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Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*

I. M. SOTO, V. P. CARREIRA, E. M. SOTO & E. HASSON

Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires, Argentina

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Abstract

As in most insect groups, host plant shifts in cactophilic *Drosophila* represent environmental challenges as flies must adjust their developmental programme to the presence of different chemical compounds and/or to a microflora that may differ in the diversity and abundance of yeasts and bacteria. In this context, wing morphology provides an excellent opportunity to investigate the factors that may induce changes during development. In this work, we investigated phenotypic plasticity and developmental instability of wing morphology in flies on the cactophilic *Drosophila buzzatii* and *Drosophila koepferae* raised on alternative breeding substrates. We detected significant differences in wing size between and within species, and between flies reared on different cactus hosts. However, differences in wing shape between flies emerged from different cactus hosts were not significant either in *D. buzzatii* or in *D. koepferae*. Our results also showed that morphological responses involved the entire organ, as variation in size and shape correlated between different portions of the wing. Finally, we studied the effect of the rearing cactus host on developmental instability as measured by the degree of fluctuating asymmetry (FA). Levels of FA in wing size were significantly greater in flies of both species reared in non-preferred when compared with those reared in preferred host cacti. Our results are discussed in the framework of an integrative view aimed at investigating the relevance of host plant shifts in the evolution of the guild of cactophilic *Drosophila* species that diversified in South America.

Introduction

Morphological variation in nature and the evolution of size and shape of organs have been central research themes since the foundation of evolutionary biology (Darwin, 1859). In a modern context, investigations of morphological variation should necessarily involve the simultaneous analysis of genetic and environmental factors causing intraspecific variation and interspecific divergence (Mackay, 2004).

In this sense, phytophagous insects provide excellent model systems to study the genetic and ecological basis of adaptation and morphological divergence, as host plants constitute the most immediate environmental factor affecting early life cycle stages. Shifts to new host plants often involve the challenge to exploit a new food source, face chemically diverse environments (sometimes including potentially toxic compounds), new mating environments, parasitoids, bacteria and fungi (Kircher, 1982; Fogleman & Abril, 1990; Via, 1990). Therefore, host plant shifts may accelerate divergence in features associated with performance in new hosts, such as developmental time, oviposition schedule and survival (Mitter & Futuyma, 1983; Etges, 1990; Jaenike & Holt, 1991; Fanara & Hasson, 2001; Jaureguy & Etges, 2007) and sensory systems, like those involved in smell and taste (Dambroski *et al.*, 2005; McBride, 2007). Likewise, changes in morphology associated with host plant shifts are well documented in insects (Jones, 1998, 2004; Hawthorne & Via, 2001; Dambroski *et al.*, 2005).

Correspondence: Ignacio M. Soto, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II (C1428 EHA), Buenos Aires, Argentina.
Tel.: +54 11 4576 3348; fax: +54 11 4576 3354;
e-mail: soto@ege.fcen.uba.ar

Furthermore, adaptation to a new host plant may also cause, either as a direct consequence or as a byproduct, the evolution of sexual isolation, highlighting the evolutionary role of host plant shifts in cladogenesis (e.g. Etges *et al.*, 2006; see also Coyne & Orr, 2004 for a review).

The *Drosophila* wing is an excellent model for studying morphological evolution for several and complementary reasons: homology is applicable to many landmarks across a large number of species (Debat *et al.*, 2003); wing development is well understood (reviewed in De Celis, 2003) and overall wing size is a highly plastic trait and reaction norms have been described in several species exposed to different sources of environmental variation (David *et al.*, 1994; Moreteau *et al.*, 1998; Morin *et al.*, 1999). It has been suggested that the wing in *Drosophila* might be considered as an ontogenetically modular structure, primarily divided into anterior and posterior compartments (Fig. 1, Guerra *et al.*, 1997; Pezzoli *et al.*, 1997; Birdsall *et al.*, 2000; Zimmerman *et al.*, 2000). However, Klingenberg & Zaklan (2000) challenged this view and argued in favour of a more integrated view of wing morphology.

There is considerable evidence indicating that different aspects of wing morphology (both size and shape) are targets of natural selection. Parallel latitudinal clines in sympatric species and parallel and reciprocating clines in different continents in the same species provide evidence of the adaptive nature of variation in wing form (Powell, 1997). Studies in the widespread species *Drosophila subobscura* revealed unexpected subtleties in the response of wing morphology to environmental variation. This species invaded the Americas, in recent times, and a few years after its first detection reciprocating latitudinal clines in wing length evolved independently in the southern and northern Pacific coasts (Gilchrist *et al.*, 2004). Interestingly, in each cline wing length variation involved different veins (Gilchrist *et al.*, 2004).

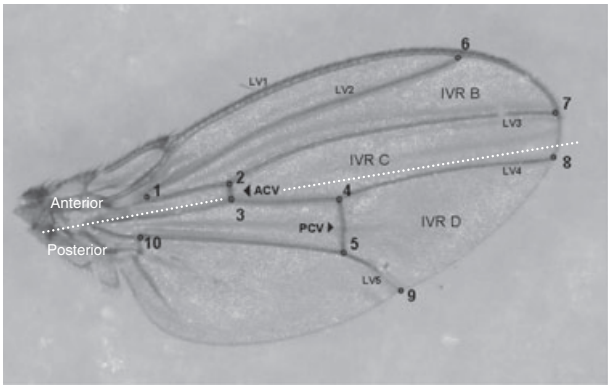

Fig. 1 Dorsal view of a right wing and landmark positioning. LV, longitudinal veins; ACV, anterior cross-vein; PCV, posterior cross-vein; IVR, intervein regions. Dotted line delimit anterior and posterior wing compartments (following Debat *et al.*, 2003).

The *Drosophila buzzatii* cluster (*repleta* group, *mulleri* subgroup, *buzzatii* complex Ruiz & Wasserman, 1993) comprises, at least, seven cactophilic species that inhabit the arid and semi-arid lands of South America (Manfrin & Sene, 2006). This guild of cactophilic species is an ideal model to investigate the role of host plant shifts in cladogenesis as it includes pairs of species in different stages of divergence (Manfrin & Sene, 2006; Soto *et al.*, 2007a). *Drosophila buzzatii* Patterson and Wheeler and *Drosophila koepferae* Fontdevila and Wasserman are sibling species that belong to the *D. buzzatii* cluster (Ruiz & Wasserman, 1993) and have partially overlapping distributions in the arid lands of north-western Argentina and southern Bolivia (Fontdevila *et al.*, 1988; Hasson *et al.*, 1992). Emergence records from naturally decaying cacti have shown that *D. buzzatii* breeds primarily on prickly pears (genus *Opuntia*) and *D. koepferae* on columnar cacti of the genera *Cereus* and *Trichocereus* (Hasson *et al.*, 1992), although some degree of niche overlap exists in nature (Fontdevila *et al.*, 1988; Hasson *et al.*, 1992; Fanara *et al.*, 1999). In addition, these fruit fly species exhibit quantitative differences in survival, body size, developmental time and oviposition preferences, which, apparently, evolved as adaptations to exploit resources characterized by diverse temporal and spatial predictabilities (Fanara *et al.*, 1999). More recent work has shown that cactus hosts have also a strong influence on wing size (Carreira *et al.*, 2006); however, the effect on overall venation pattern is still unknown.

Morphological analyses of wing traits are rarely combined with experimental manipulation, specially of the rearing hosts. The aim of our study is to investigate the effect of an experimentally induced host shift on wing morphology, using geometric morphometrics (Bookstein, 1996; Dryden & Mardia, 1998; Klingenberg, 2002), by rearing *D. buzzatii* and *D. koepferae* in primary and secondary host plants. Besides the evaluation of general patterns of phenotypic plasticity, we, specifically, address the following basic questions: (i) are wing size and shape differentially affected by the cactus hosts?; and (ii) is this effect restricted to any particular wing portion or affects the whole organ?

However, shifts to alternative hosts may involve stressful environments for the flies causing the destabilization of development. Developmental stability is an important clue to understand how traits are regulated to achieve their phenotypic target value. Linked to this issue are the concepts of phenotypic canalization and plasticity (Schlichting & Pigliucci, 1998). The former refers to the mechanisms that generate a phenotype insensitive to genetic and/or nongenetic perturbations (Waddington, 1942) and, thus, constrain morphological evolution (Charlesworth *et al.*, 1982; Maynard Smith *et al.*, 1985). The latter refers to the ability of a genotype to produce different phenotypes in response to

alternative environments (Schmalhausen, 1949). Stressful environments may modify the integration of morphological regulatory systems causing alterations of normal development (Badyaev, 2005). These events may effect a relaxation of canalization, and lead to evolutionary change because of the release of previously unexpressed genetic variation accumulated during periods of 'normal' developmental conditions (Flatt, 2005).

The degree of asymmetry in bilateral traits is often interpreted as indicative of the degree of perturbation that stressful conditions impose on development. Specifically, fluctuating asymmetry (FA), i.e. nondirectional random deviations from bilateral symmetry (Van Valen, 1962), has been proposed as a proxy for developmental instability in bilateral organisms (Zakharov, 1992). FA has been studied in a variety of taxa, both vertebrate and invertebrates, comparing conspecific populations inhabiting different geographic areas (Kark, 2001), or exposed to different ecological conditions (Nicieza *et al.*, 2006; Cuervo & Restrepo, 2007) and/or used as a biomonitor of crowding (Gibbs & Breuker, 2006), inbreeding (Réale & Roff, 2003) and nutritional stress (Stige *et al.*, 2004). In *Drosophila*, most studies explored the effect of the rearing temperature on wing development (e.g. Debat *et al.*, 2003; Faurby *et al.*, 2005; Santos *et al.*, 2006), and, more recently, the relationship between interspecific hybridization and FA (Rego *et al.*, 2006; Carreira *et al.*, 2007).

However, even in species with a well-known ecology, as *D. buzzatii*, little is known about the effect that ecologically relevant variables, such as natural substrates, may have on developmental stability. In this paper, we measured FA in wing size and shape to examine the hypothesis that rearing in a secondary host may represent a stressful environment for the flies, causing destabilization of wing development. In this sense, our prediction is that developmental instability, i.e. more asymmetric individuals, may be greater when a species is forced to grow in an unpreferred host, as a sign of environmental stress (Polack, 2003).

Materials and methods

Collection and maintenance of stocks

Strains of *D. buzzatii* and *D. koepferae* were founded with flies collected by net sweeping on fermented banana baits in late summer 2003 in a locality of western Argentina (33.3°S, 66.5°W) where both species coexist. In this sampling site, the rotting cladodes of *Opuntia sulphurea* and the stems of *Trichocereus candicans* are the main breeding and feeding resources for both species.

Captured flies were sexed in the laboratory and isofemale lines (lines hereafter) were set up by placing single wild-caught females in vials containing 5 mL of David's killed yeast culture medium (David, 1962). Each line was identified to species by the inspection of the genitalia of one progeny male as females of both species are indistinguishable. All lines were reared under identical conditions in vials with 5 mL of laboratory standard medium for 20 generations before the onset of the experiments described below.

Fresh and rotting materials of both *O. sulphurea* and *T. candicans* were also collected in the sampling locality for the preparation of two types of cactus media. Pieces of fresh cactus were stored at −20 °C and cactus necroses of both species were maintained in the laboratory adding 10 g of fresh cactus (*O. sulphurea* or *T. candicans*) every 2 weeks.

Experimental design

Ten isofemale lines of each species were randomly chosen from the initial set of isofemale lines. For each line, 100 pairs of sexually mature flies were placed in oviposition chambers (two chambers per isofemale line) as described in Fanara *et al.* (1999). Eggs were allowed to hatch and batches of 30 first instar larvae were transferred to culture vials containing a 'semi-natural' medium prepared with fermented pieces of *O. sulphurea* or *T. candicans*. A detailed account of the protocol employed in the preparation of the cactus media can be found in Fanara *et al.* (1999). Briefly, 10 mL of grinded cactus of each species were poured into standard glass vials, autoclaved and inoculated, after cooling, with 0.1 mL of fermenting juice obtained from naturally occurring rotting materials of the corresponding cactus species.

Four replicated vials were set up for all combinations of cactus and line. Larvae were raised at 25 ± 1 °C with a 12 : 12 light/dark photoperiod.

Three to five males emerged from each vial were randomly chosen and both wings of each individual were removed and mounted on microscope slides. Wing images were captured using a binocular microscope (10×) and an attached digital camera connected to a computer. In each wing, we scored 10 landmarks (Fig. 1) using tpsDig (Rohlf, 2003a).

Size variation

Centroid size was computed as a measure of overall wing size. It is defined as the square root of the sum of the squared distances of each landmark from the centroid of the configuration (Dryden & Mardia, 1998). Differences between species and the effect of cactus media on wing size were investigated by means of an ANOVA with Species (two levels: *D. buzzatii and D. koepferae*), Cactus (two levels: *O. sulphurea* and *T. candicans*) and Line (random, 10 levels) nested in Species as main sources of variation.

Shape variation

Shape variation was investigated using the Procrustes technique where all wings are superimposed for the examination of differences in the position of landmarks. Shape coordinates were computed using a morphometrical approach based on least squares Procrustes superimposition method (Bookstein, 1996; Dryden & Mardia, 1998). The shape variables calculated, called partial warps, indicate partial contributions of hierarchically scaled vectors spanning a linear shape space. The matrix of partial warp scores was complemented by two uniform dimensions of shape change.

To investigate trends in shape change, the dimensionality of the matrix of partial warps and uniform component scores was further reduced by relative warps (RWs) analysis (Bookstein, 1991; Rohlf, 1993), a principal component analysis (PCA) of the partial warps and uniform components. Calculation of RWs was performed using TpsRelw (Rohlf, 2003c) and they were further used as dependent variables in a MANOVA with Species, Cactus and Line (nested in Species) as main sources of variation. All ANOVA and MANOVA assumptions were properly checked. In fact, the interspecific MANCOVA could not be performed because of the rejection of parallelism of the relationship between dependent variables and the covariable between species (see Results).

We examined the correlation between the Procrustes (shape space) and the Euclidean (tangent space) distances using tpsSmall (Rohlf, 2003b) to validate the shape variables created with the geometric methodology. We performed the thin-plate spline RWs analysis of the coordinates of all aligned specimens (Bookstein, 1991; Rohlf, 1993) to produce graphics to visualize differences among groups.

Covariation between wing modules

Some authors suggested that intervein regions (IVR) might be considered as genetically independent units (Guerra *et al.*, 1997). To describe their respective phenotypic variation, we calculated the centroid size and partial warps of the IVRs defined between longitudinal veins 2 and 3 (IVR-B) and longitudinal veins 4 and 5 (IVR-D) (Fig. 1). By choosing these intervening regions that do not share common landmarks, we avoid superfluous covariation between modules. Besides, fly wings might be subdivided into anterior and posterior compartments (Fig. 1). These compartments correspond to distinct cell lineages and domains of gene expression (Garcia-Bellido *et al.*, 1973; Lawrence & Morata, 1976; Lawrence, 1992) and they have been considered as candidates for separate developmental modules. The IVRs assessed in our study are located in each one of the compartments.

To analyse size covariation, we performed a regression of each intervein region size (centroid size B and D) on general wing size. Afterwards, we used the residuals of both regions, devoid of allometric change because of overall increment of organ size, in a correlation analysis.

We performed a partial least-squares analysis (PLS) of the covariation between the two sets of partial warps to search for the existence of concerted shape covariation between the two intervein regions (McIntosh *et al.*, 1996). We used tpsPLS 1.14 (Rohlf, 2005) for the analysis and the significance of the correlations was tested using the sampled randomization test included in the software. We ran 10 000 permutations where the partial least-squares computations are repeated and the results compared with those based on the original correspondence.

Asymmetry assessment

For the analysis of asymmetry (matching asymmetry according to Klingenberg *et al.* (2002), 10–20 flies of four isofemale lines of each species were randomly chosen and separate landmark configurations were digitized twice in independent images of both wings of each individual for the estimation of measurement error (Palmer, 1994). The analysis included the preliminary reflection of all configurations from one body side to their mirror images (Klingenberg & McIntyre, 1998). Then, all configurations were simultaneously superimposed with the least squares Procrustes method.

We calculated skewness and kurtosis statistics for the signed differences in size between sides in each combination of species and cactus, to test for deviations from normality in the samples and discard the possibility of antisymmetry.

To investigate bilateral asymmetry in size, phenotypic variation was partitioned into among-individuals, within-individuals and error components, using a two-way ANOVA design (Leamy, 1984; Palmer & Strobeck, 1986; Palmer, 1994). In these ANOVAS, 'individual' is a random factor that stands for individual variation in size or shape; 'sides' is a fixed factor that can be considered as an estimation of directional asymmetry (DA); the Individual × Side interaction provides a measure of FA and the residual variance component among replicated measurements gives an estimation of the measurement error.

Following Palmer & Strobeck (1992), we tested for differences in FA in size among samples using the absolute value of the difference between right and left wings (R–L). This value may be biased by the presence of DA and/or simple wing size differences among samples. After correcting for DA, by subtracting the mean value of the mean R–L signed difference to each individual R–L value of the sample, we performed an ANCOVA with Species and Cactus as main fixed factors and with mean individual centroid size as covariate to statistically remove the size bias.

The ANOVA testing for asymmetry has also been adapted for the analysis of shape (Klingenberg & McIntyre, 1998). To evaluate the presence of antisymmetry,

scatterplots of left–right coordinate differences for each landmark after Procrustes superimposition were visually inspected for clustering of the values (Klingenberg & McIntyre, 1998; Debat *et al.*, 2000). The Procrustes ANOVAS for shape were calculated by summing up the sums of squares of all characters (10 coordinates) and the degrees of freedom obtained by multiplying the degrees of freedom of each factor by the total number (16 in our study) of shape dimensions (Klingenberg & McIntyre, 1998).

Because of the multivariate nature of the data, in the analysis of shape asymmetry, we decided to test for differences in FA between samples by means of *F*-tests. FA differences were tested between species within cactus and between cactus within species, using the medium squares of the Individual × Side interaction and the degrees of freedom of the corresponding Procrustes ANOVA, thus, avoiding the presence of DA (accounted by the 'Side' factor). ANOVAS testing for asymmetry in shape were performed using the program ASI (kindly provided by JP Dujardin, see Dujardin & Slice, 2007) and the other tests and assumptions using STATISTICA (StatSoft Inc., 2001), unless specifically stated.

Results

Drosophila koepferae flies reared in *O. sulphurea* and *D. buzzatii* emerged in *T. candicans* vials had the largest and smallest wings respectively (Fig. 2). On average, *D. koepferae* had larger wings than *D. buzzatii* and flies emerged in *O. sulphurea* were larger than those reared in *T. candicans* (Table 1). Differences between species and between flies reared in different cactus hosts were both significant in the general ANOVA. Likewise, lines (within species) also differed significantly in wing size, and, interestingly, differences among lines varied across cactus hosts, as indicated by the significant Line × Cactus interaction. These results suggest that phenotypic variation in wing size has a genetic basis and that the phenotype (wing size) expressed by genotypes (lines) depended on the rearing cactus.

Before proceeding to the analysis of wing shape variation, it is important to mention that the correlation coefficient between Procrustes and Euclidean distances was highly significant ($\rho = 1$, $P < 0.0001$). Such perfect approximation of shape space by the tangent space made all estimates of shape differences extremely reliable.

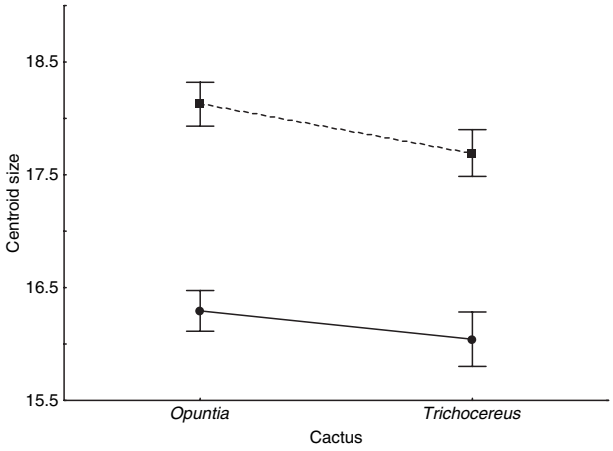

Fig. 2 Mean wing size (estimated as centroid size) and confidence intervals in *Drosophila buzzatii* (circles) and *Drosophila koepferae* (squares) reared in *Opuntia sulphurea* and *Trichocereus candicans.*

Interspecific differences in wing shape were significant; however, wing shape was insensitive to the rearing medium (Table 2). We were unable to test whether shape differences between species persisted after correcting for size by means of a MANCOVA because the data did not fulfil one of the main assumptions of the MANCOVA: the allometric relationship between size and shape had different slopes in *D. koepferae* and *D. buzzatii* (Species × Centroid size parallelism test: Wilk's $\lambda = 0.81$, $F_{16,448} = 6.41$, $P < 0.001$).

In Fig. 3, we present a plot of the mean values of RW1 and RW2 (RWs that jointly account for nearly 48% of total shape variance) of each isofemale line reared in *O. sulphurea* and *T. candicans*. Figure 3 also allows one to visualize which part of the wing is associated with shape variation accounted for by RW1 and RW2. *Drosophila buzzatii* and *D. koepferae* are clearly separated along both axes. Moreover, if we analyse the distribution of lines along the *X*-axis (RW1), it can be seen that both species differ in the orientation of the posterior cross-vein

Table 1 ANOVAS testing for differences in size (centroid size) for total landmark configuration and intervein regions (IVR).

		Total size				IVRB size				IVRD size			
Sources of variation	d.f.	d.f. error	MS	MS error	*F*	d.f. error	MS	MS error	*F*	d.f. error	MS	MS error	*F*
Species	1	19.09	560.24	52.58	10.65**	19.15	250.41	23.68	10.57**	19.17	56.37	6.96	8.10**
Cactus	1	21.43	9.44	2.14	4.41*	22.98	4.04	1.06	3.82	24.53	0.97	0.26	3.75
Species × Cactus	1	21.43	0.86	2.14	0.40	22.97	0.35	1.06	0.33	24.51	0.13	0.26	0.52
Line (species)	19	19	62.65	2.41	26.05**	19	31.28	1.27	24.57**	19	9.19	0.30	30.57**
Cactus × Line species	19	425	2.41	0.77	3.13**	425	1.27	0.39	3.26**	425	0.30	0.13	2.39**
Error	425		0.77				0.39				0.13		

MS, mean squares. *$P < 0.05$ **$P < 0.001$.

(landmark 5 is in a more proximal position relative to landmark 4 in *D. koepferae* when compared with *D. buzzatii*) and that wings in *D. koepferae* have a broader posterior distal wing margin (the distance between landmarks 8 and 9) than *D. buzzatii*. Along the *Y*-axis (RW2), both species are also clearly separated (Fig. 3). *Drosophila buzzatii* tends to have a broader intervein region D, IVRD (according to Birdsall *et al.*, 2000) which is enclosed by longitudinal veins 4, 5 and the posterior cross-vein (landmarks 4, 5, 8 and 9, Fig. 1) and an enlarged proximal wing region (distances between landmarks 1, 2, 3 and 10) than *D. koepferae*.

In contrast to wing size, and confirming the results of the general MANOVA, we did not find a consistent effect of the rearing cactus on wing shape (Table 3a). However, the MANOVAS investigating shape variation revealed, in both species, that a substantial proportion of variation is genetically determined and that shape phenotypes expressed by each line depended on the rearing cactus, as suggested by the significant Line and Line × Cactus effects (Table 3a) respectively.

The MANCOVAS, using centroid size as covariate, confirmed that observed shape differences were not entirely associated with size changes (i.e. differences were also nonallometric) as differences remained significant after statistically removing the effect of size (Table 3b). In summary, wing shape in both species was not consistently affected by the rearing substrate, although we found a size-independent genotype × environment interaction in venation change.

Table 2 MANOVA testing for shape differences between *Drosophila buzzatii* and *Drosophila koepferae* reared in two cactus media.

Sources of variation	d.f. effect	d.f. error	Wilk's value	*F*
Species	16	448	0.37	48.37*
Cactus	16	448	0.97	0.97
Species × Cactus	16	448	0.95	1.44

*$P < 0.001$.

Modularity analyses

We also investigated the degree of integration between *a priori* defined parts of the wing. First, regression analysis of the size of IVRs B and D on general wing size yielded positive and significant results in both species ($r^2 > 0.98$ and $P < 0.001$ in all cases). Secondly, the ANOVAS analysing size variation in intervein regions IVRB and IVRD yielded similar results than for general wing size, differences between Species and among lines (within species) and the Line × Cactus interaction were significant (Table 1). We also found a strong correlation between the residuals of the size of IVRB and IVRD in both species (Pearson correlation, $r = -0.37$, $N = 259$ and $r = -0.46$, $N = 208$ for *D. buzzatii* and *D koepferae*, respectively, $P < 0.05$ in both cases; Fig. 4). Overall, these results imply that the size of intervein regions B and D co-varied negatively, after correcting for overall wing size.

The analysis of the effect of the other sources of variation showed that the cactus-rearing media did not affect the shape of intervein regions B and D at variance with the results obtained for general size (data not shown). Differences in the shape of IVRB and IVRD among lines within species were significant in both species.

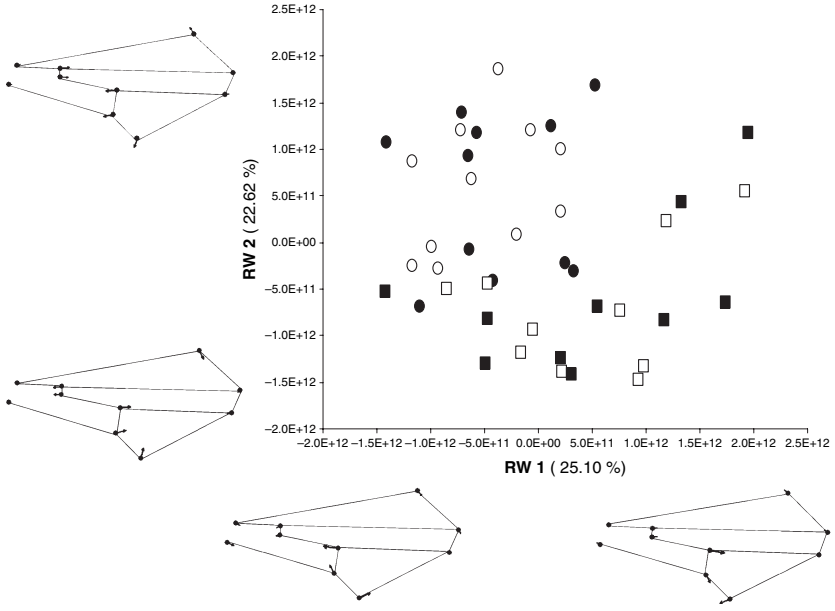

Fig. 3 Plot of mean values of the first two relative warps (RW1 and RW2, in parenthesis the percentage of shape variance accounted) of *Drosophila buzzatii* (circles) and *Drosophila koepferae* (squares) isofemale lines reared in *Opuntia sulphurea* (open symbols) and *Trichocereus candicans* (filled symbols). Graphics inserted below the *X*-axis and to the left of the *Y*-axis represent shape differences along each axis depicted as vectors indicating the displacement of the landmarks from the mean shape. The modules of the vectors are exaggerated 10 times for illustration purposes.

Table 3 Intraspecific MANOVAS (a) and MANCOVAS – using total wing size as covariate; (b) testing for shape differences among isofemale lines and between flies reared in different cactus media.

	Drosophila buzzatii				*Drosophila koepferae*			
	d.f. effect	d.f. error	Wilk's value	*F*	d.f. effect	d.f. error	Wilk's value	*F*
(a) MANOVA								
Cactus	16	222	0.94	0.84	16	173	0.91	1.05
Line	160	1915	0.03	6.37**	144	1383	0.01	7.61**
Cactus × Line	160	1915	0.39	1.38**	144	1383	0.35	1.35**
(b) MANCOVA								
Centroid size	16	221	0.89	1.76*	16	172	0.74	3.72**
Cactus	16	221	0.94	0.81	16	172	0.88	1.51
Line	160	1906	0.04	5.71**	144	1375	0.01	7.19**
Cactus × Line	160	1906	0.39	1.39**	144	1375	0.33	1.42**

*$P < 0.05$; **$P < 0.001$.

Partial least-squares analysis revealed a certain degree of concerted shape variation between intervein regions in both species. In these tests, the first three (of four) PLS axes in *D. buzzatii* and the four PLS axes in *D. koepferae* were significantly correlated between intervein regions, according to the results of permutation tests ($P < 0.04$ in all cases). The first two axes accounted for more than 99% and 98% of the covariance between IVRs (proportion of the total sum of singular values in PLS analysis) in *D. buzzatii* and *D koepferae* respectively. The correlation coefficients between pairs of corresponding PLS axes for IVRB and IVRD ranged from 0.03 to 0.52 in *D. buzzatii* and from 0.17 to 0.56 in *D. koepferae*.

Analysis of asymmetry

We examined the possible effect that breeding in primary (preferred) vs. secondary (unpreferred) cactus hosts may have on the stability of wing development by means of the analysis of FA. Descriptive statistics of asymmetry in wing size are presented in Table 4. The comparative analysis of the signed difference between the sizes of the right wing and the left wing was positive in both species (and in both cactus media) and the unsigned differences between wings were, on average, larger in *D. koepferae* than in *D. buzzatii*. However, it is interesting to note that the value of the signed difference in *D. koeferae* reared in cardón was more than three times larger than the values estimated for *D. buzzatii* and for *D. koepferae* reared in prickly pears (Table 4).

We did not find evidence of antisymmetry neither in wing size (Table 4) nor shape. Directional asymmetry was significant only in the case of *D. koepferae* reared in *T. candicans*. Flies of both species emerged in both cactus hosts exhibited signs of FA (significant Individual × Side interaction) (Table 5).

Further analyses revealed an interesting effect of the cactus hosts on the degree of FA. In both species, FA was greater in flies emerged in the unpreferred cactus host (Fig. 5) as suggested by the significant Species × Cactus interaction ($F_{1,425} = 10.83$, $P = 0.001$). In *D. koepferae*, individuals reared in *Opuntia* were significantly more asymmetric in size than those grown in cardón (Tukey's *post hoc* comparison $P = 0.024$), whereas in *D. buzzatii* flies reared in cardón were apparently more asymmetric than in *Opuntia* (Fig. 5), although in this case the difference was not significant (Tukey's *post hoc* comparison, $P = 0.56$).

The Procrustes ANOVAS testing for asymmetry in wing shape revealed significant DA in *D. koepferae* reared in *Opuntia* (Table 5) and significant FA in all cases (Table 5). Comparisons among samples by means of *F*-tests showed

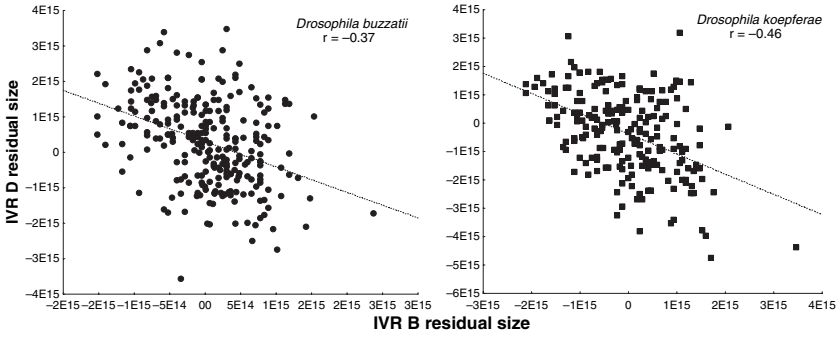

Fig. 4 Correlation analyses between the residuals of the regressions of the size of intervein regions (IVR) B and D on general wing size in *Drosophila buzzatii* and *Drosophila koepferae*. $P < 0.05$ in both cases.

that the degree of FA in wing shape in *D. koepferae* emerged in different cacti was not significantly different ($F_{912,736} = 1.07$; $P = 0.16$), whereas *D. buzzatii* showed increased amounts of FA when reared in *Opuntia* ($F_{992,736} = 1.18$; $P < 0.01$). However, it is important to note that these results are in partial contradiction with the results of the MANOVA (Table 3) showing that the cactus host did not affect general wing shape.

Discussion

A common conclusion underlies our present study on wing morphology and previous reports investigating life-history traits in the pair of sibling species *D. koepferae* and *D. buzzatii*, the remarkable influence that cactus hosts have on intra and interspecific patterns of variation (Fanara *et al.*, 1999, 2004; Fanara & Hasson, 2001; Carreira *et al.*, 2006; Soto *et al.*, 2007b). A similar effect of the cactus host on developmental time, viability and wing morphology has also been reported in *D. gouveai* and *D. antonietae*, a relatively younger pair of sibling species, that also belong to the *D. buzzatii* cluster (Soto *et al.*, 2007a,c).

Our work shows that wing size, but not wing shape, has a consistent plastic response to the cactus-rearing media, suggesting that these aspects of wing morphology may be differentially constrained during wing development. Moreover, patterns of intra- and interspecific variation suggest that wing size and shape may be relatively independent aspects of general wing morphology. In this line, our results also show that size-dependent shape changes, namely the allometric component of shape variation, comprise only a small proportion of total shape variation in *D. buzzatii* and *D. koepferae* suggesting that an important portion of shape variation is independent of size. Nevertheless, both traits were involved in genotype × environment interactions in both species, providing evidence of the importance of environmental heterogeneity, as represented by different cactus hosts, in the maintenance of genetic variation.

Despite being siblings, *D. buzzatii* and *D. koepferae* exhibit quantitative differences in both wing size and shape. Moreover, the geometric morphometric approach employed, allowed us to recognize that different wing compartments evolve at, apparently, different rates. In effect, IVRD is the portion of the wing that best discriminates between *D. buzzatii* and *D. koepferae*. Actually, variation in the morphology of IVRD is philogenetically informative in the *D. buzzatii* cluster (Moraes *et al.*, 2004; Soto *et al.*, 2007c), indicating that IVRD, which is part of the posterior portion of the wing, may be the less constrained wing part. This observation is coincident

Table 4 Descriptive statistics (and standard errors in parenthesis) of the analysis of wing size asymmetry.

Species	Cactus	No. of individuals	Mean wing size	Mean R–L signed differences	Normality–Shapiro Wilk's test	Skew	Kurtosis	R–L unsigned differences
Drosophila buzzatii	*Opuntia*	63	1631.82 (9.63)	1.95 (1.37)	W = 0.99, *P* = 0.26	0.25 (0.22)	−0.09 (0.43)	12.38 (0.83)
	Trichocereus	47	1604.58 (6.10)	1.71 (1.88)	W = 0.99, *P* = 0.46	−0.18 (0.25)	0.25 (0.49)	14.43 (1.16)
Drosophila koepferae	*Opuntia*	58	1793.90 (7.72)	1.67 (2.08)	W = 0.98, *P* = 0.10	−0.17 (0.22)	0.27 (0.45)	16.95 (1.36)
	Trichocereus	47	1821.51 (7.07)	6.22 (2.07)	W = 0.99 *P* = 0.54	0.04 (0.25)	−0.09 (0.49)	16.51 (1.33)

Table 5 ANOVAS and Procrustes ANOVAS testing for differences in asymmetry in wing size and wing shape respectively.

	Drosophila buzzatii						*Drosophila koepferae*					
	Opuntia			*Trichocereus*			*Opuntia*			*Trichocereus*		
Trait	d.f.	MS	*F*	d.f.	MS	*F*	d.f.	MS	*F*	d.f.	MS	*F*
Size												
Individual	62	3786.03	160.7**	46	1414.34	37.9**	57	2791.30	56.2**	46	1896.47	47.4**
Side	1	23.99	1.1	1	13.74	0.4	1	16.17	0.3	1	181.90	4.5*
Side × Individual	62	23.56	5.2**	46	37.33	10.5**	57	49.69	5.9**	46	39.99	5.2**
Error	126	4.55		94	3.56		116	8.46		94	7.70	
Shape												
Individual	992	6.50E−05	9.3**	736	6.00E−05	10.0**	912	8.20E−05	8.2**	736	8.60E−05	8.6**
Side	16	8.00E−06	1.1	16	3.00E−06	0.5	16	3.60E−05	3.6**	16	1.10E−05	1.1
Side × Individual	992	7.00E−06	8.5**	736	6.00E−06	11.1**	912	1.00E−05	14.2**	736	1.00E−05	18.3**
Error	2016	1.00E−06		1504	1.00E−06		1856	1.00E−06		1504	1.00E−06	

MS, mean squares. *$P < 0.05$, **$P < 0.001$.

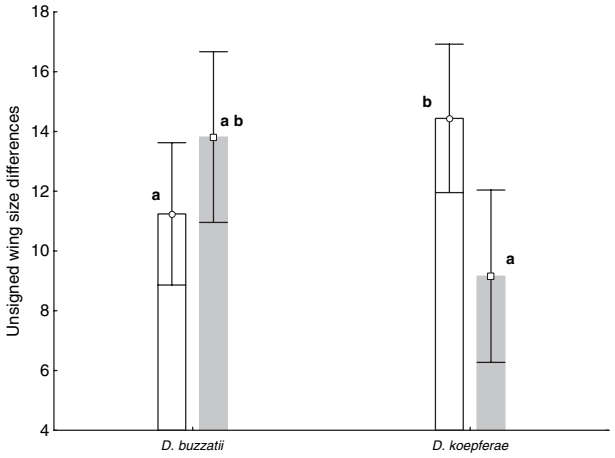

Fig. 5 Mean unsigned differences (and standard errors) in size between right and left wings in *Drosophila koepferae* and *Drosophila buzzatii* reared in *Opuntia* (white bars) and *Trichocereus* (grey bars) after correction for DA. Letters denote homogeneous groups according to Tukey's *post hoc* comparisons ($\alpha = 0.05$).

with intraspecific patterns of variation showing that IVRD exhibited the more plastic response to the rearing cacti. In this sense, our results are concordant with studies in *D. melanogaster* showing that the posterior portion of the wing exhibited the most plastic response to environmental variation in temperature (Cavicchi *et al.*, 1985).

However, we may ask whether these changes are independent of the responses of other wing portions. This issue is related to a long-standing debate between two views of wing development: the integrative and modular conceptions. However, modularity vs. integration in wing development is, actually, a matter of degree rather than an all-or-nothing issue (Klingenberg & Zaklan, 2000). In general terms, all wing regions are expected to accompany global increments in wing size. By contrast, form variation independent of wing size (the nonallometric component of wing form variation) would not necessarily be correlated among wing regions, especially if the portions under consideration are not adjacent and intermediate regions may serve as 'buffers' that compensate small disparities. In our study, the size of intervein regions B and D, two wing regions that do not share landmarks, were significantly correlated (although not tightly) for both size and shape variation, in both *D. buzzatii* and *D. koepferae*. These results give support to the idea that different wing compartments do not behave as completely independent units and that cellular growth in one part may be compensated by (and coordinated with) changes in another wing portion.

The evolutionary success of the *Drosophila repleta* species group in the New World seems to be related to their ability to utilize cactaceae as feeding and breeding resources. This ability allowed them to invade desertic regions, which are unfriendly to most *Drosophila* (Wasserman, 1982). As in most insect groups, new host plants, in cactophilic *Drosophila*, may represent a challenge as flies must adjust their developmental programme to the presence of different chemical compounds and/or to a different microflora. To measure to what extent growing in different host cacti may affect developmental stability, we measured FA in *D. buzzatii* and *D. koepferae* reared in primary and secondary cactus hosts. Our *a priori* expectation was that FA may be more pronounced in secondary (or unpreferred) than in primary (preferred) cactus host. This idea is based on previous studies showing that the performances of both species are not independent of the cactus hosts (Fanara *et al.*, 1999). Our results point in this direction, at least in *D. koepferae*, as flies reared in its unpreferred cactus host (*O. sulphurea*), were significantly more asymmetric in wing size than in the primary host (cardón). It is worth noting that a similar (although nonsignificant) trend was also observed in *D. buzzatii*. Moreover, and to put the effect of the rearing cactus in a broader perspective, it is worth mentioning that the disturbance that growing in the 'wrong' host causes on the development (as measured by FA) is even greater than in interspecific hybrids between *D. buzzatii* and *D. koepferae*. In fact, the admixture of diverged genomes in interspecific hybrids is expected to cause major problems during development (Carreira *et al.*, 2007).

Another observation that points out the effect of the cactus host is the nearly threefold increment of DA observed in *D. koepferae* in cardón (the primary host) when compared with individuals grown in *O. sulphurea* and to *D. buzzatii* (in both cacti), that were not accompanied by an increment in FA. Similar results were reported by Graham *et al.* (1993b) in *D. melanogaster* exposed to increasing concentrations of benzene, which the authors explained as a transition from FA to DA. Following this line of reasoning, we suggest that the observed increase in directional asymmetry might be interpreted as evidence of a host-driven developmental perturbation not expressed as FA (Graham *et al.*, 1993a,b; and see Palmer & Strobeck, 1992 for a different point of view).

If FA is to constitute a good biomonitor, it has to be correlated not with stress but with fitness (Floate & Fox, 2000). Interestingly, in our case the difference in the degree of FA between flies reared in preferred vs. unpreferred host plants correlates with previous evidence showing that *D. buzzatii* reared in *Opuntia* has larger body size, develop faster and are more viable than in the alternative hosts (Fanara *et al.*, 1999). Furthermore, the pattern detected in *D. koepferae* is in agreement with the estimates of general performance showing that this species is, usually, less plastic (Fanara *et al.*, 1999; Carreira *et al.*, 2006) and specialized in the use of a host that is relatively stressful to other *Drosophila* species as rearing media because of its high content of alkaloids and other potentially toxic compounds as other columnar Cactaceae (Kircher, 1982; Fogleman & Abril, 1990).

Which are the possible fitness implications of the increased FA observed in flies reared in unpreferred when compared with preferred cactus hosts? In this sense, there is evidence that FA in wing length is negatively correlated with male mating success in *D. buzzatii* (Santos, 2001 and references therein). However, as this author acknowledges, such a trend may be attributed to a poorer phenotypic condition of unmated when compared with mated males, as there is no evidence of a genetic basis of FA in *D. buzzatii*. In our study, FA was measured in the same sets of lines (genotypes) of both species in two cactus hosts, thus assuring a similar 'genetic quality' of flies reared in alternative host cacti. Whether the poorer phenotypic condition of flies reared in unpreferred hosts affects male mating performance is currently under investigation in our laboratory.

Finally, it is important to point out that the statistical methodology employed may overestimate FA (and thus developmental instability) if directional asymmetry is present (Graham *et al.*, 1998). In fact, directionally asymmetric (and/or antisymmetric) traits cannot be used as measures of developmental instability because an unknown proportion of the asymmetry variance has a genetic basis and reflect normal development (Palmer & Strobeck, 1992). Graham *et al.* (1998) proposed several methodologies to correctly estimate FA. Following these authors, we, first, established that growth between right and left wings is isometric and, second, that the residuals of the mayor axis of the regression of right on left wing measurements are homoscedastic suggesting an additive error model. Therefore, we may assure that the methodology recommended by Palmer (1994) is adequate in the present case and thus, our results are reliable.

In conclusion, the evolution and divergence of *D. buzzatii* and *D. koepferae* seems to be tightly linked to the evolutionary history of host plant use (Hasson *et al.*, 1992; Fanara *et al.*, 1999) as it was also shown in another pair of species of the *D. buzzatii* cluster (Soto *et al.*, 2007a,c). Moreover, our study shows that adaptation to new hosts is expected to have relevant consequences on development, adult morphology and fitness. Thus, understanding the evolutionary history of the *D. buzzatii* cluster would necessarily imply the expansion of our knowledge of the host plants that surely had a profound impact in a group of species in active cladogenesis.

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