Integration of Wings and Their Eyespots in the Speckled Wood Butterfly *Pararge aegeria*

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ABSTRACT   We investigated both the phenotypic and developmental integration of eyespots on the fore- and hindwings of speckled wood butterflies *Pararge aegeria*. Eyespots develop within a framework of wing veins, which may not only separate eyespots developmentally, but may at the same time also integrate them by virtue of being both signalling sources and barriers during eyespot development. We therefore specifically investigated the interaction between wing venation patterns and eyespot integration. Phenotypic covariation among eyespots was very high, but only eyespots in neighbouring wing cells and in homologous wing cells on different wing surfaces were developmentally integrated. This can be explained by the fact that the wing cells of these eyespots share one or more wing veins. The wing venation patterns of fore- and hindwings were highly integrated, both phenotypically and developmentally. This did not affect overall developmental integration of the eyespots. The adaptive significance of integration patterns is discussed and more specifically we stress the need to conduct studies on phenotypic plasticity of integration. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:454– 463, 2007.*   © 2007 Wiley-Liss, Inc.

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It is often hypothesised that developmental integration evolves adaptively to match functional integration, which would allow for faster adaptive evolutionary change (Wagner and Altenberg, '96; Griswold, 2006), but how well are they really matched in biological systems (for an overview, see Breuker et al., 2006)? The pattern elements on Lepidopteran wings are an excellent system to study this (Beldade and Brakefield, 2002; McMillan et al., 2002; Brakefield, 2006). The wing pattern as a whole is clearly an important functional morphological component of the phenotype (reviewed in Brakefield and French, '99),

and is as such subject to selection in the context of interactions with predators (Lyytinen et al., 2003; Srygley, 2004; Stevens, 2005), the resting background (Nijhout, 2001), with potential mates (Warzecha and Egelhaaf, '95; Breuker and Brake-

field, 2002; Robertson and Monteiro, 2005) or with the thermal environment (like degree of melanisation, e.g. Kingsolver and Wiernasz, '91; Van Dyck and Matthysen, '98). Although it has been rarely explicitly tested, individual wing pattern elements are therefore often assumed to be to a large degree *functionally* integrated (Brakefield, 2001). It has been suggested, however, that wing pattern elements might not show the same degree of *developmental* integration, despite the often large (positive) phenotypic (and genetic) covariances found among them and despite the fact that they are often serial homologues and produced by the same developmental mechanisms (Nijhout, '91; Paulsen, '94; Beldade and Brakefield, 2002; Beldade et al., 2002a,b; McMillan et al., 2002). The objective of this study is to investigate this hypothesis of weak *developmental* integration of eyespots despite large phenotypic covariation.

We consider traits to be developmentally integrated when they together act as an integrated and context-insensitive component of development, even when subjected to (random) developmental perturbations (Schlosser, 2004; Schlosser and Wagner, 2004). If the development of two traits is independent, then a random deviation in one of them will not be consistently associated with a deviation in the other trait. A popular means of evaluating the response to developmental perturbations is by quantifying the difference between the right and a left side of a bilaterally symmetrical trait (Van Valen, '62; Palmer and Strobeck, '86). In a sample of individuals, therefore, the signed asymmetries (R-L) of two independently developing traits will be uncorrelated. Signed asymmetry not only refers to how much a right and left side differ, but also whether right or left was the larger side. If the two traits are, however, developmentally linked, then the effects of the perturbations can be transmitted directly between the traits. This would produce a statistical relationship between the signed asymmetries of the traits. The covariances between signed asymmetries of traits result therefore from their developmental connections, but not from parallel variation of independent pathways, and can therefore be used to infer developmental integration (Klingenberg et al., 2001; Klingenberg, 2003). We consider traits to phenotypically covary, or in other words to be phenotypically integrated, when a particular size or shape of a trait consistently corresponds with that of another trait.

The idea that wing pattern elements are developmentally separate stems from comparative morphological studies by Schwanwitsch and Süffert conducted in the 1920s and 30s. They established a framework of butterfly wing patterning, the so-called "nymphalid groundplan" (Schwanwitsch, '24, '35; Süffert, '27; Nijhout, '91). According to this groundplan, a butterfly wing may consist of three paired vertical bands, called the symmetry systems, while (horizontal) wing veins further subdivide the wing into distinct wing cells, which then can contain one or more pattern elements (Fig. 1). It is these wing cells and the wing pattern elements they contain that are hypothesised to be developmentally separate from other such wing cells (see review in Nijhout et al., 2003).

The best studied and understood wing pattern elements are those in the wing cells from the border ocelli symmetry system, and it has been confirmed that there is a large degree of wing cell independence (Beldade and Brakefield, 2002; McMillan et al., 2002). The border ocelli will also be the focus of our study. Although the functional role of a large number of upregulated developmental genes in the wing cells, most notably Distal-less, and successive reaction-diffusion and diffusion-threshold steps in eyespot development have been inferred (Brakefield, '98; Sekimura et al., 2000; Brunetti et al., 2001; Koch and Nijhout, 2002; McMillan et al., 2002; Nijhout et al., 2003), the role of the wing cell borders (i.e. the wing veins and wing margins) in eyespot development is somewhat poorly understood. Wing veins do not determine the presence or absence of individual eyespots, but it seems that they may contribute to the inductive signalling for the position and morphology of each wing pattern element within the wing cell. Furthermore, in a number of butterfly species, they can also act as boundaries for developing wing pattern elements, either by acting as a sink, destroying morphogenetic substances, or because they don't allow for cell-to-cell communication of signals (Koch and Nijhout, 2002; Nijhout et al., 2003; Reed and Gilbert, 2004; Reed and Serfas, 2004; Reed et al., 2007). In these butterfly species, eyespots are incapable of developing across wing veins, which is easily observable.

Although the wing veins may compartmentalise and separate the development of eyespots, wing veins may actually also integrate eyespots by acting as both inductive signalling sources and barriers for the diffusion of morphogenetic substances. As eyespots are laid down in this network of wing veins it is feasible that a change in the overall wing venation pattern will affect all wing pattern elements simultaneously, which could potentially increase both phenotypic and develop-
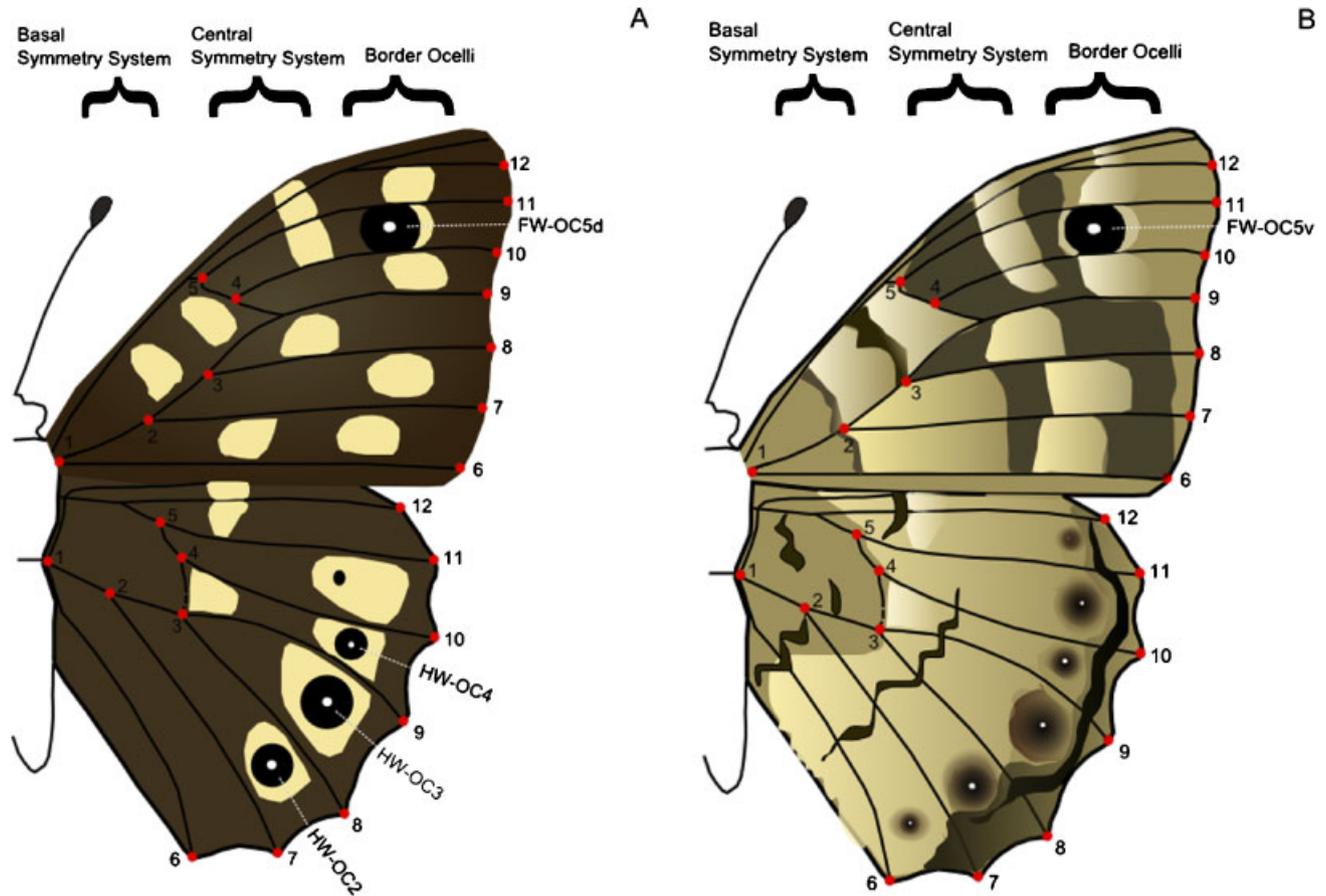
Fig. 1. The dorsal (**A**) and ventral (**B**) wing surface of a *Pararge aegeria* butterfly. The corresponding numbers on forewing and hindwing indicate homologous landmarks (i.e. wing vein intersections or where a wing vein meets the edge of the wing). Nomenclature of the symmetry systems and wing pattern elements (eyespots) is after Schwanwitsch ('35) and Nijhout ('91). FW, forewing; HW, hindwing; OC, ocellus; d, dorsal; v, ventral. The numbers refer to the wing cell. Only the black area of the eyespots was measured.

mental integration among the individual wing pattern elements. In this study we therefore investigated the phenotypic and developmental integration of border ocelli (i.e. eyespots) in relation to the size and shape of the wing venation pattern in both the fore- and hindwings. We inferred developmental integration of traits from the covariance patterns between signed trait asymmetries as explained earlier. Our model species is the nymphalid butterfly speckled wood *Pararge aegeria* L., which has been well studied for adaptive variation of both wing morphology and colouration in an ecological context (Van Dyck and Wiklund, 2002). Furthermore, this species is an example of a butterfly species in which eyespots can not develop across wing veins. In particular, we predicted that (1) neighbouring eyespots are more developmentally integrated with each other than with any other eyespot as they share a wing vein as their wing cell boundary, and (2) that

eyespots in homologous positions on the dorsal and ventral wing surface are developmentally integrated as they share the same wing veins as wing cell boundaries, even though the dorsal and ventral wing surface develop as single-layered epithelia that are developmentally independent. As these eyespots completely share their wing veins they may be more developmentally integrated than neighbouring eyespots, which share only one. Furthermore, we predicted that (3) as wing veins can act both as boundaries and inductive signalling sources the size and shape of a wing cell will affect the size and shape of an eyespot (after Monteiro et al., '97c).

MATERIALS AND METHODS

Experimental animals

The butterflies were derived from an outbred laboratory stock population of Belgian *P. aegeria*

butterflies, and reared under carefully controlled conditions allowing a direct development (temperature day/night: $23^{\circ}C/18^{\circ}C$, 75% humidity, light:dark photoperiod 18:6 hr) on the grass species *Poa annua* (cf. Talloen et al., 2004). Four larvae were transferred to a single grass plant (in a pot of 18×18 cm) within twelve hours of egg hatching. This density of same-aged caterpillars ensured an ad libitum food supply without unequal competition among the caterpillars, thereby minimizing variability in resource uptake, which could confound results. We thus reared 160 individuals (80 males and 80 females) to adulthood. To avoid wing wear, butterflies were killed within 24 hours of emergence, after their wings had fully hardened and were stored at $-18^{\circ}C$.

Morphological measurements and statistical analyses

Both fore- and hindwings were carefully removed from the thorax, placed in between two glass slides and digital images were then taken of the ventral and dorsal wing surface with an Olympus Camedia C-3030 under carefully controlled light conditions. Twelve homologous landmarks were digitized on both the fore- and hindwings in ImageJ (freely available on http://rsb.info.nih.gov/ij/) (Fig. 1). The landmarks measured are either wing vein intersections or locations where a wing vein meets the edge of the wing. As such, the landmarks provided an estimate of the wing venation pattern and of the overall wing shape.

Variation in shape was examined by using geometric morphometrics based on generalized least squares Procrustes superimposition methods (Goodall, '91; Dryden and Mardia, '98; Klingenberg and McIntyre, '98). Procrustes methods analyse shape by superimposing configurations of landmarks of two or more individuals to achieve an overall best fit. It involves four steps, which have been described in mathematical and descriptive detail elsewhere (see e.g. Klingenberg and McIntyre, '98): (1) reflection of either left or right configurations (i.e. so left and right are now orientated the same way), (2) scaling to unit centroid size (to remove the association between size and shape), (3) superimposing the centroids of all configurations, and finally (4) rotation of the configurations around their centroid to obtain the optimal alignment.

To estimate the amount of measurement error due to both imaging and digitizing, repeat

TABLE 1. Analysis of measurment error

Source	Sums of squares	Degrees of freedom	Mean squares $\times 10^6$
Individuals	0.1758	580	303.03***
Sides	0.002159	20	107.95***
Sides $\times$ Individuals	0.01435	580	24.73***
Error	0.003107	1200	2.58

Procrustes analysis of shape variance (Klingenberg and McIntyre, '98) of the amounts of shape variation attributable to different sources, for the forewings of a subset of 30 individuals, which were digitized twice. The measurement error consists of both the imaging and digitizing error. Sums of squares and mean squares are in units of squared Procrustes distance. ***$P < 0.001$.

photos and measurements were taken for a subset of 30 individuals, and a Procrustes analysis of variance (ANOVA) (Klingenberg and McIntyre, '98) was carried out. As developmental integration in this study was assessed by investigating covariation in asymmetry patterns, we needed to make sure that measurement error due to imaging and digitizing was negligible compared to biological shape and size variation. This was the case as the mean squares for individual, side and asymmetry between the sides (the side $\times$ individual interaction) significantly exceeded the mean squares of the error term ($P \ll 0.001$; Table 1). The Procrustes ANOVA for the hindwing and size of both wings show exactly the same pattern as those for forewing shape (not shown).

Procrustes distance summarizes shape differences (e.g. between a left and right wing or between the average shape of two sets of individuals, Klingenberg and McIntyre, 1998). The square root of the sum of the squared distances between corresponding landmarks of two optimally aligned configurations is an approximation of Procrustes Distance. In calculating Procrustes distance all aspects of shape variation are treated equally, regardless of their variability in the total sample. The underlying assumptions are that each landmark is equally variable, that the variation at each landmark is the same in all directions, and that variation is independent among landmarks. This is hardly ever the case, and the Mahalanobis distance may then be a better measure of shape variation as it quantifies the amount of variation relative to the variability in the data set (Klingenberg and Monteiro, 2005). We therefore quantified shape variation both with the conventional Procrustes and with the Mahalanobis distance. Differences in shape between sets of individuals were analysed by means of canonical variates analysis with 10,000 permutations.

The centroid size of all 12 landmarks of a wing was used as a measure of the size of that wing in this study. The landmarks bordering a wing cell were used to calculate the centroid size, and hence size, of that wing cell. Centroid size is the square root of the sum of squared distances from a set of landmarks to their centroid (i.e. mean x and y coordinate of a set of landmarks per individual) (see e.g. Klingenberg and McIntyre, '98).

To assess phenotypic integration of wing venation patterns, and therefore of shape, matrix correlations (Mantel test) between the covariance matrices of the Procrustes coordinates of homologous sets of landmarks were calculated. The covariance matrices for the signed asymmetries of these landmarks were used for investigating the developmental integration. Significances of the matrix correlation coefficients were calculated by permuting the (x, y) coordinates 10,000 times. Furthermore, analyses were carried out with and without the diagonal of the covariance matrices included (i.e. with and without the variance at each landmark). Excluding the diagonal means that only the covariation patterns among landmarks were investigated.

We measured the size (in mm^2) of the black part of five eyespots in the border ocelli symmetry system, three on the dorsal hindwing and one on each of the two surfaces of the forewing (Fig. 1). The fourth dorsal hindwing eyespot, the one located in wing cell 5 and therefore the eyespot in a homologous wing position as FW-OC5d, was unfortunately missing in 82% of the individuals, and therefore was omitted from the analyses. All eyespots were measured twice, and regression analyses of second on first measurements indicated that repeatabilities of these measurements were very high ($>97\%$). ANOVAs, similar to those used for wing shape and size, showed that the mean squares for individual, side and asymmetry between the sides (the side $\times$ individual interaction) significantly exceeded the mean squares of the error term ($P \ll 0.001$) by 20 to 40-fold. This means that observed asymmetries in eyespot size significantly exceeded measurement error.

Due to a scaling relationship with wing size, the size of each of the eyespots correlated positively with the overall size of the wing ($0.09 < R^2 < 0.13$, $P \ll 0.001$) and more specifically with the size of the wing cells each of them were situated in ($0.12 < R^2 < 0.20$, $P \ll 0.001$). We, therefore, used the residuals of the regression analyses of eyespot size on wing size to assess phenotypic integration of eyespots, and the signed asymmetry (R-L)

of these residuals to investigate developmental integration of eyespots.

In examining how wing cell size affected the shape of an eyespot, we were interested in the residuals of the regression analysis of eyespot size on wing cell size. A positive residual indicated that the eyespot is relatively big for the size of wing cell it is in, whilst a negative residual indicated a relatively small eyespot. If the wing veins indeed acted as barriers, the relatively big eyespots were predicted to be more ellipsoidal than the relatively small eyespots (i.e. squashed or "fat" (Monteiro et al., '97c)). We therefore fitted an ellipse to each of the eyespots, with one axis parallel to the horizontal wing veins (i.e. along the so-called mid vein which runs in between the two major wing veins and acts as the line of symmetry in an eyespot) and the other perpendicular to that. We measured eyespot shape as the ratio of the major and minor axis of the ellipse, with the higher values of the ratio corresponding to more ellipsoidal eyespots. We investigated the correlation between this measure of eyespot shape and the residuals of eyespot size on wing cell size by means of regression analysis.

The analyses were carried out in: (1) R (http://cran.r-project.org), (2) SAGE and MACE written by E. Marquez (http://www-personal.umich.edu/~emarquez/morph/), and (3) MorphoJ written by C.P. Klingenberg (C.P. Klingenberg, unpublished data).

RESULTS

Sexual Dimorphism

The wings of females were bigger than those of males, and differently shaped (Table 2). This sexual dimorphism for wing morphology is very common in butterflies, and has been argued to reflect the different selection pressures operating on male and female wings (Wickman, '92). Despite these morphological differences the test results on phenotypic and developmental integration patterns in the wings were highly similar. We therefore pooled males and females in all subsequent analyses. This concordance in test results indicates that under our study conditions the wings of males and females developed similarly.

Integration of a fore- and a hindwing

The size of a fore- and a hindwing were highly correlated ($R^2 = 0.90$, $P \ll 0.001$). So were the signed size asymmetries of both wings ($R^2 = 0.19$, $P \ll 0.001$). This indicates that fore- and hindwings

TABLE 2. Comparison between males and females for shape and size of forewing and hindwing

| | Shape | | CS | | |
	Mahalanobis distance	Procrustes distance	Females	Males	$F(1,318)$
FW	4.93***	0.061***	$23.92 + 0.097$	$22.73 + 0.083$	86.27***
HW	3.40***	0.042***	$23.86 + 0.11$	$22.92 + 0.084$	48.07***

Shape comparison was done by means of canonical variates analysis with 10,000 permutations. Mahalanobis and Procrustes distance summarize the shape differences between both sexes. Centroid size (CS; mean $\pm$ SE is indicated) was compared by means of an one-way analysis of variance with the factor sex. All values were highly significant.
FW, forewing; HW, hindwing.
***$P \ll 0.001$.

were both phenotypically and developmentally integrated for size. This indicates that when a left forewing is larger than the right forewing, the left hindwing is also larger than the right hindwing. The shape of the wing (venation) of the fore- and hindwing was also highly integrated, phenotypically and developmentally. The covariance matrices of the Procrustes landmark coordinates of a fore- and hindwing were significantly correlated (matrix correlation with diagonal included = 0.40, $P = 0.027$, and matrix correlation with diagonal excluded = 0.21, $P = 0.034$). Furthermore, the covariance matrices for the signed asymmetries of these landmarks were also significantly correlated (matrix correlation with diagonal included = 0.77, $P \ll 0.001$, and matrix correlation with diagonal excluded = 0.49, $P \ll 0.001$).

Eyespot shape and integration with the wing veins

Eyespot shape was quantified as the ratio of the major and minor axes of the ellipse, with the higher values of the ratio corresponding to more ellipsoidal eyespots. The horizontal axis (i.e. the axis of the ellipse fitted along the mid vein) was invariably the major axis of the ellipse for all eyespots measured. This measure of eyespot shape was positively correlated to the forewing eyespot size residuals on both wing surfaces (ventral: $R^2 = 0.12$, dorsal $R^2 = 0.21$, $P \ll 0.001$), indicating that the wing veins were acting as a barrier, as relatively big eyespots became more ellipsoidal. The eyespots on the dorsal and ventral side of the forewing correlated significantly for both eyespot shape and residual eyespot size (see also Table 3A) (shape: $R^2 = 0.12$, size: $R^2 = 0.30$, $P \ll 0.001$). Although eyespot shape was significantly affected by the position of the wing veins and eyespot size significantly covaried with that of the wing(cell), eyespot size and wing cell size were not developmentally integrated on both wing

surfaces (ventral: $R^2 = 0.0071$, $P = 0.29$, dorsal: $R^2 = 0.00031$, $P = 0.81$).

For each of the hindwing eyespots, eyespot shape also correlated positively with the size residuals, but a lot less significantly ($0.028 < R^2 < 0.051$, $P < 0.05$). Forewing eyespots were more ellipsoidal (mean shape = 1.22) than the hindwing eyespots (mean shape = 1.15) ($F_{1,1598} = 82.8$, $P \ll 0.001$). The signed size asymmetries of the three hindwing eyespots were, like the forewing eyespot, not significantly correlated with the signed asymmetries of the size of the wing cell they were situated in ($0.00012 < R^2 < 0.011$, $P \gg 0.05$). This indicates that, just like on the forewing, the development of the hindwing eyespots was separated from that of the wing cells.

Integration of eyespots

The forewing eyespots covaried significantly in size with each other and with the eyespots on the hindwing, with phenotypic integration being the highest among the hindwing eyespots (Table 3A). Despite this and the large developmental integration of shape and size of the fore- and hindwings the eyespots do not show the same degree of developmental integration (Table 3B). The hindwing eyespots in general seem to be more developmentally integrated with each other for size than each of them with the forewing eyespots, but only neighbouring eyespots on the hindwing are significantly developmentally integrated. The eyespot on the dorsal forewing was developmentally integrated with the eyespot on the ventral wing surface.

DISCUSSION

The eyespots studied here showed a complex, hierarchical, pattern of integration. Fore- and hindwings were integrated, and the morphologies of the eyespots covaried together, most notably when located on the same wing surface. The

TABLE 3. *Correlation matrix of the eyespot size residuals (A), and signed asymmetry of the eyespot size residuals (B)*

	FW-OC5v	FW-OC5d	HW-OC2	HW-OC3
(A)				
FW-OC5d	0.55***			
HW-OC2	0.43***	0.41***		
HW-OC3	0.42***	0.36***	0.77***	
HW-OC4	0.36***	0.32***	0.69***	0.69***
(B)				
FW-OC5d	0.16 ($P = 0.041$)			
HW-OC2	−0.013 ($P = 0.87$)	0.037 ($P = 0.46$)		
HW-OC3	0.010 ($P = 0.89$)	0.068 ($P = 0.13$)	0.23** ($P = 0.0013$)	
HW-OC4	-0.042 ($P = 0.67$)	−0.041 ($P = 0.48$)	0.12 ($P = 0.20$)	0.30** ($P = 0.0032$)

All r values in (A) are highly significant ($P \ll 0.001$). The P-values in (B) are indicated next to the r values. Nomenclature of the eyespots is after the one proposed by Schwanwitsch ('35) for the genus *Pararge*. Significance is at the 0.05 level.
**$P < 0.01$.
***$P < 0.001$.

eyespots were nevertheless largely developmentally separated, except when situated in neighbouring wing cells or in a homologous wing cell on another wing surface. The location of wing veins and eyespots may have interacted with each other here. A likely explanation for the developmental integration of neighbouring eyespots is that their wing cells shared a wing vein, while the wing cells of the eyespots on the two wing surfaces of the forewing shared exactly the same wing veins (Brakefield, '98; Allen, in press). Rather interestingly, eyespots that completely shared their wing veins, the eyespots on the dorsal and ventral side of the forewing, were somewhat less developmentally integrated than neighbouring (hindwing) eyespots. An explanation for this may be that neighbouring eyespots developed on the same wing surface (i.e. part of same single-layered epithelium), whilst the two dorsal forewing eyespots developed on different wing surfaces. A study on *Bicyclus anynana* has found similar results. Allen (unpublished data) also found, using the graphical modelling technique of Magwene (2001), that neighbouring eyespots on the hindwing were developmentally integrated, as were homologous eyespots on the dorsal and ventral surface, while neighbouring eyespots showed the higher levels of developmental integration.

Artificial selection results on the morphology of eyespots in specific wing cells, and the effects of developmental eyespot mutations (either naturally occuring or artificially generated by mutagenesis as in Monteiro et al., 2003) have been very informative about the developmental processes underlying wing patterning and have been used to infer phenotypic and developmental integration patterns among eyespots (Beldade et al., 2002b; Monteiro et al., 2003). The butterflies in those studies had invariably altered eyespot patterns and/or modified patterns of phenotypic (co-)variation. Having assessed the phenotypic and developmental integration in a non-invasive way using wild-type animals, with unaltered development, the results of our study, and of Allen (unpublished data), confirm the results of aforementioned studies concerning developmental integration. Monteiro et al. (2003), for example, describe a mutant in which failure of establishing an eyespot organizing centre resulted in the deletion of a pair of adjacent eyespots. In the study of Monteiro et al. (2003), it was furthermore proposed, as an alternative to the hypothesis of wing veins developmentally integrating neighbouring eyespots, that the existence of so-called selector genes operating on pairs of eyespots may explain the high levels of developmental integration of neighbouring eyespots (Monteiro et al., 2003).

Wing veins can significantly affect the morphology of an eyespot as demonstrated especially on the forewing. Monteiro et al. ('97c) found that artificial selection on eyespot shape in *B. anynana* resulted in correlated responses in wing (cell) size and shape. The size of the wing cell affected the shape of the two forewing eyespots (FW-OC5v and FW-OC5d, see Fig. 1) more significantly than each of the hindwing eyespots. Unlike the other eyespots, the forewing eyespots stretch from wing vein to wing vein, thereby giving the impression to be flattened by the wing veins, and consequently the dorsal and ventral forewing eyespots are more ellipsoidal than the hindwing eyespots, which were more circular. The *P. aegeria*

mutant *schmidti* is interesting in this respect. The mutant allele affects all eyespots simultaneously and in the same way. The eyespots are much bigger, and much more ellipsoidal, than the wild type on *all* wing surfaces (Russwurm, '78; Barrington, '95).

What is striking from the results of this study is the high integration of a forewing and hindwing, both phenotypically and developmentally. A similar result was found in the glanville fritillary butterfly *Melitaea cinxia* (Breuker et al., 2007). Studies on flight biomechanics in *P. aegeria* have concentrated on the forewings only (Berwaerts et al., 2002, 2006). The possible adaptive significance of the strong integration of the morphology of the forewing and the hindwing remains therefore to be investigated. Even though wing discs are generally considered to be separated developmentally, a likely explanation for the observed developmental integration is an allocation trade-off between a forewing and hindwing, as growing imaginal discs seem to compete for some hemolymph-borne source, a nutrient or a growth factor (Klingenberg and Nijhout, '98; Nijhout and Emlen, '98; Nijhout and Grunert, 2002). Very few studies have investigated the phenotypic and developmental integration of the butterfly wing venation patterns directly (Reed and Gilbert, 2004). As noted earlier, eyespot mutants have been very informative about the developmental processes underlying wing patterning and have been used to infer phenotypic and developmental integration patterns among eyespots (Monteiro et al., 2003), but unfortunately wing vein mutants are extremely rare, and experimental manipulation difficult. The most interesting wing vein mutation in this respect is the one in a hybrid *Heliconius* that causes a deficiency of homologous wing veins on the forewing and hindwing (Reed and Gilbert, 2004). It is remarkable that although the integration of the wings may have contributed to the phenotypic integration of the eyespots, it did not result in an overall developmental integration of the eyespots. Furthermore, although the development of a wing cell and an eyespot may interact with each other, and develop partly simultaneously (Reed et al., 2007), they are not developmentally integrated.

The fact that eyespots are phenotypically so well integrated does mean that selection on a particular size and shape of one eyespot could result in correlated responses of other eyespots, especially when genetic covariances exist (Paulsen and Nijhout, '93). Artificial selection experiments on eyespot patterning in *B. anynana* have provided ample evidence of such a pattern (Monteiro et al., '97a,b,c). This potential for concerted evolution makes sense when together eyespots indeed form a functionally relevant trait-like wing patterning (Brakefield and French, '99). What is so remarkable, however, is that it nevertheless seems relatively easy to "uncouple" eyespots by means of artificial selection (Beldade et al., 2002a), or by the presence of single mutant alleles of major effect. Furthermore, the finding that eyespots seem to be developmentally separated to a large extent from one another potentially allows for flexibility in response to environmental heterogeneity, and therefore for independent evolution. This is most likely a significant observation to explain the complex spatial pattern of morphological variation in *P. aegeria* across its distribution range (Schwanwitsch, '35; Brakefield and Shreeve, '92). Examples of developmental flexibility of individual *P. aegeria* eyespots include the hindwing eyespots HW-OC4 and HW-OC5 (Fig. 1). Although *P. aegeria* wings in general become paler and the expression of eyespots becomes weaker in response to a resource shortage during development (Talloen et al., 2004), it is HW-OC4 that is much more sensitive to the effects of a resource shortage during development than the other eyespots (Gibbs and Breuker, 2006). There is seasonal variation in the frequency of occurrence of HW-OC5, the eyespot which was largely absent in our study animals, whereas in British *P. aegeria* this eyespot has been shown to be involved in sexual selection, seemingly independent from the other eyespots (Shreeve, '87). However, the functional significance of specific wing pattern elements relative to crypsis, predator deflection, or sexual selection has often been assumed, but rarely tested (Stevens, 2005). This needs further experimental testing, also in *Pararge*. For example, a trade-off between crypsis and the presence of conspicuous eyespots may exist and individual eyespots may therefore experience conflicting selection pressures. The net selection result will, among other things, depend on the strength of the individual selection pressures and their timing. If the opposing selection pressures are consecutive, alternative developmental pathways may be selected for, resulting in a polyphenism of wing patterning (Brakefield, '96).

Given that wing (cell) morphology explains such a significant part of the variation in morphology of

wing pattern elements, it is feasible that strong (directional) selection on wing morphology could result in correlated responses in wing patterning and vice versa (Monteiro et al., '97c). From previous work done on flight biomechanics and life-history traits in *P. aegeria* it has become apparent that different habitats and different seasons seem to select for different wing morphologies, with correlated thermoregulatory differences in wing colouration (Van Dyck et al., '97; Van Dyck and Wiklund, 2002; Merckx and Van Dyck, 2006). What has become clear is that wing and eyespot development appear to be extremely flexible in *P. aegeria*, with the possibility of following different developmental pathways to meet the varying ecological requirements (Van Dyck and Wiklund, 2002). What therefore remains to be investigated in *P. aegeria*, but also in butterflies in general, is exactly to what extent environmental and seasonal heterogeneity causes correlated changes in wing morphology and eyespot patterning and therefore what the reaction norms of integration of wing traits look like, and whether the different developmental pathways allow for an uncoupling of traits or increase integration (Schlichting, '89). Studying phenotypic plasticity of integration and quantifying the selection pressures operating on both wing shape and eyespots would be an exciting next step in unravelling the interaction between wing morphology and eyespot patterning within an ecologically relevant context.

ACKNOWLEDGMENTS

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