



Morphological integration and modularity in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) hind wings

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ABSTRACT

The morphological integration of the hind wings of the western corn rootworm *Diabrotica virgifera virgifera* LeConte was investigated to get a better insight of the undergone by this invasive species. Geometric morphometric methods were used to test two modularity hypotheses associated with the wing development and function (hypothesis H1: anterior/posterior or H2: distal/proximal wing parts). Both hypotheses were rejected and the results showed the integrated behavior of the hind wings of *D. v. virgifera*. The hypothesized modules do not represent separate units of variation, so in a similar fashion as exhibited by the model species *Drosophila melanogaster*, the hind wings of *D. v. virgifera* act as a single functional unit. The moderate covariation strength found between anterior and posterior and distal and proximal parts of the hind wing of *D. v. virgifera* confirms its integrated behavior. We conclude that the wing shape shows internal integration, which could enable flexibility and thus enhance flight maneuverability. This study contributes to the understanding of morphological integration and modularity on a non-model organism. Additionally, these findings lay the groundwork for future flight performance and biogeographical studies on how wing shape and size vary across the endemic and expanded/invaded range in the USA and Europe infested with *D. v. virgifera*.

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1. Introduction

Morphological integration (Olson and Miller, 1958; Klingenberg et al., 2001) focuses on the connection between or among an organism's morphological traits, related functionally, developmentally, genetic and/or evolutionary. It has been quantified applying several metrics (Cheverud, 1982; Pigliucci and Preston, 2004; Wagner and Mezey, 2004), which enable the estimation of the covariation level present among the sets of traits in question (Klingenberg et al., 2001, 2012; Klingenberg, 2008, 2009; Klingenberg and Marugán-Lobon, 2013). On the other hand, modularity deals with the integrated morphological traits as “modules”, units or set of traits, that are internally coherent by tight integration among their parts, but which are relatively independent of other of those

units (Wagner, 1996; Wagner and Mezey, 2004; Klingenberg, 2004, 2008, 2009; Martinez-Abadias et al., 2012; Klingenberg et al., 2012). The combined concepts of modularity and integration focus on quantifying the relative differences in the level of integration of parts within and between sets of an organism's traits and therefore can be applied to address important evolutionary questions (Wagner and Mezey, 2004; Mitteroecker and Bookstein, 2007; Zelditch et al., 2008; Martinez-Abadias et al., 2012; Klingenberg and Marugán-Lobon, 2013). The morphometric identification of modules through hypothesis testing is essential in the study and analysis of integration (Klingenberg and Zaklan, 2000; Klingenberg et al., 2001; Goswami, 2006; Drake and Klingenberg, 2010; Jammniczky and Hallgrímsson, 2011; Martinez-Abadias et al., 2012; Klingenberg and Marugán-Lobon, 2013; Klingenberg, 2013). Geometric morphometric techniques are now routinely used to study morphological integration and have been used to answer questions related to the developmental basis of morphological changes such as wing morphology and evolution in insects (Klingenberg et al., 2001; Klingenberg, 2004, 2008, 2009, 2013; Mitteroecker and Bookstein, 2007). Different analyses have

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been used to investigate and display the patterns of integration in winged insects (Klingenberg and Zaklan, 2000; Klingenberg et al., 2001; Klingenberg, 2009).

Wing morphology has been used extensively as a model trait, predominantly in *Drosophila melanogaster*, and especially due to extensive knowledge of the genetics and developmental processes in this species (Palsson and Gibson, 2000; Bai et al., 2012). Particular studies of integration and modularity have shown that the anterior and posterior wing compartments could be considered as separate developmental units, under the control of two groups of genes during development (Cavicchi et al., 1991; Pezzoli et al., 1997; Klingenberg and Zaklan, 2000; Klingenberg, 2009). This organism has been also used as a model species for combined analyses of individual variation and fluctuating asymmetry, thus showing the strength of integration throughout the wings. This phenomenon emerges because bilaterally symmetric traits shared the same genetic basis and developmental pathways, hence developing under the same, or relatively similar environmental conditions (Klingenberg and Zaklan, 2000; Debat et al., 2003; Klingenberg, 2004, 2008, 2009). Based on this background, we have applied a similar approach to another insect species: *Diabrotica virgifera virgifera* LeConte. Bilateral symmetries (i.e. matching symmetry) in left and right hind wings were analyzed in order to infer integration and modularity in this species.

By correlating the left/right asymmetries in two different traits (e.g. wings) it is possible to determine if the same disturbance has affected both traits, thus reflecting the integration patterns existing between the left/right sides of the body, and could outcome as fluctuating asymmetry (FA). The latter describes bilateral differences found between corporal traits (e.g. left and right hind wings, elytra, eyes, etc.) originated from stochastic perturbations in the developmental process of an organism (Klingenberg and Zaklan, 2000; Debat et al., 2003; Klingenberg, 2004, 2005). Therefore, FA allows a rough estimation of the effects of both the environment and genetic influences on bilateral traits (Klingenberg, 2002, 2004, 2005).

FA can be used to infer developmental interactions among morphological traits of organisms such as hind wing shape and size in insects (Debat et al., 2003; Klingenberg, 2009). Morphological integration of the insect wings is important because it can shed light on which evolutionary processes drive this phenomenon. Therefore, FA allows the direct analysis of the intrinsic and developmental components of integration within the organism's trait under research (e.g. wings) (Klingenberg and Zaklan, 2000; Debat et al., 2003; Klingenberg, 2004, 2005).

The western corn rootworm *D. v. virgifera* is one of the most important maize pests in North America and Europe (Bača and Berger, 1994; EPPO, 2012). While there have been numerous ecological (Boriani et al., 2006; Ivezić et al., 2009; Szalai et al., 2010), and population genetic studies (Kim and Sappington, 2005; Miller et al., 2005; Ciosi et al., 2008, 2010; Bermond et al., 2012; Lemic et al., 2013) of *D. v. virgifera* in the USA and Europe, only few studies have applied traditional (Li et al., 2009, 2010) or geometric morphometric (Mikac et al., 2013; Benítez et al., 2014; Lemic et al., 2014) methods to investigate how this species has changed morphologically in its invaded range. *D. v. virgifera* is a highly mobile species that undertakes the majority of its dispersal and movement via long distance flight (Coats et al., 1986; Grant and Seevers, 1989). Hence, it has been recommended that geometric morphometrics could be useful to understand how *D. v. virgifera* hind wing shape and size have changed during its invasion of North America and Europe (Mikac et al., 2013). Different studies on insect flight aerodynamics have shown the importance of wing venation patterns on wing flexibility and its effect on aerodynamic forces (Combes and Daniel, 2003; Nakata and Liu, 2011; Bai et al., 2012; Mountcastle and Combers, 2013; Le et al., 2013), which are presumably

important to highly mobile species such as *D. v. virgifera*. However, this information is currently unknown for this species.

An important starting point would be to apply the hypotheses tested in *D. melanogaster* (Klingenberg and Zaklan, 2000) but expanding their scope to take into account the functional significance of wing venation on aerodynamic force production, especially when considering that *D. v. virgifera* is a highly mobile species. Therefore, the aim of this study was to identify the morphological integration or modularity patterns present in the hind wings of *D. v. virgifera* as an initial step to get a better insight about of the evolutionary processes experienced by this invasive species. This was carried out by examining patterns of individual variation against the FA patterns, thus allowing the study of the developmental role generating the variation observed among specimens. Here we have tested two modularity hypotheses:

H1. The anterior and posterior wing compartments are separate modules

H2. The distal and proximal wing compartments are separate modules

H1 was defined based on the developmental evidence that shows that wing forms as two distinct compartments depending on the relative amount and expression timing of different morphogens (Cavicchi et al., 1991; Pezzoli et al., 1997). Research carried out on beetles has shown that their wing develops from different patterning genes of the imaginal discs that are expressed differentially between the anterior and the posterior compartments (e.g., Tomoyasu et al., 2009). On the other hand, H2 was proposed due to previous studies that have indicated that the insect wing behaves differently in a distal–proximal axis during flight in order to achieve an appropriate flight maneuverability (e.g. Combes and Daniel, 2003; Nakata and Liu, 2011; Mountcastle and Combers, 2013; Le et al., 2013).

This research provides an investigation of morphological integration and modularity using *D. v. virgifera* hind wing shape as a model system. Hence its importance lies in the incorporation of a non-model organism to study relevant phenomena associated with morphological evolution, such as integration and modularity. This is not trivial, because some authors have argued the model organisms are biasing contemporary biological research, making difficult to study any sort of biological research on organisms not standardized as model organisms (Gilbert, 2009; Ankeny and Leonelli, 2011). Furthermore, model organisms offer an extremely restricted perspective to analyze the existing natural variation in an evolutionary context.

2. Materials and methods

2.1. Data collection and shape analysis

334 adults of *D. v. virgifera* were collected in July 2011 from maize plants in Croatia. All specimens were processed as described in Mikac et al. (2013) and Benítez et al. (2014).

Fourteen 2D 'type 1 landmarks' defined by vein junctions or vein terminations were used to carry out the geometric morphometric analyses (Fig. 1) (Supplementary Table 1) (Leschen et al., 2010).

Landmarks were digitized using the tpsDIG v2.12 software (Rohlf, 2008). Once the Cartesian coordinates were obtained for all the landmarks, the shape information was extracted by performing a generalized Procrustes analysis (GPA) (Rohlf and Slice, 1990; Dryden and Mardia, 1998), using the method developed by Klingenberg and McIntyre (1998), which takes into account the symmetry of the structure. In the present study, the structure under research presented matching symmetry, which means that it exists as a pair of separate copies (i.e. mirror images of each

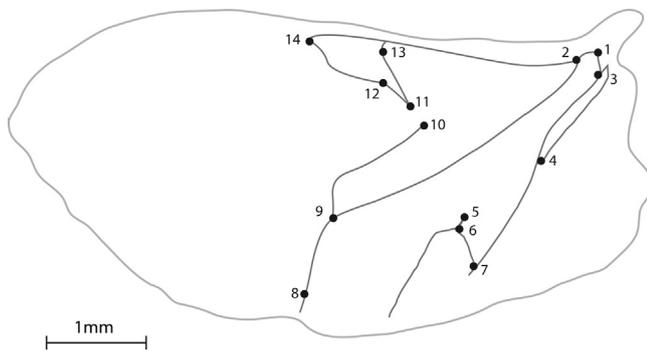


Fig. 1. Representation of the 14 morphological landmarks identified in the hind wings of *D. v. virgifera*.

other) (Klingenberg et al., 2002). In order to analyze a shape exhibiting bilateral symmetry, it is necessary to distinguish the left–right variation among individuals to the left–right asymmetries within individuals. Hence, the analysis of a symmetrical structure proceeds following several steps: initially all the configurations of one side are reflected to their mirror images, then all configurations are superimposed simultaneously using the Procrustes method. Symmetric variation and asymmetry component are then obtained from the averages and contrasts of the original and the reflected configurations (Klingenberg et al., 2002). Moreover, because of the critical importance of measurement error (ME) when analyzing asymmetries (for details see Klingenberg and McIntyre, 1998), the left and right wings of 30 specimens were digitized twice.

2.2. Morphological integration and modularity

Allometry is a key factor for both integration and modularity (Cock, 1966; Gould, 1966), therefore, allometric effects were assessed by performing a multivariate regression of shape on centroid size, pooling the dataset by sex and location of the symmetric and asymmetric component and then the covariance matrix of the residuals pooled by sex and location was computed to perform the following analyses.

Individual variation and fluctuating asymmetry (FA) were analyzed using a principal component analysis (PCA). The pattern of morphological integration between the anterior/posterior and the distal/proximal components of the wing was examined based on the variation patterns of the covariance matrix of the symmetric and asymmetric component from the regression residuals pooled by sex and locations. The percentage of total shape covariation between the hind wing modules was visualized using partial least squares (PLS) analysis (Bookstein, 1991; Rohlf and Corti, 2000). PLS axes provide new shape variables that maximize the covariance among the landmark configurations of the different modules, and therefore can be interpreted as the main features of integration among them (Corti et al., 2001).

Two different scenarios were hypothesized in order to test modularity: the first one was an anterior/posterior (A/P) subdivision; while the other was a distal/proximal (D/Pr) separation (Fig. 2). In these two hypotheses, the existence of two distinct developmental modules in the hind wings were proposed; to test this two modularity hypotheses we carried out a modularity test using the RV coefficient (Escoufier, 1973; Klingenberg, 2009). Only spatially contiguous partitions were considered when estimating the RV coefficient distribution, because the integration of landmarks within the hypothesized modules presents tissue-bound developmental interactions (Klingenberg, 2009). The RV coefficient is a measure of the strength of association between two sets of variables, and it can be considered as a multivariate analog of a squared correlation coefficient (Escoufier, 1973). A modularity hypothesis is

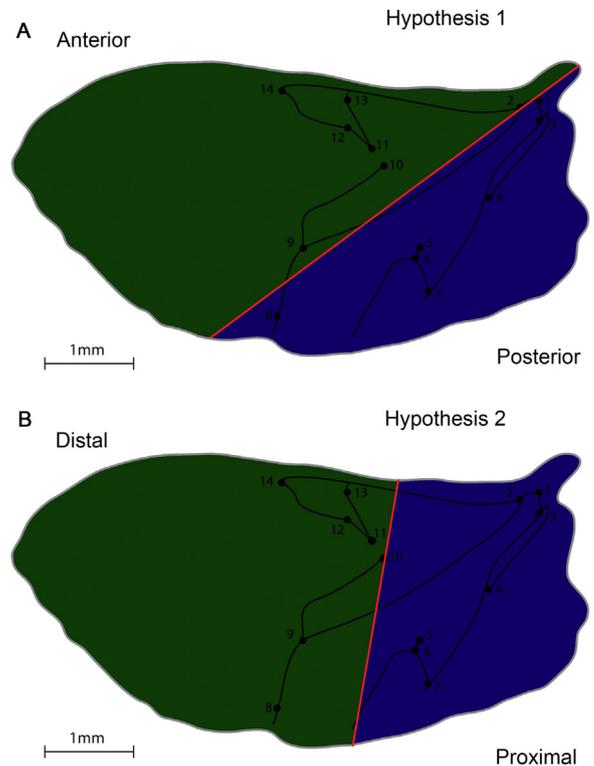


Fig. 2. Different possible subdivision hypotheses tested on *D. v. virgifera*. (A) First hypothesis tested whether the anterior and posterior (A/P) parts of the wing constitute different modules. (B) The second hypothesis tested whether the distal and proximal (D/Pr) parts of the wing constitute different modules.

accepted when the RV coefficient, for the hypothesized modularity is of the lowest value, and it is near to the lowest part of the distribution of the RV coefficients of the partitions (the left tail of the distribution). Finally, to analyze how different traits developmentally interact one another, we compared the covariance matrices of individual variation and fluctuating asymmetry with a permutation analysis (10,000 permutation runs), based on a matrix correlation and the null hypothesis of complete dissimilarity (Klingenberg, 2008, 2013; Gonzalez et al., 2011; Jojić et al., 2012). All of the aforementioned morphometric and statistical analyses were performed using Morpho J v1.04d (Klingenberg, 2011).

3. Results

3.1. Shape analysis

Procrustes ANOVA for assessing the measurement error showed that the mean square for individual variation exceeded the measurement error, such that it is negligible (Lemic et al., 2014) (Table 1). Previous studies using the same dataset have shown that the hind wing of *D. v. virgifera* exhibits significant directional and fluctuating asymmetry (Benítez et al., 2014). Even though we do not further consider the directional asymmetry in this study, we used the FA covariance matrix to estimate developmental modularity. Results confirmed the very low occurrence of an allometric effect that accounted for only 2.8% $P=0.0048$ for the symmetric component and 1.29% $P=0.0039$ for the asymmetric component of the total shape variation. The PCA analysis describing shape variation showed that the first three PCs accounted for 53.95% ($PC1 + PC2 + PC3 = 26.96\% + 18.03\% + 8.95\%$) of the total shape variation and provided a moderate approximation of the total amount of wing shape variation. The remaining 21 PCs each only accounted for no more than 7% of the variation.

Table 1
Measurement error Procrustes ANOVA for both centroid size and shape of *Diabrotica v. virgifera*, characterized by matching symmetry. Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (dimensionless).

Effect	SS	MS	df	F	P (param.)	Pillai tr.	P (param.)
Centroid size							
Individual	0.003113	0.000107	29	0.1			
Side	0.000728	0.000728	1	0.71	0.407		
Ind × Side	0.02983	0.001029	29	662	<0.0001		
Error 1	0.000093	0.000002	60				
Shape							
Individual	0.05169907	7.428E-05	696	1.15	0.03	14.91	<0.0001
Side	0.00060088	2.5037E-05	24	0.39	0.997	0.86	0.3089
Ind × side	0.04483652	6.442E-05	696	9.17	<0.0001	11.81	<0.0001
Error 1	0.01012083	7.0284E-06	1440				

Similarly, the PCA of the wing shape variation that describes the FA pattern, showed that the first three PCs accounted for 51.807% (PC1 + PC2 + PC3 = 19.98% + 18.302% + 13.51%); the remaining 21 PCs each only accounted for no more than 9.7% of the variation (Fig. 3).

3.2. Morphological integration and modularity

Concerning the morphological integration, the PLS tests between the A/P and D/Pr compartments for individual variation showed that the first two pairs of PLS axes accounted for 55.87% and 29.94% ($P < 0.001$) of the total covariance for A/P hypothesis, and 56.77% and 28.202% ($P < 0.001$) of the total covariance for D/Pr hypothesis; as for the fluctuating asymmetry they accounted for 50.67% and 33.17% ($P < 0.001$) of the total covariance for A/P

hypothesis, and 45.908% and 33.61% ($P < 0.001$) of the total covariance for D/Pr hypothesis (Fig. 4). These results showed a moderate integration between all the considered compartments. The overall strength of the relationship between A/P (RV = 0.35) and D/Pr (RV = 0.29) compartments was highly significant ($P < 0.001$), after assessing significance by performing a permutation test (10,000 runs).

Regarding the individual variation, the modularity hypothesis of A/P showed that the number of partitions considered for this hypothesis was 657, and 643 of them resulted in a lower value, the RV coefficient was 0.35, and the arrow representing it was quite on the right part of the distribution graph (Fig. 5A). This means that the covariation between the compartments considered is as different as it could be expected by random partitions, and hence these two parts do not represent different modules. Regarding the other

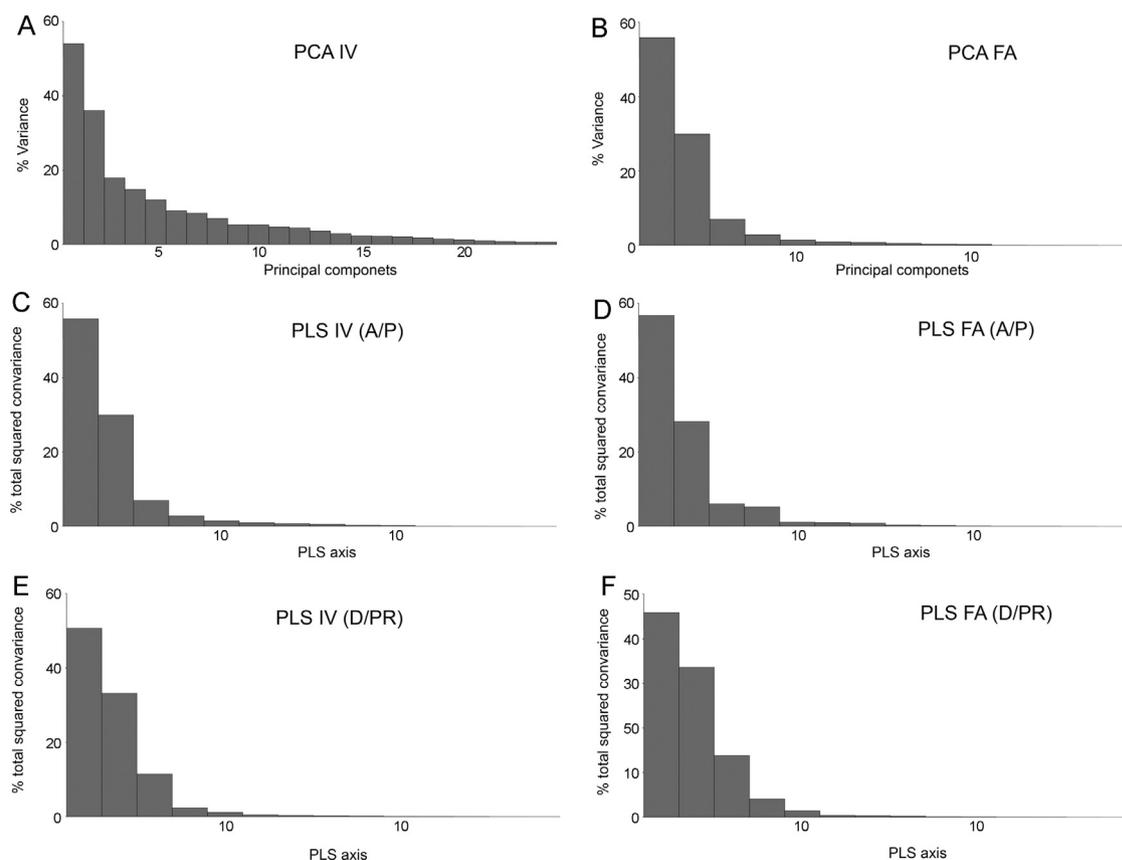


Fig. 3. Percentage of total shape variation accounted for by each axis obtained by principal component analysis (PCA) and partial least square (PLS) using covariance matrices pooled within-sex-locations. (A) PCA of individual variation (IV). (B) PCA of fluctuating asymmetry (FA). (C) PLS of individual variation (A/P). (D) PLS of fluctuating asymmetry (A/P). (E) PLS of individual variation (D/Pr). (F) PLS of fluctuating asymmetry (D/Pr).

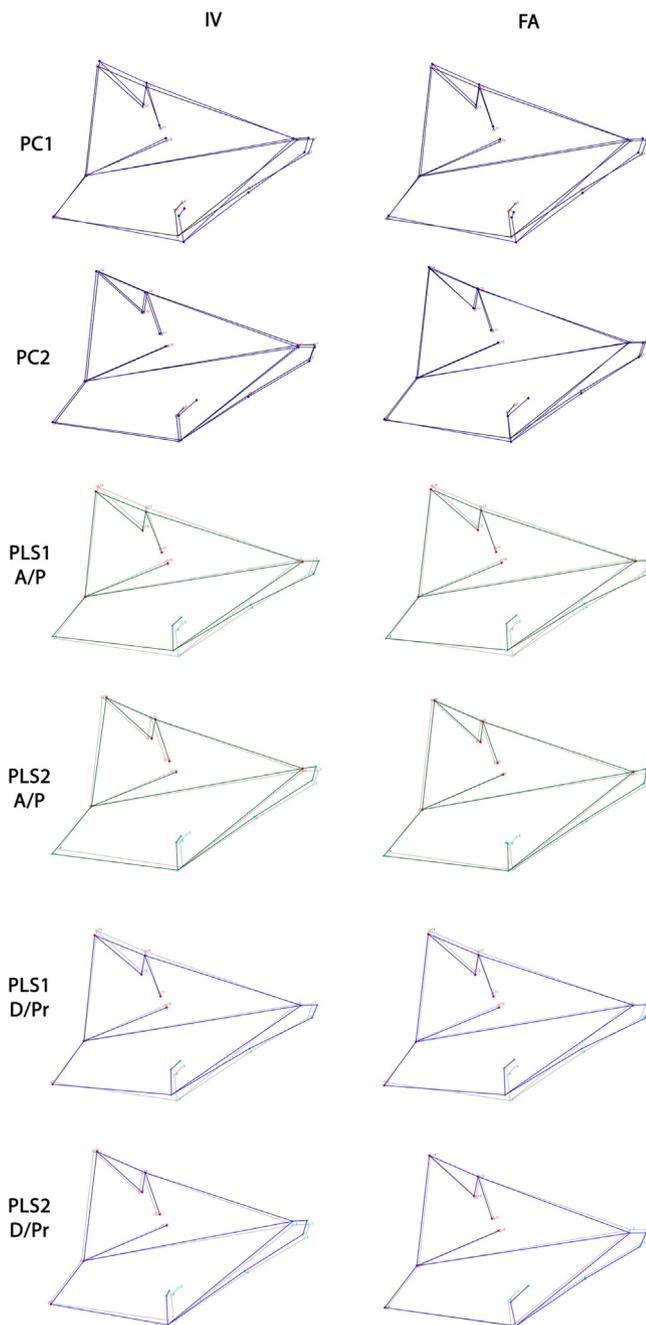


Fig. 4. The modularity hypotheses that tested the hypothesized modules of the hind wing with alternative partitions of the landmarks. (A–C) Individual variation. (B–D) Fluctuating asymmetry. The arrows indicate the RV coefficient between the anterior and posterior and distal and proximal developmental compartments. The histograms represent the distribution of RV coefficients for the alternative partitions of landmarks.

modularity hypothesis D/Pr, the number of partitions considered for this hypothesis was 657, and 308 of them resulted in a lower value, the RV coefficient was 0.28, and the arrow representing it was on the left middle tail of the distribution graph (Fig. 5C), indicating no modularity.

Regarding fluctuating asymmetry, the modularity hypothesis of A/P showed that the number of partitions considered for this hypothesis was 657, and 640 of them resulted in a lower value, the RV coefficient was 0.44, and the arrow representing it is clearly on the right part of the distribution graph (Fig. 5B). Also in this case result is not consistent with the modularity hypothesis formulated. Finally, for the D/Pr compartment, the number of

partitions considered for this hypothesis was 657, and 209 of them resulted in a lower value, the RV coefficient was 0.33, and the arrow representing it was on the left middle tail of the distribution graph (Fig. 5D), indicating no modularity.

Finally, the matrix correlation permutation test (i.e. covariance matrices of individual variation and fluctuating asymmetry), with 10,000 permutation runs emerged as highly significant (Matrix correlation = 0.90, $P < 0.001$), indicating there is a relationship between the matrices (Fig. 6).

4. Discussion

This study tested the pattern of morphological integration and modularity on *D. v. virgifera* hind wing shape using geometric morphometrics. Specifically, two wing modular hypotheses, anterior/posterior (A/P) and distal/proximal (D/Pr), were tested; they were chosen on the base of developmental morphology (Tomoyasu et al., 2009) and on studies of insect wing aerodynamics (e.g. Combes and Daniel, 2003; Bai et al., 2012; Nakata and Liu, 2011; Le et al., 2013). They were analyzed in order to investigate the developmental and/or aerodynamic importance of wing venation patterns on *D. v. virgifera* hind wing. Other studies have applied a similar approach but focusing in *D. melanogaster* (Klingenberg and Zaklan, 2000; Klingenberg, 2009). They have tested the anterior/posterior (A/P) hypothesis, which states that these compartments are separate developmental units, based on the assumption that two groups of genes control their development (Cavicchi et al., 1991; Pezzoli et al., 1997). Nevertheless, the combined analysis of individual variation and fluctuating asymmetry has emphasized the strength of integration throughout the wings of *D. melanogaster* (Klingenberg and Zaklan, 2000; Klingenberg, 2009).

The present paper has tested the A/P hypothesis and the D/Pr subdivision for both individual variation and fluctuating asymmetry (Klingenberg, 2009). Our results indicate that none of the two proposed modularity hypotheses were supported. Both the A/P and D/Pr hypotheses were rejected for both the individual variation and asymmetry, because their RV values were as different as it would be expected by random partitions. Hence, this study does not support any modular partition in the *D. v. virgifera* hind wings (based on shape analysis) when divided into A/P or D/Pr compartments. The present analyses failed to reject our two null modular hypotheses, which could be indicative, although not conclusive, of the morphological integration of the *D. v. virgifera* hind wing. Accordingly, the PLS results for both hypotheses showed a highly significant ($P < 0.001$) and moderate integration between all the considered compartments; A/P (RV = 0.35) and D/Pr (RV = 0.29). These results are in agreement with previous findings in *D. melanogaster* wings that showed a clear morphological integration of this anatomical structure (Klingenberg and Zaklan, 2000).

Consequently, it seems that hind wings are an integrated anatomical structure, thus opening several questions with regard to this integration phenomenon, taking into account that the origin of morphological integration is a central issue in evolutionary developmental biology (Klingenberg and Zaklan, 2000; Klingenberg et al., 2002, 2012; Klingenberg, 2004, 2005, 2008, 2013; Mitteroecker and Bookstein, 2007). The analysis of developmental integration in *D. v. virgifera* wings showed similar covariance patterns for FA and individual variation (Figs. 4–6). According to Klingenberg (2008), who stated that the origin of fluctuating asymmetry is from random perturbations occurred spontaneously within developmental pathways, and therefore it can be transmitted among pathways by direct interactions, here the correlated asymmetries of traits must originate exclusively from direct interactions of developmental pathways and not from parallel variations of separate pathways. In insects the

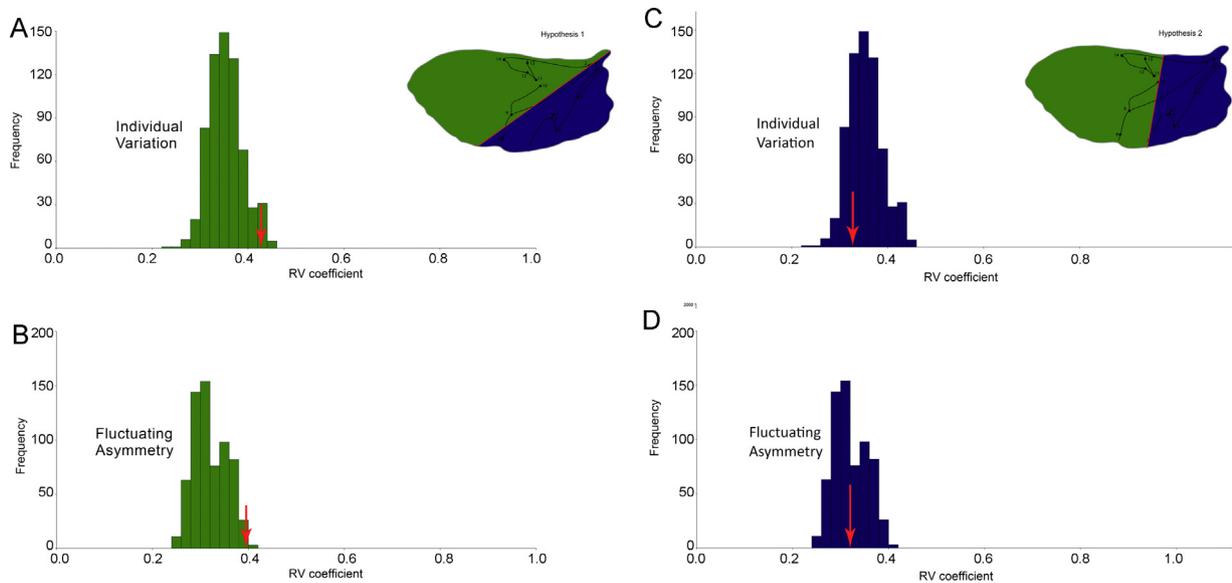


Fig. 5. Decomposition of shape variation in *Diabrotica v. virgifera*. The first two principal components (PCs) and two partial least square axis (PLS) for the individual component (IV) and fluctuating asymmetry (FA) are shown. For each PC and PLS, the diagrams show the shapes that correspond to the average shape for the respective hypothesis (A/P and D/Pr). For each pair of PLS axes, the shape changes for the landmarks of the anterior–posterior (H1) (red dots) and the distal–proximal (light blue dots) are shown in the same diagram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

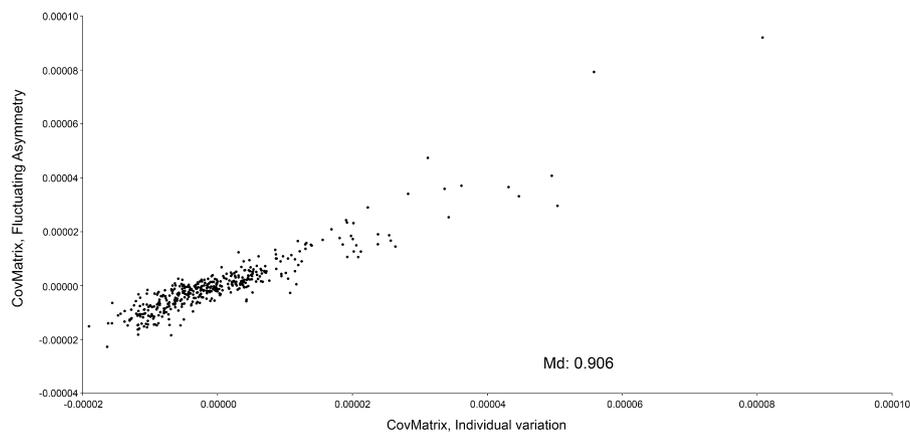


Fig. 6. Comparison of the covariance matrices of the symmetric and asymmetric component based on a matrix correlation.

aerodynamic performance of their wings with passive deformation (elastic wings) or prescribed deformation (rigid wings) models are traditionally estimated in terms of aerodynamic force, power and efficiency as was modeled in the hawk moth *Manduca sexta* L., by Nakata and Liu (2011). These authors found that wing parts (A/P and D/Pr) have different and independent functions; the flexibility can increase downwash in wake and therefore aerodynamic force. Dynamic wing bending delays the vortex lift failure, which is responsible for increasing the aerodynamic force-production. Furthermore, dynamic wing bending and twisting positively modifies the kinematics of its distal portion, enhancing aerodynamic force just before stroke reversal. For optimal flight maneuverability the integration of all the parts of the hind wing (both A/P and D/Pr) must occur, in order to decrease as many separate biomechanical movements as possible. Connections between the A/P and/or D/Pr landmarks represent optimal flight maneuverability which may assist in increasing hind wing flexibility, being therefore important factors to consider when determining the aerodynamic performance of insect wings and thus supporting our decision of analyze these two hypothetical compartments (Le et al., 2013).

Summarizing, this study showed that *D. v. virgifera* has a moderately integrated hind wing and thus the symmetric or asymmetric variation patterns found maintain the same common variation and therefore, a common origin. However, further research is required to understand the implications of this integrated behavior in the biomechanical performance of this species. For coleopterans and other insects, combine morphometric analyses with aerodynamic experiment and developmental experiment evidence and knowledge of the functioning of the whole organisms is a promising approach to elucidate the role of developmental processes in generating observable morphological variation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcz.2014.06.001>.

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