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Sensory limitations and the maintenance of colour polymorphisms: viewing the 'alba' female polymorphism through the visual system of male *Colias* butterflies

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Summary

1. Although colour polymorphisms are a widespread and conspicuous component of extant biodiversity, the selective pressures that act to maintain multiple morphs within populations remain poorly understood in most cases. In particular, the role that visual system limitations may play in maintaining multiple colour morphs is not well explored.

2. We used a female-limited colour polymorphism common to the butterfly genus *Colias*, called the 'alba' polymorphism, to investigate the hypotheses that mate-searching males may struggle to discriminate pale 'alba' females from co-occurring heterospecific white butterflies and/or heterospecific 'alba' females, or that 'alba' females may be more difficult to detect than non'alba' females in natural scenes. Such perceptual limitations may influence the relative mating rates of 'alba' versus non'alba' females, contributing to the evolutionary persistence of both morphs.

3. Based on receptor-noise-limited modelling of the male *Colias* visual system, we find that 'alba' females exhibit chromatic and luminance contrasts against background foliage that are most similar to the 'alba' females of other co-occurring *Colias* species and females of the co-occurring white butterfly *Pieris rapae*. When compared to other co-flying butterflies including non'alba' females, 'alba' females are consistently lower in chromatic contrast against background, but higher in luminance contrast.

4. When viewed side-by-side, we estimate that male *Colias* should be able to discriminate 'alba' females from other co-occurring heterospecific butterflies, including heterospecific 'albas'. However, under field conditions that involve larger distances in space or time, males are likely to face challenges discriminating between conspecific 'alba' females and co-occurring heterospecific white butterflies, particularly heterospecific 'alba' females.

5. Our results suggest that constraints arising from male visual function may be involved in the maintenance of this colour polymorphism, particularly in populations that co-occur with other 'alba'-polymorphic *Colias* species. We argue that such visual system constraints may play a larger role in the maintenance of colour polymorphism than has been empirically appreciated to date.

Key-words: *Colias eurytheme*, *Colias philodice*, colour vision, mate choice, mate discrimination, receptor-noise-limited visual model

Introduction

Polymorphisms are not only fascinating biological phenomena but they also play a critical role in major evolutionary and ecological processes, including the formation of new species, intraspecific competition, and range expansion and contraction (Forsman *et al.* 2008). Colour polymorphisms in particular are tractable phenomena for studying the evolution of polymorphisms because they are easily quantified and are common to many taxa (McKinnon & Pierotti 2010). Colour polymorphisms have been implicated in sexual selection and predation avoidance in

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poison arrow frogs (Siddiqi *et al.* 2004), speciation in *Drosophila* (Wittkopp *et al.* 2009) and *Heliconius* butterflies (Kronforst *et al.* 2006; Chamberlain *et al.* 2009), and assortative mating in lesser snow geese (Cooke & McNally 1975). The persistence of polymorphisms presents an evolutionary paradox because it suggests that multiple morphs maintain roughly equivalent fitness over long periods of time. However, in many cases, little is known about the selective pressures that impact the fitness of each morph, or how these selective forces are balanced across evolutionary time-scales.

One intriguing possibility is that colour polymorphisms persist because they exploit perceptual limitations or biases of receivers, particularly mate-searching conspecifics. A well-known example of this phenomenon is the existence of andromorph females in polymorphic damselflies (Sherratt 2008). Andromorph females are thought to visually mimic male damselflies and therefore benefit by avoiding costly male harassment (Van Gossum, Stoks & De Bruyn 2001). This benefit is counterbalanced by decreased andromorph mating rates and potentially increased predation risk of these more brightly coloured andromorph females in comparison to their more cryptically coloured gynomorph female conspecifics (Robertson 1985; Van Gossum, Stoks & De Bruyn 2001; Sirot *et al.* 2003). Similar, although less extensively studied examples can be found in other animal taxa. For instance, the Mocker Swallowtail butterfly, *Papilio dardanus*, possesses as many as 14 female-limited colour morphs (Nijhout 2003). Most of these morphs mimic different distasteful butterflies, thereby gaining protection from predation as Batesian mimics (Nijhout 2003). However, one *P. dardanus* female morph resembles the male visual phenotype. Similar to andromorph damselflies, these female butterflies are thought to mimic male coloration to avoid male courtship harassment (Cook *et al.* 1994). Polymorphism in *P. dardanus* thus appears to be maintained, at least in part, via exploitation of characteristics of male visual mate recognition.

Although limitations to male mate recognition appear to be important for the evolutionary maintenance of colour polymorphisms in a range of taxa, and theoretical treatments suggest that such scenarios are likely to be of widespread importance (Chunco, McKinnon & Servedio 2007), researchers have typically relied on human visual assessments to identify colour morphs that should present challenges for mate searching males. Such assessments have limited value because human colour vision is often dramatically different from that of focal research taxa (Kelber 2006; Land & Nilsson 2012). Thus, the more pertinent (and as yet unanswered) question is whether female morphs present discrimination challenges when viewed through the visual systems of male conspecifics. Rapid advances in vision research and visual ecology over the past several decades now enable visual system modelling for a growing number of animal species (Kelber & Osorio 2010). However, these techniques have yet to be applied to systems where males must discriminate female morphs from other similar but reproductively unprofitable visual stimuli.

We pursued this opportunity using colour measurements and visual system modelling for polymorphic butterfly species in the genus *Colias*. This group is characterized by a ubiquitous, female-limited, wing colour polymorphism, called the 'alba' polymorphism, with at least 51 of 79 species in the genus exhibiting 'alba' female morphs (L.B. Limeri & N.I. Morehouse unpublished data). Female wings are either coloured similarly to males, which are yellow or orange with black borders, or they are pale white with black borders (a form termed 'alba'). Wing coloration differences between female colour morphs are genetically determined by a dominant autosomal locus (Lorkovic & Herman 1961). 'Alba' females are thought to enjoy a metabolic advantage over non-'alba' females because they do not invest in costly yellow pigmentation, resulting in higher early-life fecundity (Graham, Watt & Gall 1980). However, researchers have suggested that this fecundity benefit is counteracted by a male mating bias against 'alba' females, leading to reduced mating opportunities and concurrent reductions in overall fitness (Watt 1995).

The argument for why males might discriminate against 'alba' females despite their higher fecundity relies on the idea that males should experience difficulties in identifying conspecific 'alba' females from other similarly coloured heterospecific females. *Colias* butterflies co-occur with a number of other butterfly species that resemble the 'alba' phenotype, at least to the human eye, including white butterflies in the subfamily Pierinae and the 'alba' females of other co-flying *Colias* butterflies. This similarity in appearance is relevant to mate selection because *Colias* males first identify mates from a distance using visual cues (Silberglied & Taylor 1978). Misdirected mating attempts should result in nontrivial fitness costs for males, ranging from losses of search time and energy to more substantial costs associated with hybrid matings (Ae 1959; Grula & Taylor 1980). The expectation is that these costs should result in a male preference bias against 'alba' females, either due to negative reinforcement based on male experiences during mate searching or the evolution of genetically determined reductions in male interest in 'alba'-like stimuli.

Behavioural evidence for a male mating bias against 'alba' females has been ambiguous to date. While some studies have reported data suggesting that males discriminate against 'alba' females (Graham, Watt & Gall 1980; Watt 1995), others have gathered neutral or contradicting evidence, in some cases reporting higher mating success for 'alba' females (Gilchrist & Rutowski 1986; Nakanishi, Watanabe & Ito 2000; Kemp & Macedonia 2007). These contradictory results suggest that we do not fully understand the determinants of male mate choice in relation to the 'alba' morph in *Colias* butterflies. In particular, we still do not know whether males should face challenges in visually discriminating 'alba' females from other reproductively unprofitable butterflies typically encountered in their environment. If such limitations to male mate recognition exist, then the observed variation in 'alba' mating success may reflect population-specific differences in the risk of misidentifying 'alba' females (i.e. due to higher or lower population densities of co-occurring white heterospecific butterflies).

We tested three related but not mutually exclusive hypotheses concerning the role that male visual discrimination may play in driving patterns of mating success for 'alba' versus non'alba' female phenotypes. First, following Nielsen and Watt (2000), we tested the hypothesis that males may face challenges visually discriminating 'alba' females for co-occurring heterospecific (and therefore reproductively nonvaluable) white butterflies, particularly members of the ubiquitous genus *Pieris*. Alternatively, we hypothesized that males may have a difficult time visually distinguishing between conspecific and heterospecific 'alba' females where multiple *Colias* species coexist. If males do visually mistake conspecific 'alba' females for pierids or heterospecific 'alba' females, reproductive interference could result that may cause males to respond differently to 'alba' females depending on community composition. Lastly, we tested the hypothesis that 'alba' and non'alba' females differ in their visual salience, either due to differences in brightness or colour contrast from the plant foliage against which they are typically viewed. Such differences in salience may result in different levels of male courtship attention focused on these two female colour morphs, leading to divergent mating rates for 'alba' and non'alba' females.

We tested these three hypotheses in a community of coflying butterflies where two *Colias* species exhibiting 'alba' polymorphisms (*Colias philodice* Godart and *Colias eurytheme* Boisduval, Fig. 1) co-occur alongside several similarly sized white butterflies, including the common butterfly *Pieris rapae* L. (Fig. 1). In western Pennsylvania, *C. philodice* and *C. eurytheme* are found in the same habitats across the growing season and appear to hybridize frequently despite documented costs associated with hybrid matings (Ae 1959). To formally test our three proposed hypotheses, we measured wing reflectance from *C. philodice*, *C. eurytheme* and *P. rapae*. We then constructed mathematical models of colour visual discrimination using data from the closely related congener *C. erate* to estimate the likelihood that the 'alba' female phenotype presents a challenge for the male visual system to distinguish from (i) white heterospecifics, (ii) congeneric 'alba' females or (iii) foliage backgrounds.

Materials and methods

REARING

Colias philodice females ($n = 5$) and *C. eurytheme* females ($n = 6$) were caught in agricultural alfalfa fields in Rochester, PA, USA (40·745664°N, 80·163377°W) and brought into the laboratory to lay eggs. Parental *C. philodice* females were a mix of homozygous ($n = 2$) and heterozygous ($n = 3$) 'alba' morphs, with the latter females producing both non-'alba' and 'alba' female offspring. *Colias eurytheme* parental females included non'alba' females ($n = 3$) as well as homozygous ($n = 1$) and heterozygous ($n = 2$) 'alba' females. In combination, these females likewise produced a mixture of 'alba' and non'alba' female offspring. Caterpillars were raised on alfalfa (*Medicago sativa* L.) in climate-controlled chambers that maintained a coincident photoperiod and temperature cycle (16:8, light/dark, 30:24 °C) with a constant vapour pressure deficit of 1·7 kPa. Alfalfa was harvested from the same field that the butterflies were collected in and surface sterilized to prevent disease introduction into the rearing programme by soaking in dilute bleach water for 30 min followed by rinsing in water. Upon eclosion and following wing hardening, males and females from each family were freeze euthanized to preserve wing coloration. Additionally, we reared *P. rapae* on laboratory-grown kale (*Brassica oleracea* L.) in the same climate chamber and freeze euthanized freshly eclosed adults for later colour measurements.

We chose to measure coloration of laboratory-reared individuals rather than from field-caught butterflies for two reasons. First, characters for reliably discriminating the 'alba' females of *C. eurytheme* and *C. philodice* have not been reported to date. Thus,

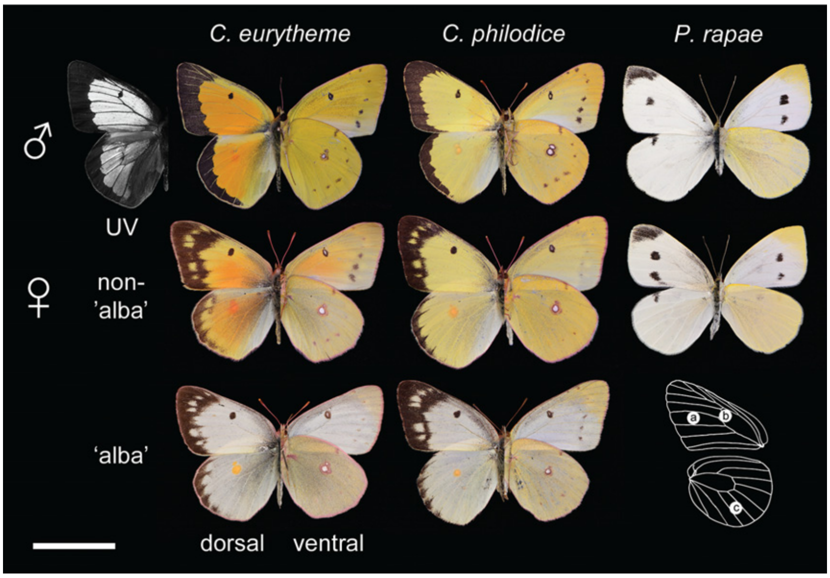

Fig. 1. Images of the focal study species, including *Colias eurytheme* (left), *C. philodice* (centre) and *Pieris rapae* (right). Males appear in the top row with females below (for *Colias* species, 'alba' females are both in the bottom row). The left side of each butterfly illustrates the dorsal wing surface and the right side illustrates the ventral wing surface. The dorsal surface of male *C. eurytheme* is also shown imaged using only ultraviolet light to illustrate male-limited ultraviolet (UV) coloration (UV, far left). White circles in the wing venation diagrams at bottom right (after Scott 1986) indicate where spectral measurements were taken on the dorsal forewing of *Colias* species (a) and *P. rapae* (b), and for the ventral hindwing surface of all species (c). Scale bar on bottom left is 2 cm.

laboratory rearing was required to unequivocally assign species identity to these butterflies. Second, as butterflies age, the scales that colour their wings are slowly lost. This gradual but somewhat stochastic process of wing wear makes comparisons between field-caught individuals of varying ages considerably more complex. We chose to control for this source of variation by focusing our measurements on fresh laboratory-reared individuals. However, we consider the relevance of age-related wing colour fading to our results in the Discussion below.

WING COLOUR MEASUREMENTS

Sample sizes for wing reflectance measurements are as follows: *C. eurytheme* males ($n = 11$), 'alba' females ($n = 8$), yellow females ($n = 10$); *C. philodice* males ($n = 12$), 'alba' females ($n = 11$), yellow females ($n = 8$); *P. rapae* males ($n = 10$), and females ($n = 10$). Wing colour measurements were taken following previously described methods (Morehouse & Rutowski 2010). Briefly, we removed wings from freshly eclosed, freeze-euthanized specimens and mounted a single forewing with the dorsal surface facing up and a single hindwing with the ventral surface facing up on matte black cardstock using archival spray adhesive (Scotch® Photo Mount, 3 M, St. Paul, MN, USA). Measurement locations were selected to best represent the dominant colour of the wing surface (Fig. 1). For *Colias*, we measured the centre of the M3 cell (wing cell identities and naming conventions are dictated by wing venation patterns, Scott 1986) of the dorsal forewing surface and directly below the discal cell in the CuA_1 cell of the ventral hindwing surface (Fig. 1). *Colias eurytheme* males exhibit structurally produced, iridescent ultraviolet (UV) patterns on the dorsal surfaces of their wings (Fig. 1, Rutowski *et al.* 2007). Light reflectance by these UV colours is highly directional such that their visibility is dependent on the geometry of incident light, wing surface and viewer. Thus, these brilliant iridescent UV patterns may often but not always contribute to the colour seen by mate-searching males (Rutowski *et al.* 2007). Therefore, we measured male *C. eurytheme* forewings in the same orientation as other wing samples (the 'UV-visible' orientation) and rotated 90° counter-clockwise (the 'UV-absent' orientation) to capture the range of male appearances during flight in the field. For *P. rapae*, the location of the black spots on the forewing made it difficult to take measurements in the same place as for *Colias*. Measurements were therefore taken at the distal tip of the discal cell on the forewing and the centre of the CuA_1 cell of the hindwing (Fig. 1).

We collected reflectance spectra in a dark room using a spectrophotometer (USB4000; Ocean Optics, Dunedin, FL, USA) calibrated with a magnesium oxide white standard. Wing surfaces were illuminated using a deuterium/tungsten/halogen light source (DH2000-BAL; Ocean Optics) positioned normal to the wing surface at the zenith above the mounted wings. Light reflected off the wing surfaces was sampled using a collimated fibre optic collector positioned at 45° below the zenith towards the proximal wing margin along the distal/proximal axis of the wing, with the exception of the 'UV-absent' orientation, where the collector was positioned above the posterior margin of the wing along the anterior/posterior wing axis. Measurements were recorded as percent reflectance from 300 to 800 nm (Fig. 2a).

VISUAL SYSTEM MODEL

We constructed a receptor-noise-limited colour opponency model based on methods originally developed by Vorobyev and Osorio (1998). The best information to date suggests that *Colias* species are likely to be pentachromatic despite the fact that their eyes contain eight distinct photoreceptor types (Ogawa *et al.* 2012, 2013). We therefore extended the Vorobyev and Osorio (1998) model to

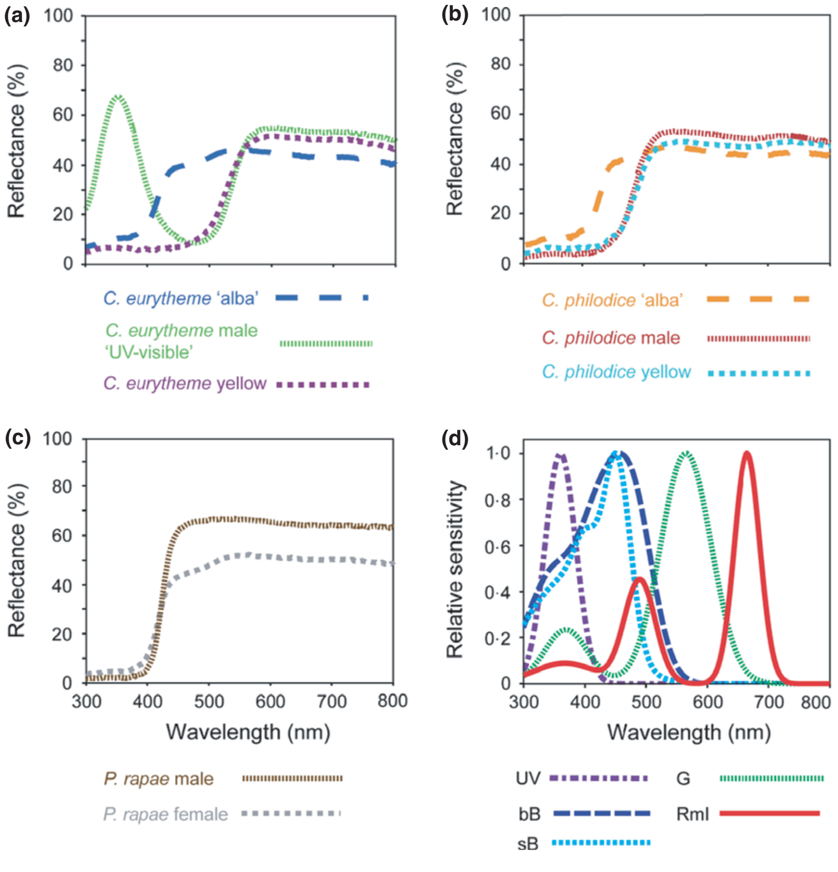

Fig. 2. Average reflectance spectra for *Colias eurytheme* males, 'alba' females, and yellow females (a), *C. philodice* males, 'alba' females, and yellow females (b), and *Pieris rapae* males and females (c). Normalized *C. erate* photoreceptor sensitivity curves for the five photoreceptors used in the visual system model are presented in (d).

pentachromacy following formulae from Morehouse and Rutowski (2010) as described below.

Both vertebrates and invertebrates interpret light reflected from surfaces using chromatic and achromatic (luminance) contrast separately (Kelber, Vorobyev & Osorio 2003; Endler & Mielke 2005). The following equation, adapted from Morehouse and Rutowski (2010), was used to calculate chromatic contrast (contrast with background; ΔS) and colour discriminability (chromatic contrast between two focal stimuli; ΔS^t):

$$\begin{aligned}\Delta S^t \,\text{or}\, \Delta S = \{ & [(e_3e_4e_5)^2(\Delta q_1-\Delta q_2)^2+(e_2e_4e_5)^2(\Delta q_1-\Delta q_3)^2 \\ & +(e_2e_3e_5)^2(\Delta q_1-\Delta q_4)^2+(e_2e_3e_4)^2(\Delta q_1-\Delta q_5)^2 \\ & +(e_1e_4e_5)^2(\Delta q_2-\Delta q_3)^2+(e_1e_3e_5)^2(\Delta q_2-\Delta q_4)^2 \\ & +(e_1e_3e_4)^2(\Delta q_2-\Delta q_5)^2+(e_1e_2e_5)^2(\Delta q_3-\Delta q_4)^2 \\ & +(e_1e_2e_4)^2(\Delta q_3-\Delta q_5)^2+(e_1e_2e_3)^2(\Delta q_4-\Delta q_5)^2] \\ & /[(e_1e_2e_3e_4)^2+(e_1e_2e_3e_5)^2+(e_1e_2e_4e_5)^2 \\ & +(e_1e_3e_4e_5)^2+(e_2e_3e_4e_5)^2]\}^{-1/2}\end{aligned}$$

For ΔS^t, Δq_i represents difference in photoreceptor output between two stimuli (e.g. q_i^1 and q_i^2). For ΔS, Δq_i is the difference in photoreceptor output between the focal stimulus (q_i) and the foliar background (q_i^B), which mathematically simplifies to q_i because q_i^B reduces to zero during von Kries transformation. q_i was determined using the following equation representing a von Kries transformation of photoreceptor quantum catches:

$$q_i = \ln(\frac{Q_i}{Q_i^B})$$

where

$$Q_i = \int_{300}^{800} R(\lambda)I(\lambda)A_i(\lambda)d\lambda$$

$$Q_i^B = \int_{300}^{800} R^B(\lambda)I(\lambda)A_i(\lambda)d\lambda$$

and λ is wavelength (nm), $R(\lambda)$ is the reflectance of a colour stimulus at a given wavelength, $R^B(\lambda)$ is the reflectance of the background, $I(\lambda)$ is the ambient irradiance in units of photon flux (μmol m^{-2} s^{-1} nm^{-1}), and $A_i(\lambda)$ is the wavelength-specific absorbance of photoreceptor i.

In contemporary populations, interactions between males and females occur most frequently in agricultural monocultures of their host plant alfalfa (*M. sativa*), where males are found searching for recently eclosed virgin females or previously mated yet receptive females. We therefore characterized background reflectance (R^B) as the average reflectance spectra from five alfalfa (*M. sativa*) leaves collected from the same field sites as our focal butterflies. In the populations we studied, *Colias* butterfly activity is concentrated around mid-day, with much higher levels of activity on sunny days (Morehouse, personal observation). We thus collected irradiance (I) data at noon on a cloudless day in Pittsburgh, PA using an Ocean Optics JAZ spectrophotometer calibrated using a standard light source (LS-1 CAL; Ocean Optics).

Photoreceptor sensitivity values (A_i) were generated from photoreceptor absorbance templates fit to intracellular recordings reported from a closely related congener, *Colias erate* (Pirih, Arikawa & Stavenga 2010; Ogawa *et al.* 2012, 2013). Species-specific data are not available for *C. eurytheme* or *C. philodice*. Thus, spectral sensitivities from *C. erate* are the closest data available for approximating the spectral sensitivity of our focal *Colias* species. In support of the implicit assumption that visual sensitivities are conserved between *C. erate*, *C. eurytheme* and *C. philodice*, we note that although visual sensitivities are evolutionarily labile across the Lepidoptera (Briscoe 2008), they also appear to exhibit relatively high levels of conservation between closely related taxa (e.g. within the Pieridae, Awata, Wakakuwa & Arikawa 2009). We also note that 'alba' females are found in *C. erate*. Nevertheless, we acknowledge that the use of visual system information from *C. erate* serves only as a first approximation for how the eyes of *C. eurytheme* and *C. philodice* might view conspecific and heterospecific coloration.

In male *C. erate*, eight photoreceptor types have been characterized from the eye: ultraviolet (UV), shouldered blue (sB), broad blue (bB), broad green (bG), green (G), red male type I (RmI), red male type II (RmII) and red male type III (RmIII) (Pirih, Arikawa & Stavenga 2010; Ogawa *et al.* 2012, 2013). We selected a subset of five for inclusion in our visual system model: UV, sB, bB, G and RmI (Fig. 2d). We excluded bG because it is a rare subclass of green receptors unlikely to contribute to colour vision beyond the inputs of the much more common G receptors (K. Arikawa, pers. comm.). RmII and RmIII were excluded because they exhibit large secondary sensitivity peaks in the short wavelengths, making them less useful for colour vision (K. Arikawa, pers. comm.).

Receptor-noise values (e_i) were calculated by dividing the Weber fraction by the square root of average photoreceptor abundances within a single ommatidium. This method assumes that effective photoreceptor noise within each colour-sensitive channel is reduced via summation within individual ommatidia, an assumption supported by behavioural data in honeybees (Vorobyev *et al.* 2001). We chose a Weber fraction of 0·01 based on empirical estimates from the butterfly *Papilio xuthus* (Koshitaka *et al.* 2008), the only butterfly species for which such estimates are available. Photoreceptor abundances were obtained from prior histological characterization of ommatidial types within the eye of male *C. erate* (Awata, Wakakuwa & Arikawa 2009).

We calculated values for ΔS (chromatic contrast against background) for each wing colour measurement for each group. We then calculated ΔS^t (chromatic contrast between two stimuli) for all possible pairwise comparisons between eight individuals per group (a total of 64 comparisons per wing colour measurement per group pair). This method of calculating ΔS^t allowed us to estimate both mean ΔS^t and its variance for each comparison. However, because the resulting set of ΔS^t values within a specific comparison are not independent from each other (i.e. they are generated by a shared set of original values), our statistical analysis of ΔS^t is restricted to qualitative comparisons of mean ΔS^t values as well as formal evaluation of ΔS^t overlap with specific numerical thresholds (see below).Values for ΔS and ΔS^t calculated using our visual system model are estimates of the discriminability of focal stimuli, scaled in units of standard deviations of photoreceptor noise. These discriminability values can be interpreted in two ways, either against an absolute threshold (the 'just noticeable difference' or jnd) or in relative terms. For the former interpretation, individuals should theoretically be able to discriminate stimuli that differ by a ΔS^t value of only 1. However, behavioural estimates derived from research using the European honeybee (*Apis mellifera*, Giurfa *et al.* 1997) suggest that a value of 2·3 is more likely to represent the threshold above which colour-based decisions can be reliably made (>75% accuracy) when stimuli are presented side-by-side. We therefore interpret all stimuli estimated to have a ΔS^t value of larger than 2·3 to be potentially discriminable by the male *Colias* eye, at least when presented simultaneously.

Discriminability values can also be considered relative to one another, with groups exhibiting lower discriminability values considered more difficult for male *Colias* to distinguish from each other. This latter method of interpretation is more likely to provide valuable insight into colour discrimination when colour stimuli are experienced at greater distances in time or space (i.e. not simultaneously) where errors introduced via processes of stimuli generalization and memory may substantially increase discrimination thresholds above the values considered reasonable for side-by-side comparison of stimuli (Kelber, Vorobyev & Osorio 2003).

The five photoreceptors identified above are those most likely to be involved in chromatic discrimination, but it is unknown which photoreceptors participate in achromatic visual discrimination in *Colias*. Many species of bees, including bumblebees (*Bombus impatiens*) and honeybees (*Apis mellifera*), use only their green photoreceptors to discriminate luminance (Giurfa & Vorobyev 1998; Hempel de Ibarra, Giurfa & Vorobyev 2002). However, recent work demonstrates that *Papilio* butterflies use the same photoreceptors for chromatic vision as for luminance discrimination (Koshitaka, Arikawa & Kinoshito 2011). We therefore chose to calculate luminance contrast scores that include input from all five photoreceptors as follows:

$$L = \sum_{i=1}^{N} \alpha_i q_i$$

where

$$\alpha_i = \frac{\eta_i}{\eta}$$

The parameter α_i weights the contribution of photoreceptor i to luminance contrast on the basis of its abundance in the eye (η_i) relative to the total number of photoreceptors (η). Rules of thumb relating achromatic contrast values to behavioural decision-making thresholds are lacking for this method of estimating achromatic contrast. Therefore, L values estimated to overlap with zero are considered nondiscriminable, but larger estimates are discussed as potentially discriminable. We calculated luminance contrast against the foliage background as well as between the wing colours of all groups. For the latter comparisons, as with estimates of ΔS^t, we compared all possible pairwise differences for each wing colour measurement for eight individuals per group (a total of 64 comparisons per wing colour measurement contrast for each group comparison).

We note that in general, luminance differences between stimuli are thought to be less reliable in terrestrial environments than chromatic contrast cues because of high temporal and spatial heterogeneity in irradiance (Kelber, Vorobyev & Osorio 2003). Nevertheless, achromatic contrast does present a source of visual information that mate-searching *Colias* males may employ when identifying potential mates. In particular, achromatic contrasts with background foliage may be relevant as wing phenotypes that exhibit higher achromatic contrasts with background are likely to be more salient across a range of illumination scenarios.

STATISTICAL ANALYSES

All statistical analyses detailed below were performed using SPSS 20 (IBM Corp., Armonk, NY, USA). Data normality and homoscedasticity were verified prior to running statistical tests. A one-way ANOVA was performed for chromatic (ΔS) and luminance contrasts (L) between the wing and the background. Post hoc comparisons were conducted using the Tukey–Kramer method to maintain an experimentwise α of 0·05. For chromatic (ΔS^t) and luminance contrasts (L) between groups, formal statistical analyses were precluded because values within these datasets were non-independent. However, we were able to qualitatively compare mean values for these metrics. In addition, we calculated 95% confidence intervals for both ΔS^t and L, and evaluated them for overlap with the critical thresholds identified above (2·3 for chromatic contrast and 0 for luminance contrast).

Results

WING CONTRAST WITH FOLIAR BACKGROUND

First, we compared chromatic contrasts between each of our focal wing colours and the foliar background against which they would typically be viewed. These values estimate a male's ability to locate a single individual within the environment using chromatic cues and present a specific test of our hypothesis that 'alba' females differ from non'alba' female morphs in their salience in the field. All estimates were well above the 2·3 threshold, indicating that male *Colias* should be able to readily discriminate all of the focal wing colours from the background. However, comparison between groups revealed significant differences in chromatic contrast to background (forewing chromatic contrast: $F_{8,82} = 64{\cdot}1$, $P < 0{\cdot}01$; hindwing chromatic contrast: $F_{7,72} = 90{\cdot}8$, $P < 0{\cdot}01$; Table 1). For both hindwing and forewing chromatic contrast comparisons, *C. eurytheme* and *C. philodice* 'alba' females were not statistically different from each other and differed from all other groups (Fig. 3a,b). These 'alba' forms exhibited the lowest chromatic contrast scores against foliage backgrounds of

Table 1. Chromatic (ΔS) and luminance (L) contrasts (means ± confidence intervals) between wing coloration and background for all groups. For chromatic contrast, values above 2·3 are potentially discriminable under optimal conditions, and for luminance contrast, values that do not overlap with 0 are potentially discriminable

		Forewing		Hindwing	
Species	Sex/Morph	ΔS	L	ΔS	L
C. eurytheme	'Alba'	75·26 ± 15·89	1·51 ± 0·06	46·98 ± 12·54	1·43 ± 0·06
	Non-'alba'	181·10 ± 14·22	0·94 ± 0·05	118·97 ± 11·22	1·16 ± 0·06
	'UV-visible' male	108·00 ± 13·55	1·55 ± 0·05	186·95 ± 10·70	1·06 ± 0·05
	'UV-absent' male	241·55 ± 13·55	0·88 ± 0·05		
C. philodice	'Alba'	81·00 ± 13·55	1·47 ± 0·05	49·43 ± 10·70	1·33 ± 0·05
	Non-'alba'	135·99 ± 15·89	1·26 ± 0·06	87·51 ± 12·54	1·10 ± 0·03
	Male	183·63 ± 12·98	1·25 ± 0·05	173·49 ± 10·24	1·09 ± 0·05
P. rapae	Female	126·21 ± 14·22	1·54 ± 0·05	81·52 ± 11·22	1·53 ± 0·06
	Male	197·60 ± 14·21	1·70 ± 0·05	128·21 ± 11·22	1·63 ± 0·06

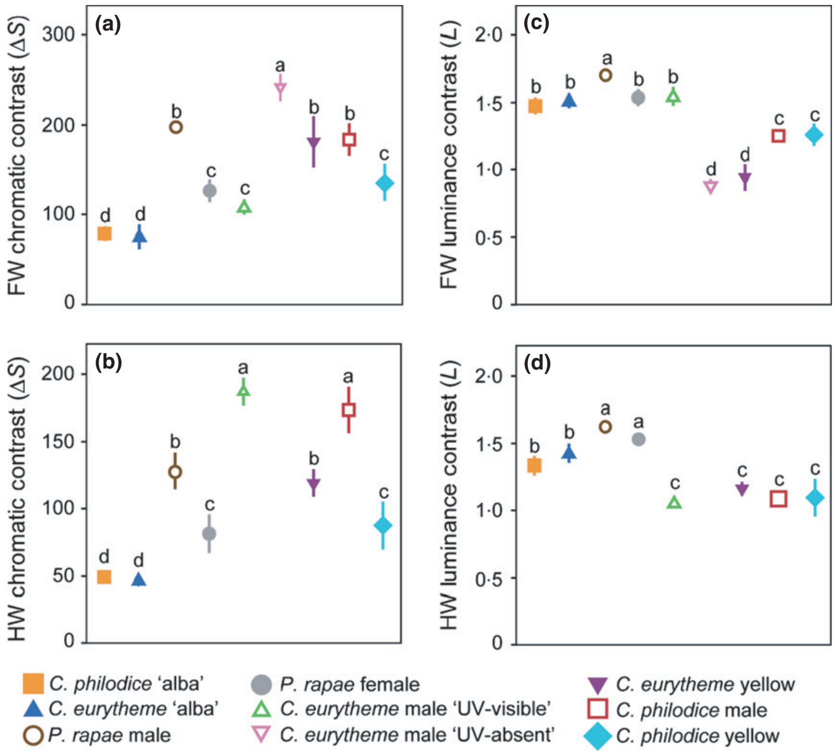

Fig. 3. Chromatic and luminance contrasts between wing and foliar background of forewing (FW) chromatic contrasts (a), hindwing (HW) chromatic contrasts (b), forewing luminance contrasts (c), and hindwing luminance contrasts (d). Error bars represent 95% confidence intervals. For symbols where no error bar is visible, error bars are smaller than the size of the symbol itself. For luminance contrasts, a positive value indicates that the butterfly wing is brighter than the background foliage. Groups with different letters are statistically distinguishable from one another.

any wing colours considered in this study. We also observed a general pattern of reduced chromatic contrast against background for ventral hindwing colours as compared to dorsal forewing colours, with more dramatic differences between dorsal and ventral wing surfaces in females compared with males (Table 1, Fig. 3a,b). The one exception to this pattern was observed for male *C. eurytheme* dorsal coloration in the 'UV-visible' position, which exhibited lower chromatic contrast scores when compared to the ventral wing coloration in those individuals.

Second, we compared luminance contrasts between each of our focal wing colours and the foliar background. These values estimate a male's ability to discriminate a focal individual against foliage using achromatic cues. All luminance contrasts were positive and significantly different from 0 (Table 1, Fig. 3c,d), indicating that male *Colias* should be able to readily discriminate all wings from the darker background foliage. Statistical comparisons between group means revealed significant differences in luminance contrast against background (forewing luminance contrast: $F_{8,82}$ = 121·0, P < 0·01; hindwing luminance contrast $F_{7,72}$ = 60·3, P < 0·01; Table 1). For luminance contrast comparisons of forewing and hindwing coloration, *C. eurytheme* and *C. philodice* 'alba' females were not statistically different from each other (Fig. 3c,d). For forewing luminance contrast, they also did not differ from *P. rapae* females and *C. eurytheme* males in the 'UV-visible' orientation (Fig. 3c). 'Alba' female colours were also consistently high in luminance contrast, with higher forewing luminance contrast scores found only for *P. rapae* males. For hindwing luminance contrast, 'alba' females were statistically brighter than all other groups (Fig. 3d).

VISUAL CONTRASTS BETWEEN SPECIES WING COLOURS

We performed chromatic and luminance contrast comparisons between the forewings and separately between the hindwings of all possible groups. These comparisons estimate the perceived differences in colour or luminance between focal stimuli (wing colours) if viewed side-by-side. Here, we report the full results from comparisons between *C. eurytheme* 'alba' females and all other groups (Table 2; Fig. 4). However, results from the same comparisons for *C. philodice* 'alba' females were qualitatively similar and are reported in full in the supplemental material (Table S1; Fig. S1, Supporting information).

When the data are considered in absolute terms, *C. eurytheme* 'alba' females were estimated to be discriminable from all other groups in forewing and hindwing chromatic contrast and hindwing luminance contrast (Table 2). However, for forewing luminance contrast, *P. rapae* females and *C. eurytheme* males in the 'UV-visible' orientation were not discriminable from *C. eurytheme* 'alba' females (i.e. 95% confidence interval estimates for these comparisons overlapped with zero, see Table 2, Fig. 4c). When these contrasts are considered in relative terms, some groups were more easily discriminable from each other than others (i.e. pairwise comparisons of some groups exhibited consistently higher ΔS^t and/or L values). *Colias eurytheme* 'alba' females were least distinguishable

Table 2. Chromatic (ΔS^t) and luminance (L) contrasts (means ± confidence intervals) between *Colias eurytheme* 'alba' females and all other groups. For chromatic contrast, values above 2·3 are potentially discriminable, and for luminance contrast, values that do not overlap with 0 are potentially discriminable. For luminance contrast, a positive mean indicates that *C. eurytheme* 'alba' females are brighter (have higher luminance) than the other group being compared

		Forewing		Hindwing	
Species	Sex/Morph	ΔS^t	L	ΔS^t	L
C. eurytheme	Non'alba'	167·69 ± 9·40	0·56 ± 0·04	93·77 ± 6·13	−0·26 ± 0·03
	'UV-visible' male	158·80 ± 6·52	−0·03 ± 0·03	168·65 ± 6·19	−0·35 ± 0·02
	'UV-absent' male	217·80 ± 7·28	0·64 ± 0·02		
C. philodice	'Alba'	25·43 ± 4·41	0·05 ± 0·03	24·55 ± 3·88	−0·06 ± 0·03
	Non'alba'	103·26 ± 6·80	0·25 ± 0·02	61·79 ± 7·39	−0·33 ± 0·04
	Male	146·11 ± 8·82	0·28 ± 0·02	152·41 ± 7·91	−0·34 ± 0·03
P. rapae	Female	60·31 ± 8·15	0·01 ± 0·05	54·48 ± 7·03	0·10 ± 0·03
	Male	134·05 ± 6·60	−0·18 ± 0·02	99·96 ± 6·61	0·19 ± 0·02

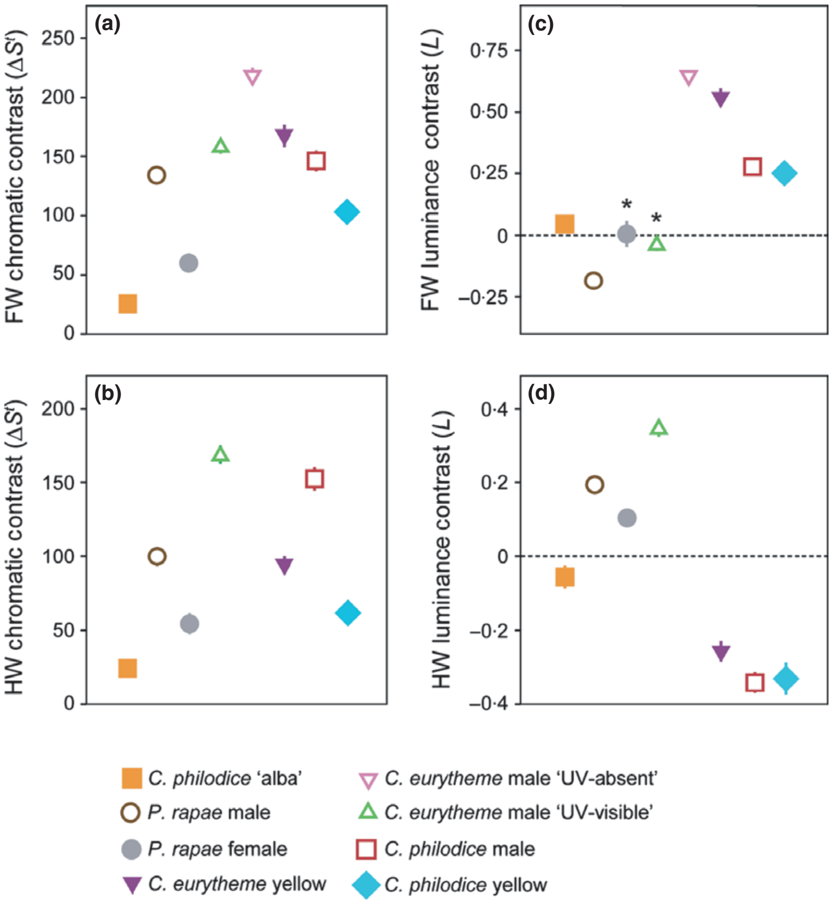

Fig. 4. Comparisons between *Colias eurytheme* 'alba' females and all other groups for forewing (FW) chromatic contrast (a), hindwing (HW) chromatic contrast (b), forewing luminance contrast (c), and hindwing luminance contrast (d). Error bars represent 95% confidence intervals. For symbols where no error bar is visible, error bars are smaller than the size of the symbol itself. For luminance contrast, a positive mean indicates that *C. eurytheme* 'alba' females are brighter (have higher luminance) than the group coded by the symbol key. A negative mean indicates the opposite. Asterisks indicate groups that overlap with 0.

from *C. philodice* 'alba' females for chromatic contrast of forewing and hindwing coloration, and luminance contrast for hindwing coloration (Table 2, Fig. 4). The next least distinguishable group from *C. eurytheme* 'albas' was *P. rapae* females, with intermediate chromatic contrast estimates for forewing and hindwing coloration, as well as low luminance contrast estimates for hindwing coloration (Table 2, Fig. 4). In addition, the forewing coloration of *C. eurytheme* 'alba' females exhibited the lowest luminance contrast with *P. rapae* females, *C. eurytheme* male in the 'UV-visible' orientation, and *C. philodice* 'alba' females (Table 2, Fig. 4c).

Discussion

We find that when viewed through the male visual system, 'alba' females of both *Colias* species should appear very bright, but not very colourful compared with a typical foliar background (Fig. 3). 'Alba' females have significantly higher luminance values than the background foliage, suggesting that it may be easy for a male to spot an 'alba' female in the field using luminance contrast. However, chromatic contrast between the 'alba' females and background foliage was lower than for non'alba' females, which may impact male ability to use colour cues to locate 'alba' females in the field. These patterns were consistent for 'alba' females of both *Colias* species. Thus, we find mixed evidence for our hypothesis that differences in mating rates between 'alba' and non'alba' females may be driven in part by challenges males face in visually detecting either form against background foliage. If anything, we find that males should be able to readily see both female forms under typical field conditions. However, little is known about whether males prioritize chromatic versus achromatic cues during mate searching. For example, if males favour chromatic cues over luminance differences when seeking mates, this may disadvantage 'alba' females. More work is needed to disentangle these possibilities.

When the wing colours of different morphs/species are compared directly to each other rather than to the background, we find that all estimated chromatic contrasts fall above the 2·3 threshold typically cited as a reasonable discrimination threshold (Fig. 4, Giurfa *et al.* 1997). This suggests that males should be able to discriminate between all focal groups based on chromatic contrast, at least if males encounter these butterflies next to each other or sequentially within a short time span. Males in high population densities may often encounter females at sufficiently high rates that a discriminability threshold of 2·3 or similar could act as a reasonable minimum. In such situations, we can tentatively conclude that males should be able to reliably discriminate between 'alba' females and other similarly coloured co-occurring species. However, for intermediate or low population densities, males should experience females at greater distances in space or time. In such situations, a discrimination threshold of 2·3 may overestimate male capacity to reliably discriminate between female wing colours. Therefore, in field scenarios where males encounter conspecific females and heterospecific individuals at greater intervals in space or time, considering the relative chromatic contrast scores may be more informative.

When our chromatic contrast estimates are interpreted in relative terms, it becomes clear that some groups may be easier for males to discriminate than others. The 'alba' females of the both *Colias* species had the lowest chromatic contrast scores when compared to each other and thus should appear the most similar to the male visual system. Conversely, the chromatic contrasts between the non'alba' females of the two *Colias* species were substantially larger than the chromatic contrasts between the 'alba' females (Fig. 4a,b, Fig. S1, Supporting information). This indicates that males should be able to more readily discriminate between conspecific and heterospecific non'alba' females than 'alba' females. As a result, males are likely to be able to visually identify conspecific non'-alba' females with greater certainty from heterospecific females when searching for mates, providing preliminary support for our hypothesis that mate-searching male *Colias* may have difficulties identifying conspecific from heterospecific 'alba' females using chromatic cues.

Similarly, we find low chromatic contrast scores when comparing the wing coloration of *Colias* 'alba' females to *P. rapae* females. However, chromatic contrast scores between 'alba' females and *P. rapae* males are of similar magnitude to chromatic differences between 'alba' females and non'alba' females. Thus, our data support the hypothesis that mate-searching males may find it difficult to visually discriminate 'alba' females from coflying white *Pieris* butterflies, but that this difficulty does not generalize to both sexes in *P. rapae*. Rather, *P. rapae* females are likely to pose the most significant source of uncertainty for mate-searching *Colias* males attempting to decide whether a white butterfly is reproductively profitable or not.

Results from our analysis of luminance contrasts between different butterfly colour phenotypes tell a similar story. We find that 'alba' females exhibit the lowest luminance contrast scores with heterospecific 'alba' females and *P. rapae* females (Fig. 4c,d). Interestingly, we also find that for dorsal forewing coloration, *C. eurytheme* males in the 'UV-visible' orientation exhibit similar luminance to 'alba' females, although these colour phenotypes differ dramatically in chromatic contrast (Fig. 4a). Thus, males should experience additional difficulties in discriminating conspecific 'albas' from heterospecific 'alba' and *Pieris* females using luminance contrast, providing further support for our hypotheses that males may experience challenges visually discriminating between conspecific 'alba' females and heterospecific white butterflies. These difficulties may be important for understanding the reproductive interactions of non'alba' versus 'alba' females.

We note that our analysis may actually provide inflated estimates of the potential discriminability of different female forms in these species, not only because of expected reductions in discriminability between stimuli experienced at distances in space and time, but also because we have focused our measurements on freshly eclosed individuals. Butterflies experience age-related fading of wing coloration due to the gradual loss of the wing scales that produce their coloration (Kemp 2006). Thus, age-related changes in coloration in any of our focal butterfly species may result in phenotypes that are even more difficult for mate-searching males to tell apart, either due to fading-related convergence of colour phenotypes and/or increases in the phenotypic variability within each morph type leading to greater uncertainty of morph identity. Future work should explore the extent to which these age-related colour dynamics influence mate attraction and male visual discrimination.

Taken together, our results provide mixed support for the idea that 'alba' and non'alba' forms exhibit dramatic differences in visual salience when viewed by males against typical foliage backdrops. However, we do find consistent evidence that males may experience difficulties when seeking to discriminate conspecific 'albas' from heterospecific 'alba' females and co-flying *P. rapae* females using visual cues. In the field, mate-searching males face a series of tradeoffs, including speed-accuracy trade-offs for visual discrimination (Wickelgren 1977) and decision-making (Chittka, Skorupski & Raine 2009). The reduced discrimination scores of visual comparisons between 'alba' females and other coflying heterospecific females may thus lead to mistakes in mate choice, resulting in loss of flight time and mating resources. If such mistakes impose regular fitness costs on males, this could favour the evolution of a mating bias against 'alba' females observed by some researchers (Graham, Watt & Gall 1980; Watt 1995). Such a bias would represent a cost to the 'alba' morph and explain why both 'alba' and yellow morphs coexist in such a variety of populations despite the potential fecundity advantage enjoyed by 'alba' females due to lower pigment-based resource allocation (Graham, Watt & Gall 1980). Although we did not explicitly test for the presence of a male mating bias, our work provides preliminary support for the idea that challenges to visual mate recognition may play an important role in how males interact with the female-limited colour polymorphism in *Colias*.

This study is among the first to explicitly ask whether visual system limitations may influence male mate recognition in a colour polymorphic species. However, we expect that sensory limitations may be of widespread importance to the evolution of traits involved in mate-attraction and mate identification, especially those exhibiting polymorphisms. For example, many species of *Heliconius* butterflies have polymorphic females that mimic different toxic species to avoid predation. This polymorphism poses a problem for visually oriented males who must discriminate multiple female forms from multiple heterospecific model species (Kronforst *et al.* 2006). In *Heliconius cydno alithea*, this challenge appears to have been 'solved' by the evolution of polymorphic mate preferences, although the visual system mechanisms underlying these assortative mating preferences are not known (Kronforst *et al.* 2006; Chamberlain *et al.* 2009).

In conclusion, our study provides a crucial first step in understanding how the male visual system might affect how males visually identify and discriminate different female colour morphs in *C. eurytheme* and *C. philodice*. Our results suggest that males may, in some scenarios, experience difficulties visually discriminating between conspecific 'alba' females, heterospecific 'alba' females, and co-flying white pierids. Such visual system constraints could lead to costly mistakes by mate-searching *Colias* males, including time lost courting heterospecifics and even higher costs associated with heterospecific mating. Thus, visual system limitations may have selected for male mating biases against 'alba' females, which, when counteracted by the higher fecundity of 'alba' females, may help to explain the evolutionary persistence of both 'alba' and non'alba' female morphs in many species of *Colias* butterflies. We suggest that greater attention to the role of sensory perception and sensory limitations in the maintenance of polymorphic mate attraction traits is likely to be a profitable area for future work.

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Data accessibility

Data deposited in the Dryad repository: doi:10·5061/dryad.nd358 (Limeri & Morehouse 2014).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. Chromatic and luminance contrasts between *Colias philodice* 'alba' females and other butterfly groups.

Table S1. Chromatic and luminance contrast means and variances for comparisons between *Colias philodice* 'alba' females and all other butterfly groups.