Quantitative-genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura* using Procrustes methods

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Abstract

Fluctuating asymmetry (FA) is often used as a measure of underlying developmental instability (DI), motivated by the idea that morphological variance is maladaptive. Whether or not DI has evolutionary potential is a highly disputed topic, marred by methodological problems and fuzzy prejudices. We report here some results from an ongoing study of the effects of karyotype, homozygosity and temperature on wing form and bilateral asymmetry using isochromosomal lines of *Drosophila subobscura*. Our approach uses the recently developed methodologies in geometric morphometrics to analyse shape configurations of landmarks within the standard statistical framework employed in studies of bilateral asymmetries, and we have extended these methods to partition the individual variation and the variation in asymmetries into genetic and environmental causal components. The analyses revealed temperature-dependent expression of genetic variation for wing size and wing shape, directional asymmetry (DA) of wing size, increased asymmetries at suboptimal temperature, and a transition from FA to DA in males as a result of increase in the rearing temperature. No genetic variation was generally detected for FA in our samples, but these are preliminary results because no crosses between lines were carried out and, therefore, the contribution of dominance was not taken into account. In addition, only a subset of the standing genetic variation was represented in the experiments.

Introduction

Developmental instability (DI) is the result of processes that disturb development along a developmental trajectory within a particular environment (Palmer 1994; Nijhout and Davidowitz 2003). A measure of within-individual variation, which is the finest level at which phenotypic variance can be reckoned (Lynch and Walsh 1998, pp. 112–116), provides a quantifiable expression of underlying DI that is referred to as fluctuating asymmetry (FA, i.e. small random deviations from symmetry in otherwise bilaterally symmetrical characters; Van Valen 1962; Palmer and Strobeck 1986; Leary and Allendorf 1989; Markow 1995; but see Palmer and Strobeck 2003, p. 281, for a cautionary remark on the relationship between FA and DI). Insofar as both sides of a symmetrical structure are uniformly controlled by the same genes (barring unusual somatic mutation or somatic recombination), the subtle nondirectional differences typically found between right and left sides of bilateral traits are expected to arise owing to inherent nongenetic developmental noise (e.g. Waddington 1957; Palmer and Strobeck 1986; Palmer *et al.* 1993). Mather (1953) was the first to suggest, however, that FA had a genetic basis. More recently, interest in FA as a putative reliable cue of the overall genetic quality of an individual has led some authors to claim that FA (and hence DI) does indeed have a positive heritability and is

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therefore biologically and evolutionary relevant on its own (reviewed in Møller and Swaddle 1997). These claims have raised strong (sometimes acid) controversies and have generated quite a number of meta-analyses on published estimates of FA heritabilities. Though one positive outcome of this debate has been more rigorous and focussed research (Polack 2003), the topic of the genetical basis of FA still remains completely unresolved (Santos 2002; Fuller and Houle 2003).

In a recent quantitative-genetic study of wing size asymmetry, one of us found low (~ 0.04) but significant heritabilities of FA in wing length and width in *Drosophila buzzatii* (Santos 2002). Some findings were, however, difficult to reconcile with those positive heritabilities, besides the fact that the additive genetic correlation for size between sides was not significantly different from +1. If variation that is due to stochasticity in development is the only real cause of FA (see Nijhout and Davidowitz 2003), there are at least three possible explanations for those results. First, they might have simply been false positives. However, the detection of similarly low (but nonsignificant) wing size FA heritability in two previous experiments (Santos 2001) casts some doubts on this explanation. Second, the random noise component is independent of genotype, but genetic variance for trait size alone could give rise to low heritabilities of FA (Klingenberg and Nijhout 1999). Although wing size in *Drosophila* usually shows intermediate heritabilities, a problem with this explanation is that there is no relationship between trait size and FA in *D. buzzatii* (Santos 2001, 2002; see also Loeschcke *et al.* 1999, 2000). Third, the sensitivity to developmental noise in a random-mating population may be genotype dependent. This alternative arises from developmental models where there is a nonlinear mapping between development and FA (see Klingenberg 2003a), and we find it particularly attractive and thought-provoking.

Stochastic mechanisms in gene expression can explain the substantial phenotypic variation usually observed in clonal populations (Elowitz *et al.* 2002). Alternatively, for bilaterally symmetrical structures controlled by the same genes, this inherent stochasticity would lower the between-side correlation in gene expression, likely resulting in FA. The important point for FA to display positive heritability is, however, whether or not the noise is genotype dependent. Models of stochastic gene expression predict that intrinsic noise should increase as the amount of transcript decreases (Rao *et al.* 2002; Swain *et al.* 2002), and recent evidence shows that the level of gene expression in eukaryotic cells is indeed strongly influenced by transcription (Blake *et al.* 2003). Although at present it is largely unknown how widespread the genetic variation in transcript levels is, recent studies in humans have identified genes whose transcript levels differed greatly among unrelated individuals (Cheung *et al.* 2003). In addition, it has already been shown that noise has a genetic compo-

nent; *recA* mutants in *E. coli* are twice as noisy as their wild-type counterparts (Elowitz *et al.* 2002). In summary, if the amount of noisiness is genetically determined, then different genotypes might exhibit different levels of DI. In this case, a reliable measure of genotype-dependent FA would clearly depend on replication, both at the genotype and at the experimental levels.

A basic problem in using FA as an estimate of an individual's ability to buffer its phenotype from genetic or environmental perturbations or both is the low signal/noise ratio. FA correlates only loosely with underlying DI because it is an attempt to estimate a variance with two data points (right and left; see Whitlock 1996, 1998; Van Dongen 1998). However, if the standard model of the relationship between FA and DI (Palmer and Strobeck 1992; Houle 1997, 2000; Leung and Forbes 1997; Gangestad and Thornhill 1999) provides a convenient approximation to reality, the putative genetical basis of FA could be detected by replicating genotypes to increase the signal. For some *Drosophila* species this can be attained from a classical and widely used technique that allows rendering individuals homozygous for virtually all genes carried by an entire chromosome, thus obtaining isogenic or isochromosomal lines (see, for example, Wallace 1981). Additional and reliable information about the relationships among genetic or environmental stresses or both with FA levels could also be gained by using the established isogenic lines (see also Woolf and Markow 2003).

An equally important problem in studies of FA is the choice of traits. For metric traits it is generally assumed that each trait value reflects an independent sample from a normal distribution with mean equal to its expected phenotypic value and variance reflecting the amount of random noise and the degree of DI (see, for example, Van Dongen *et al.* 2003). If this is the case, the presence of between-trait correlations in the unsigned FA [abs (left–right)] could be indicative of an organism-wide DI (Møller and Swaddle 1998), but the problem is the low correlation between single-trait FA and the presumed underlying DI (see above). On the other hand, if there is a developmental connection between the traits studied a correlation between the signed asymmetries of traits is expected (see Van Dongen *et al.* 1999; Santos 2002; Klingenberg 2003b), which would hamper the interpretation of between-trait correlations in the unsigned FA, thus rendering those traits unsuitable for studying organism-wide DI. In contrast, relevant knowledge on developmental interactions and morphological integration can now be gained by comparing the patterns of covariation among asymmetries of traits (Klingenberg *et al.* 1998; Klingenberg and McIntyre 1998; Debat *et al.* 2000; Klingenberg and Zaklan 2000). As pointed out by Klingenberg (2003b), this approach departs from more traditional research on FA but opens a new direction of research at the interface between evolutionary and developmental biology.

We report here some initial results from an ongoing study of the effects of karyotype, homozygosity and temperature on wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura*. This is a particularly inversion-rich species, with up to 38 natural chromosomal arrangements already reported for the largest chromosome O (Krimbas and Loukas 1980), for which a balanced marker strain has been developed (Sperlich *et al.* 1977). A large number of isochromosomal lines in an otherwise homogeneous genetic background were obtained from a stock collected at Puerto Montt (Chile), which is a New World colonizing population that harboured a total of seven chromosome O arrangements (including the recombination-derived and sporadically found O_7 arrangement; see Balanyà *et al.* 2003; Santos *et al.* 2004). Here we have used a subset of lines that belong to the O_{st} class, and have studied wing size and shape using the framework of geometric morphometrics (Bookstein 1991; Dryden and Mardia 1998). We show that the genetic variability for overall wing shape was comparable to that for wing size, although results were temperature dependent. On the other hand, directional asymmetry (DA) has been detected for wing size but not shape, and no genetic variation was observed for FA. However, it should be borne in mind that these are preliminary results because (i) no crosses between lines were carried out and, therefore, the contribution of dominance was not taken into account; and (ii) most genetic variation in New World colonizing populations of *D. subobscura* is likely apportioned among chromosome arrangements.

Materials and methods

Base stocks and fly handling

The *D. subobscura* base population originated from 93 isofemale strains derived from a large outbred stock collected by Drs J. Balanyà, G. W. Gilchrist, R. B. Huey and M. Pascual at Puerto Montt (Chile; 41°28′S) in November 1999. The isofemale lines were kept at 18°C for more than one year (~16 generations) prior to the establishment of a set of three replicated thermal selection stocks (see Santos *et al.* 2004). In November 2001, eggs were collected over three consecutive days from the populations kept at the experimental temperature of 18°C, and were placed in 130-ml bottles (~200 eggs per bottle) containing 50 ml of David's killed-yeast *Drosophila* medium (David 1962). A random sample of 300 males (100 males per replicate) from those that emerged from the bottles were used to obtain isochromosomal lines.

Extraction of O chromosomes:
The procedure used to obtain isochromosomal lines for the O chromosome (homologous to arm 3R in *D. melanogaster*; Powell 1997, p. 307) in an otherwise homogeneous genetic background is schematically shown in figure 1. Briefly, wild-type males were individually crossed to three or four virgin females from the *ch-cu* marker strain, which is homozygous for the morphological recessive markers on the O chromosome *cherry* eyes (*ch*) and *curled* wings (*cu*) (Koske and Maynard Smith 1954). The genetic background of the *ch-cu* strain is highly homogeneous and fixed for the so-called standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O_{3+4} (Lankinen and Pinsker 1977). A single F_1 progeny of each cross was backcrossed to *ch-cu* females and the scheme was repeated for five generations. After four generations of crosses the lines were identified for chromosome arrangements at all chromosomes by microscope inspection of up to five third-instar larva salivary gland squashes to look for inversion loops in polytene chromosomes. This allowed identification of the isolated O gene arrangements and to check for presence of undesirable nonstandard chromosomal arrangements on the
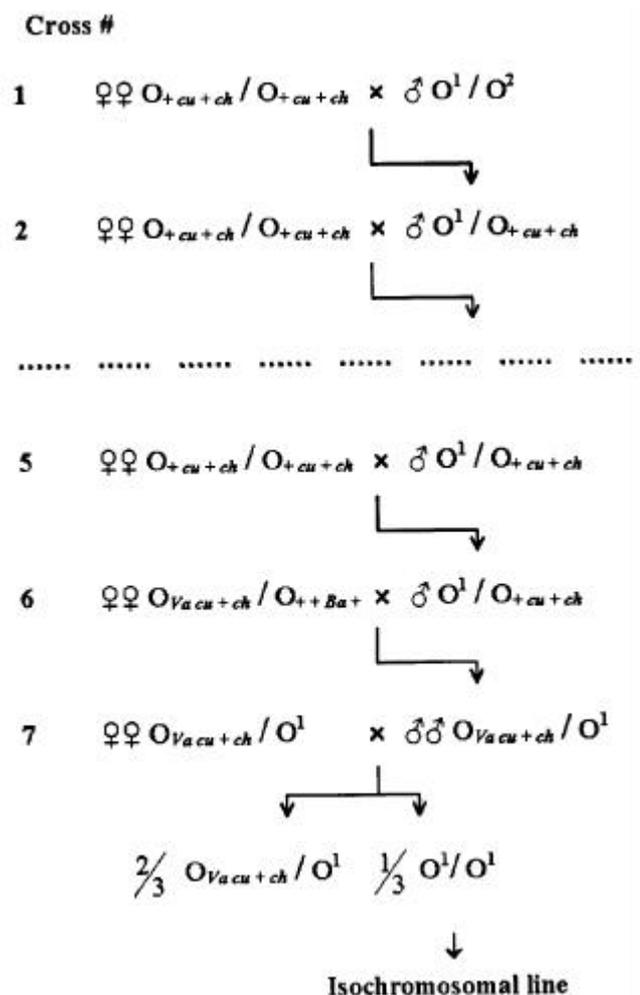
Figure 1. Mating scheme used to establish the isochromosomal lines in an otherwise homogeneous *ch-cu* genetic background.

other three major autosomes (named E, J and U; see Krimbas and Loukas 1980) carried by the founding wild-type males. To obtain the isochromosomal strains a single male from the selected lines was crossed to two virgin females from the *Va*/*Ba* balanced marker stock (Sperlich *et al.* 1977). This stock carries the dominant lethal genes *Varicose* (*Va*) and *Bare* (*Ba*) on the O chromosome and was derived from the *ch-cu* strain. Because expression of the *Ba* gene is highly variable and affected by modifiers located on the O chromosome (Alvarez *et al.* 1981), we relied only on the *Va* marker to obtain the isochromosomal lines. A total of 114 lines were obtained; their viability distributions are shown in figure 2. Viabilities were estimated after pooling the progeny raised from two replicated vials; each vial was set up with three $O_{Va\ cu+ch}/O^1$ females crossed to two $O_{Va\ cu+ch}/O^1$ males (cross # 7 in figure 1). Twelve days after crosses were made all parents were discarded. The offspring counts were continued until all flies emerged from the vials, with a total of 25,425 flies (harmonic mean = 185.7 flies per line). Viability was expressed as the ratio

$$\frac{\text{number of wild-type flies}}{\text{total number of flies}}.$$

The viability distribution for all lines (figure 2a) had the usual pattern in this kind of experiments (see, for example, Wallace 1981, p. 67), with 18 lines (15.8%) having a viability lower than 0.1. The number of lines for each chromosomal class (figure 2b) was proportional to the frequencies of chromosome O arrangements in the founding thermal stocks (Santos *et al.* 2004). As expected, those lines carrying the arrangement O_5 were lethal (figure 2b; see Mestres *et al.* 1992).

The lines used in the experiments were genotyped for microsatellite loci on the chromosome O and found to be homozygous, checked again for all chromosome gene arrangements after individually crossing the isogenic males to three or four *ch-cu* virgin females, and had a quasinormal viability (defined as >0.25). All crosses were made at 18ºC (12 h : 12 h light : dark cycle), and fly handling was done at room temperature using CO_2 anaesthesia on flies not less than 6 h after eclosion.

Experimental design: The data used here are from an ongoing study of the effects of karyotype, homozygosity and temperature on wing asymmetry in *D. subobscura*. We report results from isochromosomal lines that belong to the O_{st} class. Isochromosomal lines were kept in 130-ml bottles with low adult density to standardize the conditions of rearing before egg collections. Eggs were collected from each of 10 O_{st} lines from spoons containing nonnutritive agar with a generous smear of live yeast, and placed in three 2 cm × 8 cm vials with 6 ml of food (26 eggs per vial) at 18ºC on the same incubator shelf. Similarly, eggs from a subset of five isochromosomal

lines were placed in vials at 23ºC (suboptimal temperature). Emerging flies (no less than two or three days old) were stored in Eppendorf tubes with a 3 : 1 mixture of alcohol and glycerol at 4°C before wing measurements.

Wing size and shape

Definitions: Morphometrics involves quantitative study of form, and it is naturally understood that form consists of size and shape (Needham 1950). An important contribution of geometric morphometrics is the clear definition of size and shape (Dryden and Mardia 1998). Size is defined as any positive real-valued function from a landmark configuration (i.e. a set of points that can be precisely located) matrix X that satisfies the condition $g[aX] = ag[X]$ for any positive scalar a. The shape of a set of p landmark points is the geometrical information of the configuration of points that is invariant to translation (variation in the position of the configuration in the digital image), rotation (variation in the orientation of the configuration), and rescaling (variation in size).
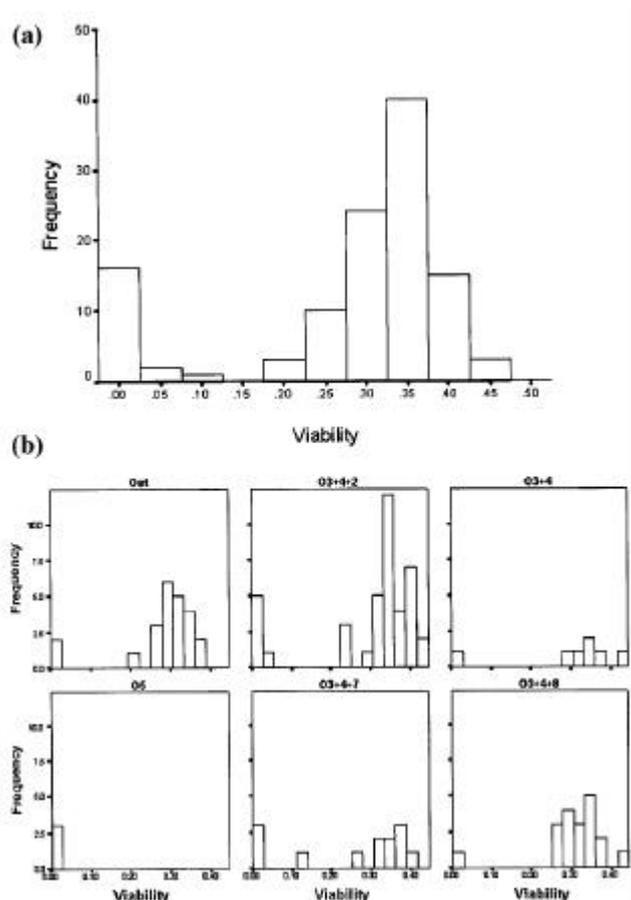

Figure 2. Viability distributions for the isochromosomal O lines: (a) all 114 lines; (b) grouped according to chromosomal arrangement.

Wing measurements: Wings from both sexes were removed and fixed in DPX under coverslips on microscope slides. The data consisted of *x* and *y* coordinates of 13 morphological landmarks (figure 3). All landmarks used are at the intersections of wing veins or at points where veins reach the wing margin and are easy to locate precisely, and can therefore be considered type 1 landmarks according to Bookstein (1991, pp. 63–67) or anatomical landmarks according to Dryden and Mardia (1998, p. 3). Wings were digitized by one of us (P. F. I.) using a compound microscope (Zeiss Axioskop) with low-power objective (2.5×) and attached video camera (Sony CCD-Iris) connected to a PC with MGI VideoWave software. Calibration of the optical system was checked in each session. All wings were digitized and measured two times in different sessions as follows. Both the left and right wings were digitized during a given session, and after an entire round on all individuals, the same process was repeated again. The *x* and *y* coordinates of the morphological landmarks were recorded for each wing in a similar fashion using the image processing and analysis program Scion Image (based on the NIH-Image for Macintosh and available at http://www.scioncorp.com). The procedure we used guaranteed that the observer was blind with respect to the results from previous measurements.

Statistical analyses of wing size and shape, and asymmetry

Overall wing size was analysed here using centroid size (defined as the square root of the sum of squared distances of a set of landmarks from their centroid or, equivalently, the square root of the sum of the variances of the landmarks about that centroid in *x* and *y* directions; Slice *et al.* 1996) as the dependent variable (referred to as CS). Wing shape (WS) was characterized by the geometrical configuration of the morphological landmarks after variation in wing size was removed.
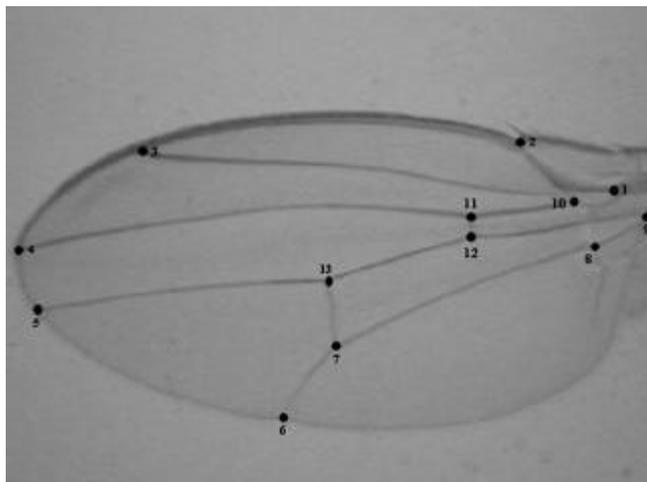
Figure 3. Image of *Drosophila subobscura* left wing indicating the thirteen landmarks used in this work.

To assess asymmetry we used the conventional mixed-model, two-way analysis of variance where individuals is a random effect and sides is a fixed effect (Palmer and Strobeck 1986; Palmer 1994). In this ANOVA the main effect of individual flies stands for phenotypic variation in the trait (CS or WS), the main effect of body sides is for directional asymmetry (DA) and tests whether or not the signed differences between the left and right wings [designated as $(L - R)$] have a mean of zero, the interaction term is a measure of fluctuating asymmetry (the variation in left–right differences among individuals) provided that there is no genetic variation for DA (see Santos 2002), and the error term provides an estimate of the measurement error. In addition, if individuals can be grouped into families or lines (as happens here), the variation among individuals can be further decomposed to test for genetic effects on the trait; and the variation due to the interaction term can also be decomposed to test for genetic effects on DA (Santos 2001, 2002). Following Sokal and Rohlf (1981, p. 337) and Leamy (1999), mean squares for individuals were tested over the error mean squares whereas mean squares for sides were tested over the individuals × sides interactions.

To test for size dependence of FA, regressions of unsigned $|L - R|$ asymmetries on centroid sizes were performed and in no case were the slopes statistically different from zero (females 18°C: $\boldsymbol{b} = -0.0211$, $F_{1,98} = 2.94$, n.s.; males 18°C: $\boldsymbol{b} = 0.0144$, $F_{1,98} = 0.47$, n.s.; females 23°C: $\boldsymbol{b} = 0.0305$, $F_{1,28} = 0.37$, n.s.; males 23°C: $\boldsymbol{b} = 0.0129$, $F_{1,28} = 0.12$, n.s.).

Generalized Procrustes analysis for quantifying wing shape asymmetry: Procrustes methods allow comparison of configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit (Rohlf 1990, 1999; Rohlf and Slice 1990; Klingenberg and McIntyre 1998). When several objects (e.g. wings) are fitted using Procrustes superimposition (as was done in the present work) the method has been called 'generalized Procrustes analysis' (GPA; see Dryden and Mardia 1998, pp. 44–47). In studies of 'matching asymmetry' (Mardia *et al.* 2000; Klingenberg *et al.* 2002), where a structure is present in the two sides of a bilateral organism, the landmark configuration of one body side has to be reflected to its mirror image before aligning the corresponding landmarks of both sides. For this purpose, we changed the sign of the *x* coordinate of every landmark for configurations from the right side, thus following our previous convenience of defining asymmetry as the left–right differences among individuals.

After reflections, our analysis used a single Procrustes superimposition to align simultaneously all the landmark configurations within each sex and isochromosomal line. For each sex we used as the 'base configuration' or refer-

ence specimen the Procrustes grand mean shape of the left wing of the species, obtained from 900 females and 1068 males derived from our thermal stocks (see Santos *et al.* 2003). This allows contrasting wing shapes between different lines or crosses. A single overall consensus configuration was computed for each isochromosomal line as the mean coordinates of corresponding landmarks in the aligned configurations (Rohlf and Slice 1990). The final iteration to minimize the sum of the squared distances between the landmarks of all objects in the sample was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden and Mardia (1998; see also Rohlf 1999). (Rescaling the coordinates of each configuration by the scaling option, $1/\cos(r)$ (see Rohlf 1999) would make very little difference, in the order of ~0.001% of the shape variation in the data sets.) The variation in the landmark coordinates that remains after Procrustes superimposition is a complete and nonredundant description of the variation in shape, and the usual linear multivariate methods focus on these coordinates (see below). The details of computation are clearly described in Klingenberg and McIntyre (1998), Klingenberg and Zaklan (2000) and Klingenberg *et al.* (2002).

In this work we used MATLAB (V.6; The MathWorks, Inc. 2002) for morphometric analyses. Some helpful functions in morphometrics from the MATLAB toolboxes Res5 and Res6 developed by R. E. Strauss were also used (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm).

***Procrustes ANOVA: extensions to analyse genetic and environmental components of shape asymmetries*:** As pointed out by Klingenberg and McIntyre (1998) calculation of Procrustes coordinates is based on the algebra of sums of squares, and the variance in the set of optimally aligned landmark configurations can be partitioned in a way analogous to the deviations from a grand mean in conventional ANOVA (Goodall 1991). The coordinates of the Procrustes-aligned configurations (all two replicates of each wing) are therefore amenable to the preceding two-factor mixed-model ANOVA.

The sums of squares for the individuals and sides main effects, individuals × sides interaction effects, and error terms are obtained after performing separated two-factor ANOVAs for each x and y coordinates of the aligned configurations and summing across all corresponding sums of squares. (Alternatively, we can perform a principal component analysis (PCA; see, for example, Jolliffe 1986) to obtain the matrix of scores from the first 22 PCs (see below) and return these scores into the space of the coordinates of the landmarks of superimposed configurations.) To decompose the resulting sums of squares in the among-isochromosomal-lines and within-isochromosomal-lines components, we proceeded in a similar way but

used lines instead of individuals as the main effect. The among-lines sums of squares for the individuals main effect are the same as those obtained after performing one-way ANOVAs to the individuals means for each landmark times the number of measurements per individual (2 wings × 2 measurements per wing; see Santos 2001). Similarly, the among-lines sums of squares for the individuals × sides interaction terms can also be obtained from one-way ANOVAs performed on signed $(L - R)$ asymmetries. In this case, L and R are estimated for each x and y coordinates of the aligned configurations as the averages of the left and right wing measurements, respectively. Lack of statistical significance among line means would indicate that there is no genetic variation for DA.

To test for presence of genetic components in wing shape FA we performed one-way ANOVAs on the unsigned $|L - R|$ estimates for each x and y coordinates of the aligned configurations (index FA1 in Palmer 1994) using lines as the main effect and summing across all coordinates to obtain the among-lines and within-lines sums of squares. There are more degrees of freedom in Procrustes ANOVA than in conventional ANOVA (Goodall 1991) because the squared deviations are summed over all the landmark coordinates. Therefore the number of degrees of freedom is that for ordinary ANOVA times the shape dimension; i.e. $2p$–4 for two-dimensional-coordinate data, where p is the number of landmarks.

Since variation around landmarks in Procrustes-superimposed biological configurations does not generally follow the isotropic model (which presumes that there is an equal amount of nondirectional variation at each landmark; see Goodall 1991; Rohlf 2000), degrees of freedom in Procrustes ANOVAs are not independent. Therefore statistical significance of ANOVA effects was tested from permutation tests (another approach would be to use conventional MANOVA; see Klingenberg *et al.* 2002). For the two-way mixed-model ANOVA randomization is a three-stage process (Good 1994; Edgington 1995): (i) random permutations within sides among individuals for the among-individuals F statistics; (ii) random permutations between sides within individuals for the between-sides F statistics; and (iii) random permutation across individuals and sides after subtracting the deviations due to both individuals and sides, and adding the grand mean (see Sokal and Rohlf 1981, p. 330). Each test used 10,000 random permutations.

***Localized variation*:** We followed Klingenberg and McIntyre (1998) and decomposed the Procrustes mean squares for each effect in the two-way ANOVAs according to the landmarks to assess how much of shape variation was due to each landmark. Thus we summed x and y mean squares of each landmark separately and computed the variance components according to the expected mean squares (Sokal and Rohlf 1981). Because the least-square

algorithm tends to spread variation from variable land-marks to the others, this approach should be taken cautiously if one or a few landmarks are much more variable than the rest (Chapman 1990; Walker 2000).

Shape variability: We used PCA to investigate patterns of covariation in the positions of landmarks, which is a usual method in the context of shape analysis (Dryden and Mardia 1998; Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000). The analyses must use covariance matrices of the coordinates of superimposed landmarks to avoid problems related to rotations of the coordinate system, and principal components coefficients can be presented graphically by drawing lines centred at the mean location of each landmark and ending at an arbitrary number of standard deviations away from that mean in the direction to which the landmark would shift.

To test for the congruence of landmarks displacements between effects (line, FA and digitizing error), both within and between samples, we followed Klingenberg and McIntyre (1998; see also Debat *et al*. 2000). Thus, within each sample we first computed matrices of sums of squares and cross-products (SSCP) for individuals (both sides and all replicated measurements averaged for each fly), for lines (averages of sides, measurements and flies within lines), for FA (fly × side interaction) from individual left–right differences (wing averages from all measurements), and for measurement error (residual variation of the replicated measurements about the wing average). After dividing the SSCP matrices by the appropriate degrees of freedom, we separated effects according to the expected mean squares by subtracting the within-line variance–covariance (VCV) matrix from the line VCV, and the measurement VCV from the fly × side VCV. For each effect, a PCA of the VCV matrix was performed, and the correlation between PCs was obtained by angular comparisons of component vectors. The statistical significance was assessed by comparing the observed values with a null distribution of absolute angles between 100,000 pairs of random vectors obtained as random points on a 22-dimensional unit sphere (see Klingenberg and Zimmermann 1992). The 0.1% quantile of the distribution of these angles was 50.3°.

Allometry: To test for size effects on shape variation we carried out multivariate regressions of Procrustes coordinates on centroid size (Dryden and Mardia 1998). These regressions generally accounted for less than 3% of total Procrustes sums of squares, and multivariate analyses using the residuals of a regression on centroid size produced results that were qualitatively identical to those of the complete variation. Therefore no size corrections were necessary and we only report the results of analyses of the total shape variation.

Temperature effects on wing size and shape

For those isochromosomal lines that were common to both experimental temperatures three-way mixed-model ANOVAs were performed with effects of temperature and sex assumed to be fixed, and line assumed to be random (for each fly we thus averaged throughout measurements and sides). Following Sokal and Rohlf (1981, p. 383), mean squares for temperature were tested over the T × L interaction, mean squares for sex over the S × L interaction, mean squares for line over the error term, the two-way interaction involving the fixed effects over the three-way interaction, and the two-way interactions involving the random and fixed effects over the error term. The sums of squares for the three-way Procrustes ANOVA were obtained after performing separated ANOVAs for each *x* and *y* coordinates of the aligned configurations and summing across all corresponding sums of squares. In this case statistical significance of ANOVA effects was tested after 10,000 permutation tests (see above).

The computer programs used for statistical data analyses were MATLAB (V.6; The MathWorks, Inc. 2002) together with the collection of tools supplied by the Statistics Toolbox (V.3; The MathWorks, Inc. 2000), and some results were checked with the statistical software packages STATISTICA V.6 (StatSoft, Inc. 2003) and SPSS V.11 (SPSS, Inc. 2001). They were run on a Pentium 4 (1.60 GHz) PC.

Results

Causal components of variation and asymmetry in wing size

The mean values of the average centroid sizes for the two repeated measures on each side are plotted in figure 4. The effects in the two-way mixed-model ANOVAs were significant at both temperatures in most cases (tables 1 and 2), including subtle directional asymmetries of wing size in all samples but males reared at 18ºC (mean centroid sizes for females at 18ºC: $\overline{L} = 0.9877$ mm, $\overline{R} = 0.9860$ mm; for males at 18ºC: $\overline{L} = 0.8934$, $\overline{R} = 0.8926$; females 23ºC: $\overline{L} = 0.9118$, $\overline{R} = 0.9069$; males 23ºC: $\overline{L} = 0.8123$, $\overline{R} = 0.8092$). DA was consistently higher at the warmest (and suboptimal) temperature and, in addition, there was some indication of genetic variation for wing size DA in males at 23ºC.

Size variation among individuals accounted for about 96% of the total variation. The intraclass correlation for size

$$\left[\frac{\boldsymbol{s}_g^2 (\text{CS})}{\boldsymbol{s}_g^2(\text{CS}) + \boldsymbol{s}_w^2(\text{CS})} \right],$$

which estimates the fraction of the total phenotypic variance due to among-lines genetic differences in O_{st} gene arrangements, was higher at 18ºC (~59%) than at 23ºC (~31%) in both sexes. The corresponding interaction components [$\boldsymbol{s}_{I\times S}^2$ (CS)] provided unbiased estimates of fluctuating asymmetry (index FA10 in Palmer 1994) in those

samples where genetic variation for DA was absent (see Santos 2002). Analyses of variance to test for genetic components of size FA (using index FA1 in Palmer 1994) only rendered statistically significant results in the males sample at 23ºC ($F_{4,25} = 3.89$, $P < 0.05$). However, this result should be taken with caution because of the biasing effect introduced by the presence of genetic variation in DA.

A close inspection of tables 1 and 2 suggests that wing size asymmetries (DA and FA) differ between temperatures, with DA being higher at the warmest temperature in both sexes but FA showing a contrasting pattern according to sex. The results cannot be ascribed to the different numbers of lines used in the experiments because similar patterns were observed when analyses were performed on the five isochromosomal lines that were common to both temperatures (results not shown). To test for the significance of the observed differences in FA between temperatures an *F*-test comparing the corresponding $s_w^2(DA_{CS})$ values in tables 1 and 2 is appropriate

since those variance components provide unbiased estimates of FA (recall that $s_w^2(DA) \approx s_{I \times S}^2$ when there is no genetic variation for DA). Approximate degrees of freedom for $s_w^2(DA_{CS})$ are

$$\frac{(MS_w - MS_m)^2}{\left[\dfrac{(MS_w)^2}{df_w} + \dfrac{(MS_m)^2}{df_m}\right]},$$

where the MSs and dfs are the corresponding mean squares and degrees of freedom for 'within (I×S)' and 'measurement error', respectively. For females FA was indeed higher at 23ºC ($F_{19,55} = 2.13$, $P < 0.05$), but no significant difference was detected for males ($F_{63,7} = 2.88$, $P > 0.05$). Overall, the results point to an increase of wing size asymmetry at suboptimal temperature but a transition from FA to DA seems to happen in males (see Graham *et al.* 2003; and below).

Causal components of variation and asymmetry in wing shape

Procrustes ANOVAs: The Procrustes ANOVAs for shape variation are also shown in tables 1 and 2. The main conclusions from the two-way mixed model are about the same as those obtained for centroid sizes but some differences are worth noticing. Thus shape variation among individuals accounted for less than 60% of the total variation. The intraclass correlation for shape, i.e.

$$\left(\frac{s_g^2(WS)}{s_g^2(WS) + s_w^2(WS)}\right),$$

which allows extracting here a univariate (but biased; see below) estimate of the proportion of shape variation accounted for by O_{st} gene arrangements, was lower at 18ºC (~24%) than at 23ºC (~43%). On the other hand, no DA was detected for overall shape and this clearly disagrees with previous claims on the putative biological significance of directional asymmetry in wing shape (Klingenberg *et al.* 1998; Klingenberg and Zaklan 2000).

Since shape is inherently multidimensional in nature, the amount of phenotypic and genetic variation of shape can best be assessed by examining the eigenvalues of the phenotypic (**P**) and genetic (**G**) variance–covariance matrices (Klingenberg and Leamy 2001). For each group, the **P** matrix here is simply the multivariate extension of the between-lines plus the within-lines variance–covariance components, and the **G** matrix is the multivariate extension of the between-lines component. The amounts of variation associated with the different dimensions in shape space are shown in figure 5. In all cases much of the variation was concentrated in the first few PCs. The ratios of the total variance of the **G** matrix to the total variance of the **P** matrix were 0.003 for females at 18ºC, 0.009 for males at 18ºC, 0.195 for females at 23ºC, and 0.171 for males at 23ºC. However, as stressed by Klingenberg and
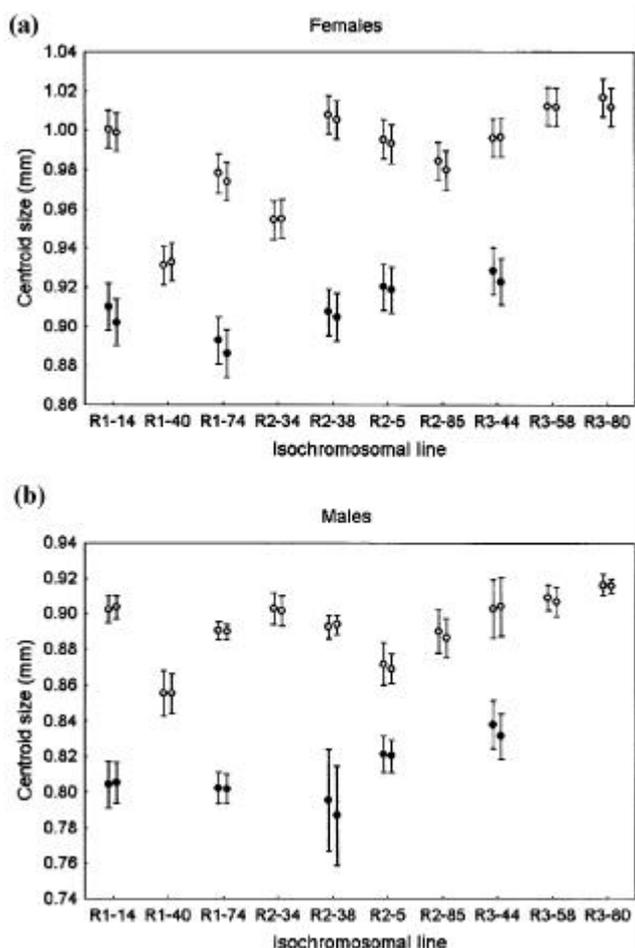
Figure 4. Averages (± 95% confidence intervals) of the centroid sizes (in a normalized form; see Dryden and Mardia 1998, p. 24) for the left and right wings, respectively, of O_{st} isochromosomal lines of *D. subobscura* reared at 18ºC (open circles) and 23ºC (closed circles).

Leamy (2001), these ratios cannot be interpreted as an estimate of the heritability of shape because the concept of heritability has no direct equivalent in the multivariate context. A better alternative would be to obtain the eigenvalues of the $\mathbf{GP^-}$ matrix, where $\mathbf{P^-}$ is the Moore–Penrose generalized inverse of $\mathbf{P}$, to assess the range of heritabilities for different shape variables. However, we have used very few lines and the estimates were subject to large sampling errors.

In contrast to what had been found for wing size, temperature did not seem to have any effect on wing shape asymmetries because no significant differences between temperatures were detected for wing shape FAs (females: $F = 1.072$, $P > 0.05$; males: $F = 1.116$, $P > 0.05$). This conclusion was reached from permutation tests (after 10,000 random permutations) because degrees of freedom in Procrustes ANOVAs are not usually independent (see above). These tests can be easily performed by noticing

Table 1. ANOVAs for assessing measurement error, directional asymmetry (sides effect), fluctuating asymmetry (individuals × sides interaction effect), and genetic components of the trait (s_g^2) and DA of the trait (s_g^2 (DA)) in O_{st} isochromosomal lines of *D. subobscura* reared at 18°C. CS refers to centroid size (values in pixels^2; 1 mm = 144 pixels), and WS to wing shape (all values × 10^4). Statistical significance for WS was determined from separate permutation tests for each effect (see text for details).

Trait	Source of variation	d.f.	Sum of squares	Mean square	Variance component	Estimate
(a) Females						
CS	Individuals (I)	99	8740.87	88.292***		
	Lines	9	5195.83	577.315***	s_g^2 (CS)	13.4481
	Within	90	3545.04	39.389***	s_w^2 (CS)	9.7996
	Sides (S)	1	6.28	6.278*		
	I × S	99	91.60	0.925***	$s_{I\times S}^2$ (CS)	0.3672
	Lines	9	10.06	1.118$^{n.s.}$	s_g^2 (DA$_{CS}$)	0.0212
	Within	90	81.54	0.906***	s_w^2 (DA$_{CS}$)	0.3576
	Measurement error	200	38.16	0.191	s_m^2 (CS)	0.1908
WS	Individuals (I)	2178	1680.026	0.771***		
	Lines	198	459.746	2.322***	s_g^2 (WS)	0.0426
	Within	1980	1220.281	0.616***	s_w^2 (WS)	0.1229
	Sides (S)	22	19.383	0.881$^{n.s.}$		
	I × S	2178	876.131	0.402***	$s_{I\times S}^2$ (WS)	0.1388
	Lines	198	56.252	0.284$^{n.s.}$	s_g^2 (DA$_{WS}$)	− 0.0130
	Within	1980	819.878	0.414***	s_w^2 (DA$_{WS}$)	0.1447
	Measurement error	4400	548.805	0.125	s_m^2 (WS)	0.1247
(b) Males						
CS	Individuals (I)	99	4147.06	41.889***		
	Lines	9	2512.14	279.127***	s_g^2 (CS)	6.5240
	Within	90	1634.91	18.166***	s_w^2 (CS)	4.4925
	Sides (S)	1	1.18	1.184		
	I × S	99	118.49	1.197***	$s_{I\times S}^2$ (CS)	0.5006
	Lines	9	5.39	0.599$^{n.s.}$	s_g^2 (DA$_{CS}$)	− 0.0657
	Within	90	113.09	1.257***	s_w^2 (DA$_{CS}$)	0.5304
	Measurement error	200	39.14	0.196	s_m^2 (CS)	0.1957
WS	Individuals (I)	2178	1630.068	0.748***		
	Lines	198	418.633	2.114***	s_g^2 (WS)	0.0376
	Within	1980	1211.435	0.612***	s_w^2 (WS)	0.1307
	Sides (S)	22	7.493	0.341$^{n.s.}$		
	I × S	2178	1061.856	0.488***	$s_{I\times S}^2$ (WS)	0.1993
	Lines	198	92.433	0.467$^{n.s.}$	s_g^2 (DA$_{WS}$)	− 0.0023
	Within	1980	969.423	0.490***	s_w^2 (DA$_{WS}$)	0.2003
	Measurement error	4400	391.298	0.089	s_m^2 (WS)	0.0889

$^{n.s.}P > 0.05$; *$P < 0.05$; ***$P < 0.001$.

that the mean square for the I×S interaction terms in the Procrustes ANOVAs are simply the sum of $\text{Var}_{(L-R)}$ (index FA4 in Palmer 1994) for each x and y coordinates of the corresponding aligned configurations divided by the shape dimension (i.e. $2p-4$; see above), and we can contrast the observed ratio of FA4 indexes with the vector of randomized ratios.

Those permutation tests do not, however, take into account the covariances among the coordinates of superimposed landmark configurations. An easy alternative is to perform a MANOVA with temperature and sex as fixed

effects, and the unsigned $|L - R|$ estimates for each x and y coordinates of the aligned configurations as dependent variables. This MANOVA test showed significant differences for temperature (Hotelling's $T^2 = 0.182$; $P = 0.034$), with sex ($T^2 = 0.067$; $P = 0.944$) and temperature × sex interaction ($T^2 = 0.123$; $P = 0.347$) effects being statistically nonsignificant. It seems therefore that wing shape FA does slightly increase at the suboptimal and warmer temperature.

Tables 3 and 4 give the variance components from Procrustes ANOVAs when apportioned by landmarks; however, we emphasize here again that these values do

Table 2. Same as in table 1, for O_{st} isochromosomal lines of *D. subobscura* reared at 23ºC.

Trait	Source of variation	d.f.	Sum of squares	Mean square	Variance component	Estimate
(a) Females						
CS	Individuals (I)	29	1237.03	42.656***		
	Lines	4	390.14	97.535*	s_g^2 (CS)	2.6525
	Within	25	846.89	33.876***	s_w^2 (CS)	8.4098
	Sides (S)	1	15.07	15.075**		
	I × S	29	47.38	1.634***	$s_{I\times S}^2$ (CS)	0.6986
	Lines	4	3.38	0.845[n.s.]	s_g^2 (DA$_{CS}$)	− 0.1525
	Within	25	44.00	1.760***	s_w^2 (DA$_{CS}$)	0.7617
	Measurement error	60	14.19	0.237	s_m^2 (CS)	0.2366
WS	Individuals (I)	638	564.236	0.884***		
	Lines	88	233.973	2.659**	s_g^2 (WS)	0.0858
	Within	550	330.263	0.600***	s_w^2 (WS)	0.1194
	Sides (S)	22	18.515	0.842[n.s.]		
	I × S	638	239.310	0.375***	$s_{I\times S}^2$ (WS)	0.1261
	Lines	88	25.508	0.290[n.s.]	s_g^2 (DA$_{WS}$)	− 0.0165
	Within	550	213.803	0.389***	s_w^2 (DA$_{WS}$)	0.1329
	Measurement error	1320	162.320	0.123	s_m^2 (WS)	0.1230
(b) Males						
CS	Individuals (I)	29	1374.18	47.385***		
	Lines	4	583.94	145.984**	s_g^2 (CS)	4.7656
	Within	25	790.24	31.610***	s_w^2 (CS)	7.8269
	Sides (S)	1	5.97	5.968**		
	I × S	29	25.72	0.887***	$s_{I\times S}^2$ (CS)	0.2925
	Lines	4	8.97	2.244*	s_g^2 (DA$_{CS}$)	0.2623
	Within	25	16.75	0.670**	s_w^2 (DA$_{CS}$)	0.1840
	Measurement error	60	18.12	0.302	s_m^2 (CS)	0.3021
WS	Individuals (I)	638	462.873	0.726***		
	Lines	88	184.569	2.097**	s_g^2 (WS)	0.0663
	Within	550	278.304	0.506***	s_w^2 (WS)	0.0817
	Sides (S)	22	23.279	1.058[n.s.]		
	I × S	638	278.756	0.437***	$s_{I\times S}^2$ (WS)	0.1288
	Lines	88	38.697	0.440[n.s.]	s_g^2 (DA$_{WS}$)	0.0005
	Within	550	240.059	0.436***	s_w^2 (DA$_{WS}$)	0.1285
	Measurement error	1320	236.808	0.179	s_m^2 (WS)	0.1794

[n.s.]$P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

not take into account the covariances among coordinates. (In addition, it should be stressed that variance components for shape effects were adjusted in each case by dividing for the number of measurements taken to make them comparable to results in tables 1 and 2 (see Santos 2001, 2002).) Landmarks 3 and 6 had the largest effects, but the among-females variation for landmark 6 seems to be mainly environmental. On the other hand, landmark 1 had relatively low amounts of variability among individual flies. DA appears to be present for landmarks 11 and 12 in females at both temperatures, which suggests that the position of the anterior cross-vein varies between sides. After plotting the Procrustes grand mean shapes of both wings in females samples it became apparent that the location of that vein was slightly more distal in the right wings. However, overall shape variation mainly displays FA because side effects were generally nonsignificant.

Patterns of shape variation: We will focus here mainly on individual and among-O_{st}-isochromosomal-lines variation. PCA of overall shape variation yielded results that were fairly consistent with those previously obtained from our thermal selection stocks (Santos *et al.* 2004).

The first three PCs for both sexes and temperatures are plotted in figure 6. The relatively high level of variability explained by a few PCs clearly suggests strong dependencies among landmarks and, hence, the isotropic model does not seem to hold (see Dryden and Mardia 1998, p. 97). The direction of PCs is arbitrary and all movements can be simultaneously reversed by 180°. Some contrasting patterns between temperatures are perceptible in both sexes, mainly involving the shifts of those landmarks that define the positions of the cross-veins. For instance, the shifts of the anterior and posterior cross-veins appear to be rather independent of each other at 18°C but not at 23°C. These results suggest that wing shape in *Drosophila* may not be as strongly resistant to environmental influences as previously thought (see Birdsall *et al.* 2000).

The features of among-lines variation associated with the dominant PCs are plotted in figure 7. The dominance of PC1 was also linked to the variability in outer landmarks 3, 4 and 6. As before, some differences between temperatures are clearly appreciated, which suggests temperature × line interactions. For instance, shifts of landmark 9 are only apparent at 23°C for both sexes. These temperature effects on wing shape genetic variation are quite
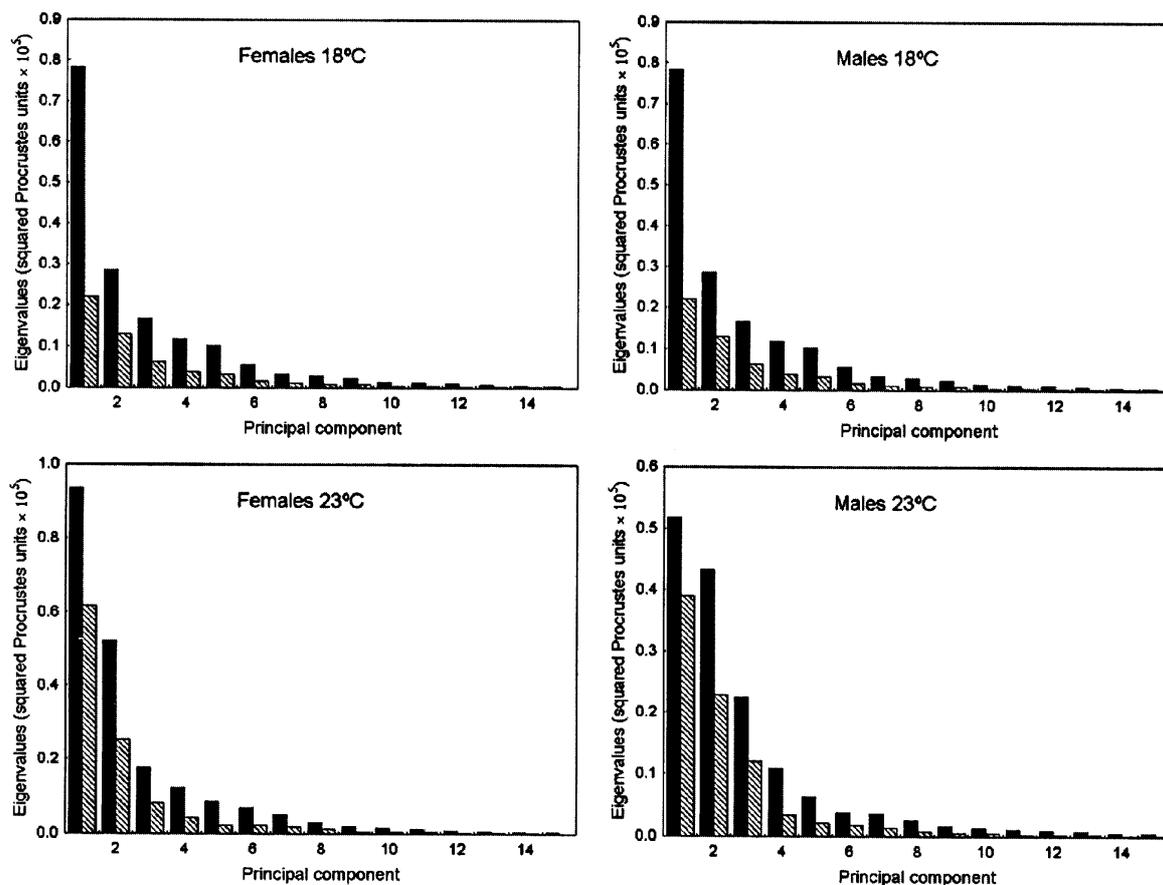
Figure 5.   Eigenvalues of the phenotypic (solid bars) and genetic (hatched bars) covariance matrices of wing shape. Note that these are only the first 15 eigenvalues out of 22 shape dimensions, but together make up more than 98% of the total shape variation.

noticeable from the Procrustes three-way mixed ANOVAs discussed below.

Within each group, the congruence of landmark displacements between effects was assessed by comparing the absolute angles between respective eigenvectors. The PC1 of line variation was generally different from the PC1 of FA (females: 72.6° at 18ºC and 36.7° at 23ºC; males: 68.3° at 18ºC and 80.8° at 23ºC); only the association for females at 23ºC was highly significant (recall that the 0.1% and 2.5% quantiles of the distribution of angles between 22-dimensional random vectors were 50.3° and 62.3°, respectively). A similar pattern was found for the association between the PC1s of line and measurement error. However, PC1s of FA and measurement error were quite similar to each other (females: 4.3° at 18ºC and 11.4° at 23ºC; males: 3.6° at 18ºC and 10.8° at 23ºC).

Considering the variation among lines between groups (by using only those five lines that were common at both

Table 3. Variance components ($\times 10^8$) for the effects of each landmark in the Procrustes ANOVAs in O$_{st}$ isochromosomal lines of *D. subobscura* reared at 18ºC (see text for details).
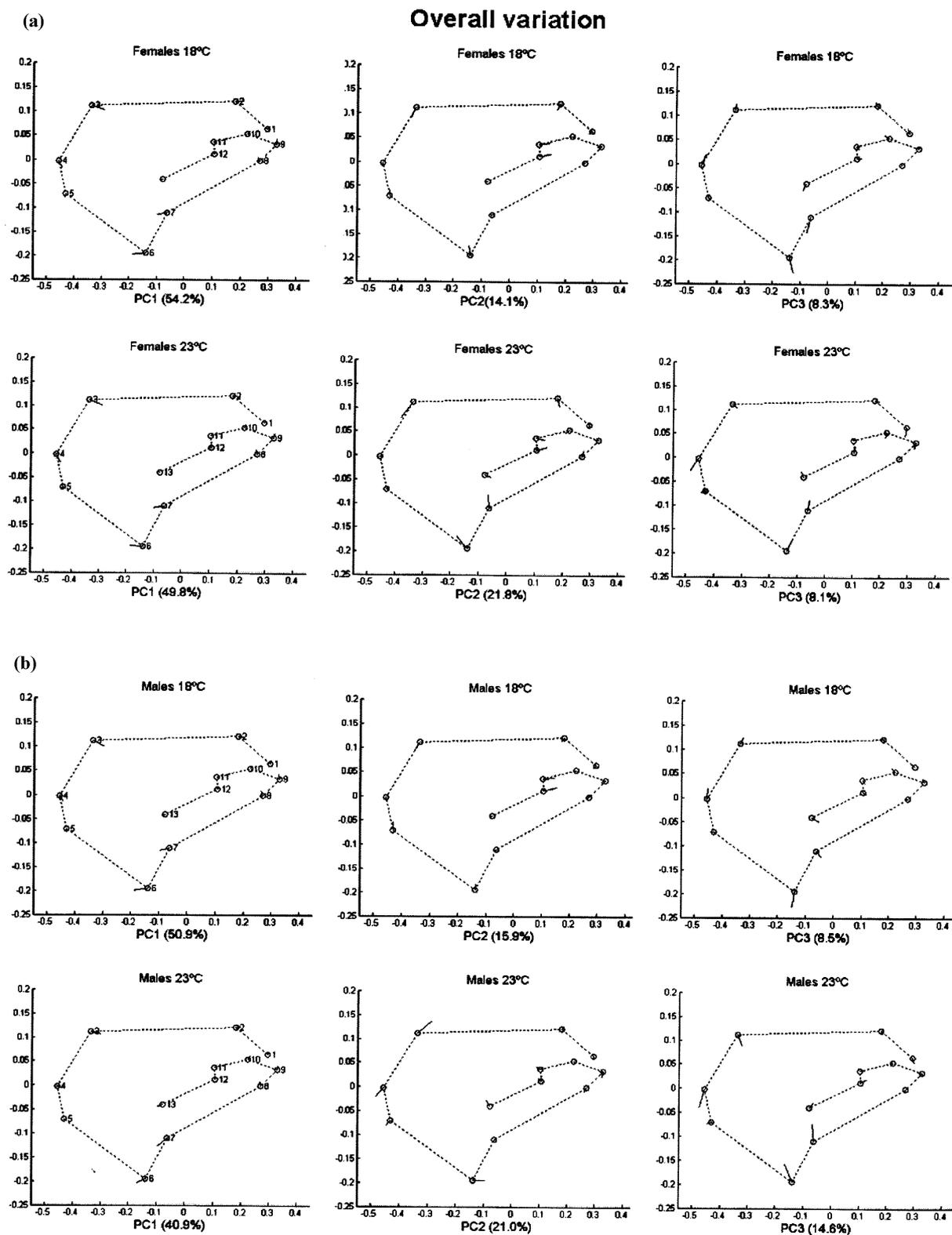
| | Landmark | | | | | | | | | | | | |
	1	2	3	4	5	6	7	8	9	10	11	12	13
(a) Females													
Individuals (I)	680***	2496***	6999***	2405***	1147***	7516***	4536***	945***	983***	1490***	2215***	2316***	1838***
Lines	198***	471***	1441***	1043***	287***	552	999***	227***	255***	888***	1075***	1377***	569***
Within	500***	2068***	5690***	1457***	886***	7014***	3628***	738***	751***	682***	1238***	1065***	1320***
Sides (S)	0	0	125	6	13	221	101	7	6	16	37**	27**	0
I × S	402***	2336***	7366***	1554***	732***	9964***	4196***	799***	616***	859***	520***	357***	829***
Lines	0	0	0	0	0	0	0	0	0	0	43	0	0
Within	431***	2396***	7760***	1636***	747***	10438***	4382***	803***	630***	905***	500***	360***	841***
Measurement	871	2977	5954	1406	642	8476	3644	774	816	967	344	169	402
(b) Males													
Individuals (I)	695***	2486***	6715***	2143***	1409***	8023***	4385***	892***	1082***	1197***	2574***	2700***	1971***
Lines	170***	118	404	636***	543***	645*	1009***	291***	410***	512***	1540***	1584***	401***
Within	540***	2379***	6348***	1565***	916***	7436***	3468***	627***	709***	731***	1174***	1260***	1606***
Sides (S)	18	111*	0	0	2	0	0	0	0	47*	8	0	0
I × S	620***	4106***	9896***	2309***	1161***	14105***	6709***	848***	821***	1168***	516***	484***	1103***
Lines	0	0	78	0	61	0	0	20	0	111	0	30	0
Within	647***	4140***	9861***	2373***	1133***	14276***	6721***	839***	840***	1118***	536***	471***	1119***
Measurement	747	2362	4053	945	476	5402	2287	725	684	1075	281	207	321

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (no Bonferroni correction was applied).

Table 4. Variance components ($\times 10^8$) for the effects of each landmark in the Procrustes ANOVAs in O$_{st}$ isochromosomal lines of *D. subobscura* reared at 23ºC (see text for details).

| | Landmark | | | | | | | | | | | | |
	1	2	3	4	5	6	7	8	9	10	11	12	13
(a) Females													
Individuals (I)	804***	3460***	9903***	3044***	1119***	8362***	5843***	1170***	1227***	1060***	1860***	2249***	1778***
Lines	121	1658***	7354***	875**	190	1562	3816***	537***	222	375**	476*	559*	1123***
Within	704***	2088***	3817***	2319***	961***	7069***	2685***	726***	1043***	750***	1466***	1786***	849***
Sides (S)	0	0	407	79	58*	561	466*	24	23	0	73*	66*	0
I × S	509***	3507***	4799***	1678***	455***	7831***	4587***	858***	873***	831***	603***	439***	764***
Lines	287*	0	0	0	0	0	0	82	67	0	0	0	266
Within	390***	3709***	5036***	1821***	460***	8208***	5121***	825***	845***	980***	685***	501***	654***
Measurement	736	2694	5891	1680	639	8320	3692	849	772	840	305	210	425
(b) Males													
Individuals (I)	505***	1405***	6341***	3170***	1057***	4386***	4991***	716***	959***	1010***	1122***	1713***	2659***
Lines	126	611**	2918**	2797***	519***	2398**	2494***	468***	293**	891***	180	238	652*
Within	401***	899***	3926***	856***	628***	2401**	2927***	329***	716***	272*	973***	1516***	2119***
Sides (S)	16	23	609	185	81	1042*	113	45	35	117	12	32	0
I × S	209	2344***	7757***	1503***	576***	7456***	4057***	849***	660***	1469***	358***	370***	718***
Lines	0	287	0	0	144	1099	0	0	224	0	32	0	0
Within	257	2225***	7766***	1535***	516**	7001***	4354***	886***	567***	1616***	345***	444***	766***
Measurement	1236	3612	8140	2609	1144	12411	5038	1012	799	1990	529	342	608

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (no Bonferroni correction was applied).

Figure 6. Principal components of the covariance patterns in landmark shifts due to overall variation for females (a) and males (b). The PC coefficients are shown as a solid line originating at the mean location of the landmark (open circles) and ending at the location to which the landmark would move at +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations (obviously an exaggeration of the variation in the dataset). The proportion of total variation accounted for by each PC is given in brackets.

Among-line variation
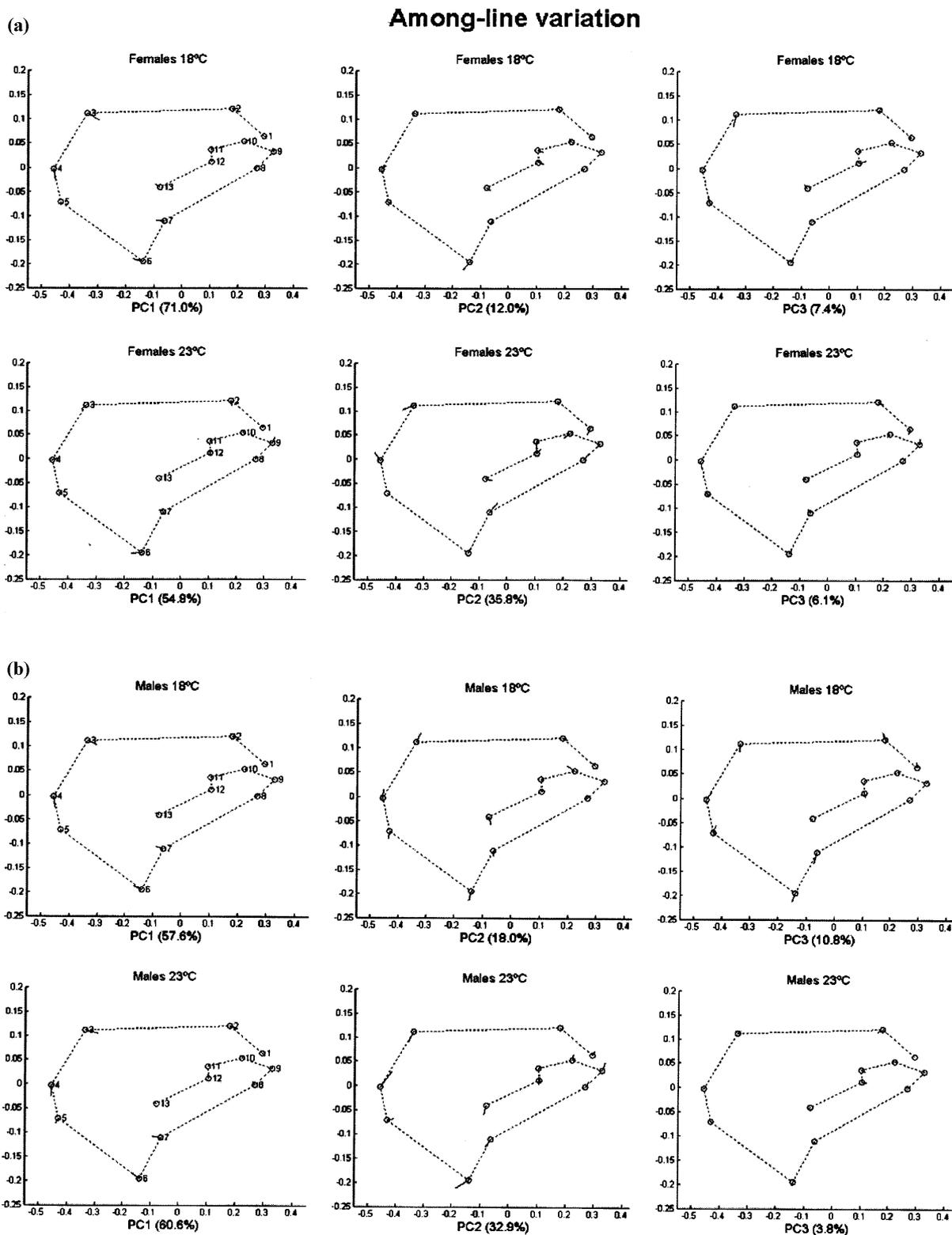
Figure 7. Principal components of the covariance patterns in landmark shifts due to among-lines variation for females (a) and males (b). The end points of the solid lines are at locations displaced +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations from the mean configuration.

temperatures), the angles between the two PC1s were 44.8° and 75.1°, and those between the two PC2s were 73.5° and 89.8° for females and males, respectively. These low associations (only significant for the PC1s of females) were somewhat surprising and suggest important temperature effects for shape during the developmental process (see below). On the other hand, the associations concerning FA were strong since the angles between the PC1s were 10.2° and 10.5° for females and males, respectively.

Temperature effects on wing size and shape

Three-way mixed ANOVAs as described in Materials and methods and summarized in table 5 were used to partition the effects of temperature, sex and O_{st} isochromosomal line, as well as all possible interaction terms. As expected, all three main effects were highly significant for centroid size, and there was also indication that the different lines responded to temperature to different extents. Sex and line effects were also highly significant for wing shape, but no temperature differences were detected for overall shape (notice, however, that $P \sim 0.07$). Nevertheless, there was a highly significant temperature × line interaction, thus suggesting that temperature had consistent but somewhat different effects on overall wing shape depending on genotype.

When all effects were apportioned by landmarks in a similar way to that described for the two-way mixed-model ANOVAs (see tables 3 and 4), temperature was found to have the largest effect on landmarks 9, 1, 10, 11 and 12; whereas temperature × line interaction mainly affected

landmarks 12 and 11 (which define the position of the anterior cross-vein) and, to a smaller extent, landmarks 7 and 13 (which define the position of the posterior cross-vein; results not shown). In conclusion, wing shape is clearly under genetic control in *D. subobscura* (as had also been found earlier in *D. melanogaster*; see Weber 1990, 1992; Gilchrist and Partridge 1999, 2001; Weber *et al*. 1999, 2001; Birdsall *et al*. 2000; Gilchrist *et al*. 2000; Zimmerman *et al*. 2000), and does not seem to be as strongly resistant to temperature effects as claimed (Birdsall *et al*. 2000).

Discussion

We have used the recently developed methodologies in geometric morphometrics to analyse shape configurations of landmarks within the standard statistical framework employed in studies of bilateral asymmetries, and have extended these methods to partition the individual variation and the variation in asymmetries into genetic and environmental causal components (Palmer 1994; Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000; Santos 2002). We have exploited the opportunity that *D. subobscura* offers for obtaining genetically homogeneous isochromosomal lines, and the work is being extended to include more chromosomal classes besides O_{st} and to performing line crosses to see whether or not increased FA is associated with homozygosity or is genotype dependent or both (some results suggest that heterozygous flies are more homeostatic than their homozygous counter-

Table 5. Three-way ANOVAs of wing size (CS: values in pixels^2; 1 mm = 144 pixels) and shape (WS: all values ×10^4) by temperature (fixed effect) and sex (fixed) considering those five O_{st} isochromosomal lines (random) that were tested at both temperatures. Statistical significance for WS was determined from separate permutation tests for each effect.

Trait	Source of variation	d.f.	SS	MS	*F*
CS	Temperature (T)	1	4447	4446.9	82.81***
	Sex (S)	1	6712	6711.5	499.44***
	Line (L)	4	224	56.0	12.48***
	T×S	1	25	25.3	2.75
	T×L	4	215	53.7	11.98***
	S×L	4	54	13.4	3.00*
	T×S×L	4	37	9.2	2.06
	Error	140	628	4.5	
WS	Temperature (T)	22	55.66	2.530	2.99
	Sex (S)	22	43.16	1.962	17.46***
	Line (L)	88	141.07	1.603	7.41***
	T×S	22	8.42	0.383	3.26*
	T×L	88	74.51	0.847	3.92***
	S×L	88	9.89	0.112	0.52
	T×S×L	88	10.34	0.118	0.54
	Error	3080	665.93	0.216	

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

parts; see Pfriem 1983). Here we attempt to interpret the initial results.

Not surprisingly the intraclass correlations for wing size and shape were relatively high but within the range of the heritabilities reported in the *Drosophila* literature for these traits (e.g. Roff and Mousseau 1987; Weber 1990, 1992; Leibowitz *et al.* 1995; Birdsall *et al.* 2000). However, contrasting patterns were observed for CS (centroid size) and WS (wing shape) intraclass correlations according to developmental temperature, and it is worth noting that the higher intraclass correlation for WS at 23ºC was mainly brought about by an increase in the genetic component (c.f. tables 1 and 2). Hoffmann and Merilä (1999) have recently reviewed some *Drosophila* studies that point to an increase of heritability under unfavourable thermal conditions, but results are generally inconsistent and in most cases the works have focussed on size-related traits. In addition, heritability can be a misleading concept because it is the ratio of two variances in a given environment. In the case of WS, it is quite clear that this trait is more resistant to environmental influences than size-related traits (Weber 1990; Birdsall *et al.* 2000; table 5 here), and the observed increase of genetic variation at the suboptimal temperature of 23ºC could be made consistent with the idea that WS has a past history of selection for canalization (Waddington 1961; Hoffmann and Merilä 1999). In any case, line effects were quite important for WS and it is likely that these effects would be larger if different chromosomal classes in addition to O_{st} were included in the analyses. Since strong and consistent latitudinal clines for chromosome arrangement frequencies are observed worldwide in *D. subobscura* (Krimbas and Loukas 1980; Prevosti *et al.* 1985, 1988; Menozzi and Krimbas 1992; Balanyà *et al.* 2003), together with rapid microevolution for the O chromosomal inversion polymorphism as a putative response to shifts in environmental temperature (Rodríguez-Trelles *et al.* 1996; Rodríguez-Trelles and Rodríguez 1998), it would be very interesting to see how particular aspects of wing shape are affected by different chromosome O arrangements.

Some qualitatively different patterns were also observed for CS and WS asymmetries. Thus a significant level of DA was generally found for centroid size with left wings slightly bigger than the right ones (figure 4). This result closely agrees with previous findings in *D. melanogaster* (Klingenberg *et al.* 1998; Klingenberg and Zaklan 2000), which apparently raises sound warnings against the conventional wisdom in *Drosophila* (Maynard Smith and Sondhi 1960; Coyne 1987; see Tuinstra *et al.* 1990 for a review). Conversely, no DA was found for overall wing shape and this is against some claims suggesting that DA has been evolutionarily conserved in fly wings (Klingenberg *et al.* 1998). As far as we are aware, this is the first study dealing with the putative genetic basis of

wing shape DA in *Drosophila*, and the conclusion is that there seems to be no genetic variation for this trait (tables 1 and 2). Adding to the conundrum, genetic variation was detected for centroid size DA in males raised at 23ºC, together with an increase of wing size asymmetry at this suboptimal temperature and a transition from FA to DA. According to Graham *et al.* (2003), the classical linear theory of DI can successfully account for both normally distributed error distributions and leptokurtic distributions caused by admixture of individuals with different levels of DI, but cannot account for transitions between FA and DA. We are not, however, totally convinced that this is indeed the case. Thus, let us focus on centroid sizes of males at both temperatures and assume for the time being that genetic variation for DA (which has often been thought of to occur and has occasionally been found in other organisms but not in *Drosophila*; see Palmer *et al.* 1993; Palmer 1994; Leamy *et al.* 1997, 2000) is present and changes as a direct response to environmental conditions in which it is expressed. FA estimated from index FA10 ($s^2_{I\times S}$) would be biased whenever genetic variation for DA is expressed (Santos 2001, 2002; table 2b here), and signed ($L - R$) asymmetries will not necessarily have to be centred at zero (there can be, however, genetic variation for DA in traits that exhibit nonsignificant DA; see Leamy *et al.* 1997). It seems to us, therefore, that a transition from 'ideal' FA (i.e. a normal distribution of left–right scores whose mean is zero; see Palmer 1994) to a distribution showing DA could be made entirely compatible with what is already known from classical quantitative genetics.

Anyway, the important question here is to know whether or not there is genetic variation for DA in *Drosophila* as suggested by Klingenberg *et al.* (1998). Taking all the information together, what seems clear is that *Drosophila* traits do exhibit DA at least under some circumstances, although genetic variation for DA has almost never been detected. The DA asymmetry in particular aspects of shape (i.e. the slight variation in anterior cross-vein of females) is subtle and these small shifts of wing veins are unlikely to impede aerodynamic properties. Likewise DA of wing size was quite small in our samples and substantially less than nondirectional asymmetry (of the order of 4% FA) at 18ºC, but accounted for a sizeable proportion of overall wing size asymmetry at 23ºC that would likely represent a burden in flight performance. Transitions from FA to DA could be easily explained if we assumed environment-dependent genetic expression for DA but, for the time being, no definitive answer on the putative genetical basis of DA in *Drosophila* can be given. Accordingly, we parsimoniously adhere to the conventional wisdom that left and right are not distinguished in *Drosophila* development (but see Ligoxygakis *et al.* 2001).

One of the main aims of the ongoing work is to test for genetic effects on FA. No genetic variation was generally

detected for FA in our samples from O_{st} isochromosomal lines, but we have already pointed out that these are preliminary results because no crosses between lines were performed and that most genetic variation in New World colonizing populations of *D. subobscura* is likely apportioned among chromosome arrangements. Thus, bottleneck effects are quite obvious when comparing chromosome, allozyme and microsatellite diversities among original Palaearctic and colonizing populations from the New World with stronger allozyme-inversion disequilibria in the latest populations (e.g. Prevosti *et al*. 1983; Balanyà *et al*. 1994; Pascual *et al*. 2001). These disequilibria are also very strong between microsatellite alleles and chromosome O inversions; all but two isochromosomal lines used here are fixed for the same allele at two microsatellite loci on this chromosome (unpublished results). Anyhow, our initial results strongly suggest that FA only reflects developmental noise and that O_{st} chromosomes from Puerto Montt seem to exhibit similar levels of DI.

Finally, it is worth mentioning that the general lack of correspondence between line variation and FA (as assessed by using the angles between corresponding PCs) suggests that developmental processes influencing wing shape do not necessarily generate random differences between body sides, contrarily to what has been previously suggested on empirical (Klingenberg and McIntyre 1998) and theoretical (Klingenberg and Nijhout 1999) grounds. This is clearly an important problem that needs to be addressed in more detail with a larger data set than the one we have used here.

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