

RESEARCH ARTICLE

Multidimensional analysis of *Drosophila* wing variation in Evolution Canyon

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Abstract

Environmental stress has been suggested to be a major evolutionary force, both through inducing strong selection and because of its direct impact on developmental buffering processes that alter the evolvability of organisms. In particular, temperature has attracted much attention because of its importance as an ecological feature and the relative ease with which it can be experimentally manipulated in the lab. Evolution Canyon, Lower Nahal Oren, Israel, is a well studied natural site where ecological parameters are suspected to drive evolutionary differentiation. In this study, using *Drosophila melanogaster* isofemale lines derived from wild flies collected on both slopes of the canyon, we investigated the effect of developmental temperature upon the different components of phenotypic variation of a complex trait: the wing. Combining geometric and traditional morphometrics, we find only limited evidence for a differentiation among slopes. Investigating simultaneously phenotypic plasticity, genetic variation among isofemale lines, variation among individuals and fluctuating asymmetry, we could not identify a consistent effect of the stressful conditions encountered on the south facing slope. The prevailing structuring effect is that of the experimentally manipulated temperature which clearly influences wing mean size and shape. Variability, in contrast, is not consistently affected by temperature. Finally, we investigated the specific relationship between individual variation and fluctuating asymmetry. Using metric multi-dimensional scaling we show that the related patterns of wing shape variation are not identical, supporting the view that the underlying developmental processes are to a certain extent different.

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Introduction

Understanding the micro-evolutionary bases of macro-evolutionary change has been challenging evolutionary biologists for decades. Advent of new research fields such as evolutionary developmental biology (evo–devo) and generalization of new methodological tools, focussing either on the molecular level such as genomics or on the phenotype such as geometric morphometrics, has generated a renewal of enthusiasm for the task and new hope of success (Carroll *et al.* 2001; Foster *et al.* 2008). Among the questions that have recently attracted fresh attention is the origin and control of

morphological variation (Hallgrímsson and Hall 2005). This question is of interest since variation provides the raw material for natural selection, and thus an understanding of the factors promoting or buffering it is needed for understanding how morphological evolution proceeds. The patterns of variation occurring in a population not only provide an insight into the possible effects of further selection, as suggested by quantitative geneticists (e.g., Lande 1979), but on the developmental processes generating it as well: modularity, integration, canalization, plasticity and developmental stability are some of such processes (e.g., Hallgrímsson and Hall 2005). These mechanisms are of special interest since their change is likely to alter the variational properties of organisms, and therefore their evolvability.

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In particular, it has repeatedly been suggested that adverse environmental conditions can promote adaptation by uncovering genetic variation through the impairment of buffering processes (Hoffmann and Parsons 1991). This hypothesis, which traces back to work on canalization by Waddington in the mid-twentieth century (Waddington 1942, 1957), was readvocated in the past 10 years with the discovery in *Drosophila* and *Arabidopsis* of the role of Hsp90 in the hide and release of cryptic genetic variation of putative adaptive value (Rutherford and Lindquist 1998; Queitsch *et al.* 2002; Sangster *et al.* 2008). Alternative to such molecular processes buffering genetic variation, recent theoretical genetic studies have suggested that robustness might be a generic property of complex genetic networks (Bergman and Siegal 2003; Hermisson and Wagner 2004). Regardless of the process involved, it has been suggested that stressful environmental conditions, as well as pleiotropic mutations, might destabilize the developmental process and lead to the uncovering of cryptic genetic variation (Gibson and Dworkin 2004; Schlichting 2008). This effect, however, is not universal, since the link between stress and genetic variation is not straightforward (reviewed by Hoffmann and Merilä 1999). The question as to which component(s) of phenotypic variation are the most sensitive to adverse environmental conditions, and what should be the consequences for potential adaptation to these conditions, remains an open one. For example, it is not known whether phenotypic plasticity, rather than genetic variation, is likely to be first promoted by such changes. This relates to the contentious debate on the relative status of plasticity, canalization and developmental stability (Debat and David 2001). In particular, the relationship between canalization, as estimated by among-individual variation, and developmental stability as measured by fluctuating asymmetry is controversial (Klingenberg and McIntyre 1998; Debat *et al.* 2000; Santos *et al.* 2005; Breuker *et al.* 2006). Are canalization and developmental stability distinct buffering processes or two complementary aspects of a single mechanism?

Local adaptation to micro-environmental conditions has been relatively rarely tested in the wild. A particularly well documented situation is the case of the so called 'Evolution Canyon', at Lower Nahal Oren, Mount Carmel, Israel. The opposite slopes of this canyon which are separated by 100 and 400 m at the bottom and top, respectively, are subjected to strongly different micro-climatic influences due to the higher illumination on the south-facing slope (SFS) than on the north-facing slope (NFS) (Pavlicek *et al.* 2003). The slopes also differ in temperature and aridity: NFS has comparatively lush vegetation of European origin, whereas the SFS is an open park forest or xeric savanna, primarily of African and Asian origin. These contrasting climatic influences were repeatedly found to be associated with a sharp differentiation in terms of species composition and population genetic structure in diverse organisms, including several *Drosophila* species (Nevo 1997, 2001; Nevo *et al.* 1998;

Pavlicek *et al.* 2003). Differences in habitat choice, thermotolerance, desiccation resistance and life-history traits were recorded (Nevo *et al.* 1998; Rashkovetsky *et al.* 2000; Iliadi *et al.* 2002; Lupu *et al.* 2004), all corresponding to the prevailing microclimate. These populations also differ in sexual behaviour, including mate choice (Korol *et al.* 2000; Iliadi *et al.* 2002). Moreover, these differences have been found to be stable through time despite year-to-year climatic variation and inter-slope migration (Korol *et al.* 2006). Such a situation has been interpreted as a case where local adaptation overrides genetic drift and the homogenizing effects of migration, thereby emphasizing the strength of natural selection. The dryer, warmer and UV exposed SFS is generally assumed to be more stressful for organisms than the NFS, which provides a suitable natural situation to investigate the effects of stress upon the components of phenotypic variability.

The wing of *Drosophila* has emerged as a particularly interesting and intriguing evolutionary model. The repeated and fast evolution at a continental scale of latitudinal clines for wing size and shape suggests strong selection on these traits (Gilchrist *et al.* 2004). Besides, those clines are paralleled by the variation of phenotypic plasticity in response to temperature, which is believed to be adaptive (David *et al.* 1994, 2006). However, the precise nature of the selective pressure allegedly acting is unclear. Flight and courtship songs are the most obvious functional hypotheses but their link with wing size and shape remains to be proven (Houle *et al.* 2003; Routtu *et al.* 2007). Besides these selective considerations, the *Drosophila* wing is particularly amenable to morphometric studies because its highly stereotyped two-dimensional structure allows the location of many well defined landmarks (Breuker *et al.* 2006; Debat *et al.* 2006). Finally, *D. melanogaster* is easily raised in the lab and experimental genetic, and environmental manipulations are routinely performed, which makes it a convenient model to investigate phenotypic plasticity (David *et al.* 1994).

In this study, we used isofemale lines derived from wild caught *D. melanogaster* from the two slopes of Evolution Canyon, and then raised them under three developmental temperatures to test the occurrence of inter-slope differentiation on wing size and shape. Using classical and geometric morphometrics coupled with multivariate statistical treatments, we analysed phenotypic plasticity, genetic variation, canalization and developmental stability for both slopes. The slopes are found to be largely similar: the differentiation, although detectable in some cases, is relatively weak. This shows that despite some evidence for differentiation among slopes, the main factor affecting wing size and shape variation in our study is rearing temperature. Using multi-dimensional scaling, we further investigated the patterns of shape variation, specifically focussing on variation among individuals and fluctuating asymmetry. While some similarity undeniably exists, FA and individual variation matrices are clearly not identical suggesting the existence of some differ-

ences in the underlying processes, namely canalization and developmental stability. We suggest that systematic application of metric multi-dimensional scaling could be fruitful in fields dealing with comparable datasets such as multivariate quantitative genetics and geometric morphometrics.

Materials and methods

Flies

The flies used in this study are from 20 different isofemale lines established from individuals collected on both slopes of Evolution Canyon in April 2006. Wild-living females were isolated in culture vials to initiate isofemale lines. The F₁ progeny were used as the parents of the experimental flies. The parents were allowed to oviposit on killed-yeast medium which is a very rich food preventing any larval crowding effect (Karan *et al.* 1999). This operation was repeated three times and the vials were distributed at three different constant temperatures i.e. 14, 21 and 28°C. Ten lines per slope were used leading to a total of 60 groups. After emergence, the adults were kept for few days on normal food and then preserved in 70% alcohol prior to measurement. The right and left wings of 10 females per group were dissected and mounted on slides using 50% glycerol and 50% lactic acid medium. Thousand two hundred individual wings were thus used in this study. Images of the wings were acquired with an Imasys U-eye digital camera (Imasys, Suresne, France) mounted on a Leica DMRB microscope (Leica Wetzlar, Germany). We digitized 15 landmarks per wing (figure 1) using an ImageJ routine (C. P. Klingenberg personal communication). To assess the measurement error and its amplitude relative to individual variation and asymmetry, both left and right wings of five individuals were digitized five times.

Data: traditional and geometric morphometrics

Generalized least-squares Procrustes superimposition was used to extract shape variation from the landmark data (Dryden and Mardia 1998). This superimposition consists of three successive steps: (i) all configurations are scaled to

a unit centroid size (i.e. the square-root of the sum of the squared distances from each landmark to the centroid of the configuration) by dividing all coordinates by the corresponding centroid size, (ii) the centroids (centres of gravity) of the configurations are superimposed through translation, and (iii) the configurations are rotated around their centroids so as to minimize the sum of squared distances between homologous landmarks, i.e., to optimize the superimposition. The new coordinates (Procrustes coordinates) are used as shape variables. Wing size was measured as the centroid size of each configuration. To allow more direct comparisons with traditional studies involving *Drosophila* wing measurements, we derived estimations of wing length and width from the landmark coordinates, as well as an elongation index computed as the ratio of length to width (wing length was measured as the distance between landmarks 2 and 13 and wing width as the distance between landmarks 12 and 15; figure 1).

Measurement error analysis

Measurement error (ME) is of critical importance when analysing fluctuating asymmetry (Palmer 1994). To assess the significance of FA relative to ME, we applied a conventional analysis of variance (ANOVA) to size-related traits (centroid size, wing length and wing width, as well as their ratio) and a Procrustes ANOVA to shape data (see below), using individual, side and their interaction as effects. In addition to the estimation of ME, this procedure allows testing for the significance of the individual effect and for the occurrence of directional asymmetry (DA). Both individual and side mean squares (MS) are tested against the interaction (individual × side) MS, and the interaction MS is tested against the residual MS (i.e. variation due to digitizing). In both cases, the model used was:

$$W_{ijk} = \mu + I_i + S_j + (I \times S)_{ij} + \varepsilon_{ijk},$$

where W is the wing parameter (either centroid size or the matrix of shape variables), I is individual (random effect) and S is side (fixed effect). The details of the Procrustes ANOVA

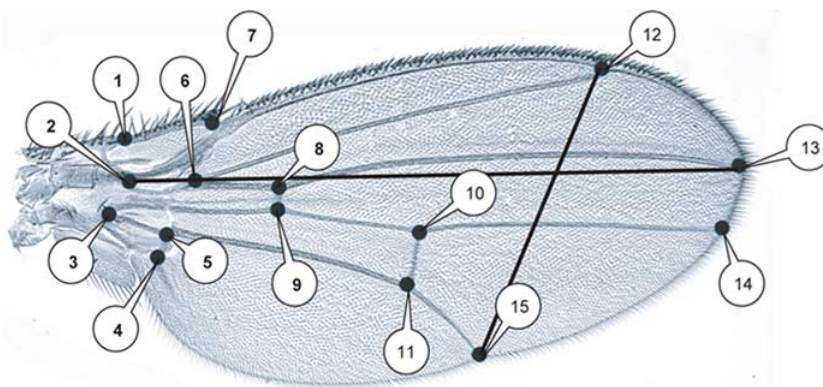


Figure 1. *Drosophila* wing landmarks digitized and distances measured in this study.

can be found in Klingenberg and McIntyre (1998). Briefly, the procedure is as follows. For each of the effects, the matrix of sums of squares and cross products (SSCP) was computed and then divided by the appropriate degrees of freedom (i.e., the usual degrees of freedom time, the number of dimensions minus four), producing the matrices of mean squares and cross products (MSCP, a multivariate analogue of MS) related to individual, side and the interaction effects. The loss of degrees of freedom is due to the superimposition process (Klingenberg and McIntyre 1998). For each effect, the sum of the diagonal elements (corresponding to the variances of the shape variables) provided us with a global estimator of the related component of variance, the so-called Procrustes mean squares. These Procrustes mean squares were then tested against each other through classical *F*-tests as in the standard ANOVA for size.

Mean size and shape

The effects of slope, temperature and isofemale lines on mean wing size and shape were investigated using ANOVAs on size-related traits and MANOVAs on the shape variables, with slope, and temperature and their interaction as fixed effects, and line as a random effect nested in slope. In both cases, the following model was used:

$$W_{ijk} = \mu + S_i + T_j + (S \times T)_{ij} + (T \times L(S))_{jk} + \varepsilon_{ijk},$$

where *W* is the wing parameter (either centroid size, wing length, wing width or their ratio, or the matrix of shape variables), *S* is slope, *T* is temperature and *L* is line. Reaction norms of all lines across temperatures were investigated for size related traits, and a discriminant analysis considering slope and temperature as factors was applied to the shape dataset.

Within lines size and shape variation and FA

To investigate the effects of temperature and of slope on within-group variation, we measured both FA and among individual-variation for each line within each temperature. For size-related traits, FA was assessed as the variance of the right minus left values (FA4 in the nomenclature of Palmer (1994) and Palmer and Strobeck (2003)) and among-individual variation as the within-line coefficient of variation of $(R + L)/2$ values. For shape data, the same procedure as used for measurement error was applied to each group, but including only a single measurement per side. The amounts of individual variation and of FA were assessed using Procrustes ANOVAs considering individual, side and the individual \times side interaction as the effects (same model as for ME analysis). The Procrustes MS related to the main effect of individual was used as an estimator of individual variation (and is noted CV in table 3 for convenience), and the Procrustes MS related to the interaction (individual \times side) was used as an estimator of FA.

As a first test of the relationship between canalization and developmental stability, we computed the correlation between individual variation and FA levels across lines for each trait, both within and among temperatures.

Size and shape variation among lines

Average values of FA and CV were computed by slope and temperature across lines. Similarly, a coefficient of variation among lines was computed for FA and CV. The effect of temperature and of slope on genetic variation for wing size and shape was estimated by the intraclass correlation coefficient (ICC) (David *et al.* 2005) as follows:

$$ICC = (MS_{\text{among}} - MS_{\text{within}}) / (MS_{\text{among}} + n \cdot MS_{\text{within}}),$$

Where *n* is the number of lines, and MS_{among} and MS_{within} are the main effect and residual mean squares from an ANOVA including line as a factor. A shape intraclass correlation coefficient was similarly derived from a Procrustes ANOVA on the shape variables.

Effects on the patterns of shape variation

We then investigated the qualitative effects of temperature and of slope upon the directions (i.e. the patterns) of shape variation. In other words, we tested whether temperature and slope might influence the combination of traits responsible for the differences among individuals or among-sides within-individuals for each line. To do so, we used a metric multi-dimensional scaling (also known as principal coordinates analysis or PCO, Mardia *et al.* (1979)); an application to a similar data set can be found in Debat *et al.* (2006). The procedure is as follows. First, we computed the correlations among all pairs of individual and FA MSCP matrices previously obtained for each line at each temperature and for both slopes (a total of 60 matrices). As an input for the metric multi-dimensional scaling we used a distance matrix composed of values of one minus these squared matrix correlations. The principal coordinates were then computed as axes that successively account for the maximum amount of the information contained in the distance matrix. Such an ordination allows one to visualize the relationship among matrices. Simply put, the closer two matrices are in the PCO plan, the more they are correlated, and the more similar the patterns of landmark variation.

A PCO was first applied to the whole dataset including all matrices from both slopes at each temperature. To check the occurrence of more subtle differentiation, we also applied this procedure on each slope separately and at each temperature, either including both individual variation and FA matrices or focusing on one type of variation at a time. In particular, we investigated the relationship between matrices of among-line variation for both slopes at each temperature.

It has been suggested that Procrustes superimposition might alter the structure of covariance matrices due to the spread of variation across landmarks related to the least squares criterion (Walker 2000). This effect is nevertheless

believed to be of limited importance when shape variation is low (Marcus *et al.* 2000; Rohlf 1999, 2003), which is clearly the case for *Drosophila* wings. Moreover, any covariance matrix computed from the superimposed configurations should be equally affected — if at all. There is, thus, no reason to suspect that the superimposition procedure might affect the relationship among covariance matrices.

All morphometric and statistical treatments were conducted using Rmorph library (M. Baylac personal communication) running with an R package version 2.7.2 R Development Core Team, 2007 (R: a language and environment for statistical computing, vienna, <http://www.R.project.org>).

Results

Measurement error

The Procrustes ANOVA and the standard ANOVAs on the size-related traits yielded similar results. Individual and interaction effects were all found to be highly significant at $P < 0.005$ (shape, $MS_{ind} = 6.86 \times 10^{-5}$; $MS_{inter} = 1.48 \times 10^{-5}$;

$MS_{res} = 0.23 \times 10^{-6}$; centroid size: $MS_{ind} = 6.02 \times 10^{-2}$; $MS_{inter} = 2.93 \times 10^{-4}$; $MS_{res} = 3 \times 10^{-6}$; results for length and width were similar and are therefore not shown). This suggests that there is significant variation among individuals and that FA is stronger than measurement error. In contrast, the side main effect was not significant (shape, $MS_{side} = 1.92 \times 10^{-5}$; centroid size, $MS_{side} = 1.28 \times 10^{-3}$), providing no evidence for directional asymmetry. Note that the interaction MS was found to be respectively more than 60 times higher than the residual MS for size and a 100 times higher for shape, suggesting that it can be used as a reasonable proxy for fluctuating asymmetry. Yet FA values are potentially slightly overestimated by this method.

Mean size and shape

The mean values for the size-related traits are shown in table 1. As repeatedly reported in the literature, wing size decreases when temperature increases. Figure 2, a illustrates the reaction norms of the lines for centroid size. Overall, the reaction norms of isofemale lines derived from the SFS and the

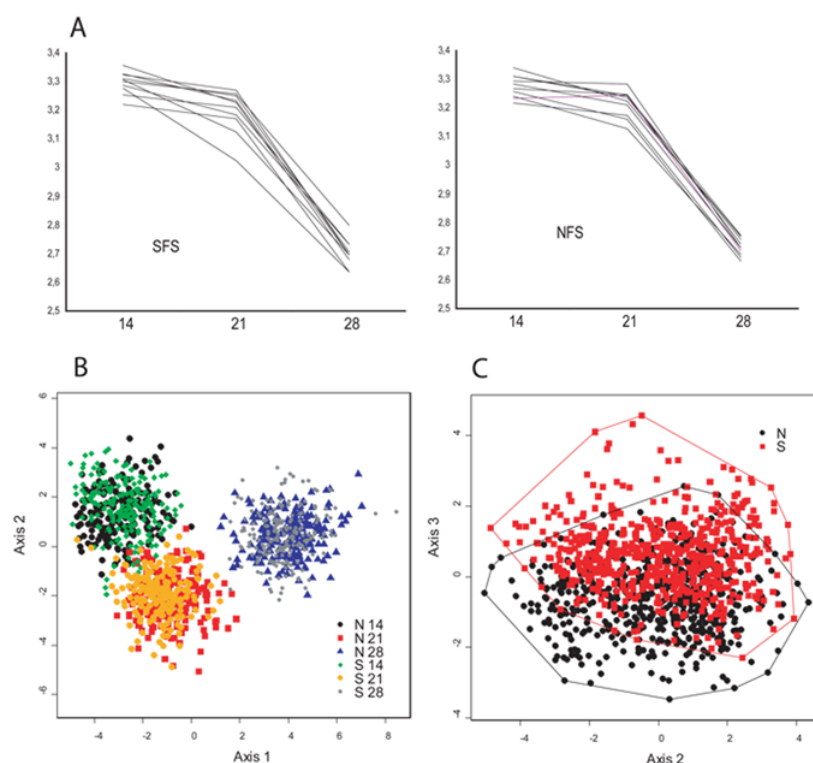


Figure 2. (A) Reaction norms for centroid size. Left, south facing slope (SFS); right, north facing slope (NFS). (B). Discriminant analysis on shape data with temperature and slopes as grouping factors. (C) Axes two (21%) and three (9%) of the same analysis focussing on the effect slope. Note the shift along the third axis. Dark symbols are for the NSF, light symbols for SFS. The three temperatures are clearly discriminated.

Table 1. Mean values and standard deviations for wing length, wing width, length to width ratio (L/W) and centroid size at each temperature for the two slopes. SD are computed across isofemale lines.

Slope	Temperature	Wing length	Wing width	ratio L/W	Centroid size
NFS	14	2.35 +/- 0.06	1.20 +/- 0.04	1.96 +/- 0.04	3.27 +/- 0.08
	21	2.3 +/- 0.06	1.176 +/- 0.04	1.95 +/- 0.04	3.21 +/- 0.09
	28	1.94 +/- 0.04	0.978 +/- 0.03	1.99 +/- 0.04	2.71 +/- 0.06
SFS	14	2.36 +/- 0.06	1.22 +/- 0.04	1.94 +/- 0.04	3.3 +/- 0.08
	21	2.28 +/- 0.08	1.175 +/- 0.03	1.94 +/- 0.05	3.19 +/- 0.11
	28	1.93 +/- 0.06	0.977 +/- 0.03	1.98 +/- 0.05	2.7 +/- 0.08

Table 2. ANOVA on wing length, wing width, the wing length to width ratio and centroid size. MANOVA on Procrustes coordinates. Temperature and slopes are fixed effects; the line effect is random and nested into slope. Pillai is for Pillai's trace.

Trait	Effect	Df	SS	MS	F	P	
Wing length	Temperature	2	38.95	19.475	7006.523	< 2.2e-16 ***	
	Slope	1	0.01	0.01	3.4795	0.062401	
	Temperature:slope	2	0.044	0.022	7.8715	0.000404 ***	
	Temperature:slope/line	54	1.199	0.022	7.9903	< 2.2e-16 ***	
	Residuals	1092	3.035	0.003			
Wing width	Temperature	2	12.0271	6.0135	8259.917	< 2.2e-16 ***	
	Slope	1	0.0089	0.0089	12.196	0.000498 ***	
	Temperature:slope	2	0.0253	0.0126	17.374	3.73E-08 ***	
	Temperature:slope/line	54	0.4935	0.0091	12.553	< 2.2e-16 ***	
	Residuals	1092	0.795	0.0007			
Ratio length/width	Temperature	2	0.2839	0.14195	106.0313	< 2.2e-16 ***	
	Slope	1	0.06309	0.06309	47.1255	1.12E-11 ***	
	Temperature:slope	2	0.00634	0.00317	2.3679	0.09416	
	Temperature:slope/line	54	0.77392	0.01433	10.7055	< 2.2e-16 ***	
	Residuals	1092	1.4619	0.00134			
Centroid size	Temperature	2	75.675	37.837	7052.356	< 2.2e-16 ***	
	Slope	1	0.005	0.005	0.8983	0.343435	
	Temperature:slope	2	0.091	0.046	8.495	0.000218 ***	
	Temperature:slope/line	54	2.456	0.045	8.4784	< 2.2e-16 ***	
	Residuals	1092	5.859	0.005			
Shape		Df	Pillai	Approx F	Num Df	Den Df	Pr (>F)
	Temperature	2	1.682	217.194	52	2136	< 2.2e-16 ***
	Slope	1	0.372	24.260	26	1067	< 2.2e-16 ***
	Temperature:slope	2	0.263	6.216	52	2136	< 2.2e-16 ***
	Temperature:slope/line	54	6.929	7.347	1404	28392	< 2.2e-16 ***
Residuals	1092						

*** significant at $P < 0.001$.

NFS are very similar, although the interaction (temperature \times slope) is significant (table 2). This is more or less consistent across the size-related traits, which generally display non-significant slope effect but a significant interaction of slope with temperature, suggesting that the two slopes tend to differ in terms of phenotypic plasticity.

The effects of temperature and of slope on wing shape are illustrated in figure 2,b & c: the dominant effect is that of

temperature which clearly induces a change in wing shape. The discriminant analysis shows that individuals reared at 28°C cluster on the first canonical axis separately from the two other temperatures which in turn are distinguished from one another on the second axis. This is in agreement with the size reaction norms, displaying a larger effect at 28°C compared to the other temperatures, suggesting an allometric effect on this first canonical plan. Only the third axis can

identify the effect of slope on wing shape (figure 2,c). All these differences are significant in the MANOVA applied on the shape variable, as displayed in table 2. As in the case of size, the significant slope \times temperature interaction suggests a difference in plasticity among slopes for wing shape. Interestingly, the slope effect is highly significant showing that in contrast with size, wing shape as measured by geometric morphometrics differs among slopes.

Variability of wing size and shape

Table 3 summarizes the information on individual variation and fluctuating asymmetry obtained for all traits, both within and among lines. The main feature of these results is that there is no consistent effect of slope or temperature on the levels of individual variation and of FA. Some trends can nevertheless be tentatively identified.

Concerning FA for wing shape, when considering the means across lines, both 28°C and 14°C samples appear to be more asymmetrical than the intermediate 21°C sample, for

both slopes. Note, however, that this effect is not found for all lines. The patterns of size FA are even less clear. For the NFS, FA decreases when temperature increases for centroid size, wing length and the ratio L/W, but not for wing width. A similar effect occurs on the SFS, but only for centroid size and the ratio L/W. Again, this concerns the mean FA across lines, but does not systematically apply within lines. No consistent difference among slopes is detected.

Concerning variation among individuals (CV), results are congruent for size and shape for mean line values. The CV tends to increase with temperature for the SFS. In contrast, for the NFS, the highest CV values are found at 21°C, and the lowest at 28°C. However, as for FA, the results are extremely heterogeneous across lines. Noticeably, within lines, the relative levels of CV are very stable across size-related traits, but rather different from shape ones.

For each trait, we also computed the correlation across lines between the FA value and the CV, first on the overall dataset (i.e., the two slopes and the three temperatures

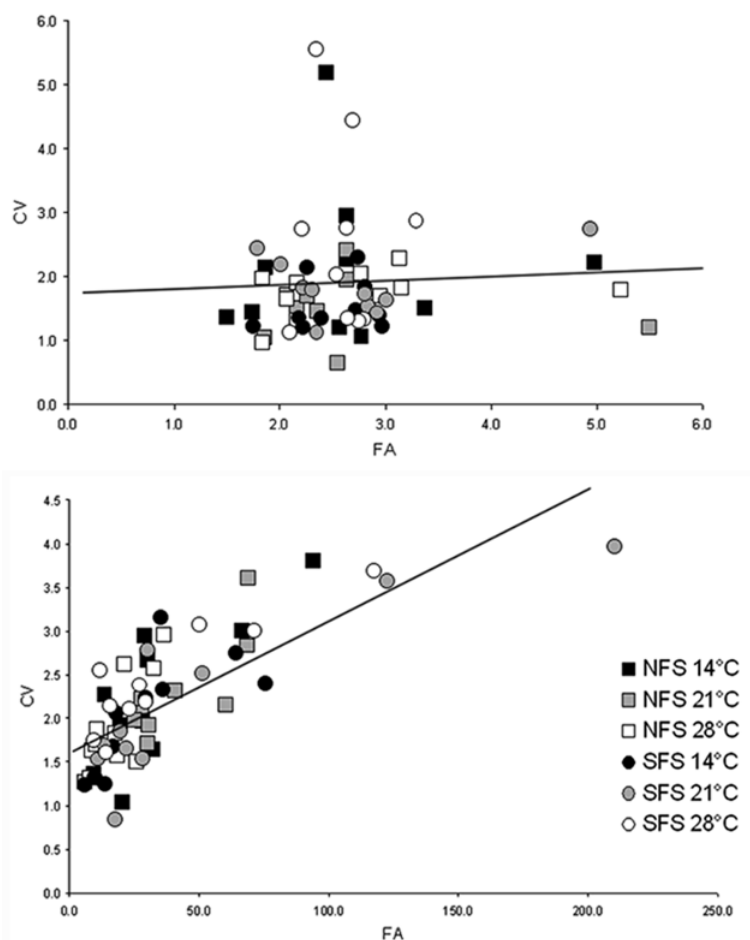


Figure 3. Relationship between FA and CV values for (A) wing shape, and (B) wing width.

Table 3. Within and among line variation. Fluctuating asymmetry (FA) values are provided together with the coefficient of variation (CV) for each line. Although a single notation was used for isofemale lines for convenience, the lines are not the same between slopes (nested within slope) and thus cannot be directly compared. Mean FA and CV were computed across lines, as well as for within line FA and CV. ICC, intraclass correlation. All FA entries but for shape were multiplied by 10^5 . Shape FA values were multiplied by 10^6 and CV by 10^5 . See text for details of calculation. ICCs are real values.

Trait	Slope	Temp	Within line variation												Among lines variation													
			line 1		line 2		line 3		line 4		line 5		line 6		line 7		line 8		line 9		line 10		mean		CV			
			FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV	FA	CV
Width	NFS	14	66.6	3.0	29.0	2.9	94.1	3.8	20.5	1.0	28.3	2.0	19.8	1.9	32.3	1.6	30.1	2.7	9.4	1.4	13.8	2.3	34.4	2.3	76.0	37.4	0.39	
		21	30.5	1.9	60.4	2.2	28.0	2.1	68.7	2.8	30.1	1.7	40.8	2.3	69.0	3.6	27.9	2.2	25.3	2.0	6.2	1.3	38.7	2.2	53.8	28.5	0.66	
		28	21.1	2.6	18.5	1.6	8.6	1.6	36.8	3.0	8.2	1.3	17.9	1.8	32.6	2.6	10.4	1.7	10.8	1.9	25.9	1.5	19.1	2.0	53.0	28.4	0.45	
	SFS	14	6.0	1.2	64.2	2.7	16.8	1.7	13.9	1.2	10.0	1.3	35.4	3.2	36.0	2.3	18.3	2.1	29.5	2.2	75.6	2.4	30.6	2.0	76.1	32.3	0.47	
		21	13.8	1.7	22.2	1.7	51.4	2.5	17.8	0.8	19.8	1.9	10.9	1.5	30.2	2.8	28.4	1.5	122.6	3.6	210.4	4.0	52.8	2.2	122.1	45.3	0.56	
		28	11.9	2.5	9.4	1.7	71.4	3.0	23.1	2.1	50.5	3.1	14.4	1.6	27.1	2.4	15.8	2.1	29.6	2.2	117.6	3.7	37.1	2.4	92.5	26.2	0.51	
Length	NFS	14	37.1	2.7	7.3	2.9	26.1	3.9	16.5	1.0	8.5	2.0	6.9	1.9	8.0	1.7	11.9	2.5	3.6	1.4	8.0	1.8	13.4	2.2	78.5	38.2	0.30	
		21	5.8	2.1	12.5	2.5	8.0	2.3	4.9	3.4	4.5	1.9	4.9	2.6	22.4	3.6	3.4	2.2	12.1	1.8	1.7	1.2	8.0	2.4	77.0	30.2	0.33	
		28	9.6	2.3	3.6	1.7	1.6	1.6	11.8	2.9	2.9	1.3	4.8	2.1	5.6	2.8	2.4	1.7	2.7	1.8	8.2	1.8	5.3	2.0	64.7	26.6	0.32	
	SFS	14	2.0	1.1	7.6	3.2	4.2	1.5	4.8	1.1	4.8	1.2	7.8	3.1	10.5	2.4	4.5	2.0	15.3	2.1	13.8	2.8	7.5	2.1	58.6	39.1	0.29	
		21	6.4	1.5	6.1	1.7	10.0	2.8	8.9	0.9	2.7	1.9	2.0	1.5	5.4	2.8	6.3	1.8	25.9	4.1	22.8	5.0	9.6	2.4	84.3	53.1	0.55	
		28	4.4	2.3	5.2	1.5	5.2	3.8	8.6	2.0	8.9	3.3	4.4	1.6	10.4	2.3	3.2	2.7	7.1	2.4	13.8	4.4	7.1	2.6	46.4	35.9	0.37	
Ratio	NFS	14	147.7	2.2	103.3	1.6	154.5	2.8	18.2	1.0	48.8	1.6	51.1	1.5	33.4	1.6	95.3	1.6	24.2	1.0	92.5	1.0	76.9	1.6	64.1	35.9	0.43	
		21	37.7	1.7	42.1	2.3	42.0	1.6	54.0	2.7	29.9	1.7	42.7	2.1	142.6	2.3	56.7	1.7	53.4	1.3	25.8	0.8	52.7	1.8	62.9	30.5	0.51	
		28	86.3	1.4	34.6	1.6	27.8	1.1	82.6	1.9	21.3	1.0	35.8	1.8	40.3	2.0	29.3	1.1	33.4	1.2	13.7	1.7	40.5	1.5	60.2	24.5	0.50	
	SFS	14	20.6	0.7	57.7	2.5	45.9	1.2	29.4	1.1	33.4	1.0	120.8	1.8	54.0	1.8	58.7	1.3	72.2	1.5	44.1	2.5	53.7	1.5	52.5	41.2	0.50	
		21	45.3	1.1	30.5	1.4	54.8	2.2	9.3	1.0	39.3	1.4	26.6	1.0	82.2	1.7	20.7	1.6	101.3	3.4	83.0	4.8	49.3	2.0	61.8	61.7	0.50	
		28	65.0	1.1	39.5	0.9	25.1	3.2	43.3	1.5	57.2	2.5	24.9	1.2	52.1	1.6	15.9	1.9	32.1	1.8	58.1	4.1	41.3	2.0	40.2	50.5	0.36	
CS	NFS	14	15.0	2.7	2.9	2.9	4.8	4.0	18.1	0.9	9.3	2.0	2.6	1.9	5.5	1.8	2.0	2.6	2.1	1.4	4.1	1.8	6.7	2.2	85.4	39.5	0.27	
		21	2.0	2.2	3.9	2.6	6.6	2.2	2.5	3.3	1.3	1.9	1.0	2.6	6.2	3.5	2.3	2.2	2.0	2.0	2.0	1.0	1.2	2.9	2.4	71.3	29.1	0.35
		28	1.4	2.5	0.8	1.6	0.9	1.6	1.3	3.0	0.9	1.3	3.7	2.0	1.6	2.8	2.8	1.6	0.9	1.8	5.2	1.8	1.9	2.0	76.2	28.7	0.30	
	SFS	14	2.3	1.1	1.5	3.1	3.6	1.6	3.7	1.2	3.6	1.2	3.2	3.1	8.0	2.4	2.1	1.9	7.0	2.1	3.1	2.8	3.8	2.1	55.0	37.6	0.30	
		21	2.1	1.5	2.1	1.7	4.6	2.6	2.5	1.0	1.6	1.9	1.8	1.6	1.2	2.7	2.4	1.9	7.1	4.1	6.5	5.0	3.2	2.4	66.2	51.6	0.57	
		28	2.1	2.3	2.8	1.5	0.5	3.6	3.2	2.1	1.4	3.4	0.5	1.7	2.8	2.4	2.8	2.4	2.4	2.3	2.5	4.5	2.1	2.6	45.9	35.5	0.41	
Shape	NFS	14	5.0	2.2	1.9	2.1	2.3	1.7	5.5	1.2	3.1	2.3	2.3	2.1	2.4	1.3	2.8	1.5	2.8	1.7	2.5	2.0	3.1	1.8	0.4	0.2	0.11	
		21	1.5	1.4	2.4	5.2	2.6	0.6	2.1	1.7	2.2	1.9	2.7	2.3	1.8	1.2	1.8	2.4	2.0	2.2	2.8	1.3	2.2	2.0	0.2	0.6	0.09	
		28	3.4	1.5	2.6	2.3	1.9	1.1	2.8	2.0	2.2	1.3	2.2	1.2	2.7	1.5	2.2	1.8	2.1	1.1	2.6	1.3	2.5	1.5	0.2	0.3	0.07	
	SFS	14	2.6	1.2	2.6	2.9	2.6	2.0	3.2	1.8	2.1	1.6	2.9	1.4	3.0	1.2	2.9	1.4	2.6	2.8	2.2	2.7	2.7	1.9	0.1	0.3	0.05	
		21	2.8	1.1	2.2	1.5	2.4	1.5	1.8	2.0	1.8	1.0	2.2	1.4	2.3	1.1	2.3	1.8	3.3	2.9	2.3	5.6	2.3	2.0	0.2	0.7	0.05	
		28	1.7	1.5	2.8	1.5	2.6	2.4	5.2	1.8	2.9	1.7	2.8	1.8	3.0	1.6	4.9	2.7	2.8	1.3	2.7	4.4	3.2	2.1	0.3	0.5	0.02	

pooled), and then on the different samples separately. The correlation is highly heterogeneous across traits and samples, ranging from an overall correlation for NFS lines of 0.03 for shape (figure 3,a), to 0.89 for wing width from SFS flies reared at 28°C (figure 3,b). The correlation is high and significant for all samples for wing width (ranging from 0.72 for SFS at 21°C to 0.89 for SFS 28°C), with a global correlation of 0.76. For wing length, the overall correlation is 0.54 ($P < 0.01$), but the heterogeneity among groups is stronger, with values ranging from 0.17 (n.s.) (SFS 21°C) to 0.82 ($P < 0.01$) (SFS 28°C). Similar results were found for the ratio (not shown). Finally, the correlations for centroid size and for shape are very low, with overall values of 0.045 (n.s.) and 0.054 (n.s.), respectively.

Results for the intraclass correlation coefficient (ICC) are displayed in table 3 (last column). The mean ICC values for each trait, computed over the temperatures and slopes, are the following: 0.36 for wing length, 0.51 for wing width, 0.47 for

the ratio L/W, 0.37 for centroid size and 0.06 for wing shape. Again, no consistent trend for the difference among slopes or temperatures could be detected.

Finally, we applied an ANOVA with temperature and line as effects on each slope separately, and computed the interaction term as an index of interline differences in temperature sensitivity (i.e. a crude estimator of the genetic variation for plasticity). All effects were significant for all size-related traits. Noticeably, the interaction MS was always higher for the SFS: 3.45 times higher for centroid size ($P = 0.005$), 1.18 times higher for wing width ($P = 0.36$), 3.38 times higher for wing length ($P = 0.006$), and twice as high for LW ($P = 0.07$).

Patterns of shape variation

The results of the PCO applied on all matrices of individual variation and fluctuating asymmetry are shown in figure 4,a,

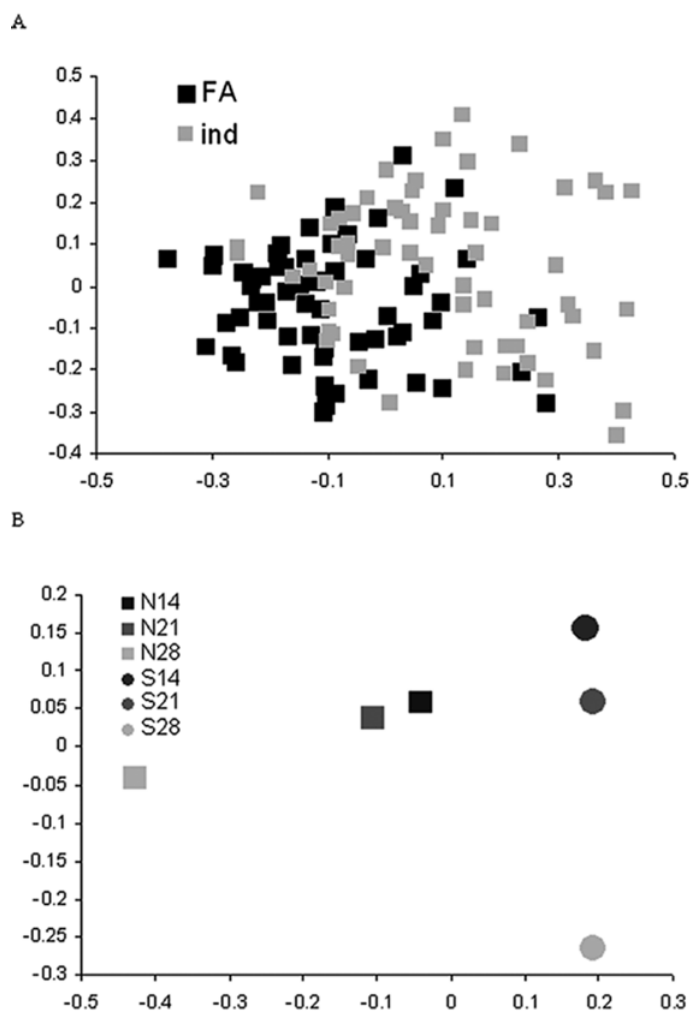


Figure 4. (A) Principal coordinate analysis (PCO) displaying the relationship between shape individual variation and FA matrices. All isofemale lines of both slopes at the three temperatures are shown. (B) PCO on line effect matrices for both slopes at the three temperatures.

which displays the first PCO plan (25% of the total variance). We examined this plan looking for any sign of clustering according to slope or temperature but failed to find any such structure (not shown). The only apparent signal is represented on the graph (figure 4,a) and consists of a shift between the individual variation matrices (grey squares) relative to the fluctuating asymmetry matrices (black squares) on the first axis, suggesting that patterns of shape variation among individuals are to some extent different from those related to shape FA.

Figure 4,b shows the results of a PCO applied on the matrices of inter-line variation. Each matrix was computed as the effect MSCP matrix in a model including line as a single effect applied independently on each slope at each temperature. On the PCO plan shown on the figure (87% of the total variance), the matrices of among-line variation seem to cluster according to the slope mostly along the first axis. Interestingly, for both slopes, the 14°C and 21°C matrices appear closer to one another relative to the 28°C matrices.

Discussion

Methodology

The methodology used in this paper is not new. Metric multi-dimensional scaling is a classical tool of ecologists (James and McCulloch 1990) and has been known by biometricians for decades (Mardia *et al.* 1979). Its nonmetric version is commonly used in population genetics to assess among-population divergence. Here, we promote its use in the context of the investigation of the relationship between multiple covariance matrices. We suggest that it is an elegant way of visualizing the similarity and differences among many matrices at once, which is extremely appealing and might prove useful for large datasets such as the one used in this study.

Among slopes differentiation

Local differentiation among individuals from the two slopes of Evolution Canyon has frequently been interpreted as evidence for the occurrence of strong selection related to the sharply contrasting climatic and ecological conditions between slopes (Nevo 2001). However, in order to override gene flow, selection would have to be particularly strong on dispersing organisms like *Drosophila*, given the extreme proximity of the two slopes. In *Drosophila*, previous studies have reported an increased thermotolerance in SFS flies (Korol *et al.* 2006), suggesting an adaptation to the stressful thermal conditions encountered on this slope. It was even proposed that such an adaptation might induce sufficient divergence to initiate sympatric speciation (Korol *et al.* 2000, 2006; Nevo 2001), although this hypothesis remains controversial (Schlötterer and Agis 2002; Panhuis *et al.* 2003). Our study focussing on wing traits of isofemale lines from NFS

and SFS provides mixed results regarding this question. Concerning mean trait values, our analysis failed to detect any difference between slopes for wing length and wing centroid size. In contrast, a significant—although subtle—difference between slopes was found for shape (figure 2,c), wing width and the ratio L/W. The most convincing effect is the temperature \times slope interaction (table 2), which is significant for all traits, suggesting a consistent difference among slopes in phenotypic plasticity to temperature. However, when investigating the reaction norms, it appears that the difference is somewhat limited, and mainly due to heterogeneity across isofemale lines (figure 2,a). This point is itself of interest, since differences among lines for temperature sensitivity denotes some genetic variation for plasticity. The higher MS for the temperature \times line effect found for the SFS might thus indicate a higher genetic variation for plasticity for this slope's flies. This is, in turn, not straightforward to interpret. If one considers plasticity to be of adaptive value in the context of a stressful thermal environment, selection for plasticity might indeed have the contrary effect, i.e. depleting the population's genetic variation for the selected trait. One should however not overinterpret this particular result since the temperature \times line interaction MS is only a crude estimate of the genetic variance for plasticity.

It has been repeatedly proposed that stressful conditions tend to disrupt developmental buffering processes including canalization and fluctuating asymmetry (Hoffmann and Parsons 1991). A very abundant literature documents in particular the effect of extreme environmental conditions on fluctuating asymmetry (reviewed by Moller and Swaddle 1997; Hoffmann and Woods 2003). From this perspective, the lack of clear difference between slopes in terms of fluctuating asymmetry and individual variation suggests that although the SFS is considered more stressful than the NFS, this does not show up in our results. This might mean either that flies are adapted to the thermal conditions encountered on SFS and that it does not constitute stress anymore, or that developmental stability of wing traits is not altered by this stress, which is in agreement with the view that FA is highly trait dependent (Hoffmann and Woods 2001, 2003). An alternative, more trivial explanation is that FA is not heritable, and that FA levels of individuals reared in the lab do not relate to those of wild individuals from which they originate. This is no groundbreaking hypothesis since the heritability of FA is typically extremely low when significantly different from zero (reviewed by Fuller and Houle 2003).

The previous consideration on stress applies to the genetic variation as measured by the intraclass correlation coefficient: the lack of difference among slopes suggests that either the thermal stress does not alter genetic variation (but see Hoffmann and Parsons 1991), or that SFS thermal conditions are no more stressful for the flies. The latter hypothesis is in agreement with the adaptive view suggesting that a strong selection is acting and promotes the adaptation to the local conditions.

Effects of growth temperature

Besides these considerations on slope effects, the impact of developmental temperature on wing-trait variation is difficult to interpret as well. The effect on mean traits is in agreement with what has classically been obtained in studies of temperature-induced plasticity in *Drosophila* (e.g. David *et al.* 1994, 2006; Imasheva *et al.* 2000; Debat *et al.* 2003), with a decrease in size with temperature. Wing shape is altered as well, as clearly shown by the discriminant analysis (figure 2,b) which is also an expected effect (Imasheva *et al.* 2000; Debat *et al.* 2003) An interesting, albeit relative, effect of temperature on shape is on FA: for both NFS and SFS, shape FA increases when the temperature departs from the optimal 21°C. This is in agreement with the prediction of reduced developmental stability in adverse environmental conditions. This interpretation should however be considered with caution since the effect is observed on the mean FA across isofemale lines but is not pervasive within lines.

Finally, the results of the multi-dimensional scaling on the matrices of among lines shape variation suggest that for both slopes the patterns of genetic variation are rather stable between 14°C and 21°C and more divergent at 28°C. This is shown in figure 4,b by the relative proximity of the corresponding matrices. This could be interpreted as the occurrence of some special genotype × temperature interaction at 28°C for both slopes. One limitation of this interpretation is the use of isofemale lines to estimate a genetic variance. Another drawback is the low number of datapoints ($n = 6$) used to infer a trend. Although interpreting patterns is tempting, one should remain cautious as this effect could possibly be a random artifact.

Fluctuating asymmetry and individual variation

The relationship between canalization and developmental stability has been discussed often in the past 10 years (Klingenberg and McIntyre 1998; Debat *et al.* 2000; Reale and Roff 2003; Santos *et al.* 2005; Breuker *et al.* 2006). Some authors have suggested that the two are distinct processes, each buffering a specific component of phenotypic variation, namely developmental noise as measured by fluctuating asymmetry for developmental stability, and genetic and environmental effects as measured by among individuals variation for canalization (e.g. Debat *et al.* 2000; Santos *et al.* 2005). An alternative view is that there is no need for more than a single mode of developmental buffering to account for the observed patterns of variation (e.g. Klingenberg and McIntyre 1998; Breuker *et al.* 2006). The debate has typically focussed on the strength of the association between individual variation and fluctuating asymmetry across a range of genetic and environmental conditions, and for a diversity of organisms and traits. No consensus has been achieved yet. In this study, we provide some mixed results concerning this association. The significant and high correlation between FA and CV for wing width (figure 3,b), and to a lesser extent

for wing length, contrasts with the very low and nonsignificant correlation found for centroid size and wing shape (figure 3,a). This contrast, which is congruent with the mixed results reported in the literature, suggests that there might be no simple rule applying to all organisms and traits, and that the link between FA and individual variation could thus be trait-specific, as is often the case in biology. The results of the multidimensional scaling (figure 4,a) show that the patterns of individual variation are in some respect different from the patterns of shape FA (i.e. the shift in the positions of the two types of matrices in the PCO plan). This had already been found in a previous study investigating the relationship between FA and individual variation matrices of *Drosophila* wing shape (Debat *et al.* 2006), and interpreted as a sign of some differences in the developmental processes generating variation. This obviously calls for an explanation in terms of mechanisms generating and/or buffering morphological variation both among and within organisms. Unfortunately, very little is known about the genetic control of symmetry and asymmetry in bilaterians in spite of recent progress (e.g. Speder *et al.* 2006, reviewed by Levin and Palmer 2007), and this incomplete knowledge sets limits to the interpretation of our results. Additional experimental investigations of the developmental bases of morphological variation are therefore needed.

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