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Drosophila wing modularity revisited through a quantitative genetic approach

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To predict the response of complex morphological structures to selection it is necessary to know how the covariation among its different parts is organized. Two key features of covariation are modularity and integration. The *Drosophila* wing is currently considered a fully integrated structure. Here, we study the patterns of integration of the *Drosophila* wing and test the hypothesis of the wing being divided into two modules along the proximo-distal axis, as suggested by developmental, biomechanical, and evolutionary evidence. To achieve these goals we perform a multilevel analysis of covariation combining the techniques of geometric morphometrics and quantitative genetics. Our results indicate that the *Drosophila* wing is indeed organized into two main modules, the wing base and the wing blade. The patterns of integration and modularity were highly concordant at the phenotypic, genetic, environmental, and developmental levels. Besides, we found that modularity at the developmental level was considerably higher than modularity at other levels, suggesting that in the *Drosophila* wing direct developmental interactions are major contributors to total phenotypic shape variation. We propose that the precise time at which covariance-generating developmental processes occur and/or the magnitude of variation that they produce favor proximo-distal, rather than anterior-posterior, modularity in the *Drosophila* wing.

KEY WORDS: ***Drosophila* wing, modularity, multilevel approach, proximo-distal axis.**

Organisms are complex cohesive systems made of several coordinated parts. In a morphological context this coordination, defined as the covariation among different traits of the same organism, is known as morphological integration (Olson and Miller 1958). Because integration is not homogeneous among all parts of an organism but is compartmentalized, a property inherently associated to integration is modularity (Wagner and Altenberg 1996; Klingenberg 2014). Thus, organisms are composed of several hierarchical and partially autonomous units called modules, which are sets of tightly integrated traits that are loosely related to traits of other such assemblies (Wagner et al. 2007; Klingenberg 2010).

Precisely knowing the patterns of integration and modularity is crucial to predict the evolutionary outcome of complex morphological structures because they can determine the adaptive peak that the species will reach (Steppan et al. 2002). Covariation, which at the genetic level arises because of pleiotropy and linkage disequilibrium (Klingenberg 2010; Wagner and Zhang 2011), is crucial in the evolutionary process because together with natural

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selection determines the direction and rate of evolution (Steppan et al. 2002; Klingenberg 2014). On the one hand, integration can deflect the response to selection toward the direction of maximum genetic variation by concentrating variation on some specific directions and limiting it in other directions (Schluter 1996; Merilä and Bjorklund 2004; Klingenberg 2014). On the other, modularity can increase evolvability by enabling changes in certain traits to happen with minimal interference with other traits (Schlosser and Wagner 2004; Hallgrímsson et al. 2009).

The wing of *Drosophila*, which is one of the most studied systems in developmental biology and quantitative genetics (de Celis 2003; Mezey and Houle 2005; Dworkin et al. 2011; Mackay 2010; Matamoro-Vidal et al. 2015), is a proper model to study the evolution and development of quantitative morphological variation. The modular organization of this complex morphological structure has been repeatedly assessed to understand the mechanisms that generate covariation and predict the response of wing shape to natural selection (Klingenberg and Zaklan 2000; Klingenberg 2009). In these studies the *Drosophila* wing has been divided into an anterior and a posterior compartment. Both compartments have been considered promising candidates for being separate developmental modules because they originate from separated cell lineages representing different domains of gene expression (García-Bellido et al. 1973). These morphological studies have failed to detect such an anterior-posterior (AP) modular organization and as result the *Drosophila* wing is usually presented as an example of a single, fully integrated structure (Klingenberg and Zaklan 2000; Klingenberg 2009).

Modularity along the proximo-distal (PD) axis of the *Drosophila* wing has not been thoroughly assessed (see Klingenberg 2009) despite accumulating evidence suggesting that some degree of autonomy may exist between different regions along this axis. At the developmental level, the PD axis of the *Drosophila* wing is determined during the larval stage by a set of genes that are expressed in nested circular domains centered in the distal region of the wing imaginal disc from where the wing everts (Terriente et al. 2008). Following these gene expression patterns the *Drosophila* adult wing is divided along the PD axis into two main regions, the wing blade and the wing base (del Álamo Rodríguez et al. 2002; Perea et al. 2009), which is also known as proximal wing (Whitworth and Russell 2003) or wing hinge (Matamoro-Vidal et al. 2015). The wing blade is defined as the area that corresponds to the distal domain of expression of the gene *vestigial* (*vg*), whereas the wing base roughly corresponds to the domain of expression of the *zinc finger homeodomain* (*zfh2*) gene (Terriente et al. 2008; Perea et al. 2009). Later in pupal development, the wing disc folds and extends in the evagination process and the wing is elongated along the PD axis (Matamoro-Vidal et al. 2015). At 15 hours after pupation the wing is evenly divided into the proximal hinge and the distal blade. To acquire the adult wing morphology, these two different cell populations are subject to different genetic regulation and mechanical forces that will lead to hinge contraction and blade elongation along the PD axis (Aigouy et al. 2010; Ray et al. 2015).

Evidence at the functional-biomechanical level also suggests that the *Drosophila* wing is regionalized along the PD axis. Though the main function of the whole wing is flight, the wing base and the wing blade differ in their precise function during flight, and this fact is mirrored in their particular structures. While the wing base is the region that transmits the forces generated by the flight muscles, the wing blade generates the aerodynamic forces that will produce the lift (Dudley 2002). Vein mass and density are highest at the wing base, where wing bending moments during flapping are greatest (Dudley 2002). Consequently, flexural stiffness is also highest at the wing base, decreasing toward the wing tip (Combes and Daniel 2005). Nevertheless it is worth mentioning that transmission and distribution of forces along the wing during flight is far from simple. For instance, a transverse flexion line occurs in *Drosophila* crossing at the base of the wing along a curved path from the costa to the posterior margin. This flexion line occurs in an otherwise stiff region allowing the wing to bend transversely downwards and it is associated with stress-reduction, with maintenance of favorable angles of attack during flight and with nonsteady aerodynamics mechanisms (Brodsky and Ivanov 1983; Wooton 1992). At the evolutionary level some degree of autonomy has also been detected between different regions along the PD axis. Several studies have shown that the latitudinal changes of wing size observed in the European and North American populations of *Drosophila subobscura* are due to continent-specific modifications of the wing along the PD axis. Whereas in Europe the size cline was due to changes in the length of the proximal part of the wing (Huey et al. 2000), in the New World the cline was due to changes in the distal part (Gilchrist et al. 2001). Thus, genetic, developmental, functional, and evolutionary evidence supports testing the hypothesis that the adult *Drosophila* wing may be composed of a proximal and distal module.

Uncovering whether the *Drosophila* wing is modular rather than fully integrated can have profound implications in understanding how the wing has evolved in a model species. This, in turn, can leverage our knowledge of the evolution of the wing shape in other species, helping us to understand how the insect wing has adapted to different environments and functions such as flight, sound-production, visual communication, crypsis, or mechanical protection (Wooton 1992). Therefore, the main purpose of this study is to test whether the wing blade and the wing base are different modules, or if, as it is currently considered, the *Drosophila* wing is a fully integrated structure. To achieve this goal, here we use a subset of lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) to perform a multilevel analysis

of morphological integration and modularity of the wing. Morphological integration arises from any process producing joint variation in multiple traits, from within-individual variation to long-term evolution (Klingenberg 2010, 2014). Because comparing integration/modularity at different levels can provide insight into the kind of processes driving morphological evolution, we take advantage of the design of this study and the DGRP sample to examine four interrelated levels of integration/modularity: static, developmental, genetic, and environmental (Klingenberg 2014). Static integration is due to the covariation among individuals of the same species and the same ontogenetic stage (Klingenberg 2014), and can be interpreted as a global phenotypic integration. Developmental integration is due to direct interactions between the developmental processes that originate different traits, and can be inferred by analyzing the patterns of covariation in fluctuating asymmetry (Klingenberg and McIntyre 1998; Debat et al. 2000; Klingenberg 2014; Martínez-Vargas et al. 2014). Developmental integration is crucial for the evolution of shape. Since genetic and environmental effects on phenotypic covariation are expressed through the developmental pathways that produce the phenotype, their role on phenotypic integration depends on the integration at the developmental level (Klingenberg 2008, 2010). The genetic and environmental levels can be obtained by combining the methods of geometric morphometrics and quantitative genetics (Klingenberg et al. 2010; Klingenberg 2014). Genetic integration/modularity determines the potential for evolutionary change of morphological structures and is due to pleiotropic effects of single loci and to linkage between loci with effects on different traits (Klingenberg 2008, 2010; Wagner and Zhang 2011). Environmental integration originates from the coordinated response of traits to different environmental factors acting on the developmental pathways (Klingenberg 2008, 2014). The simultaneous analysis of these four levels of covariation will provide deeper insight into the role that developmental, genetic, and environmental factors may play in shaping the patterns of phenotypic integration and modularity of the *Drosophila* wing, and therefore into the role that they have in the evolution of the wing shape.

Materials and Methods

SAMPLE COMPOSITION AND DATA COLLECTION

The DGRP consists of 205 inbred lines created by 20 generations of full-sibling mating of progeny of wild-caught, gravid females from a single population in Raleigh, North Carolina (Mackay et al. 2012; Huang et al. 2014). In this study, we used the subset of 40 DGRP lines that were first originally sequenced, obtained from the Mackay lab (NCSU, Raleigh, NC). Flies were raised in controlled laboratory conditions (25°C, 75% relative humidity, 12-hour light-dark cycle) until onset of the experiments in vials with cornmeal-agar-molasses medium. About 100 reproductive pairs per line were placed in egg-collecting chambers with a Petri dish containing agar 2% and commercial yeast to promote oviposition. Petri dishes were removed 8 hours later, inspected for the presence of eggs and incubated for another 24 hours to allow larval hatching. Batches of 30 first instar larvae were seeded in vials containing cornmeal-molasses agar medium and reared under the mentioned controlled laboratory conditions until emergence. To estimate the environmental component of variation four vials were set as replicates for each isogenic line. Once the larval development was completed, five adult emerged flies of each sex from each vial were randomly chosen. A total of 1600 flies (40 lines $\times$ 4 replicates $\times$ 10 specimens) were dissected and mounted on glass microscope slides for image acquisition. Since some wings were broken during the preparation process a total of 1528 (777 females, 751 males) specimens were used for shape analyses. Digital images of the two wings of each individual were obtained using a Leica MZ6 stereomicroscope equipped with a Canon Powershot S50 camera. A set of 15 landmarks covering the wing surface was digitized in each wing (Fig. 1) using the TPSdig software (Rohlf 2001). To assess measurement error, a subsample of 96 specimens was digitized three times by the same person in three separate sessions.

SHAPE ANALYSES

Shape and size information were obtained from the configurations of landmarks using geometric morphometrics methods as implemented in the MorphoJ software, version 1.06a (Klingenberg 2011). Wing size was estimated as centroid size (CS), which is defined as the square root of the sum of squared distances of each landmark to the centroid of the landmarks configuration (Dryden and Mardia 1998). Wing shape was extracted with a generalized full Procrustes fit and a projection to tangent shape space (Dryden and Mardia 1998). The Procrustes fit removes variation in the landmark coordinates that are due to size, position, and orientation. In this study, we also included appropriate reflections to account for landmark configurations from both left and right wings (Klingenberg and McIntyre 1998).

To quantify the components of variation due to the different factors of the experimental design (sex, line, environment, individual, asymmetry, and measurement error) we conducted a preliminary Procrustes analysis of variance (ANOVA) on the replicated configurations of landmarks (Klingenberg and McIntyre 1998). The Procrustes coordinates were entered as the data, the individuals as a random effect, the body side as a fixed factor, and the sex, the line, and the vial as extra factors. In this ANOVA, the individual factor stands for individual variation, the side factor for directional asymmetry, the interaction between these two factors represents fluctuating asymmetry (FA), and the variance between replicated configurations of landmarks represents measurement error (Klingenberg and McIntyre 1998).

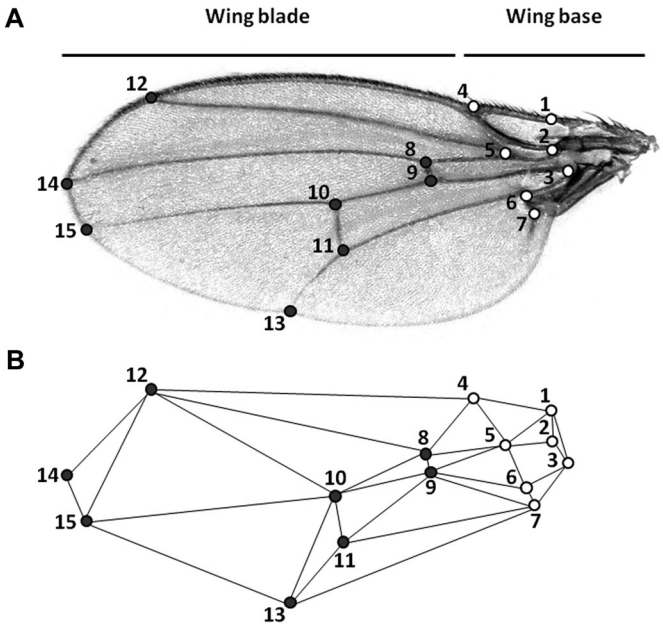

Figure 1. **Digitized landmarks and adjacency graph. (A) Layout of the landmarks digitized on each wing. The lines on the top set the limits of the wing base and the wing blade. Landmarks 1–7 (white dots) were assigned to wing base and landmarks 8–15 (gray dots) to the wing blade. (B) Adjacency graph defining spatially contiguous partitions of landmarks.**

Since measurement error in size and shape was significantly lower than variation in FA (see Results), and thus negligible, the rest of the analyses were based on a single digitization of landmarks.

For the multilevel analysis of morphological integration, we first decomposed the total shape variation into its symmetric and asymmetric components by conducting a Procrustes ANOVA on the total dataset. The symmetric component is the variation between individuals in terms of the averages of the left and right configurations and corresponds to the phenotypic variation (Klingenberg et al. 2002). The asymmetric component is the variation within individuals in terms of differences between configurations from the left and the right sides of each individual and corresponds to variation arising from direct developmental interactions (Klingenberg et al. 2002). Afterwards, principal component (PC) analyses were carried out separately for the symmetric and asymmetric components of shape to assess patterns of variation across wings (Jolliffe 2002) and to extract shape variables for the quantitative genetic analysis.

QUANTITATIVE GENETICS ANALYSES

Quantitative genetics parameters for the symmetric component of wing shape were estimated by performing a nested MANCOVA (Bégin and Roff 2004). In this analysis, the scores of the PCs with eigenvalues greater than zero were entered as wing shape variables, so that the entire dimensionality of the tangent shape space was preserved in the analysis. Sex was entered as a fixed factor, whereas Line and Vial were included as random factors. Because allometry is a strong integrating factor that tends to produce covariation throughout the entire structure and can obscure the modular organization, CS was entered as a covariate allowing correcting for size-dependent shape variation (Klingenberg et al. 2010). As flies from a given isogenic line in the DGRP have identical genotypes, the genetic, and environmental components of wing shape variation were estimated from the variation among isogenic lines and among vials of the same line, respectively (Harbison et al. 2013). The resulting covariance matrices for the random effects Line and Vial were converted from the PCs back to the original coordinate system for all further analyses, and were considered respectively as the genetic (G) and the environmental (E) covariance matrices (Klingenberg et al. 2010). To assess the patterns of genetic and environmental variation, PC analyses were performed on the G and E matrices. The eigenvectors and eigenvalues of the matrix GP^{-1}, where G and P are respectively the genetic and phenotypic covariance matrices, were computed with MorphoJ (Klingenberg 2011). The GP^{-1} matrix is considered a multivariate analog of the univariate heritability (Roff 2000), and can bring useful information on the inheritance of shape (Klingenberg and Leamy 2001). With the experimental design employed here the measure of genetic variation includes additive, but also nonadditive genetic variation, such as dominance and epistasis (Bégin et al. 2004). However, previous studies indicate that effects of dominance and epistasis are small or absent in the shape of *Drosophila* wing (Gilchrist and Partridge 2001).

The quantitative genetic parameters for the asymmetric component of wing shape were estimated by performing the same

model of nested MANCOVA performed for the symmetric component, but using the PC scores of the individual asymmetries of wing shape (i.e., the asymmetric component) as dependent variables. The 26 PCs with nonzero eigenvalues were included in the analysis. Genetic effects on asymmetry may produce differences in the average asymmetry of families and consequently it may bias the analyses of FA (Stige et al. 2006). To correct for genetic and environmental effects, the residual covariance matrix from the nested ANCOVA was computed and transformed back from the coordinate system of PC scores to that of the aligned landmark coordinates (Klingenberg et al. 2010). The converted residual covariance matrix was used to study integration and modularity in the variation produced by direct developmental interactions (Klingenberg 2008).

Similarity of covariance matrices was tested by computing pairwise matrix correlations. Matrix correlation is a measure of the overall similarity of covariance matrices and has been widely used in geometric morphometrics (e.g., Klingenberg et al. 2010). Matrix correlations were computed with and without the diagonal blocks, which correspond to the variances and covariances of the coordinates of single landmarks (Klingenberg and McIntyre 1998). Statistical significances were determined through matrix permutation tests, with 10,000 iterations, against the null hypothesis of complete dissimilarity between the covariance matrices concerned (Klingenberg and McIntyre 1998).

MODULARITY ANALYSES

Evaluations of the hypotheses of modular organization of the wing were conducted for the phenotypic (P), genetic (G), environmental (E), and developmental (FA) components of wing shape. The main hypothesis of modularity in this study is that the *Drosophila* wing is organized in two modules along the PD axis, the wing base and the wing blade. To test this hypothesis, the set of digitized landmarks was subdivided into two subsets of seven and eight landmarks, respectively (wing base: landmarks 1–7; wing blade: landmarks 8–15; Fig. 1). For comparative purposes, we also assessed the division of the wing into an anterior and a posterior compartment, the previously proposed modular scheme (Klingenberg and Zaklan 2000; Klingenberg 2009). The landmarks were subdivided into two subsets of seven and eight landmarks, respectively, corresponding to anterior (landmarks 1, 2, 4, 5, 8, 12, and 14) and posterior (landmarks 3, 6, 7, 9, 10, 11, 13, and 15) compartments (Klingenberg 2009).

The magnitude of integration between the subsets of landmarks was quantified as the RV coefficient (Escoufier 1973). To assess the hypotheses of modularity, the resulting RV coefficients were compared with the distributions of RV coefficients obtained from all the alternative subsets of landmarks. These subsets were required to include the same number of landmarks as the tested modules. By definition, subsets of landmarks resulting from a subdivision consistent with an actual modular organization are expected to show weaker covariation, and thus lower integration, than subsets not corresponding with actual modules (Klingenberg 2009). Accordingly, when the RV coefficient for the two tested subsets of landmarks was lower than 95% of the distributional values, it was considered to be statistically significant ($P < 0.05$) and the hypothesis of modularity was confirmed (Klingenberg 2009). In addition to analyses with all the alternative subsets of landmarks, we also conducted comparisons that were limited to those partitions that separated the landmarks into spatially contiguous subsets (Klingenberg 2009), that is, connected by the edges of the adjacency graph (Fig. 1B).

Recently, it has been shown that the RV coefficient can be affected by attributes of the data such as the sample size and the number of variables (Adams 2016). The covariance ratio (CR) has been proposed as an alternative to the RV coefficient for quantifying the modular structure of morphological data (Adams 2016). The CR coefficient is a ratio of the covariation between modules over the covariation within modules, and consequently it ranges between zero and positive values (Adams 2016). For random sets of variables the CR has an expected value of one. While CR values lower than one will indicate some degree of modularity within the structure, CR values higher than one will indicate greater covariation between regions than within them (Adams 2016). Thus, in addition to the RV coefficients we also calculated the CR values for the P, G, E, and FA matrices for the two hypotheses of modularity. The hypotheses of modularity were evaluated by comparing the observed CR values with permutational distributions of 999 CR values obtained by assigning the landmarks randomly to modules. The proportion of permuted values lower than the observed CR value was used as the significance of the test (Adams 2016).

Results

PHENOTYPIC, GENETIC, AND ENVIRONMENTAL VARIATION

Procrustes ANOVAs calculated on the replicated subsample revealed a significant effect of individual, sex, and line, but not of vial (environment), on wing shape (Table 1). There was also significant directional asymmetry, and FA was eight times higher than measurement error. Variation of all other effects was also higher than variation among replicates, so the effect of measurement error was considered negligible.

The PCA performed on the symmetric component of wing shape revealed that phenotypic variation is concentrated in few PCs. The first PC explained 27.5% of total shape variation and jointly with PC2 and PC3 62.5% (Fig. 2A). The shape changes associated to PC1 affected the entire structure, but the magnitude of change was larger in the landmarks from the wing blade than

Table 1. Procrustes ANOVA conducted on a replicated subsample.

Effect	SS	df	MS	*F*	*P*
Sex	0.0426	26	1.638×10^{-03}	34.06	<0.0001
Line	0.1538	78	1.972×10^{-03}	41.01	<0.0001
Vial	0.0113	312	3.636×10^{-05}	0.76	1.00
Individual	0.0988	2054	4.809×10^{-05}	2.92	<0.0001
Side	0.0072	26	2.752×10^{-04}	16.70	<0.0001
Individual × Side	0.0407	2470	1.648×10^{-05}	8.09	<0.0001
Measurement error	0.0199	9750	2.036×10^{-06}		

SS, sum of squares; df, degrees of freedom; MS, mean squares; *F*, *F* statistic; *P*, *P*-value.

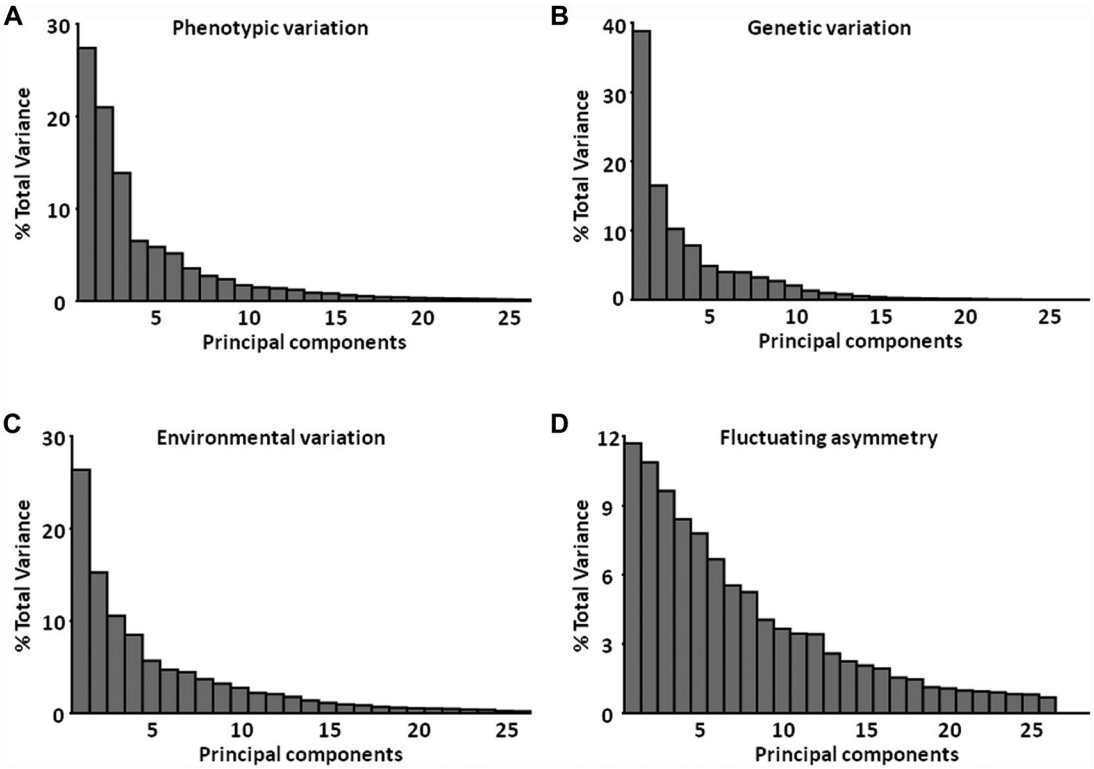

Figure 2. Percentage of total variation explained by each principal component obtained in the PCAs performed on the phenotypic (A), genetic (B), environmental (C), and fluctuating asymmetry (D) covariance matrices.

in those from the base (Fig. 3A). The PC2 involved changes in different regions of the wing, but especially in the intersections of the second and fifth longitudinal veins (L2 and L5) with the wing margin (landmarks 12 and 13, respectively). These shape changes were associated to sex differentiation, with males having broader wings than females (Additional File 1).

The genetic variation of wing shape was more concentrated in the first PC than phenotypic variation, with PC1 explaining almost 40% of the genetic variation (Fig. 2B). However, the cumulative percentage explained by the first three PCs (65.8%) was very similar to the 62.5% observed for the phenotypic variation. The shape changes associated with the first two PCs of the genetic and phenotypic covariance matrices were similar, especially those associated with PC1 (Fig. 3B). These observations were corroborated by the high correlation scores between the phenotypic and the genetic covariance matrices, both including (0.92, $P < 0.001$) and excluding (0.89, $P < 0.001$) the diagonal blocks.

The environmental variation of wing shape was distributed over PCs in a similar way as it was distributed in the phenotypic and genetic covariance matrices (Fig. 2C). However, the percentage of variation explained by the first three PCs was lower (52.4%), mainly because PC2 and PC3 in the environmental covariance matrix explained lower percentages of variation than corresponding PCs in the phenotypic covariance matrix. While the shape changes associated to PC1 were very similar to those observed in the PC1 of the phenotypic and genetic covariance

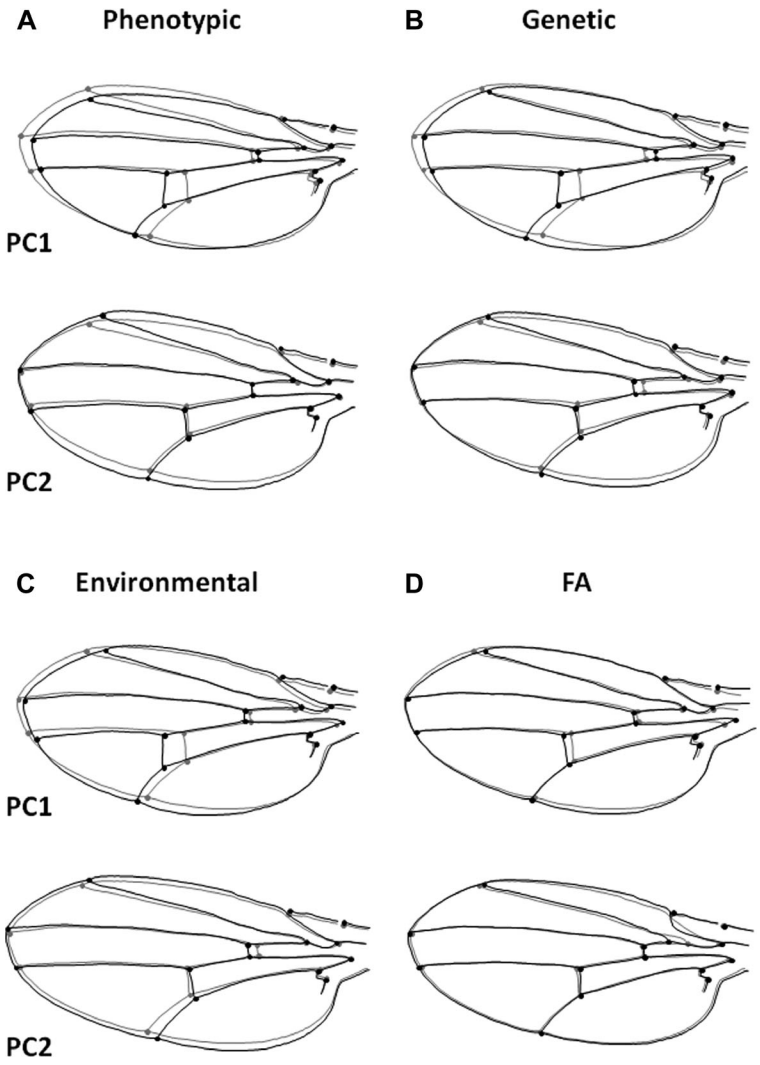

Figure 3. Shape changes associated with PC1 and PC2. Gray outlines and open circles represent the consensus configuration, and black outlines and circles represent a shape change of 0.1 units of Procrustes distance in the positive direction along the respective PC axis.

matrices, shape changes of PC2 differed slightly (Fig. 3C). The values of matrix correlation between phenotypic and environmental variation were high, but a noticeable drop was detected when the diagonal blocks were excluded (diagonal blocks included: 0.89, $P < 0.001$; diagonal blocks excluded: 0.78, $P < 0.001$). The values of matrix correlation between genetic and environmental covariance matrices were slightly lower than in the former case (diagonal blocks included: 0.84, $P < 0.001$; diagonal blocks excluded: 0.72, $P < 0.001$).

The eigenvalues of the matrix GP^{-1} decreased gradually from 0.77 to 0.007 (Fig. 4A). These values can be interpreted as heritabilities of particular shape changes represented as linear combinations of the landmark coordinates (eigenvectors). The shape changes with the maximal heritability (Fig. 4B), that is those that correspond to the first eigenvalue, were very similar to the shape changes associated to PC1 of the phenotypic covariance matrix. The eigenvector with the minimal heritability, that is the most constrained at the genetic level, involved a broadening of the wing blade, a proximal displacement of cross-veins, and a shortening of the proximal wing (Fig. 4C).

FLUCTUATING ASYMMETRY AND DEVELOPMENTAL VARIATION

The PCA performed on the covariance matrix for FA indicated that variation in FA decreased gradually from PC1 to PC26 (Fig. 2D), with no sharp decreases after the few first PCs, as shown before (Fig. 2A–C). The shape changes associated to PC1 were similar to those observed in the phenotypic, genetic, and environmental matrices, but with apparent differences in the displacement of particular landmarks (landmarks 3, 4, 6, 7, 8, 9, and 12). The shape changes associated to PC2 differed to those of the phenotypic, genetic, and environmental matrices. These results were

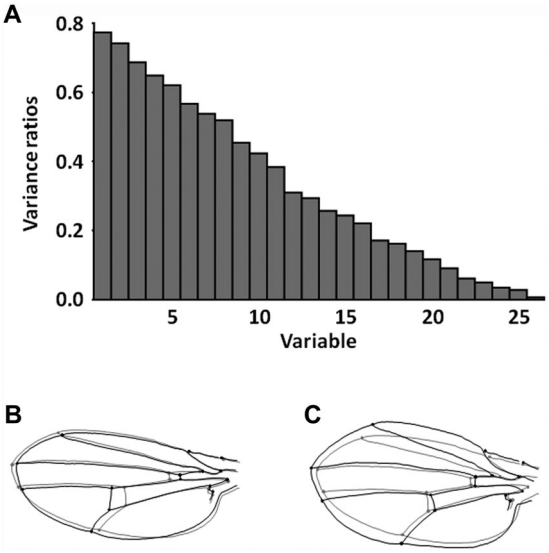

Figure 4. Results obtained in the GP^{-1}analysis. (A) Eigenvalues of the GP^{-1} matrix associated with the respective eigenvectors. (B) The shape change associated with the largest eigenvalue. (C) The shape change associated with the smallest eigenvalue.

corroborated by the values of matrix correlation. When the diagonal blocks were included matrix correlations were moderately high (FA-Phenotypic: 0.71, $P < 0.001$; FA-Genetic: 0.62, $P < 0.001$; FA-Environmental: 0.76, $P < 0.001$), but these values decreased when the diagonal blocks were excluded (FA-Phenotypic: 0.44, $P < 0.001$; FA-Genetic: 0.54, $P < 0.001$; FA-Environmental: 0.51, $P < 0.001$).

MODULARITY

The RV coefficients for the hypothesis of PD modularity were significant for all the components of shape variation (Table 2). Moreover, all the CR values for this hypothesis of modularity were significantly lower than one, except for the P matrix, which was lower than one but marginally nonsignificant (Table 2). The RV coefficients obtained for the hypothesis of AP modularity were between 1.5 and 2.0 times higher than those obtained for the PD hypothesis, and in no case were significant (Table 2). Similarly, all the CR values for the hypothesis of AP modularity were higher than one and between 1.3 and 1.7 times higher than those obtained for the PD hypothesis (Table 2).

Discussion

The results of our study reveal for the first time that the *Drosophila* wing is not a single, fully integrated structure. Our analyses indicate that shape variation is not completely coordinated across the entire wing, but it is structured to some extent into two modules that match two main regions of gene expression along the PD axis, the wing base and the wing blade (del Álamo Rodríguez et al. 2002; Perea et al. 2009). The multilevel approach allowed us to uncover that the compartmentalization of the wing shape variation occurs at the phenotypic, genetic, environmental, and developmental levels. In contrast, the partition of the wing into two modules along the AP axis was not supported in any case, confirming the results obtained in previous studies (Klingenberg and Zaklan 2000; Klingenberg 2009). The possible causes of the prevalence of the PD over the AP axis in the patterning of wing shape covariation are discussed below in a functional and developmental context.

Our analysis highlight that genetic variation (variation among isogenic lines) is the most important component of wing shape variation and that all the dimensions of the shape space have genetic variation, that is all the eigenvalues of the matrix GP^{-1} were greater than zero (Fig. 4A). These results are concordant with previous studies suggesting that in *D. melanogaster* no absolute constraint prevents the wing shape to vary (Mezey and Houle 2005; Debat et al. 2009). However, it should be noted that the eigenvalues of the matrix GP^{-1} differed considerably among PCs, indicating that the potential for evolutionary change in response to selection depends substantially on the specific shape variable. For instance, the low genetic variation associated to the smallest eigenvector of the GP^{-1} matrix (Fig. 4A) suggests that the corresponding shape changes entailing a marked change in the aspect ratio of the wing are highly constrained (Fig. 4C). Indeed, the length to width ratio is traditionally used as one of the principal measures of functional variation in wing shape (Dudley 2002; Ray et al. 2016); high AR values correspond to long and slender wings that promote slow and agile flight (manoeuvrability), whereas low AR values correspond to short and broad wings that promote fast and long distance flight (Hassall 2015; Ray et al. 2016). We conclude that in *D. melanogaster* the low genetic variation of the shape changes associated to the smallest eigenvalue might be due to stabilizing selection on wing aspect ratio resulting from a trade-off between flight duration/efficiency and manoeuvrability. If a trade-off exists between both functional capacities, covariation between these two measures of performance should be negative (Arnold 1983), as already demonstrated for the *Drosophila* wing: changes in the AR have shown to have opposite effects on several variables of flight performance characterizing the two functional capacities (Ray et al. 2016).

The patterns of integration at the phenotypic, genetic, and environmental levels were very similar, reflecting that both genetic and environmental effects contribute to phenotypic variation. The distribution of variance across the PCs, the shape changes associated to particular PCs, and the results of the modularity tests were highly coincident at the three levels, which indicate a high degree of congruence among the covariance matrices. This congruence was further confirmed by the high values of matrix correlation obtained in all comparisons. That genetic and phenotypic

Table 2. RV and CR coefficients and their associated *P*-values for the two modular hypotheses in the phenotypic (P), genetic (G), environmental (E), and fluctuating asymmetry (FA) matrices.

Hypothesis	P matrix	G matrix	E matrix	FA
Proximo-distal	RV = 0.256	RV = 0.357	RV = 0.220	RV = 0.081
	$P_{All} = 0.018$	$P_{All} = 0.016$	$P_{All} < 0.001$	$P_{All} < 0.001$
	$P_{cont} = 0.024$	$P_{cont} = 0.028$	$P_{cont} < 0.001$	$P_{cont} = 0.000$
	CR = 0.907	CR = 0.812	CR = 0.749	CR = 0.627
	$P = 0.106$	$P = 0.008$	$P = 0.007$	$P < 0.001$
Antero-posterior	RV = 0.411	RV = 0.535	RV = 0.392	RV = 0.161
	$P_{All} = 0.510$	$P_{All} = 0.286$	$P_{All} = 0.316$	$P_{All} = 0.570$
	$P_{cont} = 0.534$	$P_{cont} = 0.351$	$P_{cont} = 0.369$	$P_{cont} = 0.687$
	CR = 1.194	CR = 1.138	CR = 1.114	CR = 1.049
	$P = 0.742$	$P = 0.522$	$P = 0.620$	$P = 0.492$

All, proportion of all alternative subsets of landmarks with an RV coefficient lower than the observed; cont, proportion of contiguous subsets of landmarks with an RV coefficient lower than the observed.

correlations tend to be similar has been long observed (Cheverud 1988; Roff 1995; Waitt and Levin 1998; Kominakis 2003; Sheldon et al. 2003) and P has been proposed as a surrogate of G because in natural populations phenotypic correlations can be estimated much more accurately than genetic correlations (Cheverud 1988; Steppan et al. 2002). However, to substitute G by P without significantly affecting the results of subsequent analyses, both matrices should be proportional, which implies that the eigenvalues of the matrix GP^{-1} are all equal (Klingenberg 2003; Klingenberg and Monteiro 2005). In our study, the eigenvalues of the GP^{-1} matrix differed considerably, indicating that G and P are not proportional despite being very similar. These results add to those in other studies indicating that care should be taken when using P as a substitute of G (e.g., Willis et al. 1991; Hadfield et al. 2007; Klingenberg et al. 2010; Martínez-Abadías et al. 2012).

The covariance matrix of FA was considerably similar to that of the P, G, and E matrices, despite a different pattern of variance distribution across the PCs was observed. Covariation in FA originates from direct interactions among developmental pathways that generate the phenotypic traits (Klingenberg 2008). Genetic and environmental effects on phenotypic covariation are expressed through these developmental pathways. Therefore, it is expected that the arrangement of the developmental processes producing covariation moulds to some extent the morphological expression of covariation at the remaining levels (Klingenberg 2008, 2010). The similarity of the G, P, and E matrices to the covariance matrix for fluctuating asymmetry detected in this study indicates that direct developmental interactions have a central role in patterning the expression of wing shape variation at the genetic and phenotypic levels. These results are consistent with those obtained in previous studies of wing shape in *Drosophila* and other insect species (Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000; Breuker et al. 2006; Klingenberg et al. 2010). However, the moderate matrix correlation values observed in the comparison of the covariance matrix for FA and P, G, and E matrices indicate that other factors, such as selection, may also contribute to some extent in patterning the expression of wing shape variation (Klingenberg et al. 2010).

Our analyses of modularity indicate that the wing is divided in two modules along the PD axis, the wing base and the wing blade. We have not found previous evidence that validate testing the division of the *Drosophila* wing into three or more modules. However, since modules are hierarchically nested units (Wagner et al. 2007) the existence of two main modules does not discard that such modules could be further subdivided. For instance, in other species, such as the nonmimetic moth, *Thyas juno*, the wing is organized in a set of four modules along the proximo-distal (PD) axis that regularly correspond to the Nymphalid ground plan elements (Suzuki 2013).

In the *Drosophila* wing the hypothesis of PD modularity was confirmed at all levels of variation. However, the RV values suggested a considerable amount of covariation between both modules, especially at the phenotypic and genetic level (Table 2). Besides, the permutational distributions of RV coefficients (not shown) indicated a considerable amount of covariation across the wing. Modularity is not an all or nothing phenomenon (Klingenberg et al. 2003). Thus, the existence of modularity does not mean that no covariation exists between modules, but that covariation within them is significantly stronger than between them. In our study, the substantial amount of variation accumulated by the few first PCs suggests a considerable degree of integration (Klingenberg 2013). However, the shape changes associated to these PCs mainly affect the wing blade, which is consistent with previous studies (Klingenberg and Zaklan 2000; Debat et al. 2003). This fact could explain in part the detected pattern of

modularity, because while an important part of variation is coordinated, it mainly involves landmarks from one of the compartments. The RV and the CR coefficients of the FA matrix were considerably lower than the RV and CR coefficients of the P, G, and E matrices. Since genetic and environmental factors producing phenotypic covariation act simultaneously during development, these results suggest that the strong modular organization of the developmental covariation along the PD axis could be promoting modularity at the remaining levels. These results are coincident with those obtained in the matrix correlations and further support the hypothesis that direct interactions in developmental pathways have a central role in patterning the expression of wing shape variation at the genetic and phenotypic levels in the *Drosophila* wing.

The *Drosophila* wing has long been considered a fully integrated structure because the anterior and posterior compartments have proved to be integrated (Klingenberg and Zaklan 2000; Klingenberg 2009). The analyses of modularity performed in this study confirm that compartments along the AP axis are not modules, but in contrast they underscore that covariation is compartmentalized along the PD axis. Why covariation in the *Drosophila* wing is compartmentalized along the PD axis and not along the AP axis? The insect wing is an articulated structure organized around a hinge that shows obvious signs of polarity along the PD axis (Whitworth and Russell 2003). Its development is a complex arrangement of processes that starts with the formation of the wing disc and finishes when the imago emerges from the pupae producing important morphogenetic changes along AP and PD axes (Matamoro-Vidal et al. 2015). These processes may overlap in time and space in such a way that the effect in the covariance structure of some of them may obscure the effect of others (Hallgrímsson et al. 2009). In this context, our results suggest that the precise order of the developmental processes generating covariance in the adult wing and/or the magnitude of covariation that they produce favor PD, rather than AP, modularity. In one of the final steps, during late pupal development, the shape of the wing is profoundly altered to acquire the adult morphology. During this stage the wing hinge contracts to approximately half its initial area, while the wing blade becomes more elongated along the PD axis (Aigouy et al. 2010). Such an important process in a final step of the development could have a profound impact on the covariance structure of the adult wing shape, and could be partly responsible of PD modularity in a structure that shows obvious signs of overall integration. To assess the effect of particular ontogenetic processes, such as wing expansion, in the patterns of morphological integration of the wing, studies assessing the patterns of integration/modularity at different ontogenetic stages are needed.

Some authors have argued that developmental systems may evolve adaptively so that patterns of developmental and functional integration should match (Cheverud 1984; Wagner and Altenberg 1996). This hypothesis has been called the matching hypothesis (Klingenberg et al. 2010). In contrast, other authors argue that developmental modules are evolutionarily conservative and act as constraints on adaptive evolution (Raff 1996; Arthur 2001). The main function of the *Drosophila* wing is flight. However, during flight the wing base and the wing blade have different functions. While the wing base is the region that transmits the forces generated by the flight muscles, the wing blade generates the aerodynamic forces that will produce the lift (Dudley 2002). Since the patterns of developmental and functional integration coincide our results would support the matching hypothesis. However, flight is a complex function and different regions of the wing may develop very precise roles during this process (Dudley 2002).

Our study furthers our understanding of the *Drosophila* wing and can enhance future research about the relationship between the patterns of integration/modularity and function in the *Drosophila* wing. By revealing that the *Drosophila* wing is organized in two modules along the PD axis, the wing base and the wing blade, and that these modules occur at the phenotypic, genetic, environmental, and developmental levels, our results indicate this compartmental organization should be taken into account when assessing the effect of localized shape changes on flight performance and fitness. The effect of selection on the different wing compartments and their patterns of morphological integration and modularity should also be considered to better understand the evolutionary biology of the wing shape.

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