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Accommodating natural and sexual selection in butterfly wing pattern evolution

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Visual patterns in animals may serve different functions, such as attracting mates and deceiving predators. If a signal is used for multiple functions, the opportunity arises for conflict among the different functions, preventing optimization for any one visual signal. Here we investigate the hypothesis that spatial separation of different visual signal functions has occurred in *Bicyclus* butterflies. Using phylogenetic reconstructions of character evolution and comparisons of evolutionary rates, we found dorsal surface characters to evolve at higher rates than ventral characters. Dorsal characters also displayed sex-based differences in evolutionary rates more often than did ventral characters. Thus, dorsal characters corresponded to our predictions of mate signalling while ventral characters appear to play an important role in predator avoidance. Forewing characters also fit a model of mate signalling, and displayed higher rates of evolution than hindwing characters. Our results, as well as the behavioural and developmental data from previous studies of *Bicyclus* species, support the hypothesis that spatial separation of visual signal functions has occurred in *Bicyclus* butterflies. This study is the first to demonstrate, in a phylogenetic framework, that spatial separation of signals used for mate signalling and those used for predator avoidance is a viable strategy to accommodate multiple signal functions. This signalling strategy has important ramifications on the developmental evolution of wing pattern elements and diversification of butterfly species.

Keywords: eyespot; modularity; Nymphalidae; likelihood; *Bicyclus*; wing patterns

We may give the under surface to Mr. Wallace, but we must yield the upper surface to Mr. Darwin

(Fraser 1871, p.489)

1. INTRODUCTION

Animal species employ an array of visual signals that serve a variety of functions, including predator avoidance and mate signalling (Endler 1992). Conspicuous visual signals that make animals attractive to mates may result in increased detection by predators (Promislow *et al.* 1992), and visual signals used for predator avoidance may make individuals less attractive to mates (Burns 1966) or prevent effective mate signalling (Estrada & Jiggins 2008). Antagonism among different signalling functions on the same signal may constrain the optimization of these signals for any one function (Zuk & Kolluru 1998). Mechanisms that allow species to reduce conflict among these alternative selective pressures should increase the effectiveness of multiple signal functions and be favoured over evolutionary time (Endler 1992).

One potential solution to antagonistic selective pressures would be to separate the signals into different parts of the body, so individuals could restrict signal display to appropriate spatio-temporal conditions (Endler 1992); we

refer to this strategy as signal partitioning. By physically separating signals, and thus signal functions, animal species are better able to accommodate the various selective pressures governing the evolution of visual elements. Butterfly wing patterns serve a variety of functions, including predator avoidance and mate selection (Nijhout 1991), and provide an ideal case to evaluate the usefulness of this signal partitioning hypothesis. Because most butterflies can fold their wings together, hiding the dorsal surface, a dorsal–ventral partitioning of visual signals may present one solution to accommodating potentially antagonistic selective pressures. The speculation that dorsal wing patterns are important for mate signalling, while the ventral surface may be more subject to selection by natural enemies is, in fact, not new (Darwin 1871; Wallace 1889), although no study has directly tested this hypothesis in a comparative framework. In addition to a dorsal/ventral partition, butterflies may separate signals between forewing and hindwing, given their ability to hide the forewing behind the hindwing when at rest. These two surface axes, dorsal–ventral and forewing/hindwing, offer butterflies two spatial dimensions that may be partitioned to serve different, potentially antagonistic, signal functions.

Here we present the first phylogenetic assessment of signal partitioning in Lepidoptera, using the butterfly genus *Bicyclus* (Nymphalidae: Satyrinae). The genus comprises approximately 80 species, distributed across forests and savannahs of Africa and Madagascar (Condamin 1973; Ackery *et al.* 1995). *Bicyclus* wing patterns generally consist of eyespots and simple bands that occur on both dorsal and ventral surfaces and on the fore and hindwing (figure 1),

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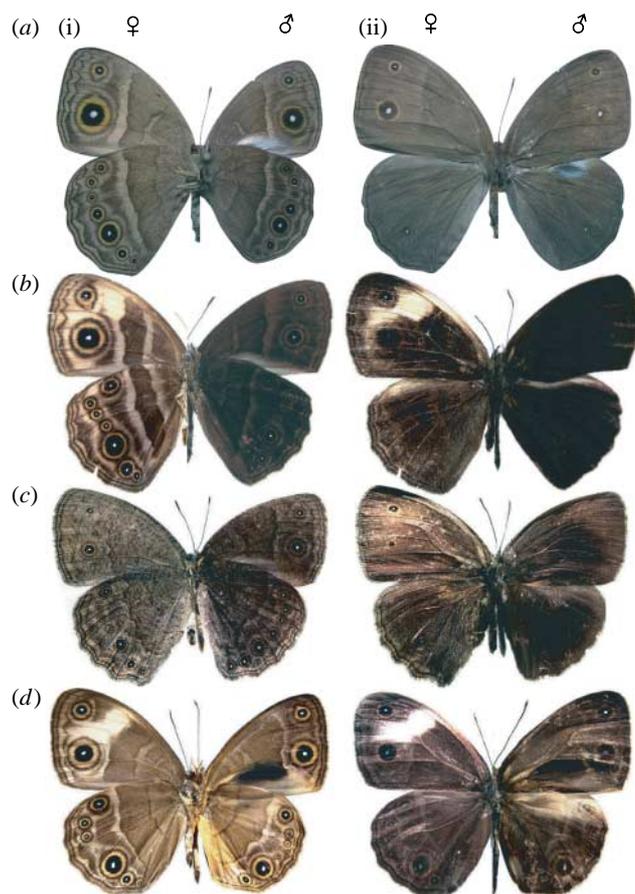


Figure 1. Representative examples of wing pattern diversity in *Bicyclus* ((i) ventral, (ii) dorsal) illustrating inter- and intraspecific variations ((a) *Bicyclus anynana*, (b) *Bicyclus evadne*, (c) *Bicyclus sandace* and (d) *Bicyclus sciathis*). Images of *B. evadne*, *B. sandace* and *B. sciathis* courtesy of T. Larsen.

and are easy to score unambiguously. One member of the genus, *Bicyclus anynana*, is also an experimental model organism in the field of behaviour and evolutionary developmental biology. Previous research on this species has provided many insights into the mechanistic basis of wing pattern evolution and the degree of evolutionary constraints surrounding this system (Beldade & Brakefield 2002; Allen *et al.* 2008). These properties, combined with available molecular sequence data (Monteiro & Pierce 2001), make *Bicyclus* an ideal candidate for the study of signal partitioning.

Support for the signal partitioning hypothesis is evidenced in behavioural and developmental studies of *Bicyclus* species. Female *B. anynana* prefer to mate with males with intact dorsal forewing (DF) eyespots (containing UV-reflective pupils); however, females do not discriminate against males with reduced, missing or incomplete (pupils removed) ventral fore- or hindwing eyespots (Robertson & Monteiro 2005). Female mate preference thus appears to be largely influenced by characters expressed on the dorsal surface of the male forewings. During courtship, *B. anynana* males and females expose their dorsal wing surface in close proximity to potential mates (Nieberding *et al.* 2008; K. L. Prudic & A. Monteiro 2008, unpublished data). This courtship display is the only time, apart from flight, when dorsal wing patterns are visible to potential receivers. In predation experiments, ventral eyespots increase the escape probability of *B. anynana* attacked by naive birds

(Lyytinen *et al.* 2004). Variation in seasonal polyphenism among wing characters provides another line of supporting evidence for signal partitioning. In five species of *Bicyclus*, 'exposed' ventral characters showed high sensitivity to temperature, while 'hidden' characters, including two DF eyespots, were much less sensitive to variation in temperature (Roskam & Brakefield 1996). The difference in reaction norms argues that different forces, such as predators and potential mates, are acting on characters expressed on different surfaces. These studies of *B. anynana* and other *Bicyclus* species concord with the hypothesis that the genus *Bicyclus* has spatially separated different signal functions among wing surfaces.

Different functions of visual signals, such as predator avoidance and mate signalling, should leave different signatures of character evolution on a phylogeny. Closely related prey species facing similar predation pressures are expected to maintain visual signals that are successful in reducing predation, while characters involved in mate signalling can exhibit rapid rates of evolution (Fisher 1930; West-Eberhard 1983; Ritchie & Gleason 1995; Seehausen & van Alphen 1999; Omland & Lanyon 2000). Additionally, barring major differences between the sexes in habitat use, signals employed for predator avoidance should display equivalent rates of evolution in both sexes, whereas characters involved in mate signalling by only one sex should show marked differences in the rates of evolution between the sexes (West-Eberhard 1983). An uneven distribution of either overall rates or sex-specific rates of character evolution among wing surfaces would support the hypothesis that butterflies are using signal partitioning as a solution to potentially antagonistic selective forces.

Based on these predictions about character evolution and selective forces, we used a comparative approach to assess support for the signal partitioning hypothesis. We inferred the phylogeny for 54 species of *Bicyclus* and tested for evidence of signal partitioning among the dorsal–ventral and forewing–hindwing surfaces. First, we used maximum-likelihood estimates of evolutionary rates to evaluate lability of characters on different wing surfaces. We then compared rates of evolution in wing characters between the sexes to assess evidence for a role in mate signalling. Finally, to assess support for signal partitioning in *Bicyclus*, we tested for uneven distribution of overall rates and sex-specific rates of character evolution among wing surfaces.

2. MATERIAL AND METHODS

(a) Phylogenetic reconstruction

To infer the phylogeny of *Bicyclus*, we used Bayesian Markov chain Monte Carlo (MCMC) analyses of the nuclear gene elongation factor 1- α (*EF1 α*) (946 bp) and the mitochondrial genes cytochrome oxidase I and II (*COI* and *COII*, respectively) (2050 bp) (Monteiro & Pierce 2001). We used likelihood-ratio tests (Sullivan & Swofford 1997) to select an independent model for each partition (partition 1: *EF1 α* ; partition 2: *COI*+*COII*). Both partitions fit an HKY+G+I model of evolution; model parameters for each partition were allowed to vary independently during MCMC runs. Trees from two MRBAYES (Huelsenbeck & Ronquist 2001) MCMC runs of four chains each were sampled every 1000 generations for 20 million generations. Each run had one cold chain and three heated chains (temp=0.2). *Halelesis asochis*

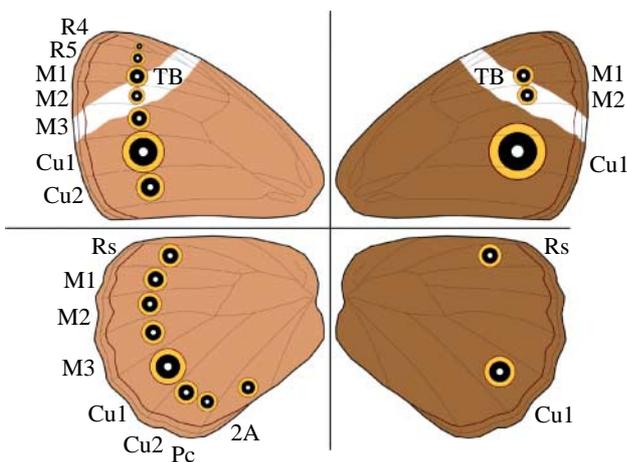


Figure 2. Schematic of *Bicyclus* ventral (left) and dorsal (right) wing characters used in this study. Eyespots are named according to wing cell location; TB, transverse band.

was designated as the outgroup (Monteiro & Pierce 2001). We used the standard deviation of the split frequencies to assess convergence between the two runs; trees sampled before convergence (determined by a standard deviation of split frequencies less than 0.02) were discarded (Huelsenbeck & Ronquist 2001). The two runs had converged by the 10 millionth MCMC generation; only trees sampled from the latter 10 million generations were used to generate a consensus phylogeny.

(b) Wing characters

Character data were collected from those specimens included in Monteiro & Pierce (2001), from specimens housed at the Yale Peabody Museum, and from specimens housed at the Royal Museum for Central Africa. We scored 22 wing pattern characters: 20 eyespots and two transverse wing bands (figure 2; see electronic supplementary material, table S1). All characters were recorded as binary (presence/absence) data because the presence or absence of eyespots significantly affects mate signalling (Robertson & Monteiro 2005) and predator avoidance (Vallin *et al.* 2005), although eyespot size may contribute to signal efficiency in predator avoidance (Lyytinen *et al.* 2004; Stevens *et al.* 2008). Males and females were scored separately; variable characters were scored as polymorphic. For those species that are known to display seasonal polyphenism, we scored only wet season forms. Although predator avoidance and mate signalling may still affect wing evolution in dry season forms (Brakefield & Reitsma 1991), seasonal polyphenism in *Bicyclus* typically results in changes in eyespot size, not the presence/absence (Condamine 1973).

(c) Evolutionary lability and wing surface

Using the consensus Bayesian phylogeny, we compared rates of character evolution on different wing surfaces using BAYESTRAITS (Pagel *et al.* 2004). For each sex, we first estimated the rate of change for all wing characters ('one-rate model') using a symmetrical Mk1 maximum-likelihood model. For each surface axis (dorsal–ventral and forewing–hindwing), we then categorized characters into two partitions: a dorsal partition and ventral partition for the dorsal–ventral axis ('two-rate dorsal–ventral model') and a forewing partition and a hindwing partition for the

forewing–hindwing axis ('two-rate forewing–hindwing model'). We again estimated the rates of character evolution, allowing the two partitions to have independent rates of change. To determine whether the rates between the two surfaces were significantly different, we compared likelihood scores of character reconstruction between the one- and two-rate models; rates were considered significantly different if the two-rate model was at least two log likelihoods better than the one-rate model (Pagel 1999). Three eyespot characters (ventral forewing R4, DF M2 and dorsal hindwing Rs; figure 2) were excluded from the analysis of male wings because eyespots were not observed at these positions in any male *Bicyclus* species included in this study.

(d) Sex-based differences in evolutionary rates

To determine which characters display sex-based differences in evolutionary rates, we compared models of evolution using likelihood-ratio tests. Briefly, using the Bayesian phylogeny with consensus branch lengths, we first estimated the maximum-likelihood model of evolution for each wing character, and for each sex in BAYESTRAITS (Pagel *et al.* 2004) and MESQUITE (Maddison & Maddison 2007). We used likelihood-ratio tests to select either a one-parameter rate of evolution (equal rates of gain and loss) or a two-parameter, asymmetrical rate of evolution (different rates of gain and loss) (Lewis 2001). Using the model of evolution estimated for a specific character and sex (where f_i and m_i are the observed character data for character i in females and males, respectively; and R_{f_i} and R_{m_i} are models of character i in females and males, respectively), we calculated the character likelihood on the Bayesian phylogeny (where $L_i(f_i, R_{f_i})$ and $L_i(m_i, R_{m_i})$ are the likelihoods of character i for females and males, respectively, using models estimated from observed character data for that sex). We then calculated the character likelihood of observed data of one sex using the model of evolution estimated from the observed data of the opposite sex (where $L_i(f_i, R_{m_i})$ and $L_i(m_i, R_{f_i})$ are the likelihoods of character i for females and males, respectively, using the model estimated for the opposite sex). We assessed differences in rates using likelihood-ratio tests, comparing likelihood scores for a particular character and sex based on the two different models of evolution: $L_i(f_i, R_{f_i})$ versus $L_i(f_i, R_{m_i})$ and $L_i(m_i, R_{m_i})$ versus $L_i(m_i, R_{f_i})$, for females and males, respectively. In cases where the data best fit a symmetrical (one-parameter) model in one sex and an asymmetrical (two-parameter) model in the opposite sex, we compared estimated asymmetrical models for both sexes. Models were considered significantly different between the sexes if the likelihood scores differed by more than two log likelihoods (Pagel 1999). Six eyespot characters were invariant in only one sex (ventral forewing R4, DF M2 and dorsal hindwing Rs were invariant in males; ventral hindwing Rs, M1 and Pc were invariant in females) and were excluded from these analyses.

To determine whether characters displaying sex-based differences in rates of evolution were unevenly distributed among the wing surfaces, we first categorized each character as displaying the same or a different model for each sex, based on the results of likelihood-ratio tests. We then used linear regression on the logit-transformed model (same or different rates of evolution between the sexes) to determine the relative importance of the two wing surface axes (dorsal–ventral and forewing–hindwing) in predicting whether a character would display sex-based differences in the model of evolution.

All analyses were carried out using the R software package (R Development Core Team 2007).

To test the hypothesis that specific eyespots have been regained, we compared unconstrained likelihood models of character evolution to a model prohibiting regain of that eyespot. Models allowing regain of eyespots were considered significantly better if likelihood scores were two log likelihoods better than the model prohibiting regain (Pagel 1999). We performed our analysis on two DF eyespots, M1 and Cu1, because these characters demonstrated high rates of change in males and females and play a significant role in mate signalling in *B. anymana* (Robertson & Monteiro 2005). For each eyespot, we analysed female and male data separately because likelihood-ratio tests indicated significant differences in evolutionary rates between the sexes for these two characters (see §3b).

3. RESULTS AND DISCUSSION

(a) *Evolutionary lability and wing surface*

The reconstructed Bayesian phylogeny of *Bicyclus* was topologically congruent with that presented in Monteiro & Pierce (2001) with the exception of the placement of *Bicyclus taenias* and *Bicyclus pavonis* (see figure S1 in the electronic supplementary material). Likelihood-ratio tests of rates of character evolution based on the Bayesian phylogeny showed significant differences in character evolution among wing surfaces (figure 3). Dorsal rates of change were higher than ventral rates of change in wing characters for both sexes, and the two-rate dorsal–ventral model fit the data better than a one-rate model (females: $\Delta \ln L = 8.950$; males: $\Delta \ln L = 18.748$). Additionally, forewing characters evolved at a higher rate than hindwing characters, and the two-rate forewing–hindwing model was a better fit than the one-rate model (females: $\Delta \ln L = 28.767$; males: $\Delta \ln L = 27.185$). In summary, dorsal characters and forewing characters are more labile through evolutionary time than those on the ventral surface and hindwing.

(b) *Sex-based differences in evolutionary rates*

Likelihood-ratio tests identified five characters with significantly different rates of evolution between females and males (figure 4). These characters displaying sex-based differences in evolutionary rates were unevenly distributed among the wing surfaces: dorsal characters were more likely to display different rates between the sexes than were ventral characters ($F_{1,12} = 9.00$, $p = 0.011$), and sex-based rate differences were more likely in forewing characters than in hindwing characters ($F_{1,12} = 6.300$, $p = 0.027$) (figure 4). This uneven distribution of sex-based rate differences among the wing surfaces are expected under a model of signal partitioning where visual signals involved in mate signalling are more likely to occur on the dorsal and forewing surfaces than on the ventral and hindwing surfaces.

In both sexes, models allowing regain of the M1 and Cu1 dorsal eyespots were significantly better than models prohibiting regain of those eyespots, indicating that once a complex character, such as the eyespot, is lost in a lineage, it can be regained (figure 5; Marshall *et al.* 1994; Whiting *et al.* 2003; Chippindale *et al.* 2004). These reconstructions also imply that eyespots appeared multiple independent times, suggesting the relative ease at which complex pattern elements can appear in novel

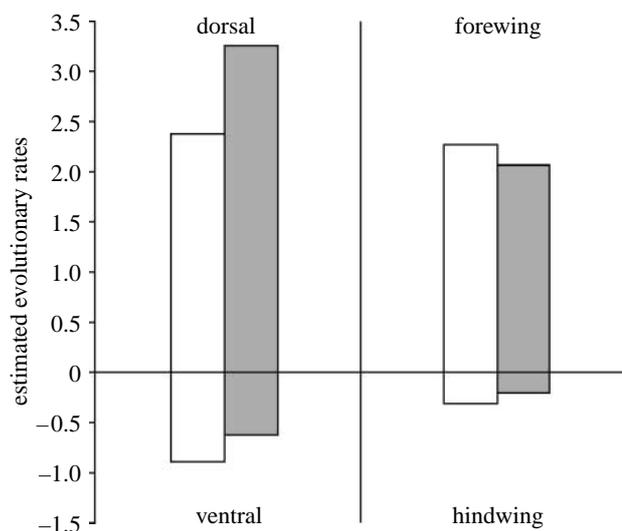


Figure 3. Estimated rates of change for two-rate models of character evolution for females (white bars) and males (grey bars).

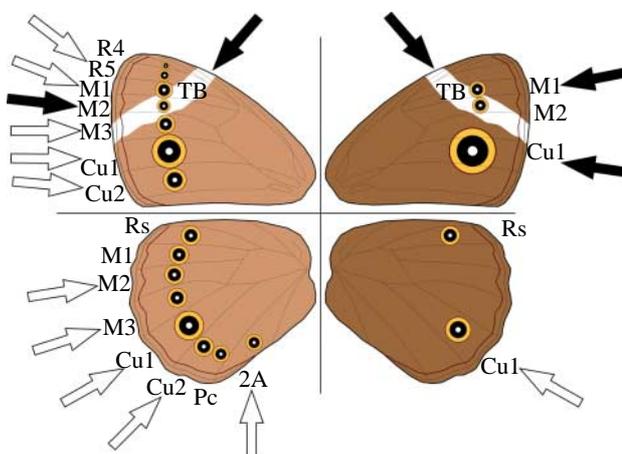


Figure 4. *Bicyclus* wing schematic displaying results of likelihood comparisons of evolutionary rates between sexes. Black arrows indicate characters with significantly different rates of evolution between females and males and white arrows indicate identical rates between the sexes. Dorsal and forewing characters were more likely to display sex-based rate differences than ventral or hindwing characters, respectively. Characters unmarked by arrows were excluded from rate comparison analyses (see §2 for details).

positions. The loss of sexually selected traits is not unprecedented (reviewed in Wiens 2001), and regains are not unexpected when sexual communication relies on relatively few signals (Ritchie & Gleason 1995; Omland & Lanyon 2000). This finding is congruent with the hypothesis that *Bicyclus* species are using different wing surfaces for different signalling functions. These observations fit a scenario where traits on the forewing are sexually selected and rapidly evolving, whereas those on the hindwing are under strong stabilizing selection, probably due to natural enemies.

(c) *Signal partitioning: other considerations*

Butterfly species may employ signal partitioning to reduce conflict among signalling functions, but complete partitioning among the wing surfaces may not be optimal or possible. Although characters inferred to be involved in

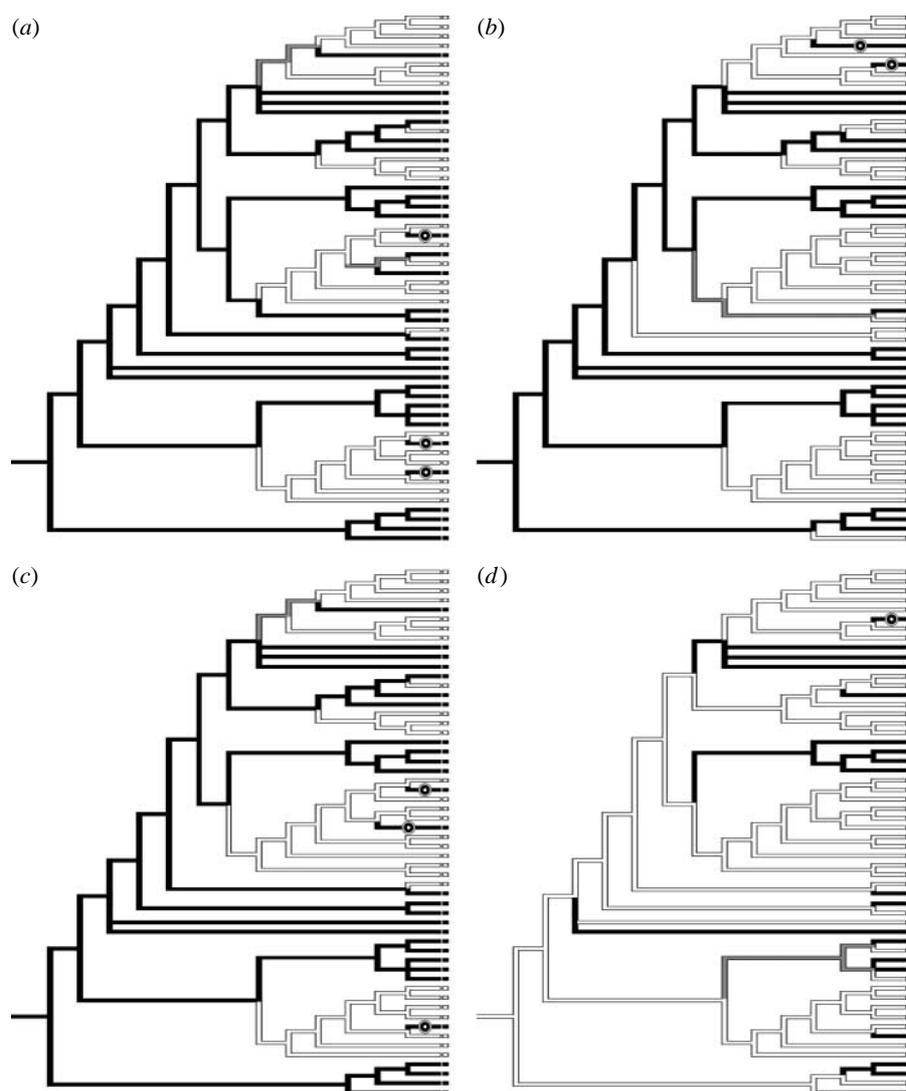


Figure 5. Re-evolution of eyespots in *Bicyclus*. Maximum-parsimony reconstructions of ancestral character states indicating presence (black) or absence (white) of specific eyespots: (a) female DF eyespot M1 ($\Delta \ln L = 4.013$), (b) male DF eyespot M1 ($\Delta \ln L = 3.481$), (c) female DF eyespot Cu1 ($\Delta \ln L = 5.389$) and (d) male DF eyespot Cu1 ($\Delta \ln L = 3.013$). Eyespot symbols highlight branches on which single-most parsimonious reconstructions indicated regains of featured eyespot. Grey branches represent equivocal ancestral state reconstructions. For all four eyespots shown, likelihood models allowing re-evolution of eyespots were significantly better than models prohibiting the re-evolution of eyespots ($\Delta \ln L$ is the difference in log-likelihood scores between models allowing and prohibiting regain of eyespots, respectively). Tree topology is identical to that in figure S1 in the electronic supplementary material, excluding outgroups.

mate signalling were more likely to occur on the DF, two ventral forewing characters (the M2 eyespot and the transverse band) also displayed different rates of evolution between the sexes. Female mate choice experiments in *B. anynana* did not find evidence for a role of ventral characters in determining preference (Robertson & Monteiro 2005), but these characters may still play a role in mate signalling in other species of *Bicyclus*. Alternatively, ventral characters may be important for male mate choice, as observed in some lycaenid butterflies (Fordyce *et al.* 2002). We analysed only the presence/absence of characters, and variation in other parameters of visual signals, such as size and colour, including ultraviolet wavelengths, may also factor into signal content. Our results indicate that signal partitioning occurs in *Bicyclus*, although some characters or wing surfaces may still serve multiple signalling functions.

Additional information concerning predator responses to eyespots is necessary for a complete understanding

of the efficacy of signal partitioning; studies that manipulate the presence/absence of eyespots on different wing surfaces would be particularly useful. Laboratory studies of predator responses to butterfly eyespots demonstrate their usefulness in predation avoidance (Lyytinen *et al.* 2004; Vallin *et al.* 2005). Field studies of lepidopteran models with eyespots also show increased survival relative to models lacking eyespots (Stevens *et al.* 2008), indicating the selective advantage these wing characters provide. Information on the natural enemies of *Bicyclus* is lacking, but additional studies of the cognitive and sensory capabilities of predators would allow a more thorough examination of the signal partitioning hypothesis in butterflies (Endler 1992; Stevens *et al.* 2008).

(d) *Signal co-option and developmental independence*

Despite partitioning signals among the wing surfaces, *Bicyclus* species are using the same serially homologous

eyespot patterns for different signalling functions. Specifically, eyespots on the ventral surface appear to be used in predator avoidance (Lyytinen *et al.* 2004) while dorsal eyespots are used for mate signalling (Robertson & Monteiro 2005). This co-option of the same signal for different functions is particularly evident when comparing the rates of evolution in homologous dorsal and ventral forewing eyespots. The M1 and Cu1 spots on the DF surface (commonly called the anterior and posterior eyespots in *B. anynana*) are among the most labile characters as measured in likelihood estimates and both are implicated in mate signalling in comparisons of sex-specific evolutionary rates (figures 3 and 4). However, on the opposite surface (ventral forewing), these same eyespots (M1 and Cu1) are invariant—the eyespots are present in both sexes of all *Bicyclus* species included in this study. Although these results do not preclude a role of mate signalling by these ventral eyespots, they do suggest different modes of selection acting between the ventral and DF surfaces.

An alternative explanation for the difference in evolutionary rates among wing surfaces would be that developmental constraints prevent changes on some wing surfaces, but not others. However, artificial selection experiments and laboratory mutants in *B. anynana* suggest otherwise. First, eyespot size readily responds to artificial selection on both ventral and dorsal wing surfaces (Holloway *et al.* 1993; Monteiro *et al.* 1994), even when selection on different eyespots is applied in opposite directions (Beldade *et al.* 2002; Allen *et al.* 2008), arguing against insurmountable developmental constraints among eyespots regarding size. Spontaneous mutations in laboratory populations affecting eyespot size, colour or shape indicate that wing patterns may change due to one or a few genetic changes (Brakefield *et al.* 1996; Brakefield 1998), and X-ray induced mutants demonstrate the relative ease, genetically speaking, of eliminating individual eyespots on the ventral hindwing (Monteiro *et al.* 2003). Although developmental correlations among eyespots exist (Holloway *et al.* 1993; Monteiro *et al.* 1994, 1997; Beldade *et al.* 2002; Allen *et al.* 2008), the evolutionary potential for independent changes in eyespot size and presence/absence, evidenced by selection experiments and laboratory mutants, argues that different selective forces, not developmental constraints, are the main drivers affecting eyespot size and number evolution in *Bicyclus*.

(e) Evolutionary consequences of signal partitioning Signal partitioning provides a solution to accommodating potentially antagonistic selective pressures, as demonstrated by *Bicyclus* wing pattern evolution. This strategy of spatially separating signal functions has predictable evolutionary consequences, which deserve future attention. Signal partitioning is expected to work similarly in lepidopteran species that rest with the dorsal surface exposed, such as some species in the nymphalid genus *Hamadryas*; dorsal surface characters should be involved in predator avoidance, while ventral characters would be used in mate signalling. The signal partitioning hypothesis also predicts that if signals used in mate recognition are important for reproductive isolation, then sister taxa would be more likely to differ in dorsal wing patterns than ventral wing patterns. Although we are unable to quantitatively assess support for this prediction in our study due to incomplete taxon sampling, at least four pairs of *Bicyclus*

species reconstructed as sister taxa (*buea-sanaos*, *dentatus-anisops*, *graueri-sebetus* and *italus-zinebi*) display wing morphologies consistent with this prediction. The two species of each pair differ in dorsal surface characters but not ventral surface characters measured in this study. A bias towards dorsal surface differences in sister species should be especially evident in sympatric species pairs, such as *buea-sanaos* and *graueri-sebetus* in *Bicyclus* (Condamin 1973; Ackery *et al.* 1995), where reinforcement against heterospecific matings would be expected (Lukhtanov *et al.* 2005). Finally, by releasing butterflies from the necessity of using the same signals for multiple functions, signal partitioning may increase the opportunity for signal evolution. This release may allow for rapid evolution of wing patterns used in mate signalling, increasing the potential for species diversification.

Our results support the model of signal partitioning in *Bicyclus* butterflies, but the phenomenon of signal partitioning itself deserves to be studied in a broader evolutionary framework. When and in which lineages did butterflies start using this strategy of optimizing their wing patterns for different functions? How does signal partitioning affect the evolution of serially homologous elements, such as eyespots, when they serve multiple signalling functions, as in *Bicyclus*? Are particular signal functions, such as mate signalling, especially important in generating novel pattern elements? Future studies targeting a broader sample of species across Lepidoptera should provide insight into the evolution of signal function and the mechanisms responsible for generating and maintaining signal diversity.

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