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Nymphalid eyespot serial homologues originate as a few individualized modules

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Serial homologues are repeated traits that share similar development but occur in different parts of the body. Variation in number of repeats accounts for substantial diversity in animal form and considerable work has focused on identifying the factors accounting for this variation. Little is known, however, about how serial homologues originally become repeated, or about the relative timing of repeat individuation relative to repeat origin. Here, we show that the serially repeated eyespots on nymphalid butterfly wings most likely arose as a small cluster of units on the ventral hindwing that were later co-opted to the dorsal and anterior wing surfaces. Based on comparative analyses of over 400 species, we found support for a model of eyespot origin followed by redeployment, rather than by the conventional model, where eyespots arose as a complete row of undifferentiated units that later gained individuation. In addition, eyespots most likely evolved from simpler pattern elements, single-coloured spots, which were already individuated among different wing sectors. Finally, the late appearance of eyespots on the dorsal, hidden wing surface further suggests that these novel complex traits originally evolved for one function (thwarting predator attacks) and acquired a second function (sexual signalling) when moved to a different body location. This broad comparative analysis illustrates how serial homologues may initially evolve as a few units serving a particular function and subsequently become repeated in novel body locations with new functions.

1. Introduction

Serial homologues such as limbs, teeth and vertebrae contribute significantly to biodiversity because they vary tremendously in shape, function and number across species. These repeated traits use the same developmental module, or gene regulatory network, along the body axis, often individuated (i.e. uniquely modified) by way of signals external to the module. In other words, the gene regulatory network is sensitive to positional information, allowing differentiation among serial homologues. Numerous studies have documented how the spatially restricted expression of regulatory genes can modify or eliminate individual incarnations of these repeated structures [1–5] and several studies have examined the evolution of serial repeat number once these were established [6–9], but few have tested whether these structures arose first as single units that were subsequently redeployed along a particular body axis or as serially repeated units from their inception. This lack of origin studies could be due to the antiquity of the traits examined and, in most relevant cases, all known fossil and extant taxa exhibit multiple units [6,9,10]. One exception is the vertebrate limb, which appears to have increased in number following its origin as a paired structure: one pair of anterior appendages evolved first, and a second pair arose later, giving rise to the posterior appendages [11].
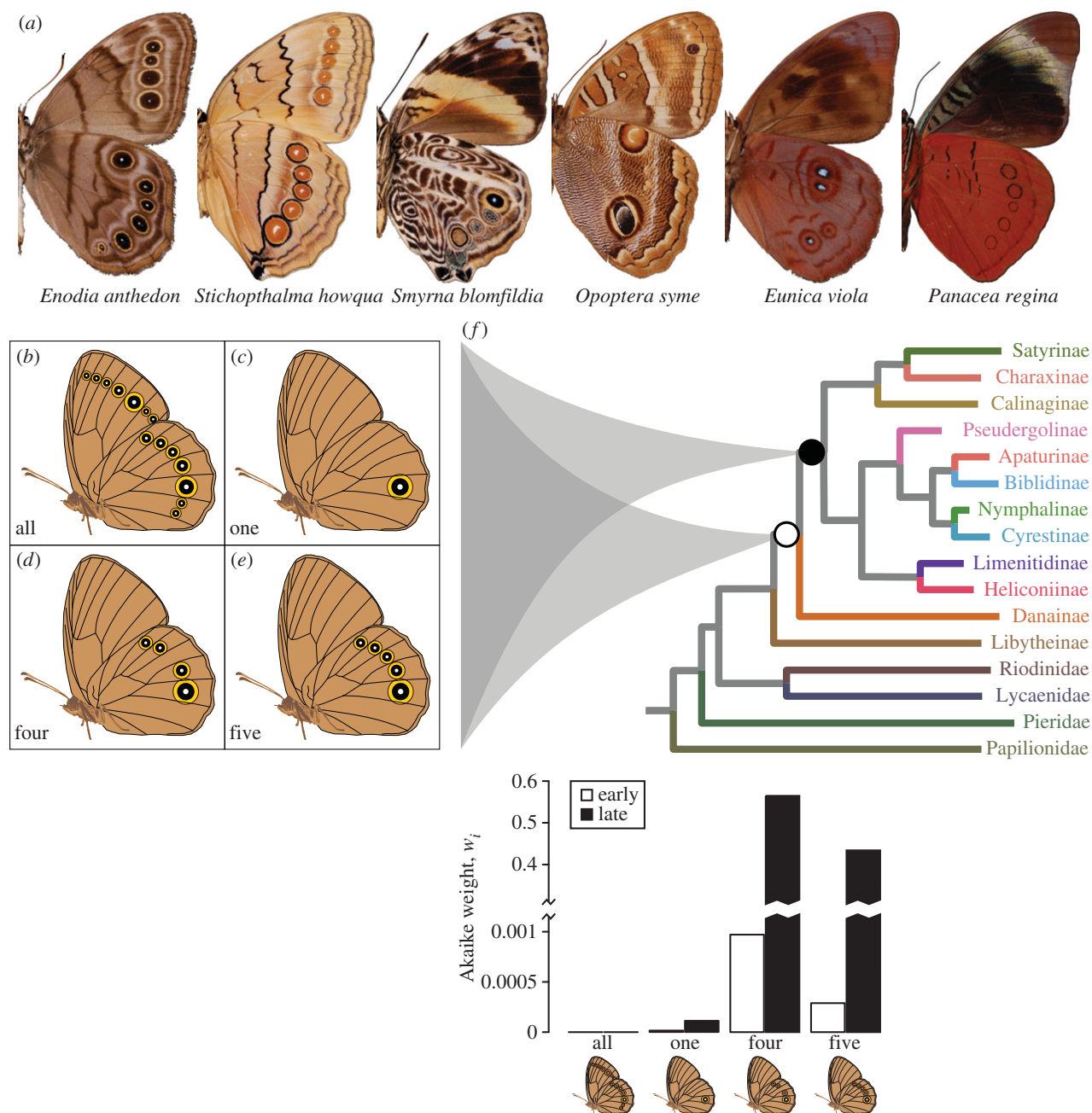
Figure 1. Diversity and origin of eyespot patterns. (a) Eyespots vary in number and location throughout the diverse butterfly family Nymphalidae. (b–e) Hypothetical patterns at the origin of eyespots: (b) eyespots in all wing sectors, (c) single eyespot in the ventral hindwing Cu1 sector, (d) four and (e) five eyespots restricted to ventral hindwing surface. (f) Relative support for each pattern as the ancestral state at two possible points of eyespot origin. 'Early' corresponds to an origin before divergence of the Danainae (open circle in phylogram) and 'late' corresponds to an origin following the diverdence of the Danainae (filled circle in phylogram). Relationships among nymphalid subfamilies based on [12].

To better understand the origin and individuation of serially repeated traits, as well as the relative timing of these two events, we focus on a more recently evolved model of serial homology: the eyespots of nymphalid butterflies (figure 1a).

Eyespots, which are characterized by a series of concentric rings of colour, likely originated early in nymphalid evolution, approximately 90 Ma [13]. These conspicuous bull's eye traits serve crucial roles in mate signalling and predator avoidance [14–17]. Although the number and locations of the original eyespots have not been investigated, the two prevailing hypotheses of eyespot origins provide contrasting views of how eyespots initially evolved. One hypothesis is based on the nymphalid groundplan (NGP), which describes pattern elements present on a butterfly wing. The NGP consists of a series of developmental symmetry systems, where eyespots belong to the most distally positioned system, the border symmetry system [18,19]. Although the NGP itself does not imply how eyespots arose, Nijhout [18,19] proposed that the border symmetry system originated from a band of colour that gradually became constricted at intersections of wing veins, forming a row of border pattern elements across the wing. These elements later gained individuation, i.e. gained the ability to disappear or be modified in colour or shape independently from the other elements in the same border system [18,19]. This hypothesis, called the symmetry hypothesis, was extended to the origin of eyespots: a band with nested stripes of coloration gradually became constricted at intersections of wing veins, forming a row of eyespots across the wing (figure 1b) [20,21]. The second hypothesis, which we refer to as the co-option hypothesis

[20], posits that eyespots originated in a single (or few) sector(s) on the wing (figure 1*c*), and were subsequently co-opted into different wing sectors. Our *a priori* test of the co-option hypothesis used the most common eyespot, found in the ventral Cu_1 sector of approximately 57% of the species we surveyed, as the original eyespot. Here, we test these two hypotheses by comparing them with maximum-likelihood estimations of when and where (on the wing) ancestral eyespot patterns first evolved.

The two eyespot-origin hypotheses also imply very different processes in the evolution of eyespot individuation. Under the co-option hypothesis, eyespots were already sensitive to positional information when they first arose, as they were not initially repeated among all wing sectors. By contrast, the symmetry hypothesis implies that the gain of positional sensitivity, necessary for individuation, occurred only after the concerted origin of all eyespots [18,19]. The two hypotheses thus present clear predictions for the ancestral state of eyespot patterns and positional sensitivity evolution: if the original eyespot(s) were restricted to one or a few wing sectors, at least some individuation was present from the outset. By contrast, if the ancestral pattern is a series of repeated units along the wing margin, then there was little positional sensitivity in the initial eyespot gene regulatory network, and individuation must have arisen later, allowing the network to be modified (or eliminated) in subsets of wing sectors. Because positional sensitivity allows individual serial homologues to become differentiated and overcome developmental correlations owing to the sharing of most network genes (the challenge of pleiotropy [22,23]), knowing when individuation evolves, relative to the timing of trait origin, is also key in understanding the evolution of functional specialization among serial homologues [24].

Here, we present broad comparative analyses to address unanswered questions about the evolution of this model of serial homology, the nymphalid butterfly eyespot. We evaluated alternative models of eyespot origin using estimations of ancestral states and explicit tests of different ancestral eyespot patterns. We also tested whether developmental independence among different eyespots arose simultaneously with or subsequently to the origin of eyespots. In addition to inferring eyespot origins, we tested potential precursor patterns to eyespots and measured the evolutionary dynamics, in terms of rates of gain and loss, following the initial appearance of eyespots. Finally, we analyse gene expression patterns and provide a developmental genetic explanation for observed evolutionary dynamics of eyespot gains and losses.

2. Material and methods

(a) Wing character data

We collected character data from 394 species of nymphalids and 29 outgroup species, based on phylogenetic sampling of previous studies [12]. Our broad sampling included 73% of all accepted genera as well as representatives of all subfamilies, tribes and subtribes of Nymphalidae. The remaining non-sampled genera are almost all considered to be closely related to sampled genera, according to Wahlberg *et al.* [12], and thus, their exclusion is not expected to significantly alter ancestral state reconstructions. The majority of specimens are from holdings in the Yale Peabody Natural History Museum, the American Museum of Natural History and the Harvard Museum of Comparative Zoology. For each species, we scored all wing surfaces (forewing, hindwing, dorsal and ventral), for a grand total of 28 wing locations of both sexes, using two specimens per sex when possible. Each wing sector was scored as having (i) no pattern, (ii) a spot, defined as a circular pattern of a single colour, or (iii) an eyespot, defined as two or more concentric rings of colour (electronic supplementary material, figure S1). Each wing sector was treated as a single binary or multistate character in all subsequent analyses of ancestral state analyses (see below). Such a treatment of wing sectors *a priori* assumes homology among the different wing patterns (e.g. a single-colour spot and an eyespot). This assumption is not unwarranted because both spots and eyespots occur in the centre of wing sectors, and both appear to differentiate from central signalling cells [25,26]. Note that the origin of eyespots has previously been associated with the origin of expression of a series of developmental genes that are not associated with simple monochromatic spots found in more basal butterfly lineages [13]. The 'eyespot' character state is thus assumed to be different from but homologous to the character state 'spot'. All patterns were scored on the LepData website (see http://www.lepdata.org) and are available in the electronic supplementary material. For the relationships among nymphalid species, we used those published in [12], and we used [27] for divergence times among butterfly families.

(b) Ancestral state estimation

We used maximum-likelihood estimation of ancestral states for 28 eyespots on the dorsal and ventral surfaces of nymphalid butterflies (electronic supplementary material, figure S1). For all phylogenetic analyses, we used the phylogenetic estimate of Wahlberg *et al.* [12], which was designed to include members from as many genera as possible (400 of 540) and to span the deep divergences in the family. The exclusion of unsampled genera, according to Wahlberg *et al.* [12], is not expected to affect the inferred relationships. This estimate of phylogenetic relationships was derived independently of any questions of eyespot evolution and represents the current best understanding of relationships within the family Nymphalidae. We tested explicit models of eyespot origins by first calculating the likelihood of an eyespot originating at specific nodes within and ancestral to the Nymphalidae using MESQUITE [28]. Calculating these likelihoods requires fixing ancestral nodes for specific states; however, as most likelihood calculations do not allow fixing internal nodes in a phylogeny, we introduced 'fossil' taxa to effectively fix states at nodes. Fossil taxa were inserted into the nymphalid phylogeny as tip taxa on very short branches (10 years long), at a node immediately ancestral to the node of interest. Because the branch connecting the fossil taxon to the tree is very short, there is little opportunity for evolutionary change, and the ancestral node is effectively fixed for the state assigned the fossil taxon. By assigning these fossil taxa a particular state (eyespot present or absent) and calculating the likelihood of the tree and the data (from contemporary and fossil taxa), we were able to calculate the model with the highest likelihood and, thus, the most likely position in nymphalid evolution where each eyespot appeared (electronic supplementary material, figure S2*a*). For the purpose of this test, the three possible states (no pattern, spot and eyespot) were collapsed into a binary character: eyespot absent (no pattern or a spot) and eyespot present (eyespot). The maximum-likelihood estimates of the presence or absence of each eyespot were then combined as the estimate of the most likely ancestral pattern for a wing surface (dorsal or ventral; electronic supplementary material, figure S2*b*). Male and female data were evaluated separately. We compared the likelihood and Akaike information criterion (AIC) scores of the most likely pattern to alternative models of eyespot origins (figure 1*b,c*). Models with likelihood scores two log-likelihood units lower than the maximum-likelihood model were rejected, whereas those within two log-likelihood units were considered equivalent [29]. This

method differed from that used in previous work [13]; in the previous study, all eyespots were considered simultaneously, without regard to the position on the wing. Taxa were simply scored as lacking or possessing eyespots, anywhere along the distal portion of the wing. This study thus differs by separately analysing eyespot evolution in the 28 different wing sectors.

(c) Evolution of eyespot individuality

To test whether gains or losses of individual eyespots were more likely towards the present, we first reconstructed gains and losses of nymphalid eyespots using marginal maximum-likelihood ancestral states in the rayDISC function of corHMM [30]. The maximally likely state at a node was considered the state at that node. We then measured the mean age of individual gains and losses, keeping the two types of change (gain, loss) separate. We compared this with a distribution of mean ages generated by bootstrapping ages of eyespot gains or losses. This distribution was drawn from a sample of all the ages where a gain or loss occurred, preserving the number of gains and losses observed in empirical data. For example, for the female ventral data, 21 gains of individual eyespots were observed. We compared the mean age of these gains with a distribution of mean ages based on samples of 21 ages drawn without replacement from the distribution of all inferred gains (a total of 28 ages), regardless of the number of eyespots gained. For each of the four combinations of sex and surface, we performed 10 000 bootstrap replicates in R [31] and rejected the individuation early model when the observed mean age of individual gains and losses was younger than the lower 95% of the bootstrapped mean distribution.

(d) Eyespot evolutionary dynamics

To test whether eyespots are gained and lost at the same rate, we performed likelihood ratio tests comparing a simple model of eyespot evolution, where eyespots are gained and lost at the same rate, with a complex model, where eyespot gains and losses occur at different rates. The complex model had one additional parameter than the simple model, so we assessed the difference of the model likelihoods using a χ^2 distribution with one degree of freedom [29]. The posterior-most wing sector (Pc) did not exhibit enough variation to permit accurate estimation of the rate matrix in complex models, so we were only able to test a subset of 24 wing sectors.

To test whether eyespots replaced spots, we compared likelihood scores of a model in which the original eyespots arose from spots with a model where the original eyespots arose from wing sectors lacking spots. We calculated the likelihood of both the four-eyespot pattern and the five-eyespot pattern (identified as the most likely original eyespot patterns) arising from spots or from sectors lacking spots (electronic supplementary material, figure S2c). Models were significantly different if the likelihood values differed by more than two log-likelihood units [29].

We tested a similar hypothesis about how eyespots evolve *after* the initial appearance of eyespots. We investigated whether, after the eyespot originated, wing sectors with spots were more likely to give rise to eyespots than were wing sectors lacking spots. We compared a simple model, where the rate of eyespot gains from sectors with spots was equal to the rate of gains from sectors lacking spots, with a complex model where the rates of eyespot gains were allowed to differ between sectors with and without spots (electronic supplementary material, figure S5). In these tests, the complex models had one more parameter than simple models, so we assessed significance using a χ^2 distribution with one degree of freedom [29]. In five wing sectors (all four Pc sectors and the dorsal anterior Cu_2), there was insufficient variation to accurately estimate the rate matrices, so we excluded these wing sectors from the analyses and performed tests on the remaining 23 wing sectors.

(e) Gene expression

We stained wing discs from fifth-instar larvae of *Bicyclus anynana* and *Junonia coenia* using the protocol of [32]. *B. anynana* larvae were collected from the Yale colony, which derived from animals originally collected in Malawi, and *J. coenia* larvae were reared from females collected in New Haven, CT. We stained for gene products of *Spalt* (*sal*, GP66-1 guinea pig polyclonal at 1 : 20 000) and *distal-less* (*Dll*, rabbit polyclonal at 1 : 200, a gift from Grace Beokhoff-Falk), with goat anti-guinea pig (Molecular Probes no. A11076) and anti-rabbit (Molecular Probes no. T-2767) secondary antibodies, respectively, at a concentration of 1 : 200. All wings were mounted with PROLONG GOLD (Invitrogen) and imaged on a Nikon 90i microscope using the NIS-ELEMENTS software (Nikon Instruments). Developmental stages of wing discs were categorized using the protocol of Reed & Serfas [33].

3. Results and discussion

(a) Original eyespot pattern

We evaluated the two hypotheses of eyespot origins by comparing their relative likelihood given phylogenetic estimates and contemporary wing patterns in nymphalid butterflies. We collected character data from 394 species of nymphalids and 29 outgroup species, based on phylogenetic sampling of previous studies [12], and estimated the ancestral state for 28 wing sectors (electronic supplementary material, figures S1 and S2). Considering all estimates for each wing sector, the most likely ancestral eyespot pattern had eyespots restricted to the ventral hindwing. There were either four (figure 1*d*) or five (figure 1*e*) original eyespots on the ventral hindwing surface, and they arose after the divergence of the Danainae (figure 1*f*), approximately 85–90 Ma. These two wing patterns are rare in contemporary taxa: of the 394 species surveyed, only two had the four-eyespot pattern (e.g. *Eunica viola* in figure 1*a*) and only six species had the five-eyespot pattern (e.g. *Panacea regina*, figure 1*a*). The rarity of the estimated ancestral state in contemporary taxa indicates that our estimates are not likely biased towards some optimal phenotype converged upon after the origin of nymphalid eyespots. This time point in nymphalid evolution was previously identified as one of two possible locations where nymphalid eyespots arose [13]. The other possibility–that eyespots arose earlier (90–95 Ma)–provided a significantly worse fit relative to the later origin implied by the best-fit model (figure 1*f* and electronic supplementary material, table S1).

The four- and five-eyespot patterns are more likely ancestral patterns than either a row of eyespots (figure 1*b*) or a single eyespot (figure 1*c*). The model implied by the symmetry hypothesis, where 14 eyespots arose in a band along the margin of the ventral wing surfaces, provided a very poor fit to the data relative to the four- and the five-eyespot models (figure 1*f* and electronic supplementary material, table S1). The most common single eyespot is in the ventral Cu_1 sector of the hindwing, found in 225 of the 394 species we surveyed, and it is often the largest eyespot in species that bear multiple eyespots. However, a model with this eyespot as the sole original eyespot provides a significantly worse fit than the four- and the five-eyespot models (figure 1*f* and electronic supplementary material, table S1). From these results, it appears that eyespots originated as a cluster restricted to a few wing sectors on the ventral hindwing. Given the
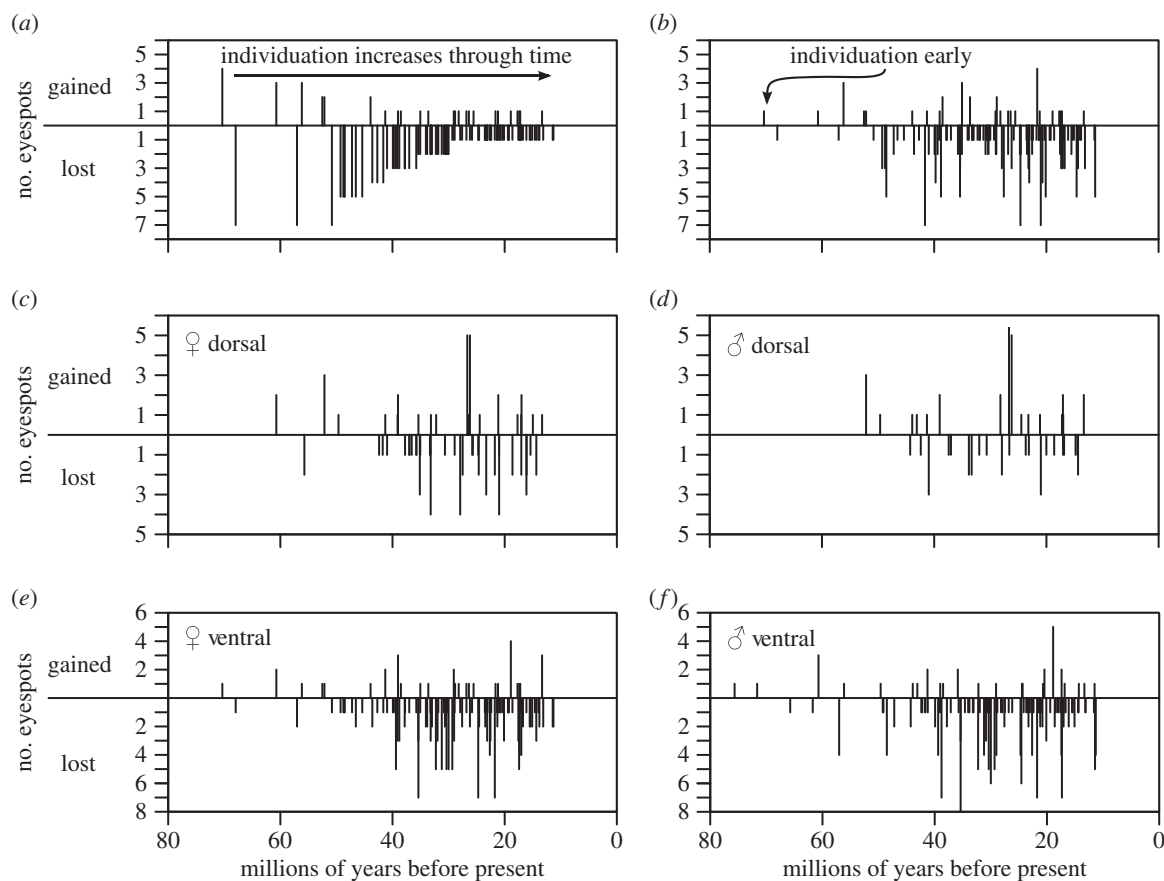
Figure 2. The relationship between time and eyespot gains and losses. In the delayed individuation model (*a*), eyespots originated as developmentally linked cluster(s), which could initially only be gained or lost in concert, so early gains and losses involved more than one eyespot (the left-hand side of *a*). Individuation among eyespots evolved later, so individual eyespot gains and losses are biased towards the present day (the right-hand side of *a*). In the individuation early model (*b*), developmental independence of eyespots among the different wing sectors arose concurrently with or soon after the initial appearance of the eyespot, so individual gains and losses are expected at any point following the initial evolution of nymphalid eyespots. Numbers of nymphalid eyespots gained and lost through time supports an individuation early model (*b*): individual gains and losses of eyespots are not biased towards the present in (*c*) female dorsal, (*d*) male dorsal, (*e*) female ventral or (*f*) male ventral eyespots.

restriction of original eyespots to a few wing sectors, the original eyespot gene regulatory network likely arose concurrently with at least some degree of individuality.

(b) Evolution of eyespot individuality

The evolution of eyespot individuality should also leave a signature in the magnitude of eyespot gains and losses over evolutionary time, i.e. in the number of eyespots that are simultaneously gained or lost in a lineage. If eyespots were not initially independent, with the ability to switch 'on' or 'off' independently of other eyespots in different parts of the wings, then we expect the early gains and losses of eyespots to occur in concert, so clusters of multiple eyespots are gained and lost early after the origin of eyespots (figure 2*a*). Only later in nymphalid evolution, after the eyespot developmental module became sensitive to positional information, would individual eyespots be gained and lost independently of eyespots in other wing locations. Conversely, if eyespots arose with developmental independence among the wing sectors, we expect no relationship between the age of the eyespot network (the time passed since eyespots originated) and the magnitude of gains and losses (figure 2*b*). That is, early independence among eyespots predicts that gains and losses occur with the same magnitude (in terms of number of total eyespots gained or lost at a particular event) at all

points in evolutionary time following the first appearance of eyespots. We evaluated the two hypotheses of eyespot individuation (individuation delayed and individuation early) by testing for a non-uniform distribution of individual eyespot gains or losses across nymphalid eyespot evolution. If individuation was delayed, then the mean age of single gains and losses should be more recent than expected from a uniform distribution, whereas no such deviation would be present if individuation arose concurrently with or early in eyespot evolution. In both surfaces and both sexes, we detected no bias in the mean age of individual eyespot gains or losses (figure 2*c–f*). Gains and losses of single eyespots were observed throughout nymphalid evolution, and for both sexes, the first gains and losses following the origin of the first eyespots involved an individual eyespot (figure 2*e,f*). Additionally, in the female data, single eyespot losses were biased *against* the present (dorsal $p = 0.012$; ventral $p = 0.045$), suggesting that some lineages may have lost some developmental independence among individual eyespots. The individuation of eyespot deployment among wing sectors thus appears to be an early event in the history of these serial repeats.

The temporal dynamics of eyespot gains on the different wing surfaces, with dorsal eyespots appearing on the dorsal surface 30–40 Myr after their first appearance on the ventral hindwing (figure 2*c,d*), indicates that eyespots may have
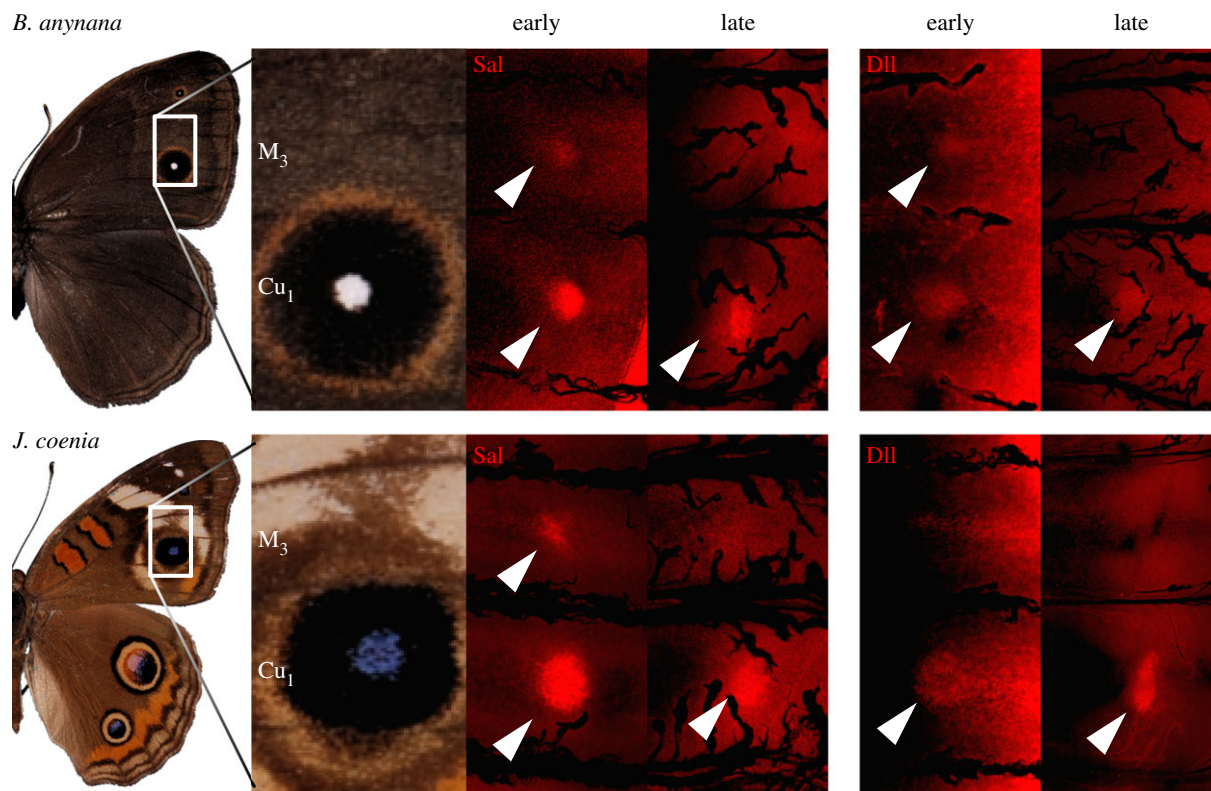
Figure 3. Differences between *B. anynana* and *J. coenia* in focal expression of eyespot-associated proteins. Adults of both species have eyespots in sector Cu_1 of the anterior wing and lack eyespots in the M_3 sector. In the Cu_1 sector, Sal and Dll show a central focus (arrowheads) of expression through early and late wing disc development in both species. In the M_3 sector, both species have early focal expression of Sal, but early focal expression of Dll is only evident in *B. anynana*.

diversified in ecological function as they were co-opted from the ventral to the dorsal wing surfaces. Comparative analyses and behavioural observations of contemporary taxa indicate that nymphalid eyespots appear to have separate functions depending on where they are located on the wing: dorsal and anterior eyespots are primarily associated with mate signalling, whereas ventral and hindwing eyespots are associated with predator avoidance [14,16,17,34]. This functional specialization may have originated in one of two different ways. Either eyespots functioned originally only in predator–prey interactions (as ventral hindwing eyespots appear to do in contemporary taxa) and evolved a secondary function in sexual signalling once they were co-opted to the dorsal surface; or alternatively, eyespots served both functions when they first originated and partitioned these functions when they later moved to the dorsal wing surfaces [24,34].

(c) Eyespot evolutionary dynamics

Our estimations of eyespot number evolution also suggest that gains and losses do not occur at equal frequencies over evolutionary time (figure 2). Indeed, most eyespots are lost at much higher rates than they are gained (electronic supplementary material, figure S3), and eyespot losses occurred at rates 2.5–91 times higher than eyespot gains. One possible explanation for this difference in gains and losses lies in the difference between the developmental changes necessary for a gain and changes underlying the loss of a trait. Gains require all necessary genes and gene interactions to occur, whereas losses can be accomplished by disrupting a single necessary interaction. However, the loss of an individual eyespot while preserving functionality of the eyespot gene regulatory network in other wing sectors necessitates the network be

sensitive to positional information. A clear example of multiple potential points of network disruption, coupled with preservation of a functional eyespot network in other locations, is evident in the expression dynamics of eyespot-associated genes in *B. anynana* and *J. coenia* (figure 3). In *B. anynana*, at least five proteins associated with adult eyespots are expressed in the future eyespot centres of larval wing discs [13], including the transcription factors Spalt (Sal) and distal-less (Dll). In the M_2 and M_3 sectors of the anterior wing, which lack adult eyespots, Sal and Dll are initially expressed, then subsequently the proteins disappear, presumably through downregulation of the respective genes. The distantly related *J. coenia* also expresses Sal and Dll in future eyespot centres of sectors that bear adult eyespots, and it too lacks adult eyespots in the M_2 and M_3 anterior wing sectors. However, in *J. coenia*, Sal protein levels are initially upregulated in these sectors, but then downregulated before central expression of Dll. This difference between the two species—downregulation after Dll expression in *B. anynana* compared with downregulation before Dll expression in *J. coenia*—shows how there may be multiple points in a developmental module that can evolve positional sensitivity without wholesale disruption of the module in all parts of the organism. Multiple points of sector-specific disruption potential in the eyespot module, as previously also demonstrated with the wing regulatory network in ants [35], might explain why eyespot losses are more likely than eyespot gains.

The flip side of this story, however, is that the presence of an interrupted network creates developmental potential for future reactivations of the network in the same wing sectors at a later stage in evolution [36]. For instance, differentiation of eyespots in the M2 and M3 sectors in *B. anynana*, the two 'interrupted' sectors normally without eyespots, was recently

shown possible via the overexpression of Dll during the late larval stages of development [37]. Importantly, while Dll levels were overexpressed across the whole wing, additional eyespots only appeared in the wing sectors that were already pre-patterned with Sal, as well as other eyespot centre marker genes, earlier in development [37]. This shows that gradual evolution in expression levels of a single gene may be sufficient to reactivate a previously interrupted network.

In summary, while the high rates of eyespot loss over eyespot gains may underscore greater genetic opportunity for disrupting rather than resurrecting a gene regulatory network, both appear to be mechanistically simple and possible. Furthermore, it is important to note that mutations that alter eyespot development happen in individuals, not populations, and ultimately, rates of eyespot evolution will depend on population-level processes of natural selection and genetic drift. It remains an open question as to how these two factors, the source of variation and the forces acting on that variation, interact to produce the distribution of eyespots in contemporary taxa.

Positional sensitivity, allowing individual eyespots to be gained or lost without disrupting patterns in other parts of the wing, could have arisen early in eyespot evolution by exploiting a pre-existing scaffold of positional information directing development of other pattern elements that were precursors to eyespots. We explored this possibility by investigating the evolutionary relationship between eyespots and simple, single-coloured spots. If eyespots derived from spots, then they should be more likely to arise from sectors with spots than from sectors lacking spots. We first tested this hypothesis for the original eyespots and found, regardless of whether there were four or five original eyespots, these first eyespots were more likely to replace spots than appear in sectors without spots (electronic supplementary material, table S2). Following the initial origin of nymphalid eyespots, a similar process occurs across ventral eyespot evolution. In the majority of wing sectors, eyespots are more likely to arise from locations bearing spots than they are to arise from locations lacking spots (electronic supplementary material, figure S4). Given that eyespots are more likely to replace spots, both at their origin and throughout nymphalid evolution, sector-specific developmental control for single-colour spots likely existed before the eyespot evolved. This ancestral independence among pattern elements belonging to different wing sectors is also evident in the absence of any delay in individuation in eyespot gains and losses (figure 2).

The replacement of spots by eyespots presents the question of whether eyespots are anything more than polychromatic spots. If so, individuation of these patterns, regardless of how many concentric circles of colour they bear, likely occurred long before the inferred origin of nymphalid eyespots. However, evidence from developmental genetics argues against the explanation that eyespots and spots are effectively the same character. Multiple studies demonstrate that a suite of genes are expressed during larval development in the locations of future eyespot centres [12,38–40], whereas expression of these same genes is conspicuously absent from larval wing locations that will bear spots in adult nymphalids and spots and eyespots in representatives of other butterfly families (see electronic supplementary material, fig. S2 B,V,W in [13] and figs 2 and 3 in [40] for examples of the absence of expression in spot locations). The origin of eyespots was shown to happen concurrently with the co-option of this suite of genes to eyespot centres, after the origin of the family Nymphalidae [13,40]. What we have now shown is that this network of genes associated with eyespot origins was likely initially only deployed in a subset of wing sectors on the ventral hindwing. Only later did the network gain novel positional information to originate in new wings (i.e. forewings) and wing surfaces over the course of evolution. The origin of eyespots and the evolution of eyespot number and eyespot individuation represents, thus, a separate evolutionary process from the origin and individuation of single-colour spots.

4. Conclusion

Eyespots provide a prime example of how serially repeated homologous traits may originate concurrently with individuation. The individuation we investigated, however, refers only to mechanisms that enable the eyespot module to be turned 'on' or 'off' in specific wing sectors. Eyespots differ in many other traits across wing sectors, including size and colour composition, and comparative analyses across a broad phylogeny will be necessary to infer when sector-specific positional information for these other aspects of eyespot individuation evolved. The potential for 'on–off' individual regulation, from the outset, has provided ample opportunity for selection to act on butterfly wing patterns and produce the diversity in eyespot number we see today. Understanding this diversity at the genetic and molecular levels still requires the identification of the 'on–off' switches, and the functional elements of the eyespot gene regulatory network [21,41]. Furthermore, given the propensity for eyespots to arise from spots, the extent of overlap in spot and eyespot development will need to be investigated further [41], along with identification of the sector-specific selector genes that are regulating the respective gene regulatory networks along the A–P axis of the wing. Isolating the developmental underpinnings of this variation will be key to understanding the evolution of specialized units in serial homologues [23].

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