ALLOMETRIC AND NONALLOMETRIC COMPONENTS OF *DROSOPHILA* WING SHAPE RESPOND DIFFERENTLY TO DEVELOPMENTAL TEMPERATURE

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Abstract.—Phenotypic plasticity of wing size and shape of *Drosophila simulans* was analyzed across the entire range of viable developmental temperatures with Procrustes geometric morphometric method. In agreement with previous studies, size clearly decreases when temperature increases. Wing shape variation was decomposed into its allometric (24%) and nonallometric (76%) components, and both were shown to involve landmarks located throughout the entire wing blade. The allometric component basically revealed a progressive, monotonous variation along the temperature. Surprisingly, nonallometric shape changes were highly similar at both extremes of the thermal range, suggesting that stress, rather than temperature per se, is the key developmental factor affecting wing shape.

Key words.—Allometric shape, *Drosophila simulans*, geometric morphometrics, growth temperature, nonallometric shape, phenotypic plasticity, stress.

Phenotypic plasticity, the ability of a genotype to produce different phenotypes in different environments, is of primary interest to evolutionary biologists (e.g., Schlichting and Pigliucci 1993; Via et al. 1995; Debat and David 2001; Pigliucci 2001; Nijhout 2003). Plasticity is an alternative to genetic change allowing populations to adapt to changing environmental conditions. Also, in loosening the relationship between genotype and phenotype, plasticity alters the response to natural selection. In spite of a large number of studies, the genetic and developmental basis of plasticity remains unclear (e.g., Via et al. 1995; but see Pigliucci 1998), perhaps because of the lack of an appropriate tool to relate phenotypic outcomes to developmental mechanisms. Quantitative genetics and the reaction norm approach have jointly provided several insights into both the genetic architecture of plastic traits and the conditions required for plasticity to evolve (e.g., Via and Lande 1985; Gavrilets and Scheiner 1993a,b; Schlichting and Pigliucci 1993; Van Tienderen and Koelewijn 1994; Pigliucci 1998; De Jong and Gavrilets 2000; David et al. 2003). Unfortunately, the relative coarseness of the measurements used so far in these approaches has impeded our ability to relate variation patterns to known developmental processes.

New morphometric methodologies focusing on shape variables derived from landmark coordinates have emerged within the past decade (Bookstein 1991; Rohlf and Marcus 1993; Dryden and Mardia 1998). These methods, collectively referred to as geometric morphometrics, allow researchers to separate the size and shape components in a morphological variation pattern and to capture shape information provided by the definition of landmarks through a relatively small number of variables (i.e., the number of landmarks multiplied by the number of coordinates). In addition to its high resolution power, geometric morphometrics offers the possibility to directly visualize shape changes, either in the form of deformation grids or as vectors depicting landmarks displacements. This latter property is obviously of primary importance in relating patterns of shape change to the known developmental processes involved in morphogenesis. Applications of geometric morphometrics in evolutionary biology are now common, ranging from paleontology (e.g., Crônier et al. 1998) to quantitative genetics (Zimmerman et al. 2000; Klingenberg and Leamy 2001; Monteiro et al. 2002; Klingenberg 2003). Only a few studies have yet used this methodology to explicitly address developmental questions (Birdsall et al. 2000; Klingenberg and Zaklan 2000; Palsson and Gibson 2000; Zimmerman et al. 2000; Klingenberg et al. 2001; Hallgrimsson et al. 2002). However, the promising results obtained through these works suggest that such an approach is particularly appropriate for analyzing phenotypic variation in a developmental framework (Klingenberg 2002).

In this context, the *Drosophila* wing appears an almost ideal model for several, complementary reasons. First, the vein pattern is highly conserved in the Drosophilid family and in other closely related groups included in the superfamily Ephydridoidea. Therefore, many landmarks can be identified that will be homologous in a large number of species. Second, the details of *Drosophila* wing development are well understood (e.g., De Celis 2003 and references therein). Third, the wing is highly plastic, especially according to growth temperature, and the reaction norms of overall size have been described in several species (David et al. 1994; Moreteau et al. 1998; Morin et al. 1999). The mean effect is a decrease in size with increasing temperature, and a maximum is found at low temperature. Significant differences are observed among geographic populations of cosmopolitan species, which are interpreted as adaptive changes (Morin et al. 1999). Different parts of the wing exhibit different reaction norms (Moreteau et al. 1998), but this point deserves more detailed analyses. Finally, the wing has an obvious function, which is flight. Evidence that the wing is a target of natural selection is provided by the observation of latitudinal clines in several species and

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sometimes independently on different continents (Gilchrist et al. 2000; Huey et al. 2000) in the same cosmopolitan species. There is an overall parallelism between genetic (cline) and epigenetic (plasticity) variation: smaller wings and increased wing loading are observed in warmer environments (Pétavy et al. 1997; Morin et al. 1999). However, the same adaptation may be reached by different means. There is some evidence that analogous clines in the same species on different continents may be achieved by different genetic architectures (Gilchrist and Partridge 2001) or by different changes in wing proportions (Huey et al. 2000).

In this paper we consider the effects of developmental temperature on the size and shape of the *Drosophila* wing using Procrustes analysis (e.g., Rohlf and Marcus 1993), providing a first application of geometric morphometrics to the analysis of phenotypic plasticity. *Drosophila simulans* was used because, in this species, the reaction norm of wing size is practically a monotonically decreasing function of temperature (Morin et al. 1999). The patterns of wing shape changes along a temperature gradient have been quantified, visually depicted, and discussed in relation with two general questions.

The first question concerns the possible independence between the two components of wing morphology, that is, size and shape. This independence has been suggested in both evolutionary genetic studies (Weber 1990, 1992; Bitner-Mahté and Klaczko 1999a,b,c; Weber et al. 2001; Hoffmann and Shirrifs 2002) and developmental studies (e.g., Resino et al. 2002). In contrast, allometric changes are commonly observed in insect wings (e.g., Baylac and Pénin 1998; for review see Stern and Emlen 1999). Additional empirical data are therefore needed. Particular attention has been paid to the relationship between wing size and shape and their changes along the temperature gradient. We provide a detailed analysis of both allometric and nonallometric components of shape variation.

The second question concerns the relative importance of integration versus modularity in the development and evolution of the wing. The emphasis of analyses of *Drosophila* wing development has recently shifted from integration to modularity (Klingenberg and Zaklan 2000). This shift is based on both developmental and evolutionary genetic studies that have suggested that the wing is composed of genetically independent developmental modules (e.g., Garcia-Bellido et al. 1973, see below; Cavicchi et al. 1981, 1985; Guerra et al. 1997; Birdsall et al. 2000; Zimmerman et al. 2000; Weber et al. 2001). However, a recent study devoted to the analysis of the patterns of integration in the *Drosophila* wing (Klingenberg and Zaklan 2000) showed that both genetic variance and fluctuating asymmetry simultaneously affect the entire wing, leading the authors to suggest that *Drosophila* wing should be viewed as fundamentally integrated rather than modular. Given these contradictory conclusions, investigating whether an environmental source of variation (i.e., growth temperature) would alter wing shape primarily within developmental compartments or across the whole wing was particularly interesting.

MATERIALS AND METHODS
Data

We used a *D. simulans* laboratory mass strain recently founded from 15 isofemale lines collected in the island of São Tomé. Experiments were done 3 months (i.e., 6 generations) after the strain foundation. Ten pairs of adults were randomly chosen and used as parents. They oviposited at 21°C for 6–8 h in culture vials containing a high-nutrient, killed-yeast food. This procedure allowed us to keep a low larval density (less than 150 per vial). Moreover we know that such a medium prevents any visible crowding effect even at much higher densities. For example, no significant wing size variation was found among densities ranging between 20 and 320 larvae per culture vial (Karan et al. 1999a). After removing the parents, the culture vials, likely containing offspring from each parental couple, were transferred to incubators regulated ($\pm$ 0.1°C) at one of seven constant temperatures (12, 14, 17, 21, 25, 28, and 30°C) covering the entire range of viable developmental temperatures. The two extremes (12°C and 30°C) are strongly stressful and 14°C and 28°C are considered mildly stressful (Pétavy et al. 2001). After emergence, adult flies were transferred to fresh food, aged a few days, and then preserved in 70% ethanol with 10% glycerol. Thirty females were randomly taken from each sample, their right and left wing removed and mounted on a microscope slide (total sample size = 210). The reaction norms of males and females are known to be similar (David et al. 1994), and thus only females were used.

Eleven landmarks were defined on the dorsal face of the wing (i.e., 22 coordinates; Fig. 1). The wings were placed under a microscope and photographed. To appraise the measurement error, the landmarks were digitized twice, using TPSdig program (available at http://life.bio.sunysb.edu/morph/). After the landmark coordinates were recorded, the configurations were superimposed onto a consensus configuration (the overall mean configuration) using the Procrustes generalized least squares procedure (GLS, Rohlf and Slice 1990; Bookstein 1991; Rohlf and Marcus 1993; Dryden and Mardia 1998). Procrustes superimposition consists of three successive steps: (1) scaling: the configurations are scaled to a unit centroid size (i.e., the sum of the squared distances from the landmarks to the centroid or center of gravity of the configuration); (2) translation: the centroid of each configuration is superimposed onto the centroid of the consensus configuration; and (3) rotation: the configurations are rotated so as to minimize the distances between the corresponding landmarks, that is, to optimize the superimposition (Dryden and Mardia 1998). The new coordinates, or Procrustes coordinates, constitute the shape variables used in the subsequent statistical treatments. The centroid size of each configuration prior to superimposition was used as the size variable. For each individual, the data consist in the landmark coordinates and centroid size averaged over the left and right wings and over the two measurement sessions, a procedure that was shown to increase the quality of the data (Arnquist and Martensson 1998).

Procrustes Analysis
Size variation

The effect of temperature on wing size was investigated by applying an ANOVA to the centroid size, including temperature as main effect. Post hoc tests were then conducted
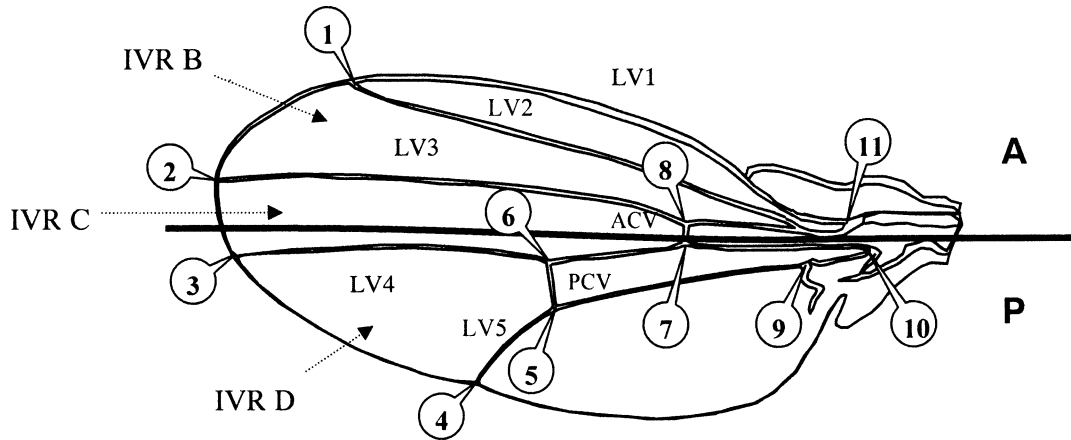
FIG. 1. Positions of the landmarks used in this study. LV, longitudinal veins; ACV, anterior crossvein; PCV, Posterior crossvein; IVR, interveins regions as defined by Birdsall et al. (2000); A, anterior compartment; P, posterior compartment.

to test whether the effect on size was consistent among treatments across the gradient.

It has been suggested that the intervein regions (IVR, Birdsall et al. 2000; Fig. 1) defined between longitudinal veins 2 and 3 (IVR-B), longitudinal veins 3 and 4 (IVR-C), and longitudinal veins 4 and 5 (IVR-D) might be considered as being genetically independent (Guerra et al. 1997; Birdsall et al. 2000; Zimmerman et al. 2001). To depict their respective behaviors across temperatures, we measured the centroid size of each IVR from the original set of coordinates prior to superimposition. The slopes of the regressions of centroid size onto temperature were compared using a *t*-test, as recommended by Zar (1996).

Overall shape variation

We tested for shape variation among samples (i.e., the occurrence of shape plasticity) by applying a multivariate analysis of variance (MANOVA) to the Procrustes coordinates with temperature as a main effect. We then conducted a discriminant analysis combined with a canonical variates analysis considering temperature as the independent factor. This approach allows to optimally visualize the relative position of the different samples in the multivariate statistical space by maximizing the among-samples variation. To test whether quantitative differences among treatments occur, we computed the generalized distances between all successive sample means in the shape space (i.e., Procrustes distances).

To appraise the respective contribution of each landmark to the overall shape variation, we summed the *x*- and *y*-coordinate mean squares due to the temperature effect for each landmark (i.e., variation of a landmark across the seven temperatures), providing a temperature effect mean squares per landmark (Klingenberg and McIntyre 1998). Finally, to visualize shape changes among samples and to discuss their developmental bases, the mean shapes were reconstructed, keeping size constant.

Relationship between size and shape: allometric and nonallometric shape variations

Allometry has been shown to occur in *D. simulans* wing shape, although accounting for a small part of shape variation

(Baylac and Penin 1998). To investigate its occurrence along the temperature gradient, we performed a multivariate analysis of covariance (MANCOVA) of the Procrustes coordinates, considering temperature as the categorical predictor and centroid size as the covariate. This procedure allowed us to test simultaneously for the effect of size on shape (allometric effect), for the effect of temperature on the shape variation independent from size, and for the consistency of the allometric effect among temperatures (interaction size × temperature).

We also conducted a multivariate regression of the Procrustes coordinates on centroid size. The predicted values were then used as shape variables accounting for the allometric component of shape variation across temperatures, and the residuals were used to investigate the shape variation independent of size, that is, the nonallometric component.

To test whether the allometric component of shape variation was influenced by temperature, we ran a MANOVA including temperature as the main effect on the predicted values (the MANCOVA was used to test for this effect on the residuals). We also performed a discriminant analysis combined with a canonical variates analysis on the residuals. To test whether the same landmarks were involved in both components of shape variation, we estimated the mean squares values related to each landmark.

To visualize the shape changes related to both the allometric and nonallometric components, the theoretical mean configurations per temperature were reconstructed using predicted values for the allometric component and the residuals added to the grand mean configuration for the nonallometric component. Finally, the Procrustes distances among these mean configurations were computed in both cases.

RESULTS

Measurement Error

We quantified measurement error (ME) through one-way ANOVAs on the repeated measures considering individual as a fixed effect (Arnquist and Martensson 1998). This was done on each landmark coordinates for shape and on centroid size. This analysis provided both absolute ME values (resid-

TABLE 1. Measurement error (ME) for each coordinate and for centroid size. Absolute and relative MEs were computed from ANOVAs considering individual as an effect, as the within group mean square (MS_{within}) and as the ratio of MS_{within} over the total variance ($MS_{within} + MS_{among}$), respectively. Absolute MEs were multiplied by 10^6.

Coordinates	Absolute ME	Relative ME
x1	4	0.041
y1	2	0.068
x2	2	0.053
y2	2	0.154
x3	2	0.114
y3	2	0.048
x4	3	0.041
y4	2	0.057
x5	3	0.107
y5	2	0.058
x6	2	0.066
y6	2	0.250
x7	2	0.071
y7	2	0.400
x8	2	0.095
y8	1	0.167
x9	3	0.231
y9	2	0.333
x10	3	0.200
y10	2	0.148
x11	2	0.200
y11	2	0.125
Centroid size	160	0.003

ual variance) and relative ME values (computed as the ratio of the residual variance over the total variance; see Table 1). The results show that the absolute ME varies only slightly among landmarks. In contrast, the relative ME appears to be landmark specific, suggesting biological differences among landmarks in their degree of variation. The impact of ME on size appears to be extremely low (Table 1).

Size Variation among Temperatures

The ANOVA on centroid size was highly significant ($F_{6,203} = 498.1$, $P < 0.001$), showing that mean size decreases as

temperature increases (Fig. 2). Post hoc comparisons showed that most of the temperatures had a significantly different effect on wing size (all $P < 0.05$), except between 14°C and 17°C (post hoc Tukey HSD $P = 0.98$). The magnitude of size differences across the thermal range does not suggest any obvious trend (Fig. 2). A closer look at the three intervein regions showed that they are similarly affected by temperature: each IVR decreases in size when temperature increases (slopes of the regressions significantly different from zero, all $P < 0.05$). No difference among slopes of each of the regressions of IVR centroid size on temperature was found (t-test for comparison of slopes: all t-values > $t_{0.05(2),409}$, $P > 0.5$; Zar 1996).

Shape Variation

Overall shape variation

The MANOVA on Procrustes coordinates was found to be highly significant (Wilk's $\lambda = 0.005$; $F_{132,1066} = 12.05$, $P < 0.001$), suggesting that wing shape varies significantly among temperatures. The discriminant analysis combined to the canonical analysis (Fig. 3) provided a clear discrimination of the samples in the plan defined by the two first canonical axes which respectively accounted for 64.6% and 28.7% of the among-temperature variance (total = 93.3%). Canonical axis 1 mainly contrasts 12°C, 14°C, and 17°C from the other samples, whereas canonical axis 2 is mostly defined by the difference among 30°C sample and the others. The combination of both axes permits a discrimination of the seven samples, whose positions appear to be congruent with the temperature gradient along a curved trajectory (Fig. 3). The Procrustes distances among successive mean shapes did not suggest any general trend along the gradient. The highest distances were found among 17°C and 21°C mean shapes and among 28°C and 30°C (results not shown). This is congruent with the statistical distances as shown in Figure 3.

The computation of mean squares per landmark provided the following results. Landmark 1 is clearly the most variable (see Fig. 4). Landmarks 2, 3, 4, and 5 are highly variable,
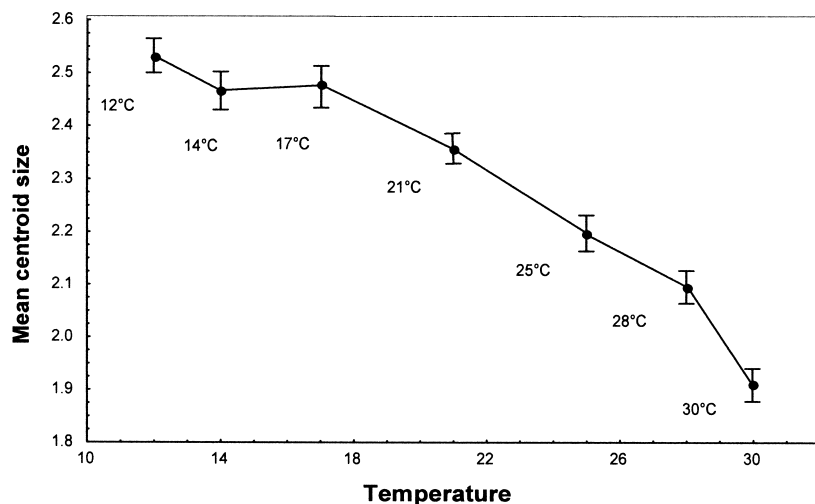

FIG. 2. Variation of mean centroid size according to growth temperature. All differences except 14–17°C are significant. Note the strong effect between 28°C and 30°C.
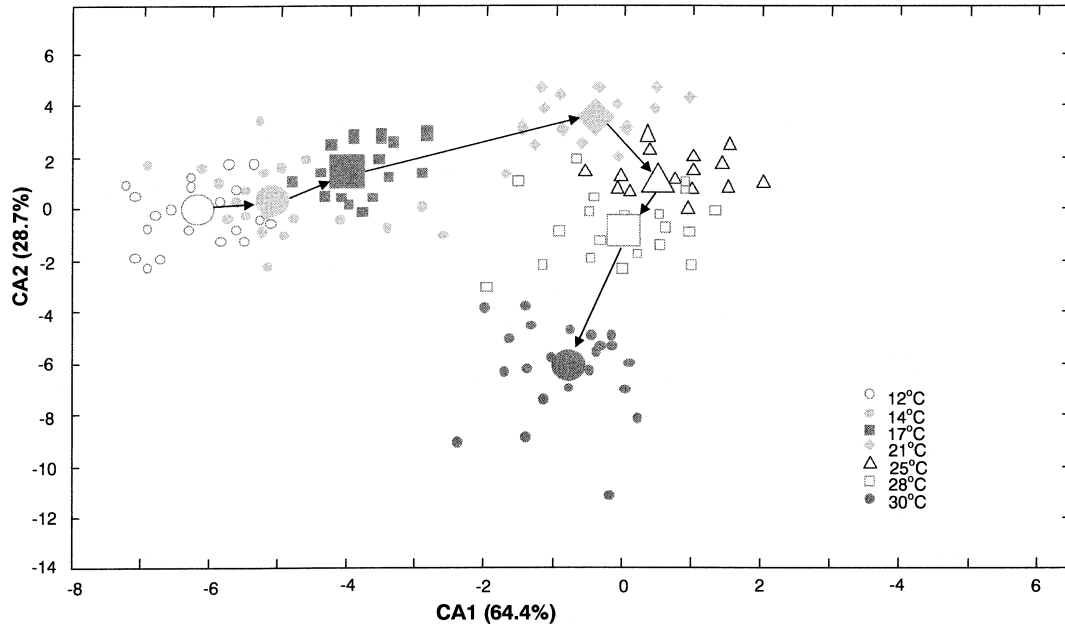
FIG. 3. Analysis of wing overall shape variation. First canonical plan computed from the canonical variates analysis on Procrustes coordinates (whole shape variation). Mean and individual values of the seven samples are shown. Arrows indicate the progression of growth temperature and show an overall curved trajectory.

although less than landmark 1. In contrast, the temperature appears to have only a small effect on landmarks 6, 7, 8, 9, 10, and 11. Such heterogeneity cannot be attributed to measurement error, which similarly affects all of the landmarks (Table 1). Whereas landmark 1 presents the highest level of absolute measurement error, its relative amplitude is among the lowest, which strengthens the biological significance of its high level of variation.

The prevailing contribution of landmark 1 to the shape variation likely induces a Pinocchio effect, namely the spread of variation from that landmark to the other landmarks due to the least squares procedure (Chapman 1990). However, this Pinocchio effect does not prevent the interpretation of our results, because it induces an underestimation of the differences among landmarks in their level of variation (Klingenberg and McIntyre 1998).

Allometric and nonallometric components of shape

Both temperature and size effects were found to be significant in the MANCOVA (respectively, Wilk's $\lambda = 0.415$, $F_{132,1025} = 1.267$, $P = 0.029$; Wilk's $\lambda = 0.79$, $F_{22,175} =$
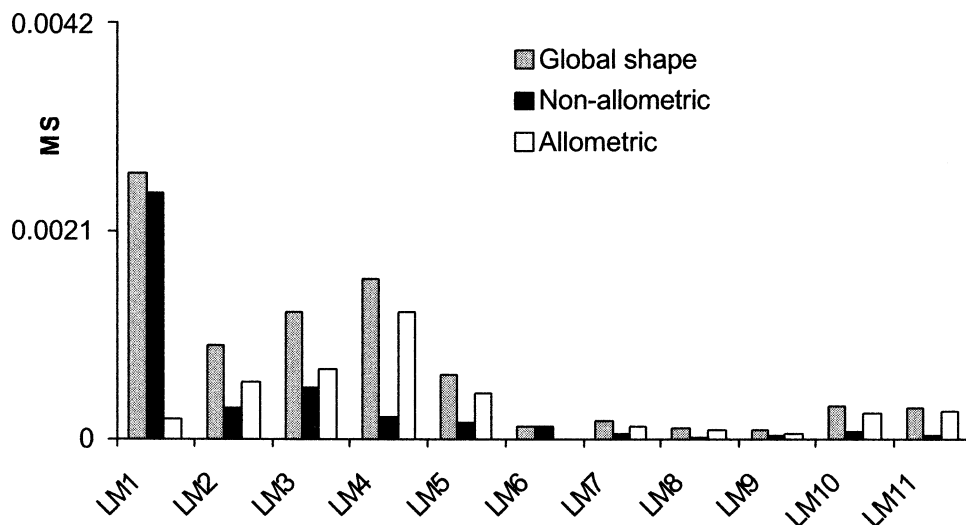

FIG. 4. Amount of variation, expressed by mean square (MS) values, for the three components of wing shape in the 11 landmarks (LM). Note the discrepancy between highly variable distal landmarks (1–5) and the mildly or poorly variable proximal landmarks (6–11). Note also that the contributions to allometric and nonallometric component of shape are highly variable among landmarks (e.g., compare LM1 and LM4).
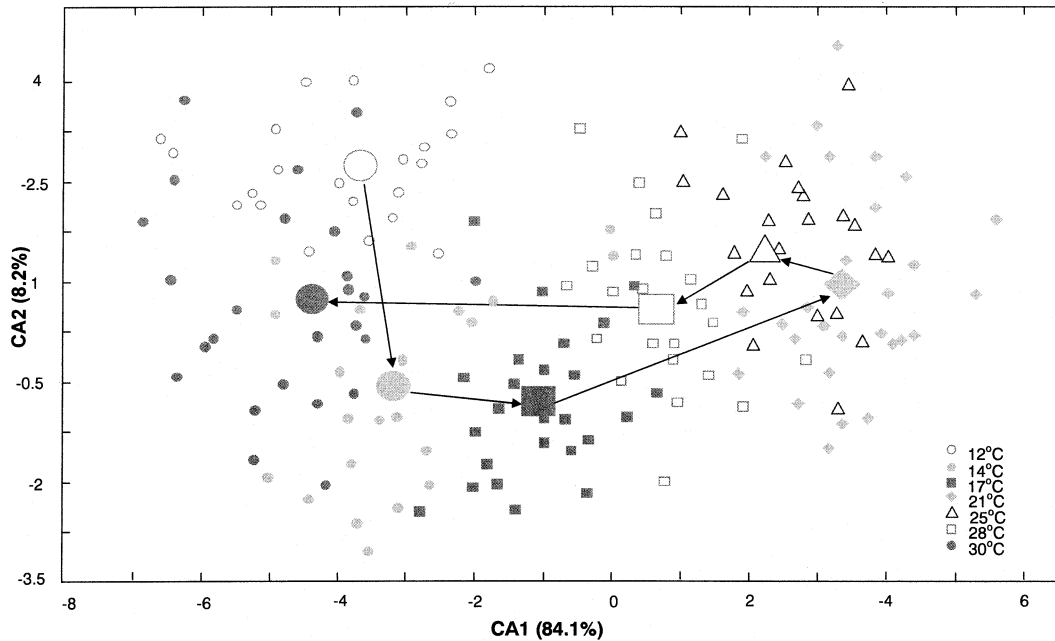
FIG. 5. Analysis of the nonallometric wing shape variation. First canonical plan computed from the canonical variates analysis on the residuals from the multivariate regression. Note the abrupt variation of shape change between 21°C and 25°C and the proximity of wing shape of flies at extreme temperatures, 12°C and 30°C.

2.111, $P < 0.01$), suggesting both an effect of temperature on the nonallometric component of shape and an allometric effect. The interaction term (size x temperature) was nonsignificant (Wilk's $\lambda = 0.432$, $F_{132,1025} = 1.203$, ns) indicating that the allometric effect remains relatively consistent along the temperature gradient.

The negative relationship between size and temperature described above (Fig. 2) could have impeded the interpretation of these results. Indeed, collinearity is a common problem in regression analysis. When predictor variates are correlated, which is the case between size and temperature, this collinearity might inflate variance levels, potentially affecting the validity of the model's parameters (Belsley et al. 1980). There are several ways for testing the impact of correlation between covariates, among which the computation of a variance inflation factor (VIF) is the most commonly used (e.g., Lande and Arnold 1983). The VIF computed for size and temperature was found to reach a value of 7, which is considered sufficiently low to not significantly affect the regression model (Belsley et al. 1980). This suggests that the relative effects of temperature and size on shape may actually be disentangled and supports the reliability of our results.

The multivariate regression of Procrustes coordinates on centroid size confirmed the occurrence of a strong allometric effect accounting for 23.8% of the total shape variance (76.2% for the nonallometric component). The MANOVA applied on the predicted values (allometric) was highly significant (Wilk's $\lambda = 0.041$, $F_{132,1025} = 5.88$, $P < 0.001$). The discriminant analysis coupled to the canonical variates analysis on the residuals (nonallometric; Fig. 5) provided the following results. The seven samples are mostly separated along the first canonical axis (CA1, 84.1% of total variance). However, the most interesting pattern is the reversal in the

direction of shape change at 21°C: indeed, while the samples from 12°C to 21°C are clearly ordinated along CA1 from negative to positive values, the reverse is observed from 21°C to 30°C, with an ordination of the sample means from positive to negative values. Remarkably, this leads to a close proximity between the 12°C and 30°C mean shapes in the first canonical plan (which accounts for 92.3% of the nonallometric variance).

Finally, for both the allometric and nonallometric components, the mean squares related to each landmark (Fig. 4) are generally in agreement with those found for the overall shape, suggesting a discrepancy between proximal and distal landmarks, the latter being more sensitive to temperature. The main difference between the allometric and nonallometric components concerns the allocation of the variation among the distal landmarks. While the nonallometric effect predominantly involves landmark 1, the allometric effect mostly concerns landmarks 2, 3, 4, and 5, landmark 4 being the most variable in this respect.

Shape changes and patterns of landmark displacements

The mean wing configurations were reconstructed separately for the overall wing shape and for both the allometric and nonallometric components. Only the 12°C, 21°C, and 30°C mean configurations are displayed on Figure 6, which makes the results easier to see. Overall shape changes can be identified by comparing these configurations along the temperature gradient. This type of graphic representation can be considered as a shape reaction norm.

It appears that shape changes related to the allometric component (Fig. 6a) are unidirectional across the whole gradient. Allometric reaction norm mainly consists of a distal con-
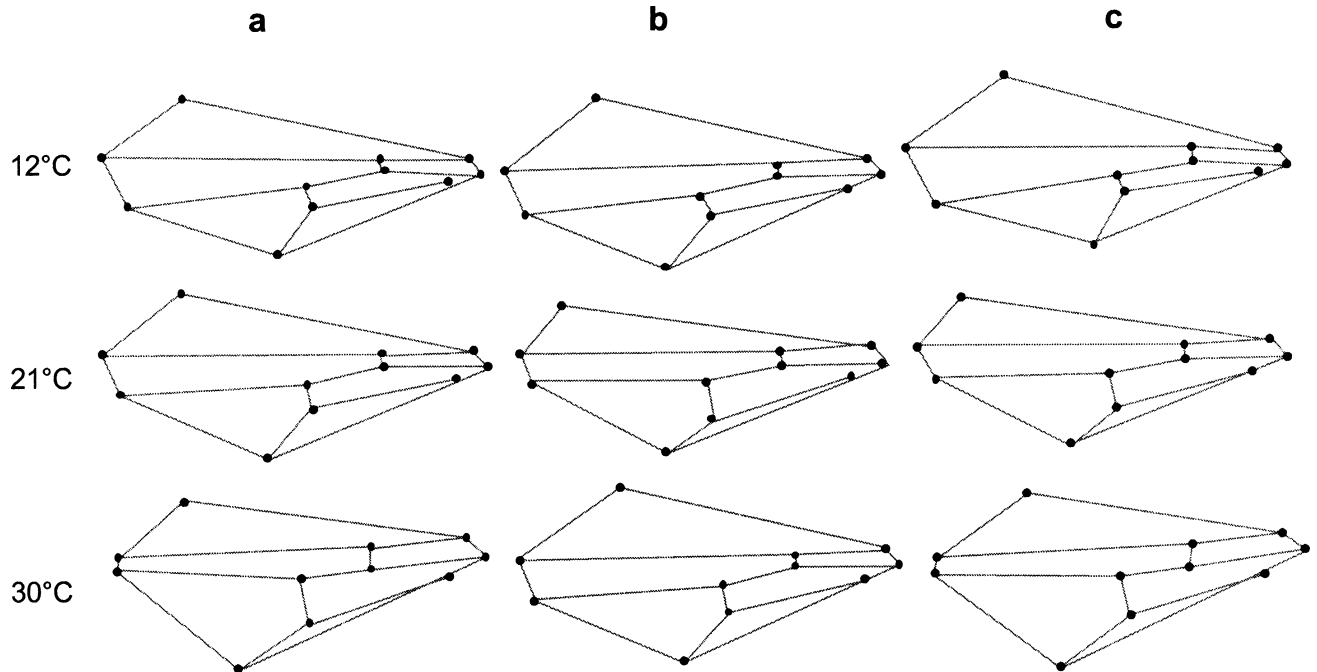
FIG. 6. Shape reaction norms: reconstruction of the mean wing shapes per temperature for the allometric component (a), the nonallometric component (b), and the global shape variation (c). The lowest temperatures are at the top, the highest at the bottom.

traction of the wing, with a strong narrowing of the intervein region defined by LV2 and LV3 (IVR-B) and a relative shortening of LV2 and LV3. This contraction of the distal part of the wing is coupled to the expansion of the posterior region and an elongation of the posterior crossvein. Note that the anterior border of the wing is not involved in the allometric change.

The nonallometric variation (Fig. 6b) involves similar but reversed shape changes below and above 21°C. This variation mainly consists in a combined anterior/posterior and proximal/distal contraction of the wing from 12°C to 21°C followed by a reverse expansion from 21°C to 30°C. This is confirmed by the Procrustes distance computed among 12°C and the other samples: it increases from 12°C to 21°C and then decreases toward 30°C (results not shown).

Overall shape changes (Fig. 6c) present a combination of both trends. The narrowing of IVR-B and the expansion of the posterior region and the posterior crossvein are consistent from 12°C to 30°C. In addition, the elongation-contraction pattern of the two median longitudinal veins recorded for the nonallometric component is observed, together with the contraction-expansion of the anterior region.

Note that the whole wing, including both the anterior and posterior compartments, is affected by temperature, regardless of whether the allometric or nonallometric component is considered. The overall discrepancy between the proximal and distal parts of the wing already suggested by the mean squares values (Fig. 4) is visually confirmed, distal landmarks being clearly more variable than the proximal ones.

Finally, to accurately depict the shifts in an individual landmark's position, the displacements of the five first landmarks (the most variable) were amplified (Fig. 7). Two observations can be made. First, the graphs provide an unam-

biguous confirmation of the general patterns of allometric and nonallometric shape changes described above. The nonallometric changes become highly similar toward low and high temperatures, while allometric changes vary quite regularly along the gradient. Second, the direction of a landmark's trajectory is clearly nonrandom. The position of landmark 1 mostly varies along the wing margin. It is noteworthy that the allometric effect is extremely low on this landmark, whereas its displacements are predominant concerning both the nonallometric component and the global shape variation. Considering its position at 21°C as a reference, any change in temperature induces a shift toward more anterior and proximal positions. Landmark 2 moves along LV2; the allometric effect on this landmark consists in a shift toward more proximal positions, from 12°C to 30°C. For the nonallometric variation, both low and high temperatures induce a shift from its position at 21°C toward more distal positions. Landmark 3 moves along the wing margin, following the anterior/posterior axis. Increasing temperature induces a shift toward more anterior positions when considering the allometric changes, while both low and high temperatures induce displacements toward more posterior positions for the nonallometric changes. Landmark 4 displacements follow LV4 rather than the wing margin. The changes in position of this landmark related to the nonallometric component are remarkably low. The allometric effect consists in displacements toward distal-posterior positions from low to high temperatures. Finally, landmark 5 moves mainly along the posterior crossvein, toward posterior positions for the allometric component when temperature increases and toward anterior positions with both increasing and decreasing temperatures for the nonallometric component.
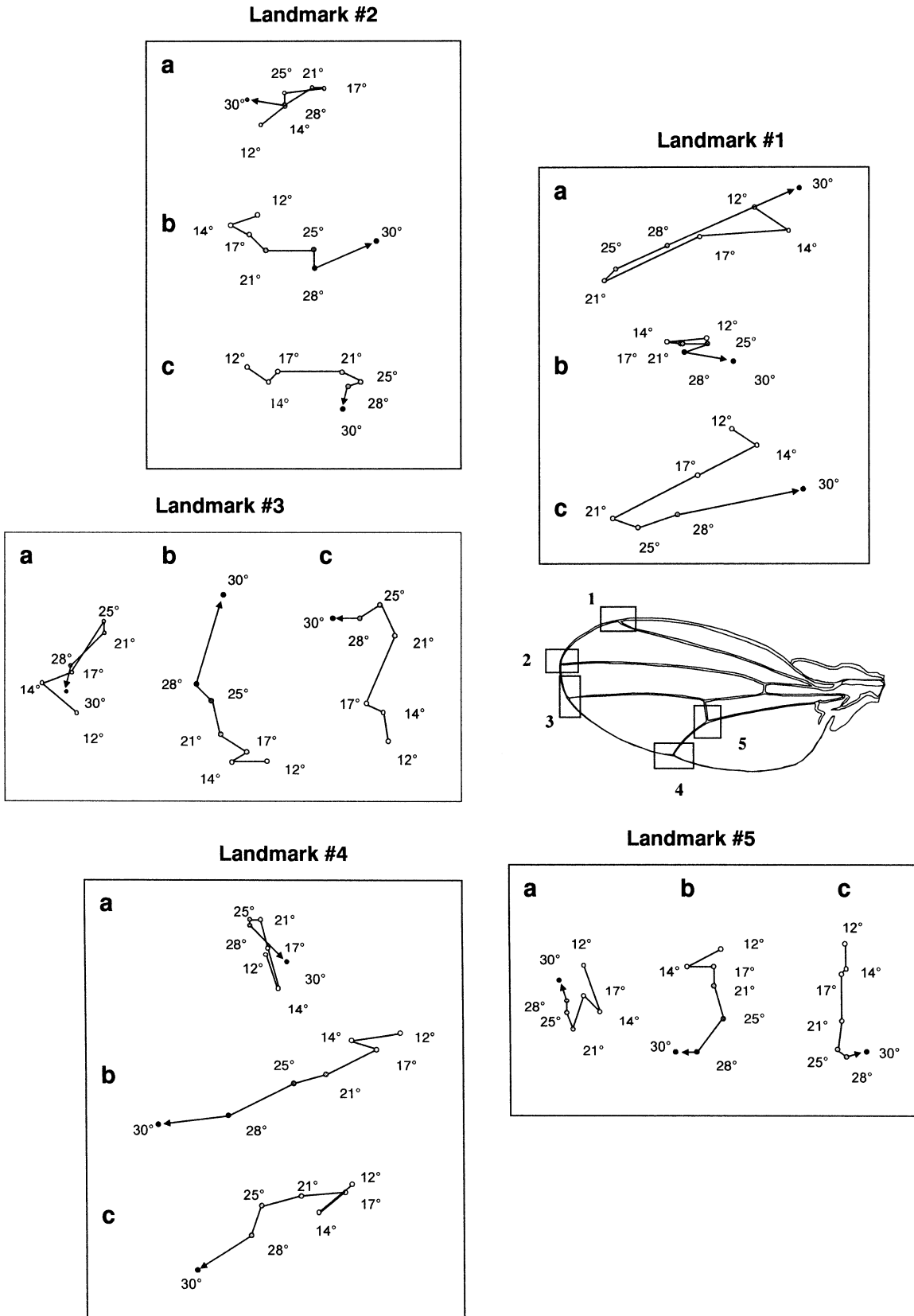
FIG. 7. Shifts in the position of the five first landmarks across the temperature range. For each graph, (a) shows the nonallometric component, (b) the allometric component, and (c) the global shape component. The graph illustrates major divergent responses in the reaction norms between (a) and (b) and the need for investigating several temperatures to define phenotypic plasticity of wing shape.

DISCUSSION

Although body size, including wing size, is highly variable among individuals of the same species, wing shape appears more stable. Investigators have tried to define shape variations in a diversity of ways. For example, taxonomists have used ratios, defining wing indices, to discriminate *Drosophila* species (Sturtevant 1942).

Recently various techniques have been used to characterize shape variations, such as the inclusion of the wing blade in an ellipse and calculation of various angles (Bitner-Mathé and Klaczko 1999a,b,c), the analysis of the wing contour with Fourier decomposition (e.g., Cavicchi et al. 1985), or the comparison of the reaction norms in various parts of the wing (Moreteau et al. 1998). To our knowledge, our work is the first application of geometric morphometrics to the analysis of the phenotypic plasticity of *Drosophila* wing.

The observation of numerous landmarks permits a multivariate quantification of shape and the construction of shape reaction norms. Still more interesting, shape variations may be subdivided into two components, an allometric one and a more important (76%) nonallometric one. Our results may be discussed in relation with both developmental processes and evolutionary adaptations.

Integration versus Modularity

It has been suggested that the *Drosophila* wing should be considered a modular structure, primarily divided into two main developmental compartments, namely, the anterior and posterior compartments (Fig. 1). Mostly analyzing distances among landmarks defined on the wing, Cavicchi et al. (1981, 1985) have suggested that these compartments are evolutionarily independent (see also Guerra et al. 1997; Pezzoli et al. 1997). However, a recent geometric morphometric study focusing on the effect of both genetic and random developmental variations on wing shape (Klingenberg and Zaklan 2000) has provided arguments against this view and concluded that shape changes should be considered as fundamentally integrated across the entire wing. Our study, focusing on an environmental source of variation, has provided less clear-cut results. On the one hand, temperature was found to affect landmarks located throughout the entire wing, including anterior and posterior positions. This is valid for both the allometric and the nonallometric components of shape change (Figs. 4, 6) and suggests that the wing reacts as a whole to an environmental change, thus supporting the view that integration is prevailing. Additionally, centroid size of the three IVRs was similarly affected by temperature, which is in contradiction with a genetic independence among IVRs (Birdsall et al. 2000; Zimmerman et al. 2000). On the other hand, the discrepancy between the allometric and nonallometric component of shape change, and particularly the respective importance of landmarks 1 and 4 in these components, might suggest a certain independence among wing regions. In addition, it is possible that different developmental pathways could be similarly affected by temperature.

Put in the perspective of other studies, our results suggest that, although primarily integrated, the *Drosophila* wing will not be free of localized and specific variation. This view is congruent with the present knowledge on developmental events responsible for wing formation. It is indeed well known that processes affecting the wing as a whole (e.g., overall wing patterning; imaginal disc eversion) and other processes acting at a more localized level (differentiation and precise positioning of the veins) may occur simultaneously (e.g., Garcia-Bellido and De Celis 1992; De Celis 1998, 2003; Klingenberg and Zaklan 2000). Both integrated and localized patterns of shape variation are thus likely to arise. Integration and modularity should then be considered complementary rather than mutually exclusive.

Proximal-Distal Discrepancy

A clear pattern of shape change reported in this paper is the discrepancy among proximal and distal landmarks in their amount of variation across temperatures: the proximal landmarks are less affected by the temperature treatment than the distal ones (Figs. 4, 6). Interpreting this pattern is obviously tentative, and several nonmutually exclusive hypotheses may be proposed. It has been shown that morphogen gradients are often involved in the patterning of the wing (e.g., Strigini and Cohen 1997; Day and Lawrence 2000; Cadigan 2002). The differential sensitivity between proximal and distal landmarks could be explained by a decreasing concentration of an underlying signal from the wing hinge to the wing tip. Genes involved in the global proximal-distal patterning of the wing such as *wingless* and *nubbin* (Ng et al. 1995; Cifuentes and Garcia-Bellido 1997; Milàn and Cohen 2000) could be good candidates for such an effect. An alternative hypothesis can be suggested. Because the first four landmarks are located on the wing margin, the observed pattern could be related to a margin effect. In other words, instead of focusing on the variation of longitudinal veins, we might consider that a shift in the position of the margin (i.e., elongation or retraction) could induce the recorded landmark's displacements. Under this assumption, an environmental effect on genes early expressed in the dorsal-ventral boundary, such as *notch* and *wingless* (De Celis and Garcia-Bellido 1994; Sturtevant and Bier 1995) could be suspected. A third and more trivial hypothesis is to relate the small amount of variation in the wing hinge to the small size of this region compared to that of the wing edge. The difference in the shape variance of both regions of the wing could then simply be due to their relative size. The position of the landmarks close to the hinge would be less variable because of the lack of available space for alternative location. The observed discrepancy along the proximal-distal axis could thus reflect a structural constraint rather than a mechanistic difference. Against this simple and trivial interpretation is the fact that basal and apical parts of the wing exhibit different reaction norms in relation with temperature (Moreteau et al. 1998).

Size, Shape, and Stress

The most striking result of this study concerns the discrepancy between size and size-related shape change on the one hand and nonallometric component of shape on the other. As stated throughout this paper, wing size and the allometric component of shape change appear to be monotonically related to temperature, although this relationship should be considered cautiously because it could be related to the almost linear

relationship between size and temperature. In contrast, the effects of low and high temperatures on the nonallometric component of shape change are strikingly similar. What do low and high temperatures have in common that might induce comparable modification of the nonallometric component of wing shape? The most straightforward answer is that both depart from the optimal developmental temperature, that is, both might destabilize developmental pathway(s) required for the production of the standard phenotype. Such an effect should thus be interpreted in terms of stress.

Although the concept of stress is not easy to delineate, its possible significance for the adaptation of natural populations to new environments is repeatedly argued (Hoffmann and Parsons 1991; Bijlsma and Loeschcke 1997; Hoffmann and Hercus 2000). The fact that low and high temperatures are symmetrically stressful for *Drosophila* is directly demonstrated by a sharp decrease in viability (Pétavy et al. 2001) and male fertility (Chakir et al. 2002) or indirectly by an increase in phenotypic variance of various traits (Imasheva et al. 1997; Karan et al. 1999a; Moreteau et al. 2003).

Two complementary genetic hypotheses have been considered to explain these patterns of phenotypic plasticity (for a review see Via et al. 1995). Genes involved in wing patterning may be affected in the same way by extreme thermal regimes, leading to the production of similar phenotypic variants. This mechanism matches the allelic sensitivity hypothesis as a genetic basis for plasticity. Alternatively, the occurrence of specific genes, reacting to the environment and controlling different phenotypes, has been suggested, leading to the plasticity genes hypothesis (Via and Lande 1995). Genes encoding for heat shock proteins (Hsp; Rutherford and Lindquist 1998; Feder and Hofmann 1999; Queitsch et al. 2002) are good candidates for such an effect. It has been shown that in standard conditions Hsp nonspecifically stabilize damaged or unstable proteins (e.g., Srivastava et al. 1998; Feder and Hofmann 1999). Stressful conditions, increasing the number of unstable proteins that will fix Hsp, could induce their titration. The usual Hsp targets, left without their stabilizer, could then be unable to play their role in various functions including developmental pathways (e.g., Rutherford and Lindquist 1998; Morange 1999). Such a phenomenon could be involved in the specific processes responsible for the patterns recorded for the nonallometric component of shape variation. Additionally, Hsp have been shown to act as a protective response against both heat and cold stress, although their role at low temperature is less well documented (but see Goto and Kimura 1998). Our data suggest that we will have to search for such genes symmetrically responding to a high or low temperature shift.

Wing Shape and Natural Selection

The occurrence of analogous latitudinal clines for wing size is documented in several drosophilid species, including *D. melanogaster* and *D. simulans* (Capy et al. 1993), *D. subobscura* (Huey et al. 2000), *D. kikkawai* (Karan et al. 1998), and *Zaprionus indianus* (Karan et al. 1999b). In all cases, larger wings are found at higher latitudes, that is under colder climates, and in this respect there is a striking parallelism with phenotypic plasticity. It is, however, generally admitted

that the mechanisms are not the same: phenotypic plasticity mostly modifies cell size, whereas genetic variations are mediated by a change in cell number (e.g., Azevedo et al. 2002). Both processes are likely to respond rapidly to directional selection, producing either smaller or bigger wings. Indeed a rapid adaptive change has been documented after the introduction of *D. subosbscura* on the American continent (Huey et al. 2000).

By contrast, shape is generally assumed to be more stable, submitted to strong developmental and phylogenetic constraints, so that significant variations expected mostly among species, to be used as taxonomic indices (Sturtevant 1942). Against this hypothesis, recent empirical observations have shown that, within a species, shape was also genetically variable (Huey 2000; Gilchrist and Partridge 2001). In this respect, geometric morphometric should be a valuable tool for the analysis of geographic populations.

Finally, are the changes in shape due to plasticity an adaptive response to a better flight capacity? For the moment, it is impossible to provide even vague hypothesis, due to the fact that the connection between flight aerodynamics and the geometric structure of the wing is very poorly understood (Gilchrist and Partridge 2001; Fry et al. 2003). What we know is that bigger wings are likely adaptive to fly in a colder environment, and that the resulting lesser wing loading is accompanied by a lesser wing beat frequency (Pétavy et al. 1997). Whether a plastic variation such as the increased distance between veins L2 and L3 at low temperatures permits a better flight efficiency is not known. However, some insight may be provided by comparing temperate and tropical species. Direct comparisons of samples differing in their flight capability should also provide insights into the adaptive value of wing shape geometry.

Perspective: Linking Phenotypic Variation and Developmental Molecular Processes

It is obvious that not only genes contribute to the formation of the phenotype. Accordingly, environmental effects should be systematically considered in the study of developmental processes (see Gilbert 2001; Sultan 2003). Geometric morphometrics provides us with an incomparable powerful tool with which to describe and quantify subtle shape variations. This power should help us to relate the growing knowledge of developmental processes to patterns of phenotypic variation significant for population and evolutionary biology (Birdsall et al. 2000; Palsson and Gibson 2000; Zimmerman et al. 2000; Debat and David 2001, 2002; Klingenberg 2002).

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