

Inferring Developmental Modularity from Morphological Integration: Analysis of Individual Variation and Asymmetry in Bumblebee Wings

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ABSTRACT: Organisms are built from distinct modules, which are internally coherent but flexible in their relationships among one another. We examined morphological variation within and between two candidate modules: the fore- and hindwings of bumblebees (Hymenoptera: Apidae: *Bombus impatiens*). We used the techniques of geometric morphometrics (Procrustes superimposition) to analyze the variation of landmark configurations in fore- and hindwings. Regression was used to correct for size-related shape variation (allometry). Principal component analysis revealed patterns of variation that were remarkably similar for individual variation and fluctuating asymmetry (FA). Because covariation of FA among parts must be due to direct transmission of the developmental perturbations causing FA, this agreement of patterns suggests that much of individual variation is also due to direct developmental interactions within each developing wing. Moreover, partial least squares analysis indicated that the patterns of shape covariation between fore- and hindwings were nearly the same as the patterns of within-wing variation. Shape covariation of FA was only found in bees that had been reared under elevated CO₂ concentration but not in bees from the control treatment, suggesting that the mechanisms of developmental interactions between fore- and hindwings are related to gas exchange. We conclude that the fore- and hindwings are developmental modules that maintain internal coherence through direct developmental inter-

actions and are connected to each other only by relatively few links that use the system of interactions within modules.

Keywords: allometry, developmental modules, fluctuating asymmetry, geometric morphometrics, morphological integration, shape.

Morphological variation of organisms is integrated; that is, the sizes and shapes of parts vary in a coordinated manner so that each organism forms a functioning whole (e.g., Olson and Miller 1958; Cheverud 1996). This coordination results from developmental interactions among the parts or modules from which organisms are built (Raff 1996, p. 326 ff.; Kirschner and Gerhart 1998, calling them “compartments”; von Dassow and Munro 1999). The integrity and coherence within a module is maintained through direct developmental interactions. The concept of modules can be used in a structural context, in which a module occupies a specific morphological domain and corresponds to a single morphogenetic field (Gilbert et al. 1996), or they can be viewed as units of gene regulation (von Dassow and Munro 1999). Although developmental modules can interact with one another to a certain degree, each one is distinct in its development and genetic control and exhibits a considerable degree of autonomy. The autonomy of modules provides the evolutionary flexibility required for adaptive changes of some parts of an organism relative to others (Raff 1996; Wagner 1996; Wagner and Altenberg 1996; Kirschner and Gerhart 1998; von Dassow and Munro 1999). Therefore, it is important for studies of organismal form to delimit the spatial domains of developmental modules and to examine the patterns of variation within modules as well as associations among them.

Because developmental interactions take place primarily within modules, it is possible to infer the spatial extent of modules from the patterns of covariation in morphometric data. Direct developmental interactions between parts generate covariation among morphological traits because they transmit variation to the different traits jointly. For instance, two parts that originate from the same developmental pre-

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cursor share variation accrued before the developmental pathways separate, or the parts may co-vary because of variable partitioning of the precursor (Riska 1986). Likewise, variation can be transmitted directly from one part to another by inductive signaling (Jacobson and Sater 1988). Phenotypic covariation can also arise in a different way, without direct interactions, if parts develop separately but co-vary because they respond to the same genetic or environmental factor. In this case, developmental variation is not transmitted between traits, and therefore this type of covariation requires a source of genetic or environmental variation that affects two or more parts simultaneously. It should, therefore, be possible to eliminate this second source of covariation and to focus exclusively on direct transmission of developmental variation by rigorously controlling for genetic and environmental variation.

A convenient way to “control” for genetic and environmental factors is to analyze fluctuating asymmetry (FA; Palmer and Strobeck 1986; Møller and Swaddle 1997). Because both body sides of an individual share the same genome and nearly the same environment, FA results from small random perturbations in developmental processes that take place on the left and right body sides (e.g., Klingenberg and Nijhout 1999). If the development of two traits is linked, the same perturbations can have an effect on both of them simultaneously and, hence, can generate a correlation between their asymmetries (note that the sign, or handedness, of the asymmetry is crucial for this reasoning). Because of the random, local origin of the developmental irregularities producing FA, signed asymmetries will only be correlated among traits if the respective parts are developmentally linked, that is, if a perturbation affecting one trait is also transmitted to the others. Therefore, correlations of FA will be confined primarily within developmental modules, whereas correlations among individuals will occur equally within and between modules.

Here we examine variation among individuals and FA in the fore- and hindwings of bumblebees, which can be expected to be distinct developmental modules because they develop from separate imaginal discs (e.g., Nelson 1924; Snodgrass 1956). The reasoning outlined above leads to the following three predictions: first, if fore- and hindwings are each a homogeneous module, then all parts throughout each wing should co-vary with one another; second, because direct developmental interactions should dominate within a module, patterns of variation among individuals should be similar to the patterns of FA within each wing (although the magnitude of variation is expected to differ); and third, to the extent that fore- and hindwings are separate modules, individual variation will co-vary, but FA will be independent between them. We test these predictions using the methodology of geometric morphometrics (Bookstein 1991; Dryden and Mardia 1998) and

its specific extensions for analyzing individual variation and FA (Klingenberg and McIntyre 1998; Auffray et al. 1999).

Material and Methods

Data

The measurements used in this study are from the fore- and hindwings of bumblebees (Hymenoptera: Apidae: *Bombus impatiens*). The bees used in this study were raised as part of an experiment to study the influence of stress through increased CO₂ concentration, a potential problem occurring naturally for these ground-nesting bees (S. M. Sowry, A. V. Badyaev, and N. J. Beckwith, unpublished data). Queens were collected in early spring, brought into the laboratory, and provided with nest boxes to start colonies.

Three treatments were administered, all providing a flow of air through the nest boxes but with different concentrations of CO₂: 10% CO₂, 5% CO₂, and a control treatment with pressurized air. Two colonies were included in each treatment. Because many bees emerging from the treatment with a CO₂ concentration of 10% had at least one deformed wing that could not be measured, we could not obtain sample sizes sufficient for our multivariate analyses. We therefore only consider bees from the control and the 5% CO₂ treatment (simply called “CO₂ treatment” below).

The wings included in this study were from worker bees. Wings were mounted on slides in Euparal. The landmark coordinates were measured from digital images of single fore- or hindwings taken with a high-resolution camera fitted to a Leica dissecting microscope. Thirteen landmarks were digitized for each forewing and six for each hindwing, which are designated F1–F13 and H1–H6, respectively (fig.

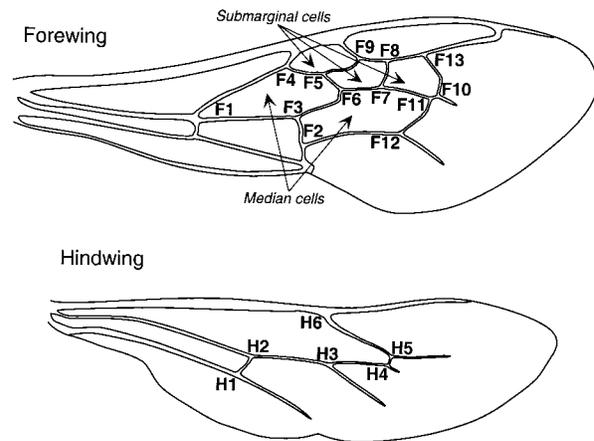


Figure 1: Landmarks digitized on the fore- and hindwings of bumblebees. Note that fore- and hindwings are not drawn to the same scale.

1). For a subset of 10 bees, an additional set of images of each wing were taken and digitized, and the landmark coordinates were analyzed to assess error caused by the imaging and digitizing procedure (Palmer 1994).

Statistical Analysis

We use geometric morphometrics based on Procrustes methods to characterize size and shape variation (Bookstein 1996; Dryden and Mardia 1998). These methods have been adapted specifically to study patterns of individual variation and FA (Klingenberg and McIntyre 1998; Auffray et al. 1999; Badyaev and Foresman 2000; Debat et al. 2000; Klingenberg and Zaklan 2000), extending earlier studies of asymmetry using Procrustes methods (Bookstein 1991, pp. 267–270; Auffray et al. 1996; Smith et al. 1997). The fore- and hindwings were subjected to these analyses separately.

As a measure of the overall size of each wing, we computed centroid size, which is the square root of the sum of squared distances of each landmark from the centroid (center of gravity) of the configuration (e.g., Dryden and Mardia 1998, p. 24). For centroid size, analyses of the variation among individuals and of asymmetry were carried out using standard univariate statistics (Palmer and Strobeck 1986; Palmer 1994).

The Procrustes approach extracts shape information from coordinate data in a set of steps that eliminate reflection (only for studies of left-right asymmetry) and variation in scale, position, and orientation (Klingenberg and McIntyre 1998). First, reflection is removed by changing the sign of the x coordinates of all left wings, that is, by flipping them horizontally to their mirror images. Then, all landmark configurations are scaled to the same size, that is, a centroid size of 1.0. Position is standardized by superimposing the centroids of all configurations on the origin (0, 0). Finally, variation in orientation is eliminated by rotating the landmark configurations around their shared centroid to achieve an optimal fit of each specimen to the consensus (average) configuration. Our procedure did this without further scaling and is, therefore, a partial Procrustes fit according to the terminology of Dryden and Mardia (1998).

The effect of measurement error was assessed for the subsample that had been digitized twice through a Procrustes ANOVA of shape (Klingenberg and McIntyre 1998) and a conventional two-factor ANOVA (Palmer and Strobeck 1986; Palmer 1994) for centroid size. For all other analyses, we used the complete set of specimens, which had been digitized only once (the “remainder” mean square, therefore, includes both the individual \times side interaction and measurement error). Procrustes ANOVAs in the two treatments were used to test for directional asymmetry and to assess the relative magnitudes of individual variation and FA (Klingenberg and McIntyre 1998). Testing of ANOVA

effects was done using the permutation methods described by Klingenberg and McIntyre (1998). Analyses were run separately for the control and CO₂ treatments. In each treatment group, the effects of the colony in which bees had been raised were eliminated by correcting for the differences in means. Finally, note that the mean squares in Procrustes ANOVAs of fore- and hindwings are not directly comparable because of the different number of landmarks.

We examined whether there was a size-dependent (allometric) component of shape variation by a permutation test (Edgington 1995). This test randomly reassigned the vectors of shape coordinates and centroid sizes (or the respective signed asymmetries) among individuals, computed the sum of squared covariances between the Procrustes coordinates and centroid size as the test statistic, and compared it with the corresponding statistic for the original sample. Each test used 10,000 randomization runs.

The allometric component of shape variation was estimated by multivariate regression of shape on centroid size (Mardia et al. 1979; Baylac and Penin 1998; Monteiro 1999). Allometric shape variation was assessed in different ways for individual variation and FA. For individual variation, left-right means of shape were regressed on left-right means of centroid size, whereas for FA, signed shape asymmetries were regressed on signed asymmetries of centroid size. For the subsequent analyses of covariation among landmarks, we used the residuals from multivariate regression of shape on size. This eliminated the effects of size, which might introduce integration through other means than the developmental interactions of interest here.

Patterns of variation within wings were examined and displayed using principal component analysis (e.g., Jolliffe 1986). This analysis extracts new shape variables, the principal components (PCs), which successively account for the maximal amount of shape variation, subject to being uncorrelated with all preceding PCs. The PCs are, therefore, an effective means of data reduction, which is especially important in the context of shape analyses, where data usually have high dimensionality (e.g., the shape space for the forewings is 22-dimensional; that for the hindwings, 8-dimensional). Moreover, the joint pattern of landmark displacements of the different PCs can be displayed graphically and interpreted as features of shape variation (e.g., Klingenberg and McIntyre 1998; Badyaev and Foresman 2000; Debat et al. 2000; Klingenberg and Zaklan 2000). The PCs for individual variation and FA were calculated from covariance matrices of the left-right averages or signed asymmetries, respectively (for further details, see Klingenberg and McIntyre 1998).

For comparisons of PCs between sources of variation (individual variation and FA), we computed the angles between the respective PCs, which can be calculated as $\alpha = \arccos [a'b/(a'ab'b)^{0.5}]$, where a and b are the two PC

vectors to be compared (e.g., Klingenberg 1996; Klingenberg and McIntyre 1998). To test differences between PCs statistically, we used a bootstrap test (Efron and Tibshirani 1993) with the null hypothesis that pairs of PCs were identical, differing only by sampling error. As the test statistic for these tests, angles between PCs were used. Resampling was carried out, with replacement, among PC scores of observations (left-right means or signed asymmetries, as appropriate for the comparison) to generate a distribution of angles under the null hypothesis of identical PCs. The test assessed how often the angles between corresponding PCs in this null distribution exceeded the angles computed from the original data. The correspondence between resampled PCs was determined by the minimum angles between them as well because PCs with clearly corresponding patterns of landmark shifts sometimes differed in the relative magnitudes of eigenvalues; we built this assignment procedure into the bootstrap routine, which therefore dealt automatically with the problem of multiple testing inherent in this situation (Westfall and Young 1993). For each comparison, we carried out 10,000 bootstrap iterations.

To assess covariation between fore- and hindwings, we ran a permutation test (Edgington 1995) with the null hypothesis that variation was independent between the two wing pairs. This test simulated the null hypothesis of independent variation by randomizing the order of hindwings, leaving the order of the forewings constant and thereby randomly reassigning fore- and hindwings to each other. As the test statistic for shape and as a magnitude of covariation in shape between fore- and hindwings, we used the sum of squared covariances between the Procrustes coordinates of fore- and hindwings (for either left-right means or signed asymmetries). For centroid size, the conventional product-moment correlation between wings was used as the test statistic.

To examine the patterns of covariance between fore- and hindwings, we used the method of partial least squares (PLS; Bookstein et al. 1990; Klingenberg and Zaklan 2000; Rohlf and Corti 2000). This method has previously been used in dose-response studies (Bookstein et al. 1990; Streissguth et al. 1993) and ecomorphology (Corti et al. 1996; Klingenberg and Ekau 1996; Adams and Rohlf 2000) and for relating morphometric data taken from different views of the same specimens (Tabachnick and Bookstein 1990) or subsets of landmarks from different parts of a structure (Baylac and Penin 1998; Klingenberg and Zaklan 2000). The PLS method is based on a singular value decomposition (e.g., Jolliffe 1986, p. 37 ff.; Marcus 1993) of the matrix of covariances between the two sets of variables, that is, the covariances between the Procrustes coordinates of fore- and hindwings. The analysis extracts pairs of PLS axes (one axis for each set of variables), which are linear

combinations of the two sets of variables that have maximal covariance between the two sets. Any PLS axis in one set of variables is only correlated with the corresponding PLS axis in the other set, but with none of the other PLS axes in that set. Finally, the first n pairs of PLS axes jointly provide a best least-squares approximation of rank n to the matrix of covariances (in this sense, PLS axes serve for data reduction just as PCs do for the variation within a single set of variables). In the context of this study, the PLS axes can be interpreted as those shape features that have maximum covariance between fore- and hindwings, and the first few pairs of PLS axes provide an optimal approximation of the total covariation between fore- and hindwings.

To compare PLS axes to within-wing PCs or vectors of allometric regression coefficients, we computed angles between them, as described above for comparisons among PCs. To test whether there was significant similarity between them, a Monte Carlo simulation of angles between random vectors in 22- or 8-dimensional space was used to establish whether the observed angles were smaller than the angles expected between a pair of random vectors (Cheverud 1982; Klingenberg and Zimmermann 1992; Klingenberg and McIntyre 1998). To make it possible to use sequential Bonferroni corrections for multiple comparisons with this test, we ran 400,000 simulations for each dimensionality.

Results

Procrustes ANOVA

The conventional two-factor ANOVAs of centroid size and the corresponding Procrustes ANOVAs of shape for the 10 specimens digitized twice gave similar results. The individuals \times sides interaction, standing for FA, was highly significant and the corresponding mean square exceeded measurement error by a substantial amount (table 1). Therefore, measurement error is not of concern for the subsequent analyses of FA in the full data set, even though each wing was digitized only once.

ANOVAs of centroid size for the complete samples revealed significant directional asymmetry in the forewings but not in the hindwings (table 2). The Procrustes ANOVAs of shape indicate that there was highly significant directional asymmetry of shape for both the fore- and hindwings (table 2). This latter observation confirms the results of Smith et al. (1997), who found substantial directional asymmetry of shape in the forewings of honeybees, and it suggests that directional asymmetry of shape may be a common feature of insect wings (Klingenberg et al. 1998). All the subsequent analyses concern the variation about the means and, therefore, automatically correct for directional asymmetry.

Table 1: Two-way ANOVAs of centroid size and Procrustes ANOVAs of shape of fore- and hindwings to assess measurement error for the 10 specimens digitized twice

	Forewings		Hindwings	
	MS	df	MS	df
Centroid size (MS in mm ²):				
Individual	.97***	9	.296***	9
Side	.0123	1	.00052	1
Individual × side	.0032***	9	.0029***	9
Measurement	.000064	20	.000017	20
Shape (Procrustes MS × 10 ⁶):				
Individual	33.3***	198	123.6***	72
Side	32.5**	22	34.7	8
Individual × side	9.15***	198	44.2***	72
Measurement	.78	440	2.17	160

Note: MS = mean squares.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

Allometric Component of Shape Variation

Permutation tests indicated that shape variation was size dependent in most cases. In both treatments and for both wing pairs, the left-right means of shape co-varied with size in a highly significant manner, as none of the 10,000 randomization runs produced covariation as strong as in the original sample for any of the tests. For FA, the tests indicated that the allometric component was significant for the forewing in the CO₂ treatment ($P < .001$) and for the hindwing in both treatments ($P < .01$) but not for the forewing in the control group ($P = .71$). Overall, therefore, there is a significant allometric component of shape variation, although it is not entirely consistent for FA. The shape features associated with allometry, from the multivariate regressions of shape on size, are presented in figure 2.

To eliminate the confounding influence of size through the allometric components of shape variation, which is not the focus of our study, the subsequent analyses used residuals from the multivariate regressions presented here (in addition to the correction for mean differences between colonies).

Patterns of Variation within Fore- and Hindwings

The first three PCs accounted for about half of the shape variation within each forewing and about three-quarters of the variation in hindwings and are, therefore, useful summaries of the 22- or 8-dimensional data in fewer dimensions. Corresponding PCs for individual variation and FA accounted for similar percentages of the total within-wing variation (given in fig. 3), indicating a similar degree of morphological integration.

The PCs revealed patterns of variation that were fairly consistent, both between individual variation and FA and between the two treatment groups (fig. 3). For the forewings, we found a clear one-to-one agreement between the PCs for individual variation and FA, both in the control (none of the bootstrap tests rejected the null hypothesis that corresponding PCs were identical: PC1, $\alpha = 46.8^\circ$, $P = .26$; PC2, $\alpha = 49.3^\circ$, $P = .27$; PC3, $\alpha = 67.7^\circ$, $P = .30$) as well as in the CO₂ treatment (PC1, $\alpha = 34.8^\circ$, $P = .44$; PC2, $\alpha = 52.7^\circ$, $P = .15$; PC3, $\alpha = 45.1^\circ$, $P = .79$).

This agreement of individual variation and FA was less clear-cut for the hindwings. In the control treatment, the bootstrap tests could not reject the null hypothesis of identical PCs for PC1 ($\alpha = 7.6^\circ$, $P = .98$) and PC3 ($\alpha = 43.6^\circ$, $P = .36$) but indicated a statistically significant difference between the PC2s ($\alpha = 55.8^\circ$, $P = .012$). In the CO₂ treatment, the PC1 of individual variation was most similar to the PC2 of FA ($\alpha = 54.8^\circ$, but the bootstrap test indicated that they were not identical, $P = .003$), the PC2 for individual variation and PC1s for FA were not significantly different ($\alpha = 38.9^\circ$, $P = .39$), whereas the difference between the two PC3s was just barely nonsignificant ($\alpha = 67.7^\circ$, $P = .052$). In sum, as the null hypothesis of identical PCs could only be rejected for two of the comparisons, there appears to be a clear, although not perfect, correspondence between FA and individual variation.

Covariation between Fore- and Hindwings

The correlation of left-right means of centroid size between fore- and hindwings was strong and highly significant in both treatment groups (control: $r = 0.84$, $P < .001$; CO₂ treatment: $r = 0.97$, $P < .001$). The correlations of signed

Table 2: Two-way ANOVAs of centroid size and Procrustes ANOVAs of shape of fore- and hindwings of the complete sample

	Control				Increased CO ₂			
	Forewing		Hindwing		Forewing		Hindwing	
	MS	df	MS	df	MS	df	MS	df
Centroid size (MS in mm ²):								
Individuals	1.019 ^{***}	64	.349 ^{***}	64	1.198 ^{***}	71	.322 ^{***}	71
Sides	.035 ^{***}	1	.000043	1	.026 ^{***}	1	.000071	1
Remainder	.00101	64	.000847	64	.00162	71	.000837	71
Shape (Procrustes MS × 10 ⁶):								
Individuals	45.7 ^{***}	1,408	127.3 ^{***}	512	31.2 ^{***}	1,562	88.2 ^{***}	568
Sides	88.6 ^{***}	22	243.7 ^{***}	8	107.9 ^{***}	22	255.1 ^{***}	8
Remainder	5.82	1,408	21.3	512	8.55	1,562	20.7	568

Note: Measurement error cannot be analyzed separately because wings were only digitized once (but see table 1). MS = mean squares.

*** $P < .001$.

asymmetries of centroid size were lower but still statistically significant in both the control ($r = 0.29$, $P < .05$) and CO₂ treatments ($r = 0.30$, $P < .05$). These correlated asymmetries of size suggest that there is developmental interaction between fore- and hindwings.

For the analyses of shape, after corrections for the effects of size and colony, the permutation test indicated significant covariance between fore- and hindwings for variation among individuals in both treatment groups (control: sum of squared covariances 2.02×10^{-9} , $P < .01$; CO₂ treatment 1.36×10^{-9} , $P < .01$). In the control treatment, there was no significant covariation between the signed shape asymmetries of fore- and hindwings (sum of squared covariances 1.53×10^{-9} , $P = .094$). In the CO₂ treatment, however, the shape asymmetries of fore- and hindwings did co-vary significantly (sum of squared covariances 2.56×10^{-9} , $P < .01$).

To identify the shape features associated with this covariation of fore- and hindwings, we ran PLS analyses for the left-right means of both treatments and FA of the CO₂ treatment. In all these analyses, the first three PLS axes accounted for the bulk of the covariances between fore- and hindwings (about 70% of the sum of singular values) and, therefore, gave a reasonable summary of the patterns of covariation. Correlations between the PLS axes were moderate, varying between 0.44 and 0.55 (but note that the PLS procedure maximizes covariances, not correlations).

The patterns of covariation between fore- and hindwings revealed by the PLS method were generally similar to the corresponding PCs, although the ordering of PLS axes did not always coincide with that of the PCs (cf. figs. 3, 4). This suggests that similar processes were involved in producing both variation within wings and covariation between wings. The most striking similarity is between the PLS1 axes for FA in the CO₂ treatment and the corresponding PC1s (the angles for the fore- and hindwings are

21.3° and 15.5°, respectively; both significantly smaller than expected for random vectors, $P < .001$). It is interesting that these same PLS1 axes also significantly resemble the corresponding components of size-dependent shape asymmetry (fig. 2; angles are 37.9° and 46.8°, $P < .05$).

Discussion

This study has examined morphometric variation in bumblebee wings to test whether the wings, each originating from a separate imaginal disc, are distinct developmental modules and to examine to what degree they interact with one another. For this purpose, we have compared patterns of covariation within and between fore- and hindwings as well as between FA and among-individual variation.

The Wings as Developmental Modules

The dominant patterns of variation within each wing are coordinated shifts in suites of landmarks that extend throughout the entire wing (fig. 3). There is no partition of the wing into subregions so that the PCs would feature only movements of landmarks located in either one or the other subregion. This means that there does not appear to be any spatial subdivision of the wings into smaller domains of integration that would be more or less independent of one another. Because this absence of parcelation of the wing also applies to FA, it appears that the small developmental perturbations that cause FA are transmitted throughout the entire wings. Therefore, each wing appears to be internally coherent with respect to the developmental processes that produce shape variation and, thus, can be considered as a single homogeneous developmental module.

Similar analyses in *Drosophila melanogaster* have shown that the entire wing behaves as a single integrated module

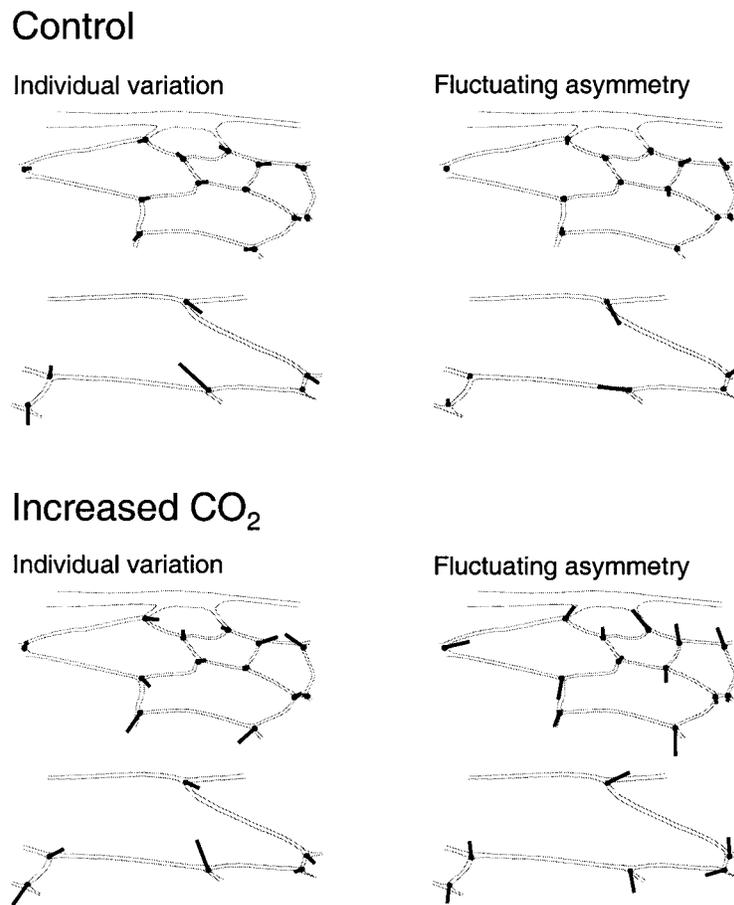


Figure 2: Size-related variation in the shape of fore- and hindwings. This component of shape variation was computed by multivariate regression of shape on centroid size (left-right means for individual variation, signed asymmetries for FA). To visualize the amounts of variation, the vectors of regression coefficients were multiplied by the standard deviation of the respective component of size variation (amplified 20-fold for individual variation and 40-fold for FA). The diagrams show parts of right fore- and hindwings (not drawn to the same scale), oriented so that anterior is up and distal is to the right.

(Klingenberg and Zaklan 2000). This module, therefore, spans across the boundary between anterior and posterior compartments, which, in *Drosophila*, is a clear delimitation both in terms of cell lineages and gene expression (e.g., Lawrence and Morata 1976). However, the compartment boundary is also the origin of morphogen gradients involved in specifying the position of wing veins (e.g., Biehs et al. 1998; de Celis 1998; Dahmann and Basler 1999; Strigini and Cohen 1999) and, therefore, actively contributes to developmental integration across the wing. Our results demonstrate that integration throughout the entire wing, whether by the same or different mechanisms, also occurs in bumblebees.

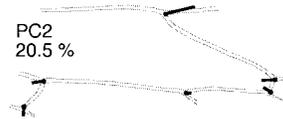
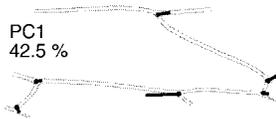
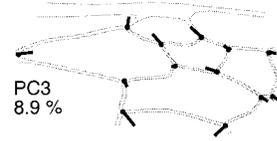
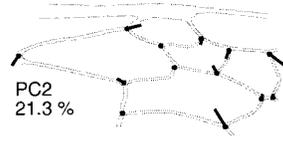
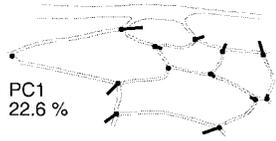
Individual variation and FA showed similar degrees of integration and clear overall correspondence in their within-wing patterns of variation. Hence, it is likely that similar

developmental processes are underlying both individual variation and FA, and that much of the genetic or environmental covariation among individuals is produced by direct developmental interactions among wing parts.

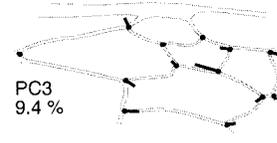
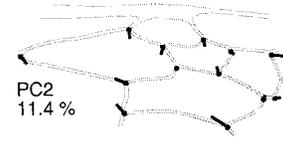
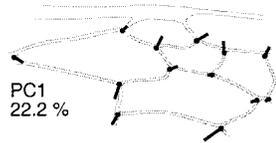
The correspondence between patterns of variation for FA and individual variation agrees with the results of previous studies of shape variation in the wings of tsetse flies (*Glossina palpalis*; Klingenberg and McIntyre 1998) and *Drosophila* (Klingenberg and Zaklan 2000). A considerable degree of integration of FA, comparable to that for individual variation, has also been reported for mouse jaws (Leamy 1993). Presumably, this correspondence between individual variation and FA is because insect wings and mammalian jaws are sufficiently compact for developmental interactions to take place throughout the entire structure. This coherence of morphological variation in-

Control

Individual variation

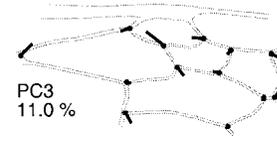
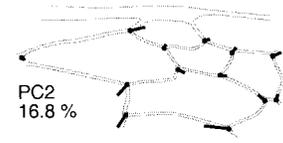
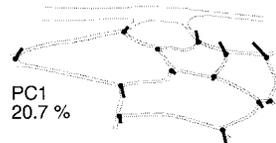


Fluctuating asymmetry



Increased CO₂

Individual variation



Fluctuating asymmetry

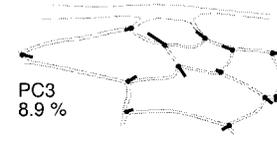
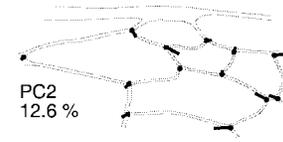
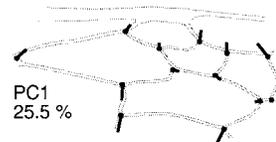


Figure 3: Principal component analysis of shape variation within wings. The analyses were carried out separately for fore- and hindwings as well as for individual variation (using left-right averages) and FA (using signed asymmetries). In each of the diagrams, the pattern of landmark shifts corresponding to the respective PC is visualized by lines that extend from the mean landmark locations (*dots*) to a PC score of 0.15 Procrustes units, which is a shape difference that far exceeds the variation present in the data set. The percentages below each of the diagrams indicate for how much of the shape variation in a sample that particular PC accounts.

dicates that these structures each constitute a single developmental module.

Covariation between Fore- and Hindwings

There was significant covariation of size and shape between fore- and hindwings for both individual variation and FA. For among-individual variation, such covariation is to be expected because any genetic or environmental source of variation can potentially generate a response in all four developing wing discs simultaneously. This also implies that the same developmental processes that produce individual variation of a given wing are also responsible for covariation of fore- and hindwings. Accordingly, it is not surprising that the patterns of within-wing variation and those of covariation between fore- and hindwings were similar, as suggested by the comparisons of PCs and PLS axes (figs. 3, 4). The covariation between fore- and hindwings for FA, however, is more remarkable because it indicates that perturbations causing asymmetry are transmitted between the developing wings, and hence, there must be developmental interactions between fore- and hindwings.

For centroid size, correlation coefficients between signed asymmetries of fore- and hindwings were about 0.3, which is comparable in magnitude to the correlations between wing and leg asymmetries in two species of moths (Van Dongen et al. 1999). Such correlated asymmetry of size may be due to physiological fluctuations that produce asymmetry in the “microenvironment” surrounding the imaginal discs within the developing organism. There is evidence indicating that localized within-organism variation in physiological factors can affect asymmetry: experiments with butterflies have demonstrated that removing a hindwing imaginal disc on one body side of a caterpillar can produce predictable asymmetry in the mass of the forewings of the emerging butterflies (Klingenberg and Nijhout 1998). These results are consistent with the hypothesis that growing structures within an organism compete for some limiting resource (Nijhout and Wheeler 1996; Nijhout and Emlen 1998), and the experimental evidence suggests that these interactions are well localized.

In agreement with this localization, previous studies have reported correlations of signed asymmetries predominantly between the sizes of parts that are morphologically adjacent. This “neighborhood effect” has been shown for the wings and legs of moths (Van Dongen et al. 1999), for different parts of mouse mandibles (Leamy 1993; Leamy et al. 1997), as well as for proximal and distal limb bones in martens and humans (Jolicoeur 1963), in mice (Leamy 1984), and in several primate species (Hallgrímsson 1998). Signed asymmetries of more distant structures, however, tend to be uncorrelated even in cases where organism-

wide correlations exist in the magnitude of FA (i.e., unsigned asymmetries; Lens and Van Dongen 1999).

Shape asymmetry is uncorrelated between fore- and hindwings in bees from the control treatment, as expected, reflecting the origin of wings from separate imaginal discs. In the CO₂ treatment, however, our study also found correlated asymmetry of wing shape even after the correction for allometric effects of size asymmetry. Whereas correlated FA in the size of organs can be easily explained by differences in the microenvironment surrounding the developing imaginal discs on the left and right body sides, it is more difficult to account for the correlated FA of shape of fore- and hindwings. This is because in order to generate correlated shape asymmetries, physiological differences between body sides must differentially affect specific parts in both the developing fore- and hindwings.

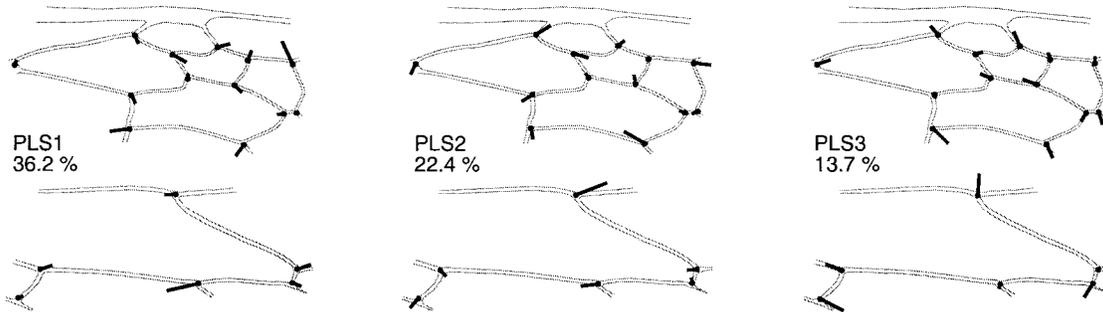
In the context of this requirement for a specific local response to a more generalized stimulus, it may be significant that the dominant pattern of covariation of shape FA between fore- and hindwings is similar to the allometric component of shape FA (cf. figs. 2, 4; CO₂ treatment). Allometric shape variation is analogous in that size is a single factor that elicits a differential response in different parts of each wing, either through the dynamics of resource uptake and growth (Nijhout and Wheeler 1996) or by signaling through hormones (Stern and Emlen 1999) and, thus, leads to changes in wing shape. It is noteworthy, however, that FA in size itself is not the cause of the correlated shape variation because the direct allometric effects of FA in size have been removed statistically. Nevertheless, the processes that produce regionally differentiated growth in response to size may also be sensitive to other physiological factors, perhaps components of the same pathway that translates size variation into changes of proportions. Subtle left-right differences in the conditions for wing development or the concentrations of some signaling factor could, therefore, generate correlated asymmetry by activating those developmental processes that are also involved in allometric shape variation. Interestingly, the lack of correlated FA of shape in the control treatment coincides with the absence of size-dependent shape FA in the forewing. It is conceivable that the mechanism translating physiological left-right differences into shape FA was inactive and, therefore, did not generate a correlation between shape FA of fore- and hindwings in the control treatment.

Possible Mechanisms for Correlated FA

Because correlated FA of fore- and hindwing shape was present in the CO₂ treatment, but not in the control, it seems likely that the processes generating the correlated FA are related to gas exchange. The respiratory system of bees may contribute to correlated asymmetry through the

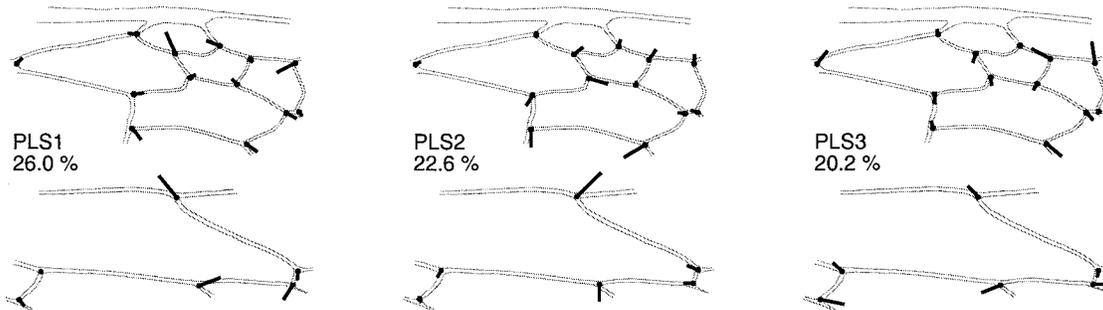
Control

Individual variation



Increased CO₂

Individual variation



Fluctuating asymmetry

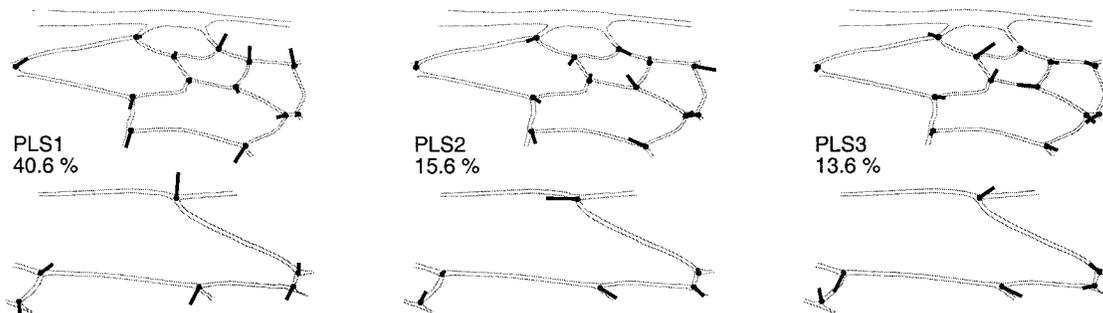


Figure 4: ANCOVA between fore- and hindwings by the partial least squares method. The diagrams represent the PLS axes, those shape features of fore- and hindwings that have maximal covariation between wings. The percentage given for each pair of PLS axes is the proportion of the sum of singular values for which it accounts, which is a measure of its share of total between-wing covariance. We did not run a PLS analysis for FA in the control treatment because there was no significant shape covariance between fore- and hindwings.

arrangement of its main tracheae (Nelson 1924; Snodgrass 1956, p. 229). The dominant feature of the respiratory system of bee larvae is a pair of lateral tracheal trunks that run along either body side and that are linked to each other by transverse ventral commissures (in the thorax these are clearly smaller than the longitudinal trunks). The

tracheal system opens to the outside by a pair of spiracles on the sides of each body segment from the mesothorax to the eighth abdominal segment, each connected by a short tracheal tube to the main lateral trunks. The wing imaginal discs are associated with branches of the tracheal system (Snodgrass 1956, p. 114). Each forewing disc re-

ceives two tracheal branches that connect to the lateral trunk of that body side near the meso- and metathoracic spiracles, respectively, and the hindwing disc is supplied through a single trachea branching from the lateral trunk near the metathoracic spiracle (Comstock 1918, fig. 32). Because the wing discs are not connected between body sides and do not have direct tracheae to spiracles opening to the outside, this arrangement of tracheae links the gas exchange of the fore- and hindwing discs on each body side. Presumably, variability in gas exchange has greater effects on developmental processes in the CO₂ treatment, where CO₂ levels may directly affect physiological condition, than in the control treatment, where CO₂ levels may not be limiting. Consequently, local variation in gas exchange should be more directly reflected in FA of the final wing shape in the CO₂ treatment, and because fluctuations in gas exchange are transmitted between the fore- and hindwings on each body size, this localized variation should also be manifest as correlated asymmetry.

An alternative mechanism, favored as an explanation for localized effects of imaginal disc removals in caterpillars (Klingenberg and Nijhout 1998), is that correlated FA of fore- and hindwings is caused by interactions among imaginal discs via hemolymph-borne factors. This mechanism may account for correlations of FA in centroid size, which were nearly identical in both treatments. However, because the hemolymph of insects is not involved in gas exchange, there is no reason why such a mechanism should not apply to the control treatment. Hence, the fact that correlated FA of shape only occurred in the CO₂ treatment makes this alternative seem a partial explanation at best. In order to establish the mechanisms responsible for correlated FA of size and shape and, thus, the processes by which fore- and hindwings interact, further experiments are clearly needed.

Conclusions

The morphometric analyses indicate that variation in the arrangement of landmarks is coordinated throughout the wings. Because this is the case even for FA, the effects of developmental perturbations responsible for FA are transmitted throughout the entire expanse of the wing. This strong integration indicates that each wing is a homogeneous developmental module. Because the patterns of individual variation and FA are remarkably congruent, it appears that direct interactions are also the prime factor defining the patterns of genetically or environmentally induced shape variation. Therefore, these direct developmental interactions are an essential determinant of the “genotype-phenotype map” (Wagner and Altenberg 1996).

While the wings are internally coherent modules, they are not entirely independent of one another but co-vary

to produce integration at the whole-organism level. Genetic and environmental variation among individuals elicits a strong coordinated response (e.g., allometry in response to size variation), whereas random developmental variation within the organism can produce more subtle correlated FA. Our analyses showed that the patterns of covariation between fore- and hindwings coincide with the patterns of variation within wings. This agrees with the expectation for a system in which organism-wide coordination among modules is achieved by a relatively small number of inputs whose specific effects on shape are then determined through the system of developmental interactions within modules.

The results of our study show that the environment can play a substantial role in the developmental interactions between imaginal discs. Because shape asymmetry co-varied between fore- and hindwings only in the CO₂ treatment, the developmental interactions between wings appear to be related to gas exchange. The arrangement of the tracheal system suggests a possible mechanism of interaction between developing fore- and hindwings. While investigators may regard such environmental effects as a nuisance because experimental results cannot be extrapolated easily from one environment to another (e.g., from laboratory to field), this condition dependence also can point to possible mechanisms and suggest further experiments. Integrating morphometric analyses with experimental evidence and knowledge of the functioning of the whole organisms is a promising approach to elucidate the role of developmental processes in generating observable morphological variation.

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