusic, 2009; Srinivasarao, 1999; Vukusic, Sambles, & Ghiradella, 2000). Butterflies have also proven pivotal in the exploration of how nanoscale architectures can generate visual effects. Investigations in this group have informed our understanding of a raft of novel phenomena (Vukusic et al., 2001; Vukusic & Hooper, 2005; Vukusic, Sambles, & Lawrence, 2000, 2004). Such work has also revealed great variation in colours and colour-producing surface architectures among closely related species (Ghiradella, 1985, 2010). This implies considerable evolutionary lability, not only in the colours themselves, but in their underlying (nanoarchitectural) proximate bases. Such lability is consistent with the action of sexual selection (Brunton, 1998), acting interspecifically (via accentuating divergence among species for mate recognition) and/or intraspecifically (via favouring runaways in species-specific aspects of the colour phenotype).

Sexual selection has been well studied in butterflies, particularly in the context of mate choice, and occasionally in relation to exaggerated structural colour-based signals. Overwhelmingly, however, most of these studies have explored preferences and signal variation at the interspecific level. This reflects an enduring interest in the empirical use of butterflies to advance theory relating to speciation and associated phenomena (such as reinforcement; Jiggins, Naisbit, Coe, & Mallet, 2001; Kronforst, Young, & Gilbert, 2007; Melo, Salazar, Jiggins, & Linares, 2009; Silberglied & Taylor, 1973). Although informative, this emphasis means that we have learned relatively little about sexual colour variation at the intraspecific level, and even less about the nature of intraspecific mating preferences. This contrasts markedly with other taxa, for which mating preferences have been characterized down to individual populations (Endler & Houde, 1995; Houde & Endler, 1990; Simmons, Zuk, & Rotenberry, 2001), or even to the level of individual females (Brooks & Endler, 2001). Only a single study (to our knowledge) has sought to contrast interspecific versus intraspecific levels of variation in colour-based butterfly mating signals (Brunton & Majerus, 1995). In terms of structurally coloured ornamentation in this group, it is therefore difficult to appraise the extent to which evolutionary innovation has been driven by interspecific versus intraspecific processes of sexual selection.

In this study we set out to explore a contrast in intraspecific mating biases and sexual signal variation in two closely related *Hypolimnas* butterflies: *H. alimena* (the blue-banded eggfly) versus *H. bolina* (the common eggfly). These species are similar in many aspects of their biology, including host plant–habitat use, life history, breeding phenology, extent of polyandry (about 1.1 matings per female) and male mating tactics (Braby, 2000; D.J. Kemp, unpublished data). They are both sexually dimorphic for dorsal coloration, wherein males exhibit variously exaggerated structurally coloured markings. As expected for the mating signals of close sympatric relatives, these markings are highly divergent, showing discrete differences in both colour (hue) and overall pattern. However, *H. bolina*'s sexual signal displays far greater visual exaggeration, primarily in terms of narrow-band brightness, and arises from a more architecturally complex surface structure (see below; Fig. 1). Female *H. bolina* are known to prefer increasingly bright male signals (Kemp, 2007; see below), suggesting that strong directional female choice drives signal exaggeration in this species. Nothing is known of intraspecific mating preferences in *H. alimena*. Putting aside questions of why species differences in preferences exist, we hypothesized that the (relative) lack of visual exaggeration in *H. alimena*'s sexual coloration may relate to the absence of directional female mate preferences. This predicts a species difference in the shape of female preference functions, which we tested via mating experiments in *H. alimena*, designed to emulate closely the preference assays used in *H. bolina* (Kemp, 2007).

In addition to investigating female preferences, we also set out to determine natural levels of phenotypic variation in the male signal characteristics of each species. This is of interest for two reasons. First, it was necessary to define the bounds of signal variation in *H. alimena* to guide the brightness manipulations used in mating experiments. Second, and as noted above, theory predicts that strong directional selection of the nature anticipated in *H. bolina* will increase phenotypic trait variation, primarily by favouring greater developmental integration and condition-dependent trait expression (Badyaev, 2004; Bonduriansky, 2007; Rowe & Houle, 1996). Stabilizing selection, by contrast, should disfavour intraspecific variation. We therefore predicted that if female preferences in *H. alimena* are stabilizing (or weakly directional relative to *H. bolina*), then variation in the male signal should be relatively reduced in this species. We expected this to apply particularly to signal brightness, rather than hue, given that brightness is the key feature of visual exaggeration that varies between these congeners.

METHODS

Study Species and Signal Traits

Our study species are medium- to large-sized nymphalid butterflies with wide, overlapping distributions throughout Australasia. We essentially used the more intensively studied species *H. bolina* as a comparative basis for investigation of mating preferences and signal variation in *H. alimena*. As noted, female *H. bolina* are known to mate preferentially with males bearing bright dorsal ultraviolet (UV) markings (Fig. 1a). A 50% reduction in peak UV brightness has been demonstrated to affect sexual attractiveness to the same extent as complete removal of these markings (Kemp, 2007). The UV markings have no analogue in conspecific females, and are extremely reflective and chromatic (Kemp & Macedonia, 2006; Fig. 1c). They arise from multilayer

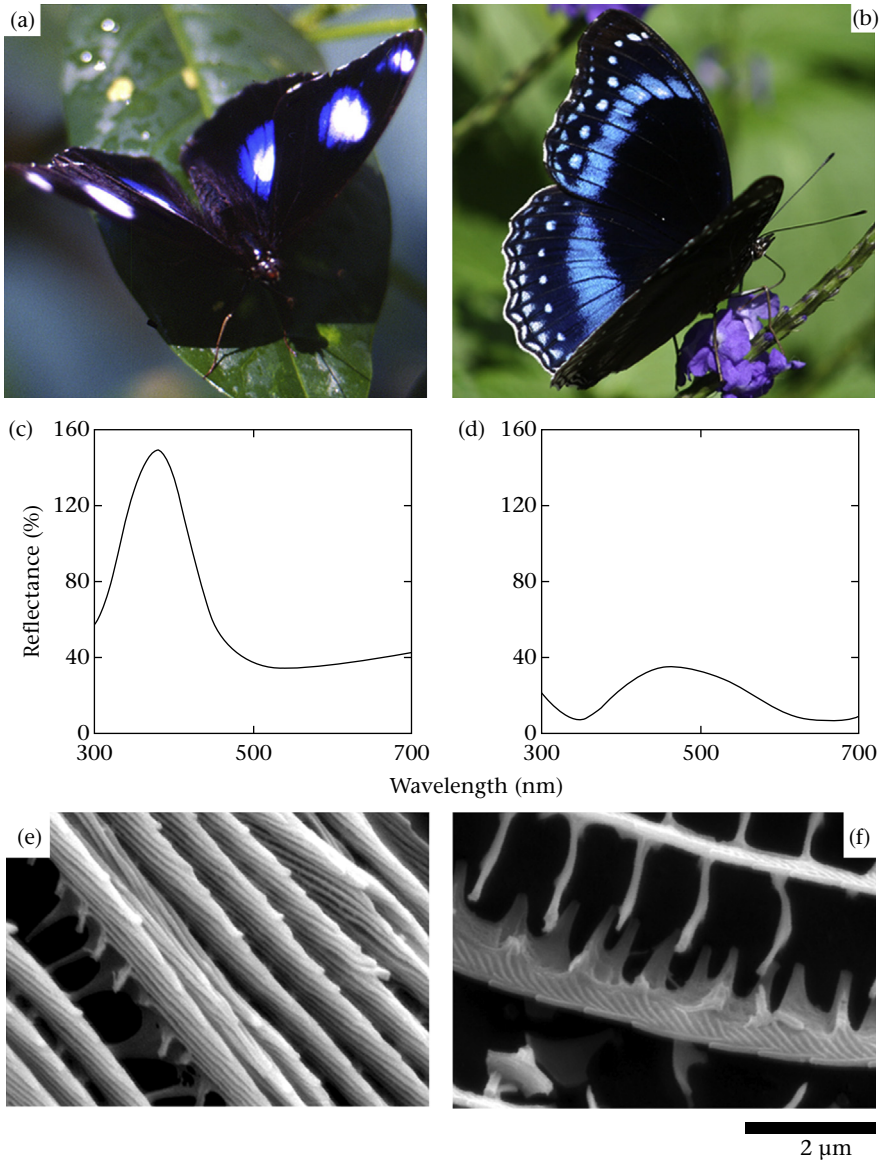

Figure 1. Male colour patterns, colour properties and production mechanisms in the two closely related *Hypolimnas* butterflies. Males of *Hypolimnas bolina* feature ovoid-shaped iridescent patches on their dorsal wings (a) that are maximally reflective in UV wavelengths (c) and generated by a complex ridge-lamellar surface architecture (e). In contrast, the dorsal wings of male *Hypolimnas alimena* display bands of metallic blue (b) which are much less iridescent, less bright and less chromatic (d) than *H. bolina*'s UV markings, and which arise from a simpler microrib structure (f). Reflectance spectra in (c) and (d) are averages for 30 freshly emerged exemplars of each species. Scanning electron micrographs in (e) and (f) were obtained from gold-coated wing scales using a Leica-Cambridge Stereoscan 360FE scanning electron microscope (acceleration voltage = 15 kV, probe size = 75–90 pA), using established protocols.

interference generated by a complex architecture of alternating air–cuticle interfaces positioned on the uppermost surfaces of wing scales (Fig. 1e; also see Ghiradella, 1974; Ghiradella, Aneshansley, Eisner, Silberglied, & Hinton, 1972). Similar to many butterflies, for example *Ancyluris meliboeus* (Vukusic et al., 2001), the multilayers are angled relative to the plane of the wing scale. This, coupled with the angled insertion of individual scales on the wing membrane, produces a greatly restricted range of viewing angles for the signal (Kemp & Macedonia, 2006). In *H. bolina*, this means that the bright UV flashes on and off as males sweep their wings through a modest angular range, which contributes a strobe effect when males are courting females (T. E. White, J. Zeil, & D. J. Kemp, unpublished data). Male *H. alimena*, by contrast, display largely noniridescent dorsal blue bands (Fig. 1b), which are weakly expressed in some conspecific females (which are polymorphic and highly polyphenic for dorsal blue). The blue of male *H. alimena* achieves less than one-third the peak brightness of male *H. bolina*'s UV (Fig. 1d), is not limited-view, and arises from architecturally less innovative arrays of surface microribs (i.e. the type III structures of Vukusic, Sambles, & Ghiradella, 2000; Fig. 1f).

Field Collections and Assessment of Colour Variation

We sampled male *H. bolina* and *H. alimena* from the same mate encounter sites around Cairns, Australia (16°53′S, 145°45′E), where the two species frequently interact (D.J. Kemp, personal observations, 1997–2010). Butterflies were collected in January 2007 and from September to March 2008, transported in glassine envelopes placed inside an ice cooler, and quickly euthanized by freezing upon return to the laboratory. We subsequently quantified the reflectance of each species' dorsal colour markings using an Ocean Optics USB-4000 spectrometer (100 ms integration time, 10 averaged

scans), PX-2 pulsed xenon light source and magnesium oxide (MgO) reflectance standard. Reflectance was captured from a circular region of 2 mm diameter, with the wing rotated on a dual-axis stage to locate the orientation conducive to maximum brightness according to well-established protocols (see, e.g. Kemp, 2006, 2008; Kemp & Macedonia, 2006). This is necessary because simply measuring each wing from a fixed angle of orientation would introduce artefacts from interindividual differences in wing orientation conducive to maximum brightness. We summarized brightness for each wing as the maximum amplitude of the unimodal reflectance peak (see Fig. 1c, d). In this paper, where we refer to brightness we do so in percentage units relative to the 100% brightness of an MgO standard. We quantified hue (in nanometres) as the wavelength corresponding to the position of this peak (i.e. λ_{max}). Right and left wing values for brightness and hue were highly correlated ($r > 0.95$), and we averaged them for each individual.

In addition to capturing males, we also collected conspecific females of each species and used them to generate laboratory-reared F1 generations. This provided animals for the experiments in *H. alimena*, and for estimating peak signal brightness of both species (as in Fig. 1c, d). Juveniles of both species were reared on *Asystasia gangetica* (Acanthaceae) at 28 ± 2 °C (day) and 23 ± 2 °C (night), in a 14:10 h light:dark photoperiod. Several hours after emergence, adult *H. alimena* were marked with a small identifying number in gold ink on their ventral hindwing. A similar numbering technique was used in Kemp's (2007) *H. bolina* experiments, and is not thought to influence individual behaviour or signalling (Morton, 1982). Before being released into a cage (see below), all subjects were stored individually in gauze-topped 650 ml plastic cups at a constant 24 ± 0.5 °C, and given access to cotton wool soaked in 15% sugar water solution.

Experimental Set-up

We conducted two mating experiments with *H. alimena*, both conducted using the same 6 × 15 m and 4 m high outdoor enclosure as used by Kemp (2007) to assay mating preferences in *H. bolina*. This cage, situated on campus at James Cook University (Cairns), was covered by 32% woven shade-cloth, and outfitted with mulch and potted palms to mimic the rainforest edge environments that frequently serve as the mating habitats of both species. Surviving subjects were captured and quickly euthanized after each experiment and stored at −30 °C as a reference source and to allow future measurements of morphology, reflectance and/or ejaculate characteristics (data not shown).

Experiment 1: Brightness Increase/Reduction

This experiment involved only two colour treatments: one in which peak brightness of male *H. alimena*'s dorsal blue band was artificially increased to a level about 1.4× that of the average newly emerged male, and the other in which the peak was reduced to about 0.67× that of the average newly emerged brightness (Fig. 2). These manipulations were achieved using stock mixtures of blue and black acrylic paint dissolved in 95% ethanol. The blue paint, so-called galactic blue (Pearlescent FW Liquid Acrylics, Daler-Rowney, NJ, U.S.A.), dried with a metallic effect similar to the weak iridescence of *H. alimena*'s natural blue markings. Both treatment solutions were opaque, which meant that they completely obscured the natural dorsal markings. However, the shape of treatment reflectance curves closely resembles that of the natural wing colour (Fig. 2), and the use of both treatments simultaneously controlled for the effects of handling, marking, and the presence of blue and black ink.

We initiated this experiment by liberating into the cage 50 virgin, 3–4-day-old laboratory-reared females, plus 25 similarly aged

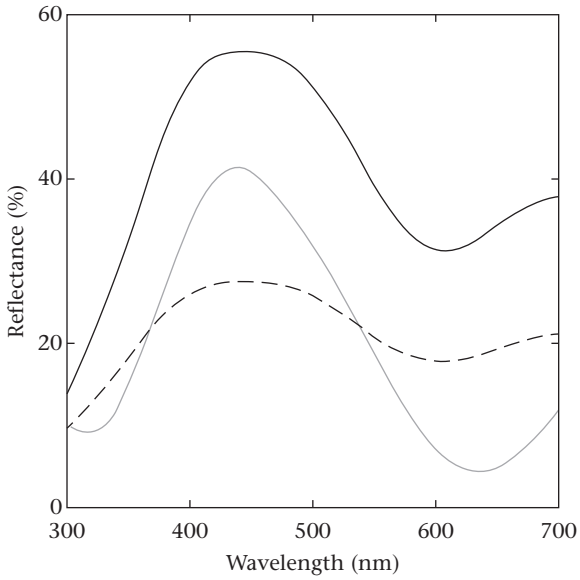

Figure 2. Reflectance spectra of the dorsal blue band of male *H. alimena* indicating the visual manipulations employed in experiment 1. The upper (solid black) and lower (dashed black) traces represent the increased (1.4×) and reduced (0.67×) brightness treatments, respectively. The halftone trace is a reflectance spectrum averaged across 30 fresh laboratory-reared males, which we used to approximate maximum male signal brightness.

males in each of the two experimental groups (1.4× and 0.67× peak brightness). The cage was monitored thereafter at 30 min intervals from 0800 to 1600 hours daily, from 11 to 21 March 2008. Mating pairs were removed on sight and replaced with a new virgin female and a single male from the corresponding experimental group. Dead individuals were also collected and replaced. In this way we maintained equal ratios of females to males, and equal numbers of males in each treatment. In this experiment, and the next one, we chose to maintain equal male:female ratios in order to match previous experiments in *H. bolina* (Kemp, 2007). Adult female *Hypolimnas* gradually become sexually receptive after emergence, over a period of 4–8 days in *H. bolina* (Kemp, 2001), hence instantaneous operational sex ratios were likely to be much less than 50:50 throughout these experiments (i.e. biased towards receptive males).

Experiment 2: Graded Brightness Reduction

In this experiment, conducted 30 November to 8 December 2007, reared males were randomly assigned upon adult emergence to one of six groups: two control groups and four treatments of decreasing blue band brightness. The treatments were achieved by painting male wing markings with stock solutions of ink/ethanol designed to decrease the peak amplitude of the blue reflectance curve to either 0.75×, 0.5×, 0.25× or 0×, relative to the peak amplitude of freshly emerged males (which is ca. 40% of the reflectance of an MgO standard; see Results). We generated these stock solutions using various ratios of black Sharpie ink (Newell Rubbermaid Office Products, Oakbrook, IL, U.S.A.) dissolved in 95% ethanol. The visual effect of these treatment manipulations on the butterfly wing is indicated by Fig. 3a, b. For the controls we treated one group by painting over the males' dorsal blue markings with 95% ethanol only, and the second group by painting the naturally black dorsal wing regions with the 0× treatment solution (i.e. opaque black). Male blue band brightness in both cases did not differ from the mean for fresh males. Both groups controlled for

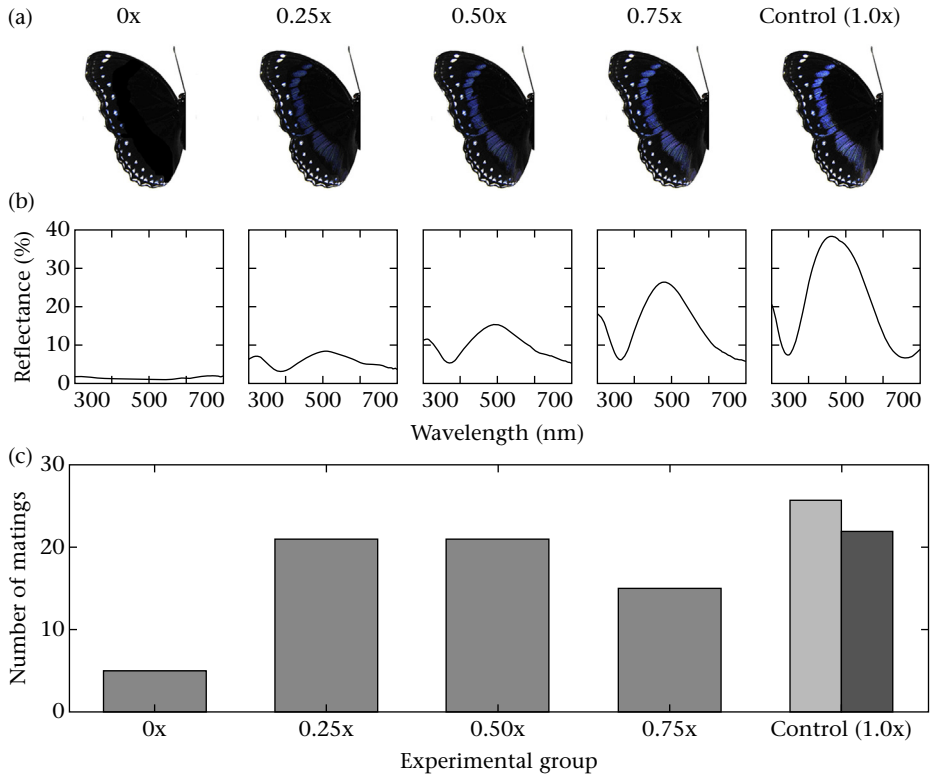

Figure 3. Summary of the visual appearance and experimental mating frequencies of the male wing colour groups in experiment 2. (a) Stylized visual representation of dorsal coloration across the experimental groups. (b) Spectral reflectance curves of the male blue bands for each group. Each curve is the average of five specimens. (c) Relative attractiveness of each group, obtained as their total frequency of matings achieved over the course of the experiment. The two control groups used in this experiment are shown separately here: males in the first control (light column) had their blue markings painted over with ethanol only; males in the second control (dark column) had black ink placed on the already black regions of their wing.

handling effects; the first group also controlled specifically for painting ethanol over the dorsal blue markings, while the second group controlled for the presence of black ink on male wings (odour, weight, aerodynamic effects, etc.). This experiment was procedurally identical to the first one except that 10 males in each of the six groups (i.e. the four brightness reduction treatments plus the two controls) were liberated initially, and subsequently maintained in equal ratios along with 50 virgin females for the next 8 days.

Statistical Analysis

Colour data obtained from field-sampled butterflies did not deviate significantly from the normal frequency distribution (Kolmogorov–Smirnov test: brightness: *H. bolina*: $d = 0.105$; *H. alimena*: $d = 0.072$; hue: *H. bolina*: $d = 0.076$; *H. alimena*: $d = 0.116$; all $P > 0.05$; Lillefors' test also $P > 0.05$ in all cases). We therefore used parametric t tests and F tests to appraise species differences in trait means and variances.

In both mating experiments we maintained equal frequencies of males across treatments in the enclosures to allow total frequencies of group matings to reveal colour-based female preferences. We assessed per-group differences in mating frequencies using Fisher's exact test (when $k = 2$) and the chi-square heterogeneity test (when $k > 2$). Experiment 2 was designed to allow a more sensitive analysis of the female preference function for male brightness. Here we defined a priori four candidate female preference functions (models 1–4 in Fig. 4). These represent biologically realistic choice functions that are potentially testable given our resolution of five male brightness treatments. Models 1–3 represent situations in which an attractiveness threshold exists, that is, a brightness level below which male attractiveness suffers. Model 4 represents a situation in which attractiveness scales linearly with brightness. We derived expected mating frequencies for each candidate preference function and appraised goodness of fit of the observed mating frequencies across experimental groups. Given the unreliability of chi-square and G tests when expected frequencies are low, we tested goodness of fit using a randomization procedure (Sokal & Rohlf, 1995) based on 10 000 iterations.

In both experiments we supplemented our per-group analyses by examining individual latencies until mating using survival regression analysis. This approach analyses the time until an event occurs, and is often used to study failure events or death, but is applicable to a range of biological situations involving latency until a discrete event (Moya-Laraño & Wise, 2000). In our case it is potentially more sensitive than the simple group contrasts in allowing the inclusion of data from males that died or that ultimately failed to mate. These censored observations may contribute usefully to the solution in the sense that such males persisted in the cage for a known length of time without successfully mating. Group survival functions were estimated using the Kaplan–Meier product limit method (Peterson, 1977), whose main assumption is that censored and noncensored individuals share equal per-time probabilities of mating during their experimental tenure. We used nonparametric significance tests for treatment differences: Gehan's Wilcoxon test (where $k = 2$, which generates a z value), and a generalization of Gehan's Wilcoxon test, Peto and Peto's Wilcoxon test and the log-rank test (where $k > 2$, which generates a χ^2 value; Mantel, 1967). For experiment 2 we also planned a series of post hoc contrasts to test sequentially each treatment group against control (unaltered brightness) males.

Of final note, in experiment 2 we included two groups to control for the effects of colour treatment in subtly different ways (as

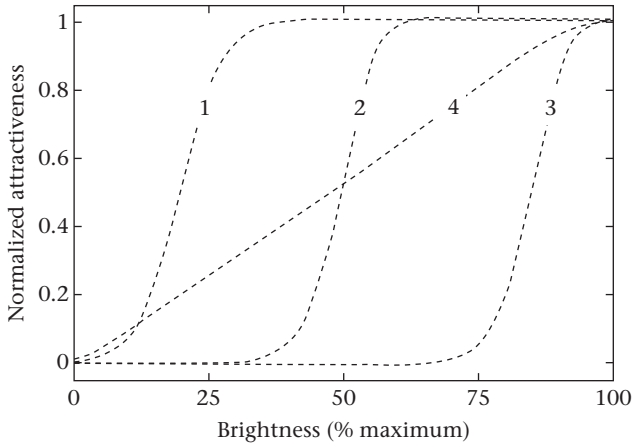

Figure 4. Four hypothetical female preference functions for the brightness of male *Hypolimnas alimena*'s dorsal blue markings. The x-axis represents a gradient of brightness increasing from completely black (0%) up to the average value for male *H. alimena* (set at 100%). The y-axis is female preference, which is equivalent to male attractiveness. Model 4 expresses this parameter as a linearly increasing function of male blue brightness, whereas models 1–3 are step functions featuring various attractiveness thresholds. Model 3, for instance, describes a situation in which male attractiveness drops off very quickly once wing brightness begins to depart from the average.

outlined above). A planned contrast of mating frequencies between these two groups showed no difference (two-tailed Fisher's exact test: $P = 0.554$; survival analysis: $z = 0.536$, $P = 0.592$). In the Results we report, and show in the figures, mating frequencies for each group separately, and retained them as two separate groups in the chi-square test and survival analysis of overall among-groups heterogeneity. The randomization-based goodness-of-fit tests to the candidate preference functions (Fig. 4) required pooling of subjects across these two groups (given that they each represent 1.0× brightness males) which we achieved by using the median mating frequency across them. Similarly, in post hoc survival analysis contrasts we used data from both controls pooled as a single group. All approaches therefore incorporate information from both control groups, which is appropriate because individuals from both groups (although statistically indistinguishable in terms of mating frequencies) were present among the pool of potential mates throughout the experiment.

RESULTS

Natural Colour Variation

Based on our field sample of male butterflies (Table 1), the wavelength corresponding to peak reflectance amplitude (λ_{max}) varied between the two species by roughly 100 nm, which describes their obvious colour difference (i.e. blue [*alimena*] versus UV-violet [*bolina*]). λ_{max} also varied intraspecifically by up to ±35 nm (or 16–18% of each species' respective mean value), and this degree of variation was consistent across species (F test of variances: $F_{40,40} = 1.73$, $P = 0.090$). In terms of brightness, the blue band of male *H. alimena* was less than one-third as bright as *H. bolina*'s UV spots ($t_{78} = 18.6$, $P < 0.001$), and roughly one-third as variable in absolute terms ($F_{40,40} = 3.57$, $P < 0.001$). The range of peak brightness exceeded 130% of the mean value for each species. The brightness range of wild male *H. alimena* (7.7–43.3%, relative to MgO reflectance) indicated that naturally occurring signal variation would be almost completely encompassed by our 0.25–1.0× (control) brightness treatments of experiment 2. Only two very worn specimens exhibited peak reflectance values lower than 10% (equating to the 0.25× brightness treatment), and these were just marginally below this level (7.7 and 8.7%). Our 'increased' treatment of experiment 1 (1.4× brightness) would just exceed that of the brightest male in this sample.

Table 1
Summary descriptive statistics for the dorsal colour signals of male *Hypolimnas* butterflies sampled from Cairns, Australia

Visual parameter		*H. bolina* (ovoid UV patches)	*H. alimena* (blue bands)
Peak brightness (%)	Mean	83.0	26.7
	Variance	285.8	80.1
	99% CI	75.8–90.3	22.8–30.5
	Min–Max	29.5–139.0	7.7–43.3
	Range (Range/Mean)	109.5 (132%)	35.6 (133%)
Hue (nm)	Mean	364	468
	Variance	203	352
	99% CI	358–370	460–476
	Min–Max	328–394	437–514
	Range (Range/Mean)	66 (18%)	77 (16%)

Peak brightness is the maximum amplitude of the unimodal reflectance peak (see Fig. 1c, d), and is presented here as percentage reflectance relative to an MgO standard. Hue is the wavelength position of this peak (i.e. λ_{max}), measured in nanometres. $N = 40$ males for both species.

Experiment 1: Brightness Increase/Reduction

We observed 67 matings in this experiment. Brightness-increased males achieved 35 (52%) of these, a proportion not significantly different from 50:50 (Fisher's exact test: $P = 0.86$). Survival analysis also indicated no difference between the 1.4× and 0.67× brightness groups in average latency until mating ($z = 0.134$, $P = 0.893$; Fig. 5a). The brightness reduction/increase treatments therefore appeared to have no measurable effect upon male attractiveness.

Experiment 2: Graded Brightness Reduction

In this experiment we aspired to greater empirical resolution by presenting female *H. alimena* with males of signal brightness decreasing in regular increments from naturally occurring peak levels (represented by the two control groups) down to total signal absence. Here we sought to estimate the female preference function for male brightness in regard to four a priori hypothesized models (Fig. 4). A total of 89 matings were observed over the course of this experiment, and these were nonuniformly distributed across the male colour groups ($\chi^2_5 = 15.8$, $P < 0.01$; Fig. 3c). Randomization tests indicated that the pattern of matings across groups differed significantly from predictions of model 2 ($P < 0.0005$), model 3 ($P < 0.0001$) and model 4 ($P < 0.0001$), but not model 1 ($P = 0.478$). The fit to model 1 suggests that the probability of mating was equivalent for all males except those in the completely blackened (0× brightness) group. This result was supplemented by survival analysis, which revealed significant between-group differences in survival (i.e. latency until mating) trajectories ($\chi^2_5 = 12.6$, $P < 0.05$; Fig. 5b). Planned post hoc contrasts indicated no difference between 1.0× brightness males (individuals pooled across the two control groups) and males with 0.75× brightness ($z = 1.021$, $P = 0.307$), 0.5× brightness ($z = 0.788$, $P = 0.431$) and 0.25× brightness ($z = 0.459$, $P = 0.647$), but highly significant divergence from the 0× brightness group ($z = 3.48$, $P < 0.005$).

DISCUSSION

Structural colours present a showcase for evolutionary innovation, not only in their visual effects, but also in terms of the optical

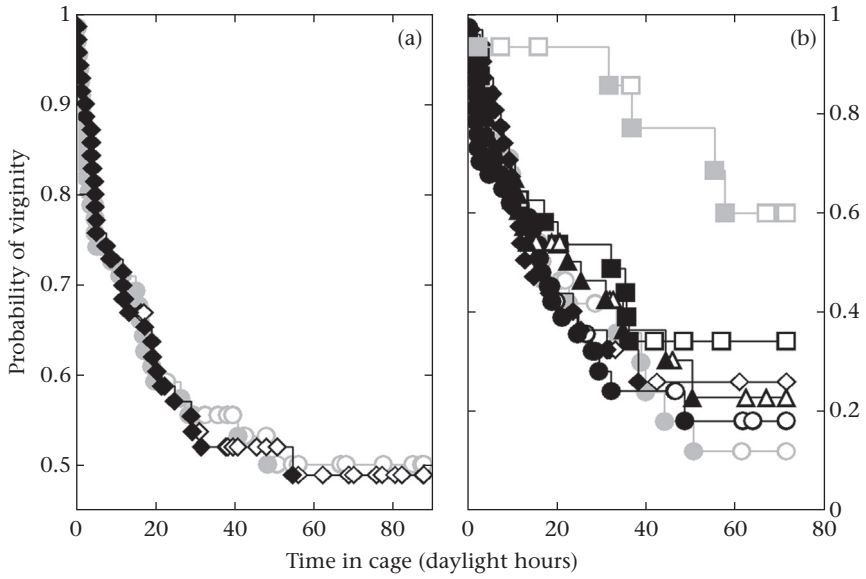

Figure 5. Survival functions indicating the cumulative probability of males in different wing colour groups remaining virgin in (a) experiment 1 and (b) experiment 2. Each step function represents the mating trajectory for each male group, with individual fates denoted by closed symbols (representing a mating) or open symbols (representing a death or an unmated result at the end of the experiment; that is, a 'censored' observation). The x-axis represents time, calculated as the number of daylight hours over which each experiment was conducted. (a) Black diamonds = brightness-increased males (1.4× brightness), halftone circles = dulled males (0.67× brightness); (b) halftone grey squares = 0× brightness males, black squares = 0.75×, black triangles = 0.5×, black diamonds = 0.25×, black circles = black ink control, halftone circles = ethanol control (see Methods).

mechanisms used to produce them. Examinations in groups such as butterflies have revolutionized our understanding of how nanoarchitectures can interact with light, and inspired highly novel photonic technologies. Against this background, it is notable that we know relatively little about the selective forces responsible for driving both functional innovation and visual exaggeration of these traits (see, for example, the contrast presented by avian studies; Maia et al., 2013). Mating experiments in butterflies have shown how interspecific preferences can favour discrete variation in the presence or patterning of structurally coloured wing markings (Fordyce, Nice, Forister, & Shapiro, 2002; Silberglied & Taylor, 1978; Sweeney, Jiggins, & Johnsen, 2003). Here we explored how intraspecific preferences may contribute to the exaggeration (or lack thereof) of butterfly structural colours, which has rarely, if ever, been examined in this group. We performed a simple two-species contrast, which has obvious limitations, but is none the less insightful given the close ecological similarity of these two congeners. Our experimental results are consistent with female choice exerting stabilizing selection on the brightness of male *H. alimena*'s blue sexual signal. This contrasts intriguingly with the directional nature of preferences in the more visually exaggerated congener, *H. bolina* (Kemp, 2007). Furthermore, as predicted for signal traits subject to stabilizing versus directional vectors of selection, *H. bolina* shows evidence for greater phenotypic variation in signal brightness. We discuss several key features of these results and experimental approach, along with the limitations to broader conclusions in light of the two-species comparative context.

As with previous experiments in *H. bolina* (Kemp, 2007), an implicit assumption of our present approach is that per-treatment mating frequencies are directly indicative of female preferences. The males of both species are territorial, which raises the possibility that differential treatment mating rates may instead be driven by competitive interactions among males in the cage. In *H. bolina* this possibility was excluded by replicating the cage results via presentations of virgin females to single, colour-manipulated males in the field (Kemp, 2007). Independent experimental and observational work also indicates that male coloration is irrelevant to tenure at high-quality territories among wild male *H. bolina* (Kemp & Macedonia, 2006; Rutowski, 1992). We therefore rely on this precedent in interpreting the present experimental results for *H. alimena*. Notably, *H. bolina* is the considerably more aggressive species of the two (Kemp, 2010), which supports the strength of this precedent. Males of each species are also seen to abandon site defence under the abnormally high male densities used in these cage experiments (sensu Alcock, 1994). On this basis, it seems very unlikely, albeit not impossible, that male–male interactions rather than female choice influenced the likelihood of mating among colour-manipulated *H. alimena*. More generally, we consider that species differences in female preference functions offer a more parsimonious explanation for their relative difference in male signal exaggeration.

Our first experiment tested for a difference in mating probabilities among two artificially coloured male treatments which varied subtly around the peak brightness level of naturally occurring males. Rather than including a separate control group in this experiment, we relied on the mutual control given by the fact that males in each treatment were handled and marked similarly, and carried equivalent amounts of ink on their wings. We therefore cannot assess the overall level of male attractiveness in this experiment; for example, it is possible that both groups were equally unappealing to females. The frequency of matings in this experiment (67 over 11 days; 6.09/day) was slightly lower than that of experiment 2 (89 over 9 days; 9.89/day), although the sex ratio was also slightly more male-biased in the latter experiment (50:50 versus 60:50 males:females). In experiment 2, where we included two control groups, mating frequencies were statistically equivalent, that is, the female preference function was flat, across both controls and treatment groups ranging down to 0.25× fresh male brightness. We therefore consider the combined results of both experiments in concluding that stabilizing rather than directional female preferences determine signal brightness in *H. alimena*. The contrast with *H. bolina* is, in any event, stark, given that 0.5× brightness males in this species are clearly less attractive than controls, and as attractive as males that have their signal entirely obscured (Kemp, 2007).

Theory expects differences in phenotypic (and genetic) variances in traits subject to stabilizing selection versus those subject to

the strong vectors of selection engendered by directional female preferences (Bonduriansky, 2007). We hypothesized stronger directional selection upon male signal brightness in *H. bolina*, and hence predicted relatively greater variation for their UV markings. Our present results on wild-caught males express good support for this, in terms of both absolute variance and range. Although males of both species show substantial brightness variation, the UV of *H. bolina* shows over triple that of *H. alimena*'s blue. This contrasts with signal hue (i.e. λ_{max}), wherein phenotypic variance was equivalent across species. Given that we used variously aged field specimens in these contrasts, the extant phenotypic variation would have incorporated any differences between individuals that existed upon adult emergence, as well as subsequent variation caused by age-based fading (Kemp, 2006). In the wild, both sources of variation would affect male colour signals, and hence influence the selective potential of female choice. Among coliadine species that display bright multilayer-generated UV similar to that of *H. bolina*, the former component of variation is known to be both highly heritable and condition dependent (Kemp & Rutowski, 2007; Kemp, Vukusic, & Rutowski, 2006). Multilayer UV in *Colias eurytheme* also fades more strongly with age than do other (pigmentary) aspects of the colour phenotype (Kemp, 2006), and thereby magnifies the visual signature of ageing. Directional female preferences for UV brightness in *H. bolina* could therefore provide multiple sources of information regarding mate quality. Our experiments suggest that signalling of this nature is unlikely in *H. alimena*, given that females do not discriminate except against males completely lacking their dorsal blue signal.

Our present experiments, coupled with those in *H. bolina* (Kemp, 2007), both implicate female choice as a selective driver of male sexual coloration within each species. The species difference in female preference functions corresponds with those in male visual exaggeration, and could explain their difference in functional signal innovation (i.e. the generation of colour using multilayers versus microribs; Fig. 1e, f). In this sense, it is interesting that either blue or UV could be produced by either species' chosen photonic surface architecture, yet the difference between them amounts to a total redesign of the nanostructures running the full length of each dorsal wing scale. This is consistent with the high evolutionary lability known to typify the instruments of butterfly structural coloration (Ghiradella, 1985, 2010). However, as is the case more generally in butterflies, we lack a solid phylogenetic basis for interpreting these differences (see, e.g. Prum et al., 2006). The two species contrasted here are known to hybridize in captivity (J. Olive, 2002, personal communication), and are therefore very closely related, but we know little about the broader patterns of evolutionary divergence in this group (emerging phylogenetic analyses at this point at least support the close relatedness of these species; see Kodandaramaiah, 2009). We have limited grounds to evaluate definitively which species (or whether both) diverged in regard to female preferences, nor can our present results inform why such divergence may have occurred. Species differences in the phenotypic target of mating preferences per se are expected, to guard against maladaptive heterospecific matings (Silberglied & Taylor, 1973), but divergence in the shape of intraspecific female preference functions need not necessarily result. Our results implicate intraspecific effects as drivers of interspecific variation in the extent of visual exaggeration, and perhaps functional innovation, in structurally coloured butterfly mating signals. This insight is valuable, given the paucity of knowledge regarding such effects, but awaits testing across a greater range of species, ideally once phylogenetic data come to hand. Similar approaches have proven productive in understanding the evolutionary innovation of structural coloration in other groups, such as birds (Maia et al., 2013; Shawkey et al., 2006).

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