



Left-right asymmetry of fly wings and the evolution of body axes

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The body plan of *Drosophila*, and presumably that of other insects, develops under the control of anterior–posterior and dorsal–ventral axes, but no evidence for a left–right axis has yet been found. We used geometric morphometrics to study the wings in three species of flies: *Drosophila melanogaster*, *Musca domestica* and *Glossina palpalis gambiensis*. In all three species, we found that both size and shape showed subtle, but statistically significant directional asymmetry. For size, these asymmetries were somewhat inconsistent within and between species, but for shape, highly significant directional asymmetry was found in all samples examined. These systematic left–right differences imply the existence of a left–right axis that conveys distinct positional identities to the wing imaginal discs on either body side. Hence, the wing discs of *Drosophila* may be a new model to study the developmental genetics of left–right asymmetry. The asymmetries of shape were similar among species, suggesting that directional asymmetry has been evolutionarily conserved since the three lineages diverged. We discuss the implications of this evolutionary conservatism in conjunction with results from earlier studies that showed a lack of genetic variation for directional asymmetry in *Drosophila*.

Keywords: development; directional asymmetry; *Drosophila melanogaster*; left–right axis; morphometrics; shape

1. INTRODUCTION

Directional asymmetry, in which left and right body sides differ consistently from each other, is widespread in the animal kingdom, and its evolution and development have attracted an increasing amount of attention (Bock & Marsh 1991; Palmer 1996; Levin 1997). The expression of directional asymmetry is mediated by a left–right axis conveying distinct positional identities to developing structures on either body side. Recent discoveries have elucidated the developmental mechanisms that establish the left–right axis in vertebrates (e.g. Hyatt *et al.* 1996; Levin 1997; Levin *et al.* 1997; Supp *et al.* 1997; Varlet & Robertson 1997), in nematodes (Wood 1991), and in sea urchins (McCain & McClay 1994). In contrast, developmental genetic studies in *Drosophila* have revealed only the existence of anterior–posterior and dorsal–ventral axes (St Johnston & Nüsslein-Volhard 1992), but not of a left–right axis. This led some authors to conclude that a left–right axis does not exist in flies and other insects, and that its absence constitutes a developmental constraint that precludes the evolution of directional asymmetry (Tuinstra *et al.* 1990; Raff 1996).

Here we study directional asymmetry of the wings in three species of flies. Using a new morphometric method (Smith *et al.* 1997; Klingenberg & McIntyre 1998) to quantify left–right differences in size and shape of wings, we demonstrate that all three species exhibit directional asymmetry. This is strong evidence that flies do have a left–right axis. Furthermore, the left–right differences in shape are similar among the three species of flies studied,

suggesting that the asymmetry has a common evolutionary origin and has been conserved for tens of millions of years.

2. MATERIAL AND METHODS

(a) Data

We examined samples of male flies reared in the laboratory. The primary sample for the fruit fly, *Drosophila melanogaster* (Drosophilidae), were $n=117$ males of the F₂ generation from a cross between the Canton-S and Oregon-R lines; to test the consistency of results, we used supplementary samples of females from the parental lines ($n=62$ and 78), reciprocal F₁s ($n=54$ and 66) and F₂s ($n=117$). For the house fly, *Musca domestica* (Muscidae), the primary sample ($n=30$ males) was from a line derived from a population in Edmonton (Alberta, Canada); we also included a supplementary sample ($n=29$) from a line from Houston (Texas, USA). Finally, we used a sample ($n=70$) of males of the tsetse fly, *Glossina palpalis gambiensis* (Glossinidae), from a line originally from Burkina Faso. For each fly, a set of landmarks was recorded for both wings (figure 1). Landmark configurations were recorded using a dissecting microscope equipped with a camera lucida and a digitizing tablet.

To quantify and minimize measurement error (Palmer 1994), all wings were digitized twice for *Drosophila* and *Musca*, and three times for *Glossina*. Unlike fluctuating asymmetry, which concerns the dispersion of individual left–right differences, directional asymmetry pertains to the mean left–right difference in a sample, and is thus statistically less difficult to estimate. Because directional asymmetry is a mean, the variance of estimates due to random measurement errors is inversely proportional to sample

size multiplied by the number of replicate measurements. Therefore, even with a moderate sample size and two replicates, random measurement error becomes negligible.

In contrast, systematic measurement errors (e.g. from optical distortion) are more serious for analyses of directional asymmetry. We are confident, however, that systematic errors do not affect the data used in this study. First, data obtained independently with different equipment produced consistent results. Second, measurements of the same wings repeated from both the dorsal and ventral sides (i.e. after reflection) were no more variable than replicates digitized from the same side. This indicates that there was no distortion in the measurements, and thus we can rule out artefacts from the digitizing procedure as a source of the observed left-right asymmetries.

It was not possible to digitize the same set of landmarks for all three species; of the 12 or 13 landmarks included for each species, nine were shared by all three species. The results presented here are from analyses using all landmarks available in each test to provide maximum information and statistical power; analyses with only the nine shared landmarks produced similar results.

(b) Analysis

Directional asymmetry is a consistent bias toward one body side, which can be analysed as the mean difference between left and right sides in either size or shape. It is tested statistically with a *t*-test for size or with its multivariate equivalent, the T^2 -test, for shape (e.g. Flury 1997). As with the within-sample variation, these *t*- and T^2 -tests used the variation of individual asymmetries around the mean, which is a measure of fluctuating asymmetry (the index FA4 of Palmer (1994)). Therefore these statistical tests assess directional asymmetry relative to fluctuating asymmetry.

As a measure of the overall size of wings, we computed centroid size (Bookstein 1996), the square root of the sum of squared deviations of landmarks around their centroid. It can be viewed as a composite of all pairwise distances between landmarks, and may thus be more sensitive than conventional measurements. Directional asymmetry for centroid size was estimated as the mean of signed left-right differences, and tested statistically with a *t*-test. Quantile plots indicated that left-right differences were close to a normal distribution, and contained no outliers that might have unduly affected the estimates of directional asymmetry or *t*-tests.

For analysing the directional asymmetry in wing shape, we used a Procrustes approach (Goodall 1991; Bookstein 1996). Landmark configurations of left and right wings were scaled to unit centroid size, those of the left wings were reflected to mirror images, and all configurations were optimally superimposed (Smith *et al.* 1997; Klingenberg & McIntyre 1998). Shape differences were then measured as the square root of the sum of squared deviations between corresponding landmarks of two shapes. This dimensionless measure of shape difference is an approximation of the Procrustes distance (Bookstein 1996). Scatter plots of individual left-right differences at each landmark gave no evidence for anti-symmetry (pairs of clusters). With regard to the multivariate distribution of asymmetries, plots of squared standard distances against quantiles of the χ^2 distribution (Flury 1997, theorem 3.3.2) revealed no outliers beyond the expectation from the multivariate normal distribution.

For each species, the existence of directional asymmetry in shape was tested with a one-sample T^2 test (Flury 1997). To avoid singularity of the covariance matrix, this test omitted four coordinate variables (Bookstein 1996).

For shape, directional asymmetry in each species can be described as a vector of average left-right differences in landmark positions (DA vector). To test whether the DA vectors differ among samples, we conducted pairwise T^2 tests (Flury 1997; dimensions adjusted as above). In addition, we calculated vector correlations, which measure the similarity of DA vectors in terms of the directions and relative magnitudes of the landmark displacements, irrespective of the overall magnitude of left-right differences. It is calculated as the inner product of two vectors normalized to unit length ($VC = \mathbf{a}'\mathbf{b} / (\mathbf{a}'\mathbf{a}\mathbf{b}'\mathbf{b})^{0.5}$, where \mathbf{a} and \mathbf{b} are DA vectors from two samples). Values can range from -1 (for opposite DA vectors) to $+1$ (for proportional DA vectors).

Statistical significance of vector correlations among DA vectors was assessed with a Monte-Carlo test. For the null hypothesis, random DA vectors were simulated as independent and identically distributed circular Gaussian perturbations at every landmark (Goodall 1991). For each iteration of the test, a pair of vectors was drawn from a $(2k-4)$ -dimensional normal distribution ($2k-4$ is the dimensionality of the shape space for k landmarks in a plane), the vector correlation between them was computed and compared with the vector correlation between the original DA vectors. For each comparison, 10 000 iterations were performed. We combined this procedure with the bootstrap to examine whether the sampling variation of DA vectors had an influence on the outcome of the Monte-Carlo tests; there was virtually no effect on the significance levels achieved, and we therefore can ignore sampling variation in this test.

3. RESULTS

Wing size showed that statistically significant directional asymmetry occurred in all three species, but was not consistent among samples (table 1). The right wing was larger than the left wing in *Musca* and *Glossina*, but the left wing was larger than the right wing in most samples of *Drosophila*.

Differences between the mean shapes of left and right wings (figure 1) were subtle but highly significant statistically (*Drosophila*, difference=0.0066; *Musca*, difference=0.0068; *Glossina*, difference=0.0056; all $p > 0.0001$ and thus significant after sequential Bonferroni adjustment (Sokal & Rohlf 1995)). All additional samples of *Drosophila* and *Musca* showed similar, significant directional asymmetry. Therefore, in all three species, wing shape displays clear directional asymmetry.

Given this directional asymmetry in wing shape, we can conduct further analyses to examine whether left-right displacements of landmarks are the same in different species. Pairwise comparisons showed that the DA vectors were significantly different among species (*Drosophila* versus *Musca*, difference=0.0058; *Drosophila* versus *Glossina*, difference=0.0068; *Musca* versus *Glossina*, difference=0.0043; all $p > 0.0001$ and thus significant after sequential Bonferroni adjustment). Some, but not all, intraspecific comparisons of DA vectors showed significant differences as well.

Although DA vectors are not identical, homologous landmarks tend to have similar left-right displacements in the three species (figure 1). For instance, landmark 2 is in a more proximal position on the left than on the right side, landmark 4 is more distal and slightly more anterior, landmarks 10 and 11 are more proximal, and landmarks 15

Table 1. Mean centroid sizes of left and right wings (\pm s.e.), directional asymmetry (\pm s.e.), *t*-statistics and directional asymmetry as a percentage of mean size for the different samples of flies

(The first samples listed for each species are the primary samples used for interspecific comparisons. Samples from reciprocal F_1 crosses are designated (paternal \times maternal strain). Significance levels: * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$ in two-tailed test.)

sample	left wing (mm)	right wing (mm)	difference (mm)	<i>t</i>	% difference
<i>Drosophila</i>					
F_2 males	2.334 \pm 0.009	2.329 \pm 0.009	0.0046 \pm 0.0018	2.53*	0.20%
F_2 females	2.537 \pm 0.014	2.540 \pm 0.014	-0.0029 \pm 0.0020	-1.42	-0.11%
F_1 (C \times O)	2.709 \pm 0.011	2.698 \pm 0.011	0.0112 \pm 0.0027	3.79***	0.41%
F_1 (O \times C)	2.760 \pm 0.010	2.749 \pm 0.010	0.0115 \pm 0.0039	2.97**	0.42%
Oregon-R	2.714 \pm 0.009	2.712 \pm 0.009	0.0011 \pm 0.0025	0.45	0.04%
Canton-S	2.482 \pm 0.014	2.479 \pm 0.012	0.0027 \pm 0.0067	0.40	0.01%
<i>Musca</i>					
Edmonton	5.708 \pm 0.038	5.717 \pm 0.039	-0.0094 \pm 0.0038	-2.48*	-0.17%
Houston	5.101 \pm 0.114	5.102 \pm 0.114	-0.0010 \pm 0.0044	-0.23	-0.02%
<i>Glossina</i>					
	6.802 \pm 0.025	6.829 \pm 0.025	-0.0262 \pm 0.0050	-5.28***	-0.38%

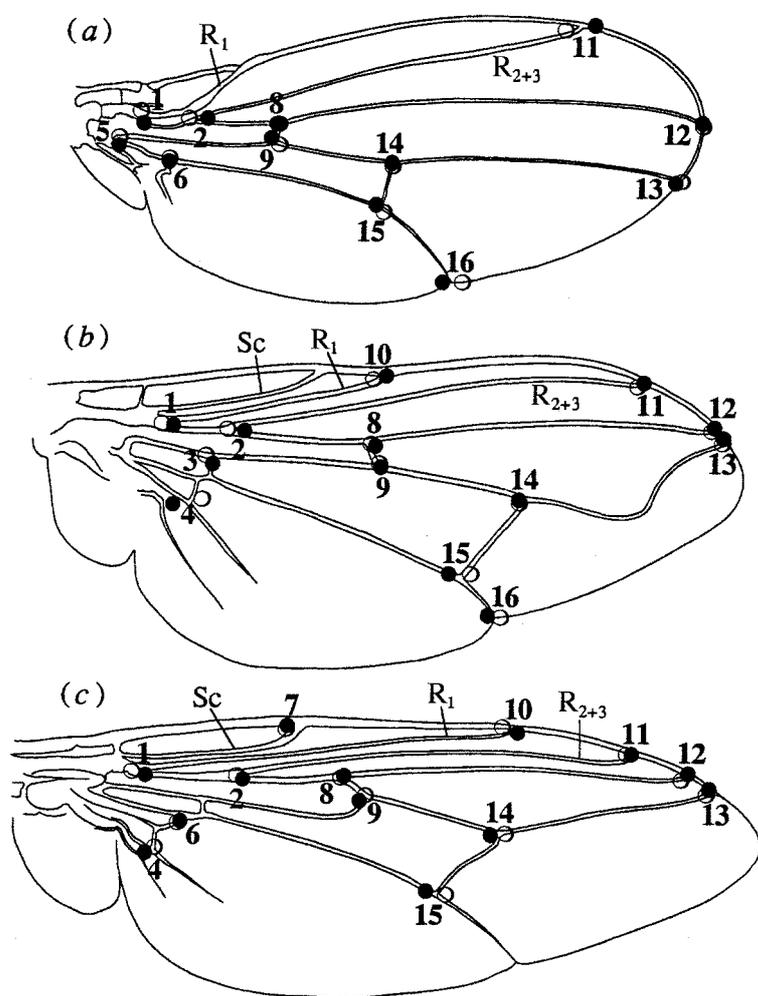


Figure 1. Directional asymmetry of shape in fly wings. (a) *Drosophila*. (b) *Musca*. (c) *Glossina*. The differences between mean positions of landmarks on the left wings (open circles) and right wings (solid circles) have been magnified tenfold for better visibility. Numbers designate homologous landmarks. Labelled wing veins are the subcosta (Sc; the Sc is reduced and does not extend to the wing margin in *Drosophila*) and the first two branches of the radius (R_1 and R_{2+3}).

The landmarks were the following (nomenclature after Colless & McAlpine (1991)): 1, branching point of veins R_1 and R_S (base of R_{2+3} and R_{4+5}); 2, branching point of veins R_{2+3} and R_{4+5} ; 3, anterior and proximal corner of the discal cell (branching point of veins M_{1+2} and M_{3+4}); 4, intersection of veins CuA and 1A, merging into vein CuA+1A; 5, branching point of veins M (continuing as M_{1+2}) and CuA (continuous distally with M_{3+4}); 6, origin of crossvein *m-cu* from vein CuA, which bends sharply posteriorwards at this landmark; 7, intersection of veins C and Sc; 8, intersection of vein R_{4+5} and crossvein *r-m* (anterior crossvein); 9, intersection of crossvein *r-m* and vein M_{1+2} ; 10, intersection of veins C and R_1 ; 11, intersection of veins C and R_{2+3} ; 12, intersection of veins C and R_{4+5} ; 13, intersection of veins C and M_{1+2} ; 14, intersection of vein M_{1+2} and crossvein *i-m* (posterior crossvein); 15, intersection of crossvein *i-m* and vein M_{3+4} ; 16, intersection of M_{3+4} and the wing margin.

and 16 are more distal on the left than on the right wing. To quantify these similarities, we calculated vector correlations between DA vectors. All the vector correlations among DA vectors were positive, both for intra- and interspecific comparisons, indicating that shape asymmetries were never reversed. Whereas all intraspecific vector

correlations were statistically significant, the results of interspecific comparisons were variable. High vector correlations were obtained between *Musca* and *Glossina* ($VC=0.79$, $p=0.0001$) and between *Musca* and *Drosophila* ($VC=0.62$, $p=0.0043$) (both significant after sequential Bonferroni adjustment). In contrast, the vector correlation

between the DA vectors for *Drosophila* and *Glossina* was lower and not statistically significant ($VC=0.33$, $p=0.090$).

Drosophila differs from the two other species considered here in that the first branch of the radial vein (R_1) meets the wing margin in a proximal location, roughly where the subcosta meets the wing margin in the other two species (landmark 7, see figure 1). Therefore, the R_{2+3} vein is the anteriormost longitudinal vein reaching the distal part of the wing, a position that corresponds to the R_1 of *Glossina* (the R_1 of *Musca* has an intermediate position). We incorporated this positional correspondence to reflect the important role of spatial arrangement (e.g. by lateral inhibition) in the development of wing veins (Sturtevant & Bier 1995). Thus we reassigned landmark 11 of *Drosophila* to landmark 10 (the corresponding location in the other species), and used the resulting DA vector to calculate vector correlations with the other two species. The vector correlation between DA vectors changed little for the comparison of *Drosophila* and *Musca* ($VC=0.63$, $p=0.0039$), but increased substantially for the comparison of *Drosophila* and *Glossina* ($VC=0.47$, $p=0.029$). Therefore, after adjustment for positional differences among species, all comparisons of DA vectors showed significant similarities (with sequential Bonferroni correction taking into account all three pairwise comparisons).

4. DISCUSSION

We have shown that the shape of fly wings, and often also size, exhibit directional asymmetry. This is clear evidence for the existence of a left–right axis in flies. Because left and right wings develop independently from separate imaginal discs, these systematic left–right differences must be based on a difference in positional identity between the left and right wing discs. Alternative explanations for the asymmetry, such as differential use of structures on the two body sides (Smith & Palmer 1994), can be ruled out because the wings are not functional until after metamorphosis. These results imply that, in addition to the well-known anterior–posterior and dorsal–ventral axes, flies do have a left–right axis affecting at least the wing discs. It is surprising that no left–right positional signalling has been discovered before in *Drosophila melanogaster*. Perhaps this is because positional signalling of the left–right axis is transient and much less prominent than that of the two principal body axes, as in vertebrates, where the left–right axis is much better known (Levin 1997; Varlet & Robertson 1997).

Other systematic left–right asymmetries have been reported from flies, such as the coiling of the gut (Strasburger 1932) or the torsion of genital segments in males (circumversion; McAlpine 1981; Colless & McAlpine 1991). These asymmetries may also rely on positional signals from a left–right axis, but they do not provide unambiguous evidence because alternative mechanisms are conceivable. For instance, mutations at the *rotated abdomen* locus in *Drosophila* cause abdominal torsion; Martín-Blanco & García-Bellido (1996) hypothesized that an intrinsic torque of muscle fibres leads to asymmetry in the overall structure of muscles of the body wall, causing them to exert force in an oblique direction, which ultimately generates the clockwise staggered

arrangement of adult abdominal segments characteristic of the mutant phenotype. Yet, because the wings develop from imaginal discs that are physically separated, such a mechanism without left–right signalling cannot account for wing asymmetry.

Asymmetries that require a left–right axis appear to be widespread among insects. For example, directional asymmetry in wing shape has also been reported from honey bees (*Apis mellifera*; Smith *et al.* 1997). More extreme examples include the consistently left-sided asymmetry of mandibles in thrips (Thysanoptera; Heming 1993) and the variable but species-specific asymmetries in the genital system of bed bugs (Cimicidae; Carayon 1966).

The positive vector correlations among the DA vectors indicate that left–right displacements of landmarks are similar among the three species (see figure 1). This similarity suggests that the developmental basis of directional asymmetry is conserved and must, therefore, have persisted since the three phylogenetic lineages diverged. The fossil record of the families represented here extends back to the Oligocene (36–23 Ma; Carpenter 1992), but Cretaceous fossils from the family Calliphoridae (Carpenter 1992), more closely related to *Musca* than to the other taxa considered here (McAlpine 1989), suggest that the evolutionary divergence of the three lineages is substantially more ancient. Hence directional asymmetry of wing shape has persisted in the three lineages independently at least for tens if not hundreds of millions of generations.

Such long-term conservation of wing asymmetry is surprising because asymmetry might be expected to impede flight performance (Møller & Swaddle 1997). Directional asymmetry of wing size is small in all three species (table 1), and probably insufficient to affect flight, as it is considerably less than the average (non-directional) asymmetry of 1.6–2.1% reported for *Musca* captured by swallows from a population with 0.5% average asymmetry (Møller 1996). Likewise, the asymmetries in shape are subtle; most likely, these small shifts of wing veins are not sufficient to cause changes of wing stiffness and hence aerodynamic properties (Ennos 1989). Still, selection should oppose mutant alleles that increase these asymmetries.

Overall, this evolutionary conservatism is consistent with the lack of genetic variation for directional asymmetry, which has been found repeatedly in *Drosophila* (Maynard Smith & Sondhi 1960; Coyne 1987; Tuinstra *et al.* 1990; Monedero *et al.* 1997). Some authors have attributed this absence of genetic variation to the lack of a left–right axis, because left–right positional signalling would be necessary for differential expression of genes on the two body sides (Tuinstra *et al.* 1990; Raff 1996, pp. 80 ff., 302 ff.). Thus they interpreted the lack of a left–right axis as a developmental constraint that precludes the evolution of external asymmetry in flies. This argument must be revised in the light of the results of our study, at least with regard to the wings. A full explanation will need to consider the genetic basis of left–right signalling as well as the developmental pathways that translate these positional signals into morphological asymmetry. In vertebrates, studies of this kind are making rapid progress (Levin 1997; Varlet & Robertson 1997). Our finding of directional asymmetry in fly wings suggests the wing discs of *Drosophila* as an additional system for these studies.

Directional asymmetry occurs throughout the animal kingdom (Palmer 1996), and a left-right axis has now been demonstrated for most major phyla, including all the principal model organisms in developmental biology. Although the left-right axis is established in different ways in different taxa, corresponding to the differences in their early embryogenesis, it is possible that the primary source of left-right asymmetry is much more ancient (Levin 1997). The fact that left-right asymmetry has been overlooked in the development of *Drosophila*, in spite of an unparalleled research effort, may be indicative of the discoveries yet to come.

We thank Lisa Meffert for providing flies, and Fred Bookstein, Jeff Marcus, Christen Mirth, Fred Nijhout, Rich Palmer, Kathleen Smith, David Stern, Steven Vogel and two anonymous referees for discussion and comments on previous versions of this paper. Financial support was provided by the Swiss National Science Foundation to C.P.K., a University of Alberta PhD scholarship to S.D.Z., and grants from NSERC (Canada) to A. R. Palmer and R. H. Gooding.

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