

Eyespot evolution: phylogenetic insights from *Junonia* and related butterfly genera (Nymphalidae: Junoniini)

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SUMMARY Butterfly eyespots have been the focus of a number of developmental and evolutionary studies. However, a phylogenetic component has rarely been explicitly incorporated in these studies. In this study, I utilize a phylogeny to trace the evolution of eyespot number and position on the wing in a group of nymphalid butterflies, the subtribe Junoniini. These butterflies have two kinds of eyespot arrangements which I refer to as *Serial* and *Individual*. In the *Serial* arrangement, eyespots are placed in a series on compartments 1–6 (counting from the anterior wing margin). In the *Individual* arrangement, eyespots are isolated on specific compartments, ranging from 1 to 4 in number. This can be divided into four subtypes based on the number and positions of eyespots. I map the evolution of these five arrangements over a phylogeny of Junoniini reconstructed with ca. 3000 base pairs

of sequence data from three genes. The results show that almost all arrangements have evolved at least twice, with multiple shifts between them by addition and deletion of eyespots. I propose a model involving genetic or developmental coupling between eyespots in specific compartments to explain these shifts. I discuss their evolution in light of existing knowledge about their development. I also discuss potential explanations for functional significance of the eyespot patterns found in the group. Differential selection for and against eyespots, both at different times over the phylogeny and in different regions, have driven the evolution of eyespot arrangements. The study throws open many questions about the adaptive significance of eyespots and the developmental underpinnings of the various arrangements.

INTRODUCTION

Butterflies, with their colorful and diverse wing patterns, have long fascinated both laymen and scientists. Not surprisingly, their ornate wing patterns have been the focus of a number of scientific studies and perhaps no single aspect of their biology has elicited as many studies as the intricate eyespots found in the family Nymphalidae. Eyespots in nymphalid butterflies have been studied from a number of perspectives including evolution, ecology, development, and genetics (e.g., Nijhout 1980; Brakefield et al. 1996; Brunetti et al. 2001; Beldade et al. 2002; Monteiro et al. 2003; Marcus et al. 2004; Robertson and Monteiro 2005; Vallin et al. 2005). Despite numerous studies on their evolutionary aspects, very little phylogenetic information has been incorporated and the need for phylogenetic input in such studies has been stressed (Brakefield 1998; Monteiro and Pierce 2001; Beldade and Brakefield 2002; McMillan et al. 2002; French and Brakefield 2004; Reed and Serfas 2004; Monteiro 2008). Research on eyespots has thus far focused on a few model species, notably *Bicyclus anynana* (Nymphalidae: Satyrinae; e.g., Brakefield and French 1993; Brakefield 1998; Monteiro et al. 2007) and *Junonia* (= *Precis*) *coenia* (Nymphalidae: Nymphalinae; e.g., Nijhout 1980, 1985; Carroll et al. 1994; Rountree and Nijhout 1995; Keys et al. 1999) and we have achieved substantial progress in understanding the *process* of eyespot formation. However, the potential of phylogenetic information to illuminate *patterns* of evolution of

eyespots has rarely been harnessed. Here, I utilize a phylogenetic framework to investigate the patterns of eyespot evolution in a group of butterflies and discuss the implications of the results within the context of their evolution and development.

The study group

The tribe Junoniini (Nymphalidae: Nymphalinae) consists of 85 species under six genera—*Precis* (17 spp.), *Hypolimnas* (26 spp.), *Salamis* (3 spp.), *Yoma* (2 spp.), *Protogoniomorpha* (2 spp.), and *Junonia* (35 spp.) (Nymphalidae Systematics Group [NSG] 2009). The majority of species in the group display distinct eyespots on the dorsal surface of their wings whereas the ventral surface tends to be more cryptic, with the eyespot patterning reduced or absent in many species. Although the number and positions of eyespots on the fore- and hindwings are identical in most species, some species are dimorphic between the two sides and in such species the hindwings usually have better-developed eyespots. There is considerable variation in the number and position of eyespots between species, making these butterflies an ideal group to study the patterns of eyespot evolution from a phylogenetic perspective.

The adaptive functions of dorsal and ventral eyespots are considered to be different (Robertson and Monteiro 2005; Stevens 2005; Oliver et al. 2009). Studies on *B. anynana* have shown that the genetic coupling between the dorsal and ventral wing pattern elements is limited (see Allen 2007, for a review).

Hence evolution of eyespots on either side of the wings can potentially take different directions under differential selection pressure. In this study I focus on the dorsal eyespot patterning of species within Junoniini and restrict myself to the hindwing patterning in order to maintain consistency in inferences.

In the nymphalid groundplan (Schwanwitsch 1924; Süffert 1927; Nijhout 1991) eyespots are part of the “border ocelli system” on the distal half of the wing. In Junoniini, they generally consist of contrasting rings surrounding a white pupil. In some species these eyespots consist of a single disc, but can be unambiguously identified as homologous to normal eyespots based on their position on the wing relative to other wing pattern elements. Each eyespot is normally confined to the region bounded by two veins, that is a wing compartment (region bounded by two veins; also called “wing-cell”). For this study, I number these compartments 0–6 beginning from the anterior wing margin (the costa) (Fig. 1). In some species eyespots on compartments 1 and 2 fuse together to form a composite eyespot. I will use the term twin-eyespot to refer to an arrangement where one large composite eyespot or two eyespots occur on adjacent compartments without being flanked by any eyespots in neighboring compartments.

The eyespot patterning found on the hindwing dorsal surface can be classified into five distinct types. These are described below with examples.

(a) *Serial arrangement*: Eyespots are arranged in a series on compartments 1–6. Some species lack eyespots in compartment 6, whereas some, like *J. terea*, have two small conjoined eyespots in the compartment. In some species, for instance, *J. atlites*, there is an additional faint eyespot in compartment 0. This arrangement is found in all species of *Precis* with eyespots, almost all *Hypolimnas* species with eyespots, *J. hedonia*, *J. sophia*, etc.

Eyespots on other species occur individually, with substantial differences in their placement and number. I will collectively refer to all of these as the *Individual* arrangement. Generally, the eyespots forming the *Individual* arrangements are more conspicuous than those comprising the *Serial* arrangement. Four further subtypes can be identified within the *Individual* arrangement.

(b) *Individual 5 arrangement*: A single eyespot is present on compartment 2. *J. ansorgei*, *J. cymodoce*, and *J. cytora* represent this type.

(c) *Individual 2-5 arrangement*: Compartments 2 and 5 have an eyespot each. For example, *J. orithya*, *J. touhilimasa*, etc.

(d) *Individual 12-5 arrangement*: A single eyespot is present on compartment 5 in addition to a twin-eyespot in 1 and 2. Examples include *J. almana* and *J. coenia*.

(e) *Individual 12-56 arrangement*: Two twin-eyespots occur on 1 and 2 and 5 and 6. For example, *H. deois*, *H. diomea*, etc.

Although seasonal polyphenism is prevalent in the group, eyespot number and positions on the dorsal hindwing remain

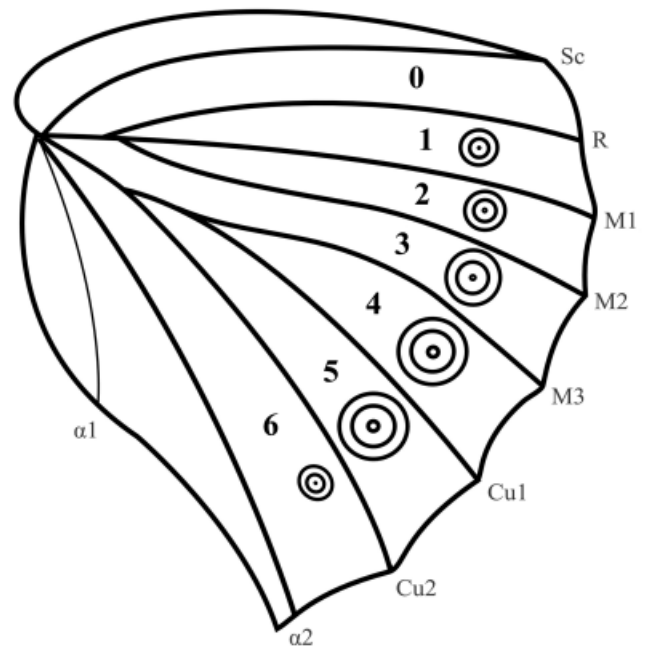


Fig. 1. Dorsal hindwing of *Junonia hedonia* depicting the positions of eyespots of a typical species in Junoniini with the *Serial* arrangement of eyespots. Numbers next to the eyespots indicate the compartment numbers used in this study. Vein nomenclature is shown to the right.

the same. Some species exhibit pronounced sexual dimorphism in eyespot patterning. For instance, in *J. orithya*, females have much larger eyespots than males (Larsen 2005; Kusaba and Otaki 2009). *H. bolina* has several subspecies and males lack eyespots in most subspecies whereas all females and males of some subspecies have the *Serial* arrangement. Intra-specific variation with respect to eyespot size is common whereas polymorphism with respect to eyespot number is rare. *J. coenia*, *J. evarete*, and *J. genoveva* possess both the *Individual* 2-5 and *Individual* 12-5 patterns (Neild 2008). Some individuals of *J. almana* and *J. lemonias* lack distinct eyespots on 5.

Objective of the study

The main objective of this study was to trace the evolution of eyespot number and position within Junoniini and determine the trends in their evolution. As a null-hypothesis, I postulated that a shift from the *Serial* arrangement to an *Individual* arrangement is evolutionarily more likely because deletion of eyespots at certain compartments can lead to such a shift. I also hypothesized that there has been no reversal to the *Serial* arrangement following a shift from *Serial* to an *Individual* arrangement, because the genetic mechanism for eyespot formation is presumably lost during the initial transition.

MATERIALS AND METHODS

The phylogeny of Junoniini was reconstructed based on 3090 base pairs of DNA sequence data from three genes—*COI* (cytochrome

oxidase subunit I), *EF-1 α* (elongation factor-1 α) and *wingless*, the former a mitochondrial gene and the latter two nuclear genes. Sequences representing 23 species of *Junonia*, six of *Hypolimnas*, eight of *Precis*, and two species each of *Protogniomorpha*, *Yoma*, and *Salamis*, which were part of published studies (Wahlberg et al. 2005; Kodandaramaiah and Wahlberg 2007) were downloaded from GenBank. *COI* sequences from *H. octocula* and *H. antilope* were also retrieved from GenBank (accession numbers EU626558 and EU626557). Additionally, exemplars of five species of *Hypolimnas*—*deois*, *diomea*, *dinarchia*, *monteironis*, and *salmacis*—were sequenced using primers and protocols described in Kodandaramaiah and Wahlberg (2007). Sequences are deposited in GenBank (accession numbers GQ240281–GQ240289). All eyespot arrangements found in respective genera were represented in the study. Figure 2 depicts photographs of all species with eyespots used in the study.

The combined dataset was analyzed under the maximum likelihood criterion using the software RAXML-III (Stamatakis et al. 2005, 2008). The GTR+G model was assumed and the dataset partitioned into three categories corresponding to the three genes. Support for individual clades was estimated using bootstrapping with 1000 replicates.

The evolution of the five arrangements over the phylogeny was traced using Mesquite (Maddison and Maddison 2008). Specimens from the voucher collection of the NSG (2009) were examined to determine the number and positions of eyespots for each species. Additionally, specimen photographs from various sources (Wynter-Blyth 1957; D'Abbrera 1982, 1990, 1997; Scott 1986; Smith et al. 1989; Larsen 1991; Corbet and Pendlebury 1992; Haribal 1992; Pringle et al. 1994; Pinratana and Eliot 1996; Tveten and Tveten 1996; Igarashi and Fukada 1997; Braby 2000; Tennent 2002; Woodhall 2005; Kehimkar 2008; Neild 2008) were examined to assess subspecific polymorphism in eyespot number. Each arrangement was treated as a character and the presence or absence of the character was coded for all species. Species with variation in the number of eyespots were coded with data from the form with maximum number of eyespots.

Yoma, *Salamis*, and some species within *Hypolimnas* lack eyespots and they were coded accordingly. The two species of *Protogniomorpha* have two distinct eyespots composed of concentric rings on 2 and 5, in addition to black patches in place of eyespots on 0 and 1. These were coded with presence for *Individual 2-5*. Characters were mapped under the parsimony criterion using the Trace Character option in Mesquite.

RESULTS

The phylogeny was generally well-supported and broadly congruent with the relationships reported in Kodandaramaiah and Wahlberg (2007) and Wahlberg et al. (2005). At the generic level *Precis* was sister to *Hypolimnas* and this clade was sister to the rest of the junoniines. *Yoma*, *Protogniomorpha*, and *Salamis* formed a clade and were together sister to *Junonia* (Fig. 3). Some nodes were weakly supported, with <50% bootstrap values. However, the inferences made in the study are robust to possible alternate placements of clades subtended by such nodes.

The results of the character optimization indicate that the *Serial* and *Individual* arrangements have evolved independently multiple times within Junoniini, with shifts between the two arrangements in both directions (Fig. 4). Three *Individual* subtypes 5, 2-5, and 12-56 have evolved at least twice over the phylogeny.

The *Serial* arrangement evolved at least once in *Hypolimnas* and *Precis*, and was lost in *Hypolimnas*. The arrangement has also evolved at least once within the “basal” *Junonia* species and again as a shift from an *Individual* pattern in the *sophia* – *westermanni* clade.

There was at least one shift from *Serial* to *Individual 12-56* within *Hypolimnas*. *Yoma* and *Salamis* lack eyespots, whereas *Protogniomorpha* has the *Individual 2-5* arrangement. *Individual 5* has appeared in *J. cytora* and the ancestor of the *cymodoce*–*ansorgei* clade. Within *Junonia*, *Individual 2-5* has evolved three times—once in the ancestor of the sister species *touhilimasa* and *artaxia*, once in *J. villida* and again in the ancestor of the *orithya*–*hierta* – *oenone* clade. *Individual 12-5* has evolved once in the ancestor *J. almana* and its sister clade. It is also found in the *coenia*–*evarete*–*genoveva* clade but it is unclear whether it evolved independently in the ancestor of this clade. The ancestor of Junoniini either had the *Serial* arrangement or no eyespots.

DISCUSSION

The results of the character optimization show that although the eyespot arrangements have a tendency to be associated with specific clades, there have been numerous shifts and multiple independent instances of evolution of various arrangements. The number of eyespots has repeatedly increased and decreased over the phylogeny with no obvious trends in either direction.

The reconstruction indicates that the ancestor of Junoniini either had no eyespots or had the *Serial* arrangement, but not any of the *Individual* arrangements. Hence, I conclude that the *Serial* arrangement is most basal among the five. The *Individual* arrangements have evolved either independently or as a result of deletion of eyespots from the *Serial* arrangement. Interestingly, irrespective of this mode of evolution, there is a distinct propensity for eyespots in the *Individual* arrangements to be found on specific compartments and combinations. Eyespot 5 is present in all cases and when found in combination with other eyespots, eyespot 2 is always part of such combinations. Furthermore, twin-eyespots are always formed from the combination of 5 and 6 or 1 and 2. These observations suggest some “rules” for the configuration of eyespot arrangements. A survey of dorsal and ventral wing patterns among nymphalid butterflies that have the *Individual* arrangement indicates that the same configurations are also found in several other species within the nymphaloid clade *sensu* Wahlberg et al. (2005), which includes the



Fig. 2. Photographs of the dorsal forewings of species with eyespots used in the study. i–xxii, *Serial*; xxiii–xxv, *Individual 2*; xxvi–xxxv, *Individual 2-5*; xxxvi–xxxviii, *Individual 2-56*; xxxix and xxxx, *Individual 12-56*. Specimens of *Junonia evarete* and *J. genoveva* pictured here have the *Individual 2-5* arrangement, but have been coded with presence for *Individual 2-56* because some specimens are known to possess an extra eyespot in 1.



Fig. 3. Maximum likelihood topology resulting from the RAxML analysis of the combined dataset. Numbers below the nodes indicate bootstrap support.

subfamilies Heliconiinae, Limenitidinae, Nymphalinae, Biblidinae, Apaturinae, Pseudorgolinae, and Cyrestinae, but not in the satyroid clade (Satyrinae, Charaxinae, and

Calinaginae). Examples from the nymphaloid clade include species of *Vindula* (Heliconiinae), *Kallimoides* (Nymphalinae), and *Vanessa* (Nymphalinae).

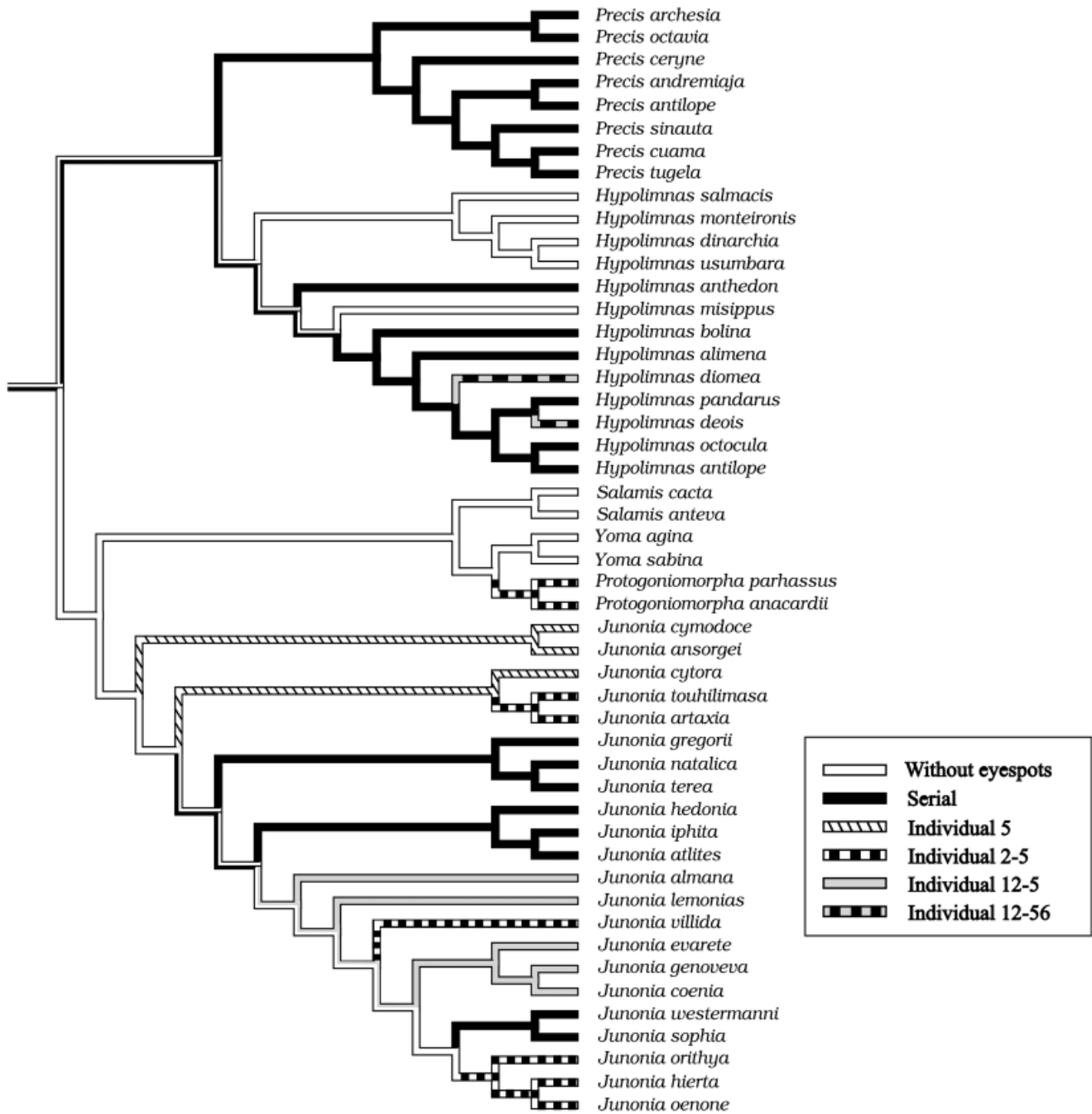


Fig. 4. Results of the ancestral character reconstruction in Mesquite. Branches with two characters indicate ambiguity.

The predisposition for certain eyespot positions and combinations within Junoniini suggests that eyespots in certain compartments are linked either developmentally or genetically. I suggest a simple model to explain the patterns found in Junoniini. There is strong coupling between compartments 2 and 5. There is also weaker coupling of compartments 1 to 2 and 5 to 6. The strength of the latter two vary across taxa. The varying strengths of linkage between specific compart-

ments and the clear-cut propensity for eyespots to be found on some compartments need further explanation.

The transition from *Serial* to *Individual* in *Hypolimnas* is unquestionable. However, whether the evolution of 12-5 in the ancestor of *J. almana* and its sister clade was another such shift is not clear from the reconstruction. The branches leading to the *terea*–*natalica*–*gregorii* and *atlites*–*iphita*–*hedonia* clades are reconstructed as having either the

Serial patterning or no eyespots at all. As all *Junonia* species possess eyespots, it is reasonable to conclude that the ancestors along these branches possessed the *Serial* arrangement, which in turn implies that there was a second shift from *Serial* to *Individual*. This conclusion is corroborated by *J. atlites*, where eyespots in 1, 2, and 5 are substantially bigger than the remaining eyespots, quite likely indicating a transitional arrangement. In all these instances where deletion of eyespots from *Serial* has given rise to *Individual*, eyespots 3 and 4 are lost, either due to large effect mutations or gradual reduction in size. Indeed, these two eyespots are never found in any of the *Individual* patterns. This again suggests that these two eyespots are coupled to each other. *Missing* and *3+4*, two mutations reported in *B. anynana* (Monteiro et al. 2003, 2007) lead to the deletion or reduction of eyespots on these two compartments. Although the same mutations are unlikely to have played a role in the evolution of eyespots within Junoniini, the coupling between 3 and 4 is likely to be a more general phenomenon in nymphalid butterflies.

Early research on *J. coenia* showed that the group of cells at the center of the future eyespot, the “focus,” can be transplanted to a different area on the wing during early pupal development to induce a fully developed ectopic eyespot (Nijhout 1980), evidence that these cells in the focus play a pivotal role in eyespot determination. Later studies revealed the expression of various genes during the late larval wing stage in the focus and regions flanking it, including *distal-less* (*dll*), *spalt* (*sal*), *engrailed* (*en*) and *cubitus interruptus* (*ci*), *notch* (*N*), *patched* (*patch*), *hedgehog* (*hh*), and *wingless* (*wg*) (Carroll et al. 1994; Keys et al. 1999; Brunetti et al. 2001; Reed and Serfas 2004). Although evidence for gene expression does not necessarily imply their role in eyespot formation (French and Brakefield 2004; Evans and Marcus 2006), it is probable that several or all of these genes are involved in eyespot differentiation. Mutational changes in any one of the genes involved can potentially lead to deletion of eyespots. However, the hypothesis of “no reversal to *Serial* after a transformation from *Serial* to *Individual*” is not supported by the results because there is one such reversal in the *J. sophia*–*westermanni* clade. The developmental mechanism is either not lost, or lost and later regained by co-option from compartments 2 or 5.

Otaki et al. (2005) presented a survey of the presence of “pupal spots” in nymphalid butterflies. These pupal spots correspond to eyespot centers in adult wings. Such spots were found to occur on compartments 1–5 in *J. almana* and *J. orithya*. They speculated that eyespot-organizing foci are present in compartments 3 and 4, but are either turned off or become increasingly weak in activity during later developmental stages. Similarly, pupal spots were found in compartments that normally lack eyespots in *Vanessa cardui*. These compartments produce eyespot-like elements which disappear in later stages of development. Moreover, experiments on *B. anynana* have shown that compartments without eyespots

have the ability to produce eyespots (Brakefield et al. 1996; Koch et al. 2000; Beldade and Brakefield 2003). Based on these lines of evidence, I tentatively conclude that irreversible mutations by which a character state cannot be restored to its previous state are unlikely to have caused the loss of eyespots on certain compartments. The genetic mechanism is retained, but developmentally turned off and can be turned on again during the course of evolution.

Several models have been put forward to explain the process of eyespot differentiation in terms of regulatory interactions between specific gene products. Such models are based on existing knowledge of spatio-temporal patterns of gene expression in developing eyespot foci (Marcus 2005). Readers are referred to Marcus (2005) for a summary of such models. Evans and Marcus (2006) used a computational simulation approach to propose a new developmental model. According to their model the balance in thresholds necessary for upregulation and downregulation of *hh* is crucial, with small changes resulting in failure to produce eyespot foci. They surmise that alterations in thresholds can lead to loss of eyespots in lineages and changes in the opposite direction can allow eyespots to reappear. Their model is currently the best explanation for the patterns observed within Junoniini. The genus *Junonia* offers an exciting opportunity to test such hypotheses.

Functional significance of eyespot patterning

One of the primary functions of eyespots is thought to be their role as antipredatory devices (Blest 1957; Lyytinen et al. 2003; Vallin et al. 2005; Vlieger and Brakefield 2007). Two hypotheses—the “intimidation hypothesis” and the “deflection hypothesis” seek to explain the mechanism by which eyespots are effective against predation (reviewed in Stevens 2005). The intimidation hypothesis posits that large and conspicuous eyespots, such as those comprising the *Individual* patterning in many of the junoniine species, frighten predators by mimicking vertebrate eyes, startle displays, neophobia, or simply by being highly conspicuous. Kodandaramaiah et al. (2009) tested this hypothesis experimentally in a predation experiment with *J. almana* as the prey and Great Tits (*Parus major*) as the predator. They showed that the presence of eyespots deterred attacks from birds, lending strong evidence in favor of the intimidation hypothesis. The intimidatory function of large eyespots has also been demonstrated in another nymphalid butterfly, *Inachis io* (Nymphalinae; Blest 1957; Vallin et al. 2005, 2007). Stevens et al. (2007, 2008a) used artificial cardboard prey with eyespot-like patterns to test what makes eyespots effective against predation. Conspicuousness and larger size of these eyespot-like patterns were shown to be critical determinants of their effectiveness. In some species within Junoniini, absence of eyespots in certain compartments has allowed remaining eyespots to become larger because they can extend into neighboring compartments (see *J. villida*, for

example). Furthermore, the absence of adjoining eyespots makes such eyespots more conspicuous. Hence, the intimidating value of such eyespots has likely been an important force in the evolution of *Individual* arrangements.

Stevens et al. (2008b) have shown that the effectiveness of intimidating eyespots is also influenced by the background of the prey, and can be counter-productive to survival under certain conditions. Additionally, the intimidating effect of eyespots is likely to vary between predators (Edmunds 1974; Vallin et al. 2007). If eyespots are not intimidating to a predator, they can make the butterfly easier to spot and increase predation. Interestingly, *J. sophia* and *J. westermanni* are descendants of an ancestor that colonized Africa from Asia approximately 5 million years ago (Kodandaramaiah and Wahlberg 2007). An explanation for the reversal to the *Serial* arrangement is that the different predatory community or habitat in Africa selected against large and conspicuous eyespots and the *Individual* arrangement was lost in favor of the *Serial* arrangement.

The deflection hypothesis states that eyespots are effective by deflecting attacks toward themselves and away from the vital body parts (Wourms and Wasserman 1985). Deflectory eyespots are thought to be smaller than intimidating eyespots, and may be serially or individually arranged. Eyespots forming the *Serial* pattern and smaller eyespots that make up the *Individual* patterns in some species may work in a similar way.

Other hypotheses for the significance of eyespots are their role in mate recognition and sexual selection. Robertson and Monteiro (2005), Breuker and Brakefield (2002), and Costanzo and Monteiro (2006) have shown that eyespots in *B. anynana* are subject to sexual selection. Strong sexual dimorphism in species such as *J. orithya* and *H. bolina* where eyespots in males are smaller or entirely absent suggests the possible role of sexual selection. Although there is little evidence to show that nymphalid eyespots are used in species recognition, Fordyce et al. (2002) have demonstrated that spots dotting the hindwings of females of *Plebejus* (= *Lycaeides*, Fam: Lycaenidae) are used by males to recognize conspecifics. The comparative work on species within *Bicyclus* by Oliver et al. (2009) has indicated that dorsal eyespots may play a role in mate recognition. The considerable variation in size, color, position, number, and structure of eyespots among species in Junoniini suggests that these eyespots might be used in mate recognition.

SUMMARY AND CONCLUSION

This is the first study to investigate the evolution of eyespot number in butterflies over a phylogeny. I have mapped the evolution of five distinct arrangements of eyespots found in the tribe Junoniini. The results show that these patterns have evolved multiple times independently. I discuss these results in light of the developmental mechanism underlying eyespot formation. I propose a simple model to explain the patterns

found in Junoniini: there is strong coupling between eyespots 2 and 5, and weaker coupling between 1 and 2 as well as between 5 and 6. Eyespots 3 and 4 are also linked to each other, and together lost or regained during shifts between *Serial* and *Individual* arrangements. I propose that during these shifts, the genetic mechanism is retained intact, but is developmentally turned off and can be switched on again. Changes in eyespot patterning have been driven largely by various selective forces and there is little developmental constraint for the production of either the *Serial* or *Individual* arrangements apart from the positions on which eyespots can occur. Mate recognition, sexual selection, and adaptation against predation are the three prominent explanations for the functional significance of eyespots. Members of Junoniini form an ideal group to understand the evo-devo and ecological significance of eyespots.

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