Developmental instability as a research tool: using patterns of fluctuating asymmetry to infer the developmental origins of morphological integration

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Abstract

This paper takes the study of fluctuating asymmetry (FA) beyond its conventional use as a measure of developmental instability, and turns it into a new research tool for evolutionary developmental biology. Covariation between morphological traits can have two developmental origins: direct developmental interactions, for instance between traits that derive from a common developmental precursor or interact by induction, and parallel variation in separate pathways that are subject to the same environmental or genetic variation. Analysis of covariances in signed FA can be used to distinguish between these two origins of trait covariation. For signed FA in two traits to be correlated, the developmental perturbations causing asymmetry must be transmitted between the developing traits, which requires a direct developmental link between them. Therefore, the analysis of covariation in signed FA can be

used as a method for delimiting developmental modules. This method is applied in two examples: *Drosophila* wings and the fore- and hindwings of bumblebees. The analyses show that the entire *Drosophila* wing is a single, fully integrated module, which includes both the anterior and posterior compartments. This is consistent with experimental results from developmental biology. In bumble bees, the fore- and hindwings are each a separate module, between which there is only a limited amount of covariation, as expected between structures derived from different imaginal discs. Whereas the method confirmed previous knowledge about the boundaries of modules in these two test cases, the promise of this method is in cases where no a priori information is available.

Introduction

In most of the research concerning developmental instability, fluctuating asymmetry (FA) has been used as a measure of stress in populations or of individual quality (e.g., Palmer and Strobeck 1986; Parsons 1990; Graham et al. 1993; Møller and Swaddle 1997). Developmental considerations have entered these studies primarily to account for the relationships of FA with stress or quality (e.g., Hallgrímsson 1993, 1998; Palmer 1996).

In this chapter, I will introduce a fundamentally different approach. Rather than giving a developmental account for FA (e.g., Klingenberg, this volume), here I will demonstrate the use of FA as a research tool to address a central issue in evolutionary developmental biology: the developmental origin of morphological integration and modularity (e.g., Olson and Miller 1958; Riska 1986; Cheverud 1996; Raff 1996; Wagner 1996; Wagner and Altenberg 1996; Arthur 1997; Kirschner and Gerhart 1998; von Dassow and Munro 1999; Mezey et al. 2000). Modules are integrated assemblages that are internally coherent and relatively autonomous from the remainder of the system. One example of a module is a group of genes tightly interconnected by many regulatory interactions, but which is linked to other parts of the overall gene network only by a few regulatory inputs and outputs (e.g., von Dassow et al. 2000). Another kind of module is a developmental precursor that is a largely self-contained assemblage of cells within which patterning processes interact to establish the spatial organization of the prospective adult structure. In developmental

biology, this sort of module has long been known as a morphogenetic field (e.g., French et al. 1976; Ingham and Martinez Arias 1992; Williams and Carroll 1993; Gilbert et al. 1996; Raff 1996, p. 333). Fields are distinct from other kinds of modules in that they are morphological units with clear spatial boundaries. The approach that I outline in this chapter identifies such developmental modules by using FA as a tool to delimit the spatial domains of direct developmental interactions. This approach is complementary to the genetic and molecular methods used in developmental biology, and opens a wide and unexplored field for studies of developmental instability.

I begin by defining two distinct ways in which development can produce covariation between morphological traits, and then explain how covariance of FA can be used to distinguish between them. That associations of developmental instability can be used to investigate developmental relationships between traits was first outlined by Sakai and Shimamoto (1965). However, their analyses confounded within- and between-genotype variation, and the idea has not been taken up by other researchers for over three decades. The approach has recently been more fully developed, and here I demonstrate it with two case studies of morphological integration and modularity in the wings of fruit flies and bumblebees (Klingenberg and Zaklan 2000; Klingenberg et al. 2001). These examples also serve to demonstrate the use of the recent techniques of geometric morphometrics to study individual variation and FA (Auffray et al. 1996; Smith et al. 1997; Klingenberg and McIntyre 1998; Auffray et al. 1999; Debat et al. 2000). Finally, I briefly discuss the possible contribution these approaches can make to the emerging synthesis of evolutionary and developmental biology.

Two developmental origins of morphological integration

Morphological integration manifests itself through the coordinated variation among the parts of organisms, and most studies have investigated it by analyzing patterns of correlation among traits (Olson and Miller 1958; Cheverud 1996; Wagner 1996). Development is a prime determinant of these patterns of correlation (e.g., Riska 1986), and therefore of morphological integration. The degree of integration determines to what extent

the phenotypic effects of variation in developmental processes will be shared among parts. But in order for integration to be observed or measured, some source of developmental perturbations, either from within the organism of from its environment, is required to generate variation.

There are two alternative ways in which developmental processes can generate coordinated variation in morphological structures (Fig. 1). The first is a direct connection or interaction between the developmental processes that generate the structures, and therefore a direct transmission of developmental perturbations to different parts. For instance, a developmental precursor can be partitioned to give rise to two or more descendant structures (Fig. 1a; Sakai and Shimamoto 1965; Riska 1986), or there can be inductive signaling from one developing part to another (Fig. 1b). In either of these cases, perturbations that occur during the interaction or variation that has accrued before can be shared or passed between parts, and therefore may become manifest as correlated variation of the final morphological structures. For the most part, these direct interactions will be confined within developmental modules.

The second origin for covariation between traits is based on parallel variation in independent developmental pathways that both respond, simultaneously but separately, to a common source of variation (Fig. 1c). One possible sources of variation are allelic variation of a gene that is part of both pathways, even though they are spatially and temporally separated, such as the *Distal-less* gene in the distal parts of the legs and in the eyespots on the wings of butterflies (Carroll et al. 1994; Panganiban et al. 1994). Another possible origin of joint variation is an environmental factor to which both pathways are sensitive. Regardless of whether it is genetic or environmental, however, if such variation is to generate a simultaneous response in separate traits of the organism, it must affect the individual as a whole. Without this kind of shared input, separate developmental pathways will not show parallel variation, but vary independently. Therefore, this mode of covariation among parts relies on extrinsic variation among individuals, like perturbations originating from the environment or genetic variation at the population level.

To distinguish between direct developmental interactions and parallel variation as origins of covariation, investigators can manipulate the input of external variation through specific experimental designs and measurement schemes. In particular, covariation by parallel variation of independent pathways can be eliminated by controlling rigorously for all genetic and environmental variation. The remaining covariation among traits should then be due to direct connection of developmental pathways.

Using FA to distinguish sources of integration

Probably the easiest way to control for both genetic and environmental variation is to use left-right asymmetry of bilaterally symmetric organisms (i.e., most animals) or symmetric parts (e.g., plant leaves). Left and right sides share the same genome, except for cases of somatic mutation or recombination (which are rare under natural conditions). Moreover, the two body sides of an individual experience very similar environmental conditions. Except perhaps for special cases like sessile organisms oriented with respect to an environmental gradient, conditions on either body side of a given individual are usually far more similar than from one individual to another. Therefore, variation among individuals in their left-right asymmetry is mostly due to random developmental perturbations (developmental noise; e.g., Klingenberg, this volume).

Because of the random nature of this variation, a correlation between the signed asymmetries of traits can be used to infer interactions between the developmental processes that produce the traits. 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. For a particular individual, if the first trait has a given direction of asymmetry, for instance being bigger on the left than on the right side, the asymmetry of the other trait may just as well have the same or the opposite direction. In a sample of individuals, therefore, the signed asymmetries of the two independently developing traits will be uncorrelated. In contrast, if there is a developmental connection between the traits, then the effects of perturbations can be transmitted directly between traits, producing a statistical relation between the directions of asymmetries of the traits (see also Van Dongen et al. 1999). The developmental

connection between traits is manifest in morphological data as covariance between their signed asymmetries. In other words, the covariances between signed asymmetries of traits result from their developmental connections, but not from parallel variation of independent pathways.

It is important to distinguish the study of developmental integration from the investigation of correlated asymmetry to estimate individual differences in organism-wide developmental instability and buffering from multiple traits (e.g., Polak et al., this volume; Lens and van Dongen 1999; Leung et al. 2000). Those studies estimate variation in the organisms' capacity for buffering from correlations in the amounts of unsigned FA among developmentally independent traits. Therefore, sets of traits between which there are direct developmental interactions (and thus correlations of signed FA) are unsuitable for those purposes, because such traits do not provide independent information to estimate buffering capacity. In contrast, if direct developmental interactions are of interest, as in this chapter, then the relevant information is obtained from the signed asymmetries.

The study of the developmental origins of morphological integration can proceed by comparing the patterns of covariation among traits for signed FA, reflecting direct connection of developmental pathways only, to the patterns of covariation among individuals, which stems from both sources of covariation. This reasoning leads to three testable predictions (Klingenberg et al. 2001): (i) within a homogeneous module, all parts should covary with one another; (ii) because direct developmental connections should dominate within a module, the patterns of variation for individual variation and signed FA should be similar (although not necessarily identical, and their relative contributions to the total variation may differ); and (iii) between separate developmental modules, signed FA should be independent. These predictions can be evaluated in studies of morphometric variation within and among individuals.

Procrustes analyses of individual variation and FA

There have been studies comparing patterns of morphological integration between FA and individual variation using conventional length measurements (e.g., Leamy 1993).

However, this approach has been developed more explicitly in the context of geometric morphometrics, in which the shape of a structure is characterized by the arrangement of a number of morphological landmarks (Klingenberg and McIntyre 1998; Auffray et al. 1999; Debat et al. 2000; Klingenberg and Zaklan 2000; Klingenberg et al. 2001). A further refinement of this approach distinguishes the "matching symmetry" of separate organs on the left and right body sides (e.g., limbs) from "object symmetry" of structures that are bilaterally symmetric in themselves (e.g., skulls; Mardia et al. 2000).

Procrustes analysis extracts shape information from the coordinate data for a set of landmarks by eliminating extraneous information on size, position, and orientation of specimens (Dryden and Mardia 1998; Klingenberg and McIntyre 1998). In addition, for studies of left–right asymmetry, reflection is removed by transforming all configurations from one body side to their mirror images (for matching symmetry; Klingenberg and McIntyre 1998) or by including both the originals and mirror images of all configurations to the analysis (for object symmetry; Mardia et al. 2000). The Procrustes procedure then scales the configurations to unit size, superimposes them by their centroids, and rotates them to an optimal fit to the overall mean shape. The coordinates of the superimposed configurations, or equivalently, the deviations of each configuration from the mean (Procrustes residuals), can then be analyzed with the techniques of multivariate statistics.

The effects of various sources of shape variation can be separated just as in the familiar decomposition of sums of squares in analysis of variance (ANOVA; Klingenberg and McIntyre 1998; Mardia et al. 2000). These Procrustes analyses use the standard type of two-factor ANOVA customary for FA studies (Leamy 1984; Palmer and Strobeck 1986). The main effects of individuals and body sides stand for individual variation (the 'factorial' component of variation in the terminology of Lajus et al., this volume) and directional asymmetry, respectively. The individual-by-side interaction provides a measure of fluctuating asymmetry. Finally, if replicate measurements of the coordinates of each specimens are available, these indicate the measurement error. Because the Procrustes analyses are inherently multivariate, however, the analysis provides a covariance matrix for each effect in place of the scalar variance components in conventional ANOVA. Subsequent

multivariate analyses identify the main patterns of variation of these matrices for graphical presentation and interpretation.

This statistical approach is remarkably powerful, and can reliably find even very subtle shape differences. For instance, studies using this approach have consistently found directional asymmetry for wing shape in bees (Smith et al. 1997; Klingenberg et al. 2001) and flies (Klingenberg et al. 1998), and thus clearly refute earlier claims that left and right sides are not distinguished in development (e.g., Tuinstra et al. 1990). Because of this sensitivity even for subtle effects and the ability to localize them in relation to the geometry of the structure under study, the Procrustes method is an excellent tool for examining patterns of integration for FA and individual variation.

Statistical methods for studying patterns of covariation

The analysis of patterns of integration consists of two more specific tasks: the analysis of overall variation throughout an entire structure and the analysis of covariation between specific parts.

Overall variation of a morphological structure: principal components

The patterns of covariance in a single set of variables, such as the landmark coordinates after Procrustes superimposition, are most often studied with principal component analysis (PCA; e.g., Jolliffe 1986; Klingenberg 1996; Dryden and Mardia 1998; Klingenberg and McIntyre 1998). This method extracts the principal components (PCs), which are features of shape variation that successively account for maximal amounts of variation, while being mutually uncorrelated. The PCs have a clear geometric interpretation, because they are the major and minor axes of the scatter of data points in the space defined by the morphometric data. Simply put, this analysis is the multivariate equivalent of two-dimensional scatter plots, where variation is often characterized by an ellipse enclosing the data points. More formally, the same idea is the basis for constructing equal-frequency or confidence ellipses (Sokal and Rohlf 1995, p. 586 ff.). Just as the directions and lengths of the major and minor axes can describe a two-dimensional ellipse, the PCs and associated

variances (eigenvalues) can characterize the directions and amounts of multivariate variation in a sample.

A particular strength of geometric morphometrics is its ability to localize and display features of variation in relation to the arrangement of landmarks on the structure under study. The directions of the PCs in shape space correspond to the dominant features of shape change and can therefore be visualized graphically as relative shifts of the landmarks. To compare these directions in shape space, for instance to assess the correspondence between analyses for FA and individual variation, it is possible to calculate the angles between corresponding PCs (e.g., Klingenberg 1996).

The shape changes corresponding to the PCs can often be interpreted in the light of biological knowledge, for instance, on the development of the structure. It is important to note, however, that these are *a posteriori* interpretations applied to the PCs as descriptors of morphological variation—therefore the PCs can suggest hypotheses, but they cannot conclusively identify the underlying causal processes.

Covariation between two parts

As a statistical test to examine whether there is any covariance between two parts, I use permutation tests (e.g., Edgington 1995). These tests simulate the null hypothesis of independent variation in the two parts by randomly reshuffling the landmark configurations for one of the parts among specimens. This step was repeated 10,000 times for each test, and the *P* value was estimated as the proportion of permutation runs in which the sum of squared cross-covariances between coordinates of the two parts exceeds the sum of squared cross-covariances in the original data (for details, see Klingenberg and Zaklan 2000; Klingenberg et al. 2001).

A possible means to identify patterns of covariation between two sets of variables is the partial least squares (PLS) technique (e.g., Bookstein et al. 1990; Bookstein 1991, p. 41 f.; Rohlf and Corti 2000). The PLS method has been used in a variety of contexts, such as ecomorphology (Corti et al. 1996; Klingenberg and Ekau 1996; Adams and Rohlf 2000), community ecology (Chessel and Mercier 1993), medical dose–response studies (Bookstein

et al. 1990; Streissguth et al. 1993), a study relating different morphometric data sets collected from the same specimens (Tabachnick and Bookstein 1990), and morphological integration (Klingenberg and Zaklan 2000; Klingenberg et al. 2001).

A PLS analysis produces pairs of linear combinations (the PLS axes) that have maximal covariance between two sets of variables, subject to the condition that each PLS axis covaries only with its counterpart, but with none of the remaining PLS axes for the other set (for details, see Bookstein et al. 1990; Rohlf and Corti 2000). In a study of covariation between body parts, for instance, each pair of PLS axes therefore represents features of shape in the two parts that vary jointly. Just as PCA summarizes the overall variation in a data set by approximating the covariance matrix with a small number of PC axes, PLS is based on an approximation of the matrix of cross-covariances between two sets of variables. The PLS axes can be interpreted in a similar manner as the PCs for overall variation, as they share a number of mathematical properties, and they also can be compared directly to the PCs (e.g., Klingenberg and Zaklan 2000, Appendix). The PLS method is similar to the more widespread method of canonical correlation analysis in that both focus on the relationship between two sets of variables, but as PLS maximizes the covariance, not correlation, between the two sets, it maintains the scaling of variables and thus the geometric structure of the data.

Applications: morphological integration in insect wings

Integration within a wing: anterior and posterior compartments of fly wings

The wings of flies are derived from imaginal discs, pieces of epidermal tissue set apart from the larval tissues, which undergo specific processes of growth and patterning (reviewed by Cohen 1993). Because of the patterning interactions that take place within them, the imaginal discs of *Drosophila* have been highlighted as examples of morphogenetic fields, and therefore also developmental modules (Gilbert et al. 1996; Raff 1996, p. 327 f., 333). Each fly wing, however, consists of anterior and posterior compartments (Fig. 2), which are separate cell lineages from the inception of the imaginal discs and throughout development (e.g., Cohen 1993; Dahmann and Basler 1999). A number of studies have

therefore suggested that the compartments, or even smaller parts of the wing, are autonomous units of morphological variation, and consequently, that each of them is a separate developmental module (Cavicchi et al. 1981; Thompson and Woodruff 1982; Cavicchi et al. 1985; Cowley and Atchley 1990; Cavicchi et al. 1991; Guerra et al. 1997; Pezzoli et al. 1997; Baylac and Penin 1998; Birdsall et al. 2000; Zimmerman et al. 2000). These studies were carried out in a number of different contexts and used a variety of methods, mainly by multivariate analyses of distance measures but also with the techniques of geometric morphometrics. Therefore, the studies are difficult to compare, and fail to answer unambiguously the question whether the entire wing is a single module or whether the two compartments are independent units.

Here, I summarize the results of a study that specifically addressed this question with the methods of geometric morphometrics outlined above (Klingenberg and Zaklan 2000). The data are the coordinates of 12 landmarks measured on both wings of 117 *Drosophila melanogaster* females (Fig. 2; the original study considered both sexes, but because the results are very similar, I only report those for the females here). The initial ANOVA indicated that both FA and individual variation were highly significant statistically, and therefore the subsequent analyses are warranted (further details in Klingenberg and Zaklan 2000). Both for individual variation and FA, most of the variation was concentrated in just a few dimensions, and I therefore only present the first three PCs and PLS axes.

The PCs for variation among individuals reveal several patterns of overall variation in the wing (Fig. 3, upper row), corresponding to variation in the shape of the distal part of the wing, forming a blunter or more pointed tip (PC1), a contraction or expansion of the distal part of the wing (PC2), or a rotation of the distal part of the wing relative to the proximal part (PC3). All of these patterns involve landmarks in both the anterior and posterior compartments jointly. The permutation tests indicated that there was highly significant covariance between the landmark configurations in the two compartments. Further evidence for anterior–posterior integration comes from the PLS analysis of only the variation that is shared between the two compartments (Fig. 3, lower row). The pairs of PLS axes, combined for both compartments, closely match the corresponding PC patterns. The angles between

corresponding PCs and PLS axes ranged from 14.7˚ to 20.9˚ and were all significantly smaller than the angles between random directions in shape space. Finally, a statistical model of complete integration, in which the patterns of covariation between compartments account for all the variation throughout the wing, could not be rejected (further details in Klingenberg and Zaklan 2000). This underscores the strength of integration between the compartments at the level of variation among individuals: the covariation between anterior and posterior compartments is nearly sufficient to account for all the variation across the entire wing.

The PCs for FA (Fig. 4, upper row) are dominated by the distal part of the wing blade (PC1), a proximo-distal shift of the posterior crossvein and narrowing of the distal wing blade (PC2), and the positioning of the anterior crossvein relative to neighboring landmarks (PC3). The permutation test for FA, just like the one for individual variation, indicated highly significant covariance between anterior and posterior compartments. The PLS axes for FA (Fig. 4, lower row) showed a clear one-to-one match to the corresponding PCs, suggesting that the patterns of FA covariation across the compartment boundary can account for most FA throughout the wing. The angles between PCs and PLS axes ranged from 10.5˚ to 51.9˚, and were all significantly smaller than expected for random directions. Moreover, the model of complete integration could not be rejected statistically (Klingenberg and Zaklan 2000). The existence of pervasive integration of FA across the anterior–posterior boundary implies that there are direct developmental connections between the two wing compartments, and that developmental perturbations are transmitted across the entire wing imaginal disc or pupal wing. Altogether, these analyses indicate strong integration across both wing compartments not only for individual variation, but also for FA, suggesting that the wing is a single, fairly homogeneous developmental module.

The compartment boundary has a special role in integration across the wing. But far from being an inert delimiter between autonomous developmental domains, it is an active center of integration, from which crucial patterning signals emanate (Lawrence and Struhl 1996; Strigini and Cohen 1999; Milán and Cohen 2000). Because these signals travel from the boundary both into the anterior and posterior compartments, they constitute a direct connection between the developmental processes responsible for positioning the various

veins (Sturtevant and Bier 1995; Biehs et al. 1998; de Celis 1998; de Celis and Barrio 2000). Consequently, any variation affecting the signaling from the boundary will have effects in both compartments simultaneously, and will thus be a source of covariation. This information on the underlying developmental processes provides a mechanistic explanation for the morphometric covariation throughout the wing blade of *Drosophila*.

Although the patterns of individual variation and FA are not identical, the respective PCs show that they share a number of common features (upper rows in Figs 3, 4). There is a considerable similarity between the PC2s for individual variation and FA, and the PC3 for individual variation corresponds to the PC1 for FA (for further details and statistical tests, see Klingenberg and Zaklan 2000). Similarities between the patterns of individual variation and FA have also been reported for the wings of tsetse flies (Klingenberg and McIntyre 1998). The correspondence between the patterns of individual variation and FA suggests that a considerable part of the environmental and genetic covariation of wing parts among individuals may be based on the same direct developmental links. These developmental interactions, demonstrated by focusing exclusively on the random perturbations responsible for FA, may therefore act as conduits for variation from other sources as well.

These results are fully consistent with the notion that each wing disc is a single coherent module. Therefore the finding of integration throughout the wing validates the morphometric method for delimiting developmental modules. The analyses have confirmed the predictions (i) that there should be covariation throughout the wing and (ii) that the patterns of individual variation and FA should be similar. However, as a demonstration of the method, this case study is incomplete because it does not include a test whether FA is uncorrelated between developmentally independent parts (prediction iii).

Integration between fore- and hindwings of bumblebees

To investigate the covariation between separate modules, I report results from a study of covariation between fore- and hindwings in bumblebees (Klingenberg et al. 2001). Because the fore- and hindwings of bees, like in other holometabolous insects, develop from separate imaginal discs (Nelson 1924; Snodgrass 1956), they clearly should be separate

modules. If each wing is a coherent developmental module, as in flies, then variation should be homogeneous within each wing, and the patterns of FA and individual variation should be similar. Individual variation and FA should differ, however, in the degree of covariation between fore- and hindwings. FA should be independent between the fore- and hindwings to the degree that they are separate modules. In contrast, fore and hindwings are expected to covary among individuals due to parallel variation of developmental pathways. This expected discrepancy provides the opportunity to test the third prediction made above.

The data are from the bumblebee *Bombus empatiens*, and consist of the coordinates of 13 landmarks on the forewings and six on the hindwings, which were digitized on each body side (Fig. 5). The study includes 65 worker bees reared under normal conditions (control treatment) and 72 bees reared under elevated CO_2 concentration (CO_2 treatment). Here, I primarily concentrate on the results for the control treatment, and then briefly present the differences in the CO_2 treatment to illustrate an additional point (for a full account, see Klingenberg et al. 2001).

The PCs for the fore- and hindwings of the bees from the control treatment are shown in Figure 6. All these PCs involve coordinated shifts of landmarks throughout the entire wing. The wings do not appear to be subdivided into regions consistently associated with different PCs, which thus would appear to vary independently. Therefore, variation appears to be homogeneous across the entire wings, as is consistent with the first prediction.

There is a clear one-to-one agreement between corresponding PCs for individual variation and for FA (Fig. 6). This agreement is not only visually apparent, but also confirmed statistically, as bootstrap tests did not find significant differences between the corresponding PCs for individual variation and FA in the forewings, and only one of three comparisons in the hindwings showed a significant difference (for details, see Klingenberg et al. 2001). Therefore, there is a clear correspondence between the patterns of integration for individual variation and FA, and the second prediction is clearly fulfilled.

For individual variation, the permutation test of covariance between fore- and hindwings showed highly significant covariance of both size and shape. The sizes of the two wing pairs were strongly correlated ($r = 0.84$; $P < 0.001$). Likewise, a permutation test

showed significant covariation of shape between fore- and hindwings ($P < 0.01$). The PLS analysis shows patterns of covariance between fore- and hindwing shapes that are similar to the respective PCs for within-wing variation (cf. Figs. 6, 7). For the genetic and environmental sources of variation among individuals, the dominant features of variation within and between wing pairs therefore coincide.

The tests of between-wing covariance for FA revealed significant covariation of size, but with a substantially lower correlation ($r = 0.29$) than for the among-individual variation. For shape FA, however, the permutation test did not reject the null hypothesis of independent variation in fore- and hindwings ($P = 0.094$). Therefore, the third prediction, that FA in different modules should be independent, appears to hold for shape.

The correlated FA for size may be due to interactions between imaginal discs, for instance, by competition for some resource limiting growth (Nijhout and Emlen 1998), which can be sufficiently localized to affect left–right asymmetries (Klingenberg and Nijhout 1998). Such a process is likely to affect each imaginal disc as a whole, and is therefore not very surprising that it affects size and not shape.

The data for the CO_2 treatment suggest a possible explanation for this correlated FA. In addition to size, in the CO_2 treatment there is also correlated FA for shape (even after correction for allometric effects of size FA on shape). Moreover, the PLS1 axes for shape FA not only coincide with the PC1s of the fore- and hindwings, but also with the allometric shape component. In short, there seems to be variation between wing discs that grow better and others that do not grow that well, even beyond the extent to which this sort of “vigor” is reflected in size. A possible mechanism to transmit developmental perturbations is the system of tracheal tubes, which closely links the developing fore- and hindwings on either body side, but does not connect the two sides. Because gas exchange is likely to limit growth performance in the CO_2 treatment, it is possible that small left-right differences of CO_2 concentrations can jointly affect the fore- and hindwings on either body side, and therefore lead to coordinated asymmetries (for detailed results and discussion, see Klingenberg et al. 2001).

Overall, this study of morphometric covariation in bumblebee wings confirms the predictions for integration and asymmetry, but also raises the caveat that these predictions do not provide an automatic and fail-safe test, and need to be considered carefully in the context of the biology of the organisms under study. In this case study, most notably, the difference between the control and CO_2 treatments illustrates that there may not be clear all-or-nothing results, but that there can be degrees of independence or interdependence between distinct developmental modules.

Discussion: modularity, integration, and developmental instability

The two studies summarized above indicate that the wings of flies and bumblebees are integrated units of morphological variation, within which covariation of parts is mostly due to direct developmental interaction. In contrast, the fore- and hindwings of bumblebees are distinct modules, although the separation between them is not entirely "impermeable" and can be overcome by environmental conditions.

Studies using geometric morphometrics have found that integration is pervasive throughout the entire wings of *Drosophila* (Figs. 3, 4; Klingenberg and Zaklan 2000), tsetse flies (although one landmark is substantially more variable than the others, and therefore to some extent separate from them; Klingenberg and McIntyre 1998), and bumblebees (Fig. 6; Klingenberg et al. 2001). This is in apparent contrast to some findings from earlier studies based on correlations among distances between landmarks in *Drosophila* wings, which mostly emphasized the relative autonomy of different wing parts, and particularly the anterior and posterior compartments (Cavicchi et al. 1981; Thompson and Woodruff 1982; Cavicchi et al. 1985; Cowley and Atchley 1990; Cavicchi et al. 1991; Guerra et al. 1997; Pezzoli et al. 1997). It is possible that these differences are primarily due to the methods used, as some distances will necessarily be uncorrelated or negatively correlated if landmarks shift in opposite directions, even if their movements are tightly coordinated (see Figs. 3, 4). Similarly, it is possible that the differences to the study of Zimmerman et al. (2000), who ran separate analyses of shape measures derived from each wing cell and found no pervasive covariation between them, are also due to the differences in the methods used.

Morphological integration across the entire fly wing is in agreement with the information about the processes involved in wing development. The compartment boundary plays a particularly important role in anterior–posterior patterning (Biehs et al. 1998; de Celis 1998; Dahmann and Basler 1999; Strigini and Cohen 1999; de Celis and Barrio 2000), and is therefore itself an active center of integration. The signaling that originates from this boundary contributes to integration in the wing, but other processes, such as vein differentiation and the morphogenetic movements during the final wing expansion are also possible contributors to overall integration (Waddington 1940; Sturtevant and Bier 1995; de Celis 1998). These developmental mechanisms provide ample opportunity for transmitting variation across different parts of the wing, and covariation throughout the wing blade seems to be the almost inevitable outcome.

The patterns of individual variation and of FA were similar. This correspondence is especially apparent for the bumblebee example (Fig. 6; Klingenberg et al. 2001), but it also applies for *Drosophila* (Figs. 3, 4; Klingenberg and Zaklan 2000) and tsetse flies (Klingenberg and McIntyre 1998). This similarity suggests that similar processes may participate in the morphological expression of the random variation responsible for FA as well as of the environmental and genetic variation between individuals. For the mouse mandible, Leamy (1993) reported correlations of FA that reached similar magnitudes as those for individual variation, and a comparable degree of overall integration. In contrast, Debat et al. (2000) report substantial differences between these patterns of individual variation and FA in the dorsal aspect of the mouse skull, and conclude that different processes are involved at the two levels of variation. The reasons for the discrepancy between the results of these studies are unclear, and clearly require further study.

In the bumblebee example, the degree of integration within each wing was similar for individual variation and FA, whereas the degree of covariation of signed FA between fore- and hindwings for both size and shape was clearly lower than the covariation among individuals. For size, these between-module correlations of signed asymmetry were comparable to those recorded between segments of different legs in moths (Van Dongen et al. 1999). Similar, low to moderate correlations between the asymmetries of different

structures have been described from mouse mandibles (Leamy 1993; Leamy et al. 1997) and from proximal and distal limb bones in mice (Leamy 1984), martens and humans (Jolicoeur 1963), and several other primates (Hallgrímsson 1998). As all these correlated asymmetries occur among structures that are located close to each other, they have been referred to as a "neighborhood effect" (Leamy 1984). They may originate from phenomena such as competition among growing structures (Klingenberg and Nijhout 1998; Nijhout and Emlen 1998), or from differential use of structures on the two body sides (e.g., Trinkaus et al. 1994). In either case, these correlated asymmetries require direct interaction between the developmental processes forming the structures in question (Fig. 1), and these interactions will usually only take place over limited physical distances.

In the bumblebee example, the relatively weak correlations between fore- and hindwings are set against the strong within-wing integration. What delimits the wings as modules is not the presence of covariation within wings versus its complete absence between wings, but the greater degree of covariation within modules. This difficulty in drawing precise limits of modules is common to a variety of concepts of modularity, regardless of whether they emphasize embryonic patterning (e.g., in the context of morphogenetic fields; Gilbert et al. 1996; Raff 1996), pleiotropic gene effects on morphological traits (Cheverud 1996; Wagner and Altenberg 1996; Mezey et al. 2000), or regulatory interactions among genes (Kirschner and Gerhart 1998; von Dassow and Munro 1999). In one way or another, modularity is defined by the greater connectivity or stronger interactions within modules and contrasted to the fewer, weaker links between modules. Inevitably, this definition by the degree of interaction leads to somewhat fuzzy limits for modules (cf. von Dassow and Munro 1999, p. 312). In this regard, the morphological approach to identifying developmental modules is no different from the approach based on genetic interactions.

In the case studies presented here, these results are not surprising, as they primarily confirm what is already known from experimental studies about the development of fly wings. Therefore, the method has given the correct results in these test examples. The principal advantage of the approach, however, is that it can easily be applied to organisms for which detailed developmental information is not available. The results from such analyses

can provide considerable insight into the developmental processes underlying morphological variation.

In conclusion, the method of identifying developmental modules through analysis of correlated asymmetry is a substantial addition to the set of tools available for morphometric studies. It is the logical consequence of defining a module by the spatial extent of direct developmental interaction, and therefore provides a direct morphological equivalent to the concept of modularity based on the connectivity of gene networks (Kirschner and Gerhart 1998; von Dassow and Munro 1999).

Coda

The reasoning presented in this chapter differs fundamentally from most other contributions in this volume, as it employs developmental instability as a tool to investigate the developmental relationships between parts of organisms and to delimit developmental modules. Most previous FA studies, in contrast, have been interested in developmental stability per se, as a measure of individual quality or of environmental and genetic stress to which organisms are subjected (e.g., Møller and Swaddle 1997). Accordingly, these more traditional lines of FA research have emphasized the magnitude of left-right asymmetry and often have ignored covariances of signed asymmetries. When correlations of signed FA between traits occur (Van Dongen et al. 1999), previous studies have considered them as somewhat of a nuisance, as these developmental relations interfere with the goal of estimating organism-wide correlations in the amounts of FA, where different structures are used to provide independent estimates of FA (e.g., Polak et al., this volume; Lens and van Dongen 1999; Leung et al. 2000). Here, I have introduced just the opposite perspective, focusing on the correlations of signed FA as a tool to investigate the underlying developmental relationships. This approach opens a new direction of investigation, where a wide field of applications at the interface of evolutionary and developmental biology still awaits exploration. I am confident that this new approach will complement and enrich the study of developmental instability.

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Figures

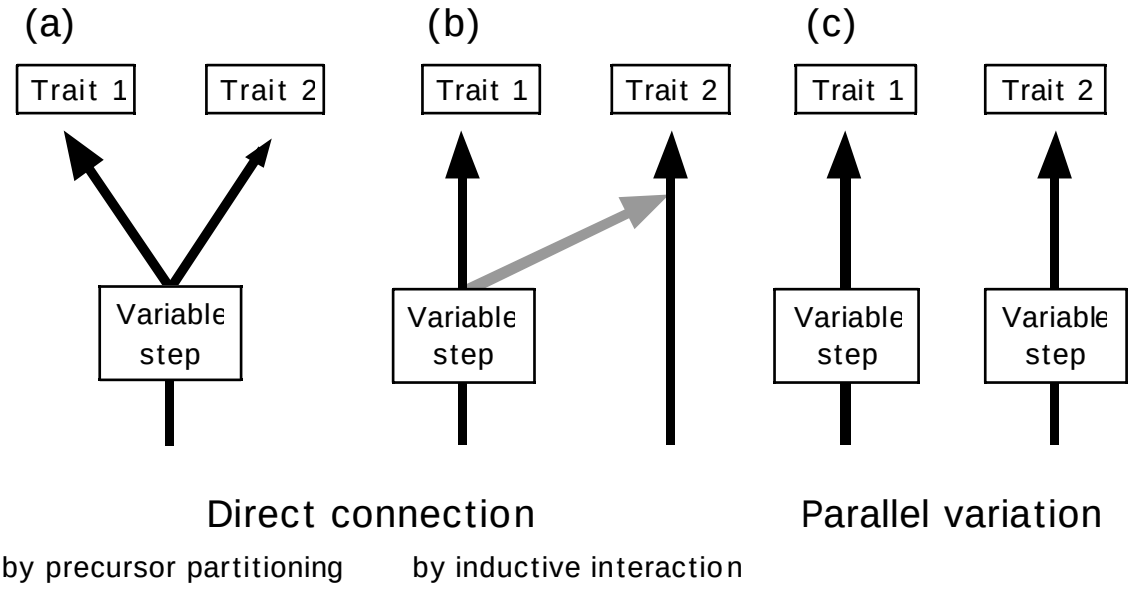

FIG. 1. Alternative origins of covariation between traits. Covariation can be due to *direct connection* between the developmental pathways that produce the traits, for instance because a developmental precursor is partitioned into two structures (a) or because there are inductive interactions between the two pathways (b). In this case, there need only be a single variable step in the developmental process, whose effects are transmitted to both traits. Alternatively, separate pathways can give rise to covariation by *parallel variation* if both pathways if both include a shared variable step, such as a gene for which there is allelic variation in the population, or sensitivity to the same environmental factor (c). In this case, each pathway must have a variable step that is subject to the same outside influence (e.g., genetic variation or environmental effect).

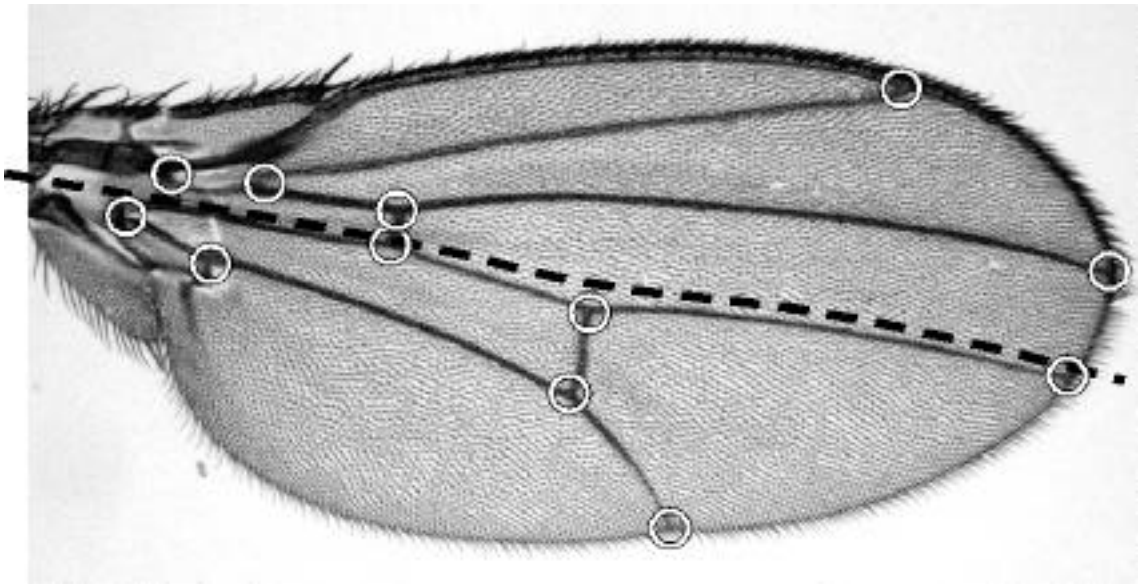

FIG. 2. A *Drosophila melanogaster* wing, with the approximate boundary between anterior and posterior compartments (dashed line) and the landmarks included in the analyses (circles).

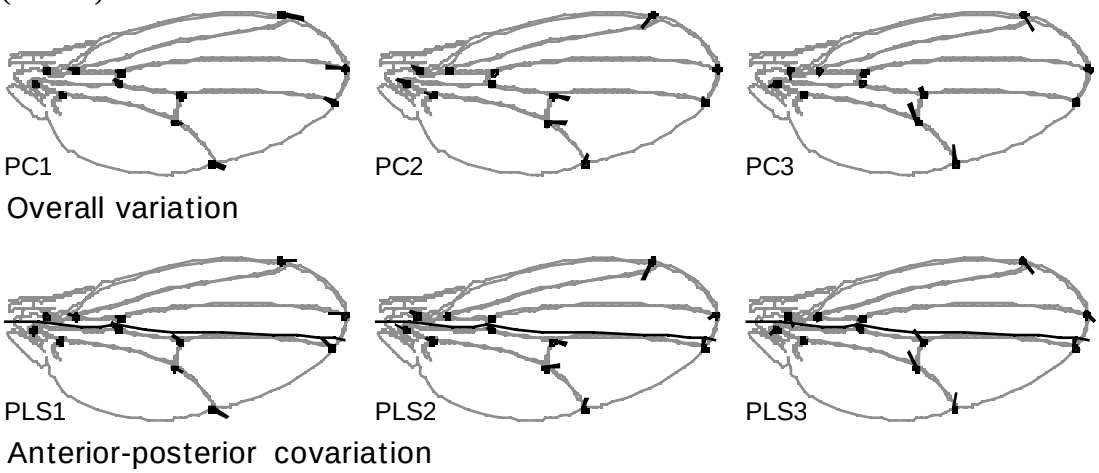

FIG. 3. Patterns of variation among individuals, as revealed by PCA of the overall variation across the entire wing (upper row) and the PLS analysis of only the covariances between anterior and posterior compartments (lower row). The dots indicate the mean landmark location, and the bold black lines point to a shape in the direction of the respective PC or PLS axis (the lengths of the lines correspond to a very large shape change of 0.1 Procrustes units). The diagrams for PLS analysis show both the PLS axes for the anterior and posterior compartments, separated by the compartment boundary (thin black line). (From Klingenberg and Zaklan 2000, © Society for the Study of Evolution.)

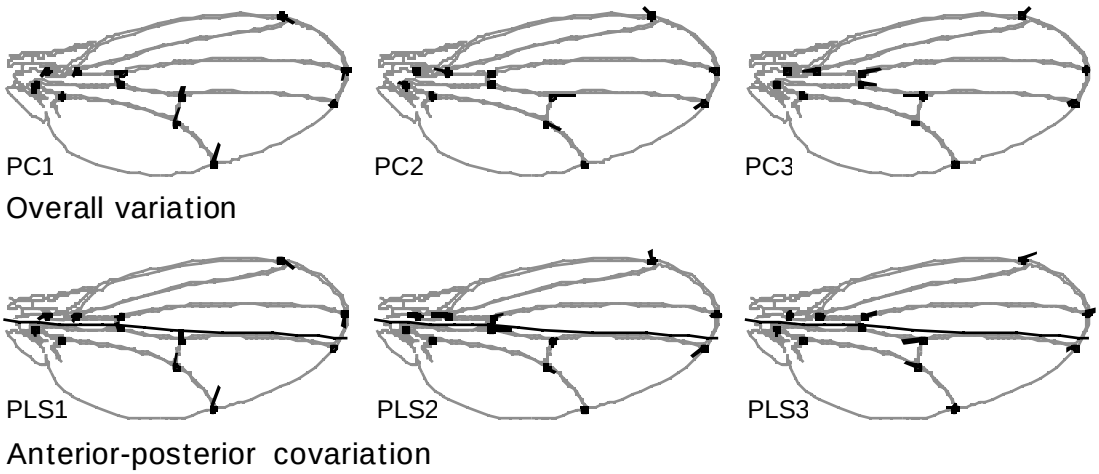

FIG. 4. Patterns of variation for FA, as obtained from PCA of asymmetry variation across the entire wing (upper row) or from PLS analysis of FA covariation between anterior and posterior compartments. For further explanation of the graphs, see Fig. 3. (From Klingenberg and Zaklan 2000, © Society for the Study of Evolution.)

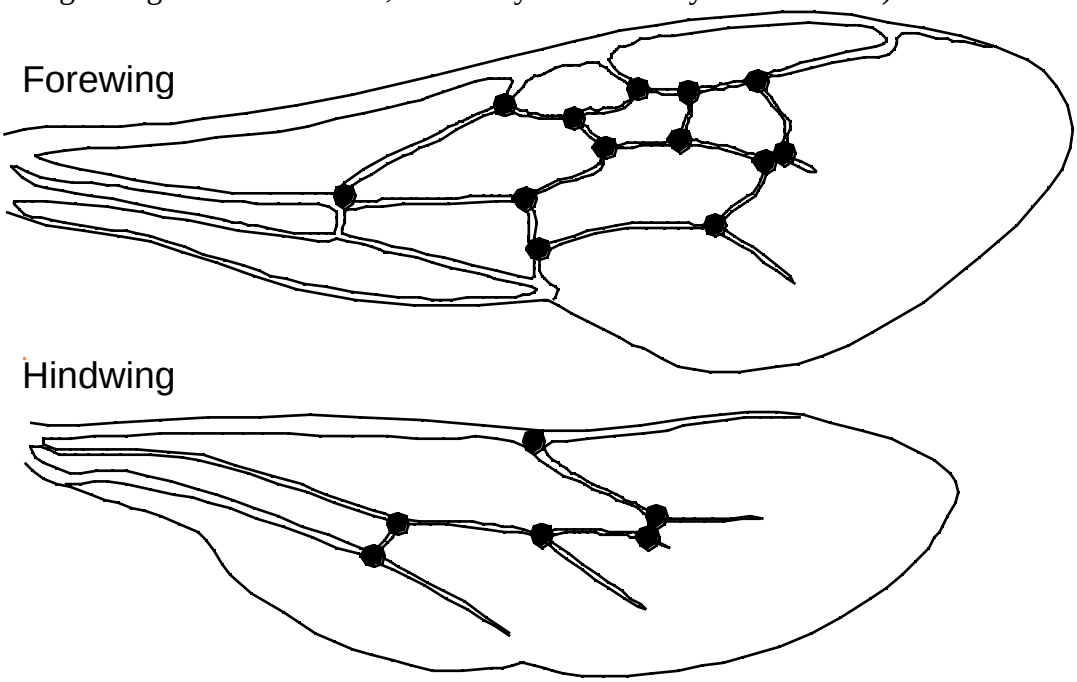

FIG. 5. Landmarks digitized on the fore- and hindwings of bumblebees (the diagrams of the two wings are not drawn to scale). (Modified from Klingenberg et al. 2001, © The University of Chicago.)

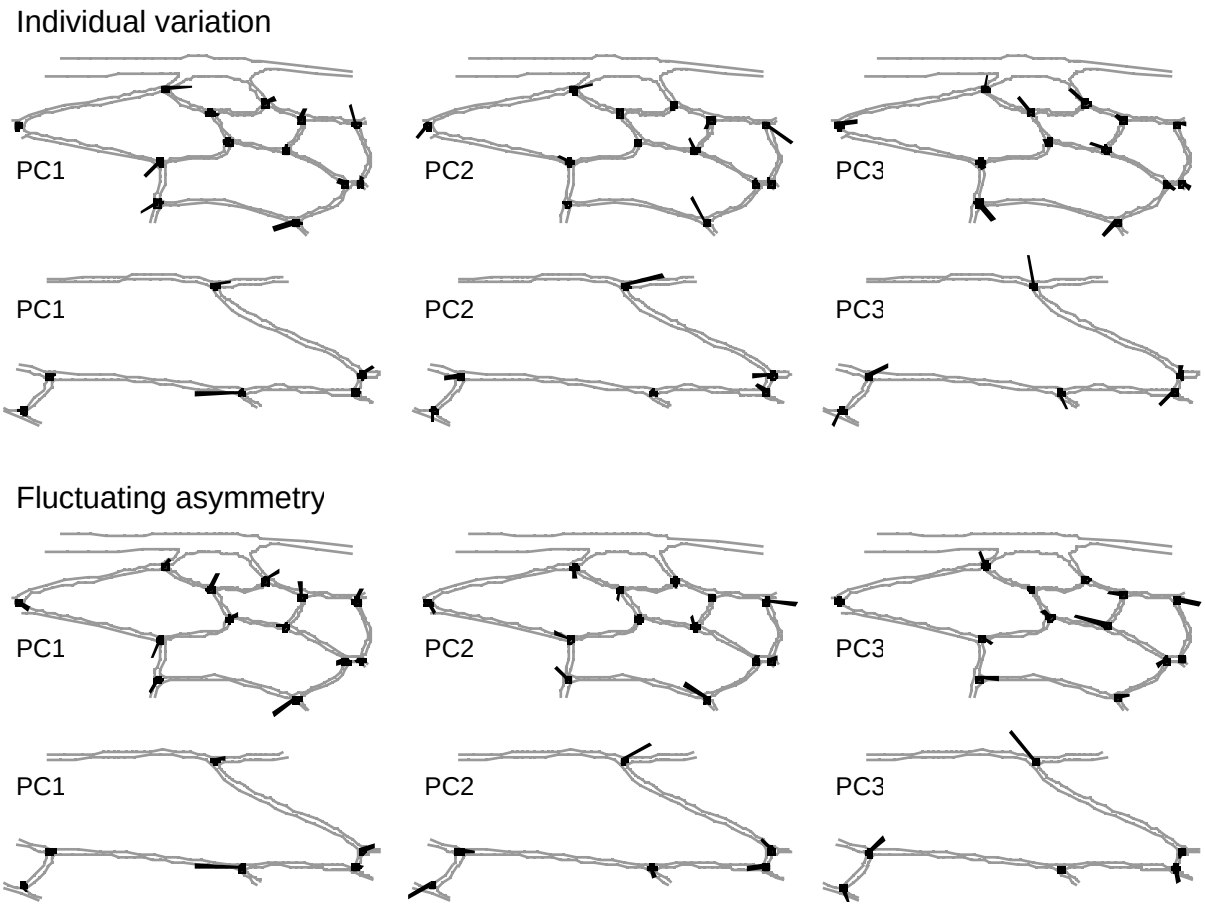

FIG. 6. PCA of variation within the fore- and hindwings of bumblebees for individual variation (upper row) and FA (lower row) in the control treatment. The dots represent the mean landmark locations, and the black lines point to a shape corresponding to a PC score of 0.15 (a very large shape change). (From Klingenberg et al. 2001, © University of Chicago Press.)

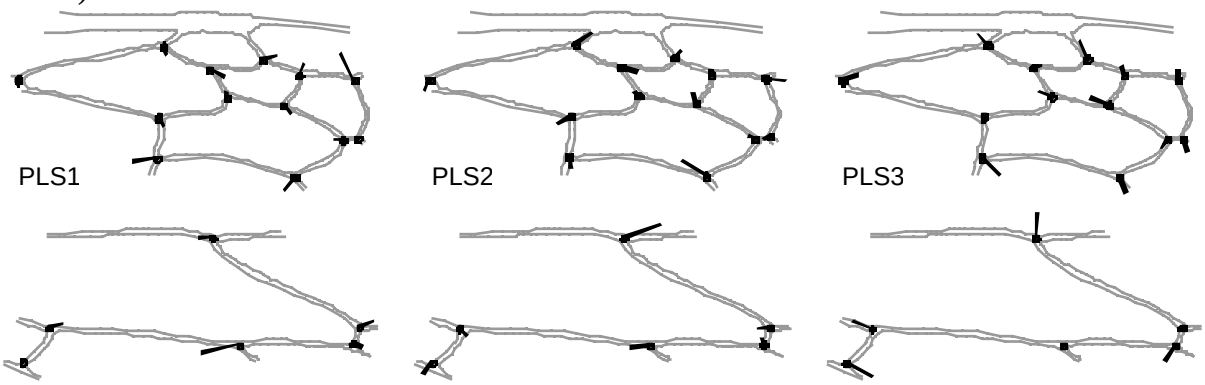

FIG. 7. PLS analysis of covariance between fore- and hindwings for among-individual variation in the control treatment. (Modified from Klingenberg et al. 2001, © The University of Chicago.)