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The use of fluctuating asymmetry and phenotypic variability as indicators of developmental instability: a test of a new method employing clonal organisms and high temperature stress

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ABSTRACT

Developmental instability, as estimated by two measures – fluctuating asymmetry and phenotypic variability – was examined using sternopleural bristle number and two wing traits in a clonal strain of *Drosophila mercatorum*. Eggs were exposed to short-term (30 min) heat stress in water baths at different temperatures (35–40°C in 0.5°C steps) or to a control temperature regime at 25°C. Fluctuating asymmetry and phenotypic variability in sternopleural bristle number were not affected to a significant extent by heat stress, whereas the fluctuating asymmetry and phenotypic variability of both wing measures were significantly higher in adults developed from heat-stressed eggs than in adults developed from eggs kept at 25°C. For both wing measures, there was a tendency for the highest fluctuating asymmetry and phenotypic variability to be observed at temperatures of 37–39°C, suggesting that individuals who experienced the greatest developmental instability at very high temperatures (39.5–40°C) did not survive the heat stress. For the two wing measures, the fluctuating asymmetry and phenotypic variability were significantly correlated, but this was not the case for sternopleural bristle number. Based on a new method, we quantified the effect that environmental variability had on fluctuating asymmetry and phenotypic variability, but found no correlation with the temperatures at which the eggs were stressed. This shows the unpredictability or the impossibility of controlling environmental variability, even in laboratory experiments. We suggest that the method introduced here may in part explain why non-reproducible results have been obtained in developmental instability studies.

Keywords: clonal organism, developmental instability, *Drosophila mercatorum*, environmental variation, short-term heat stress.

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INTRODUCTION

Developmental stability refers to the production of a specific phenotype under a given set of environmental conditions (Zakharov, 1992; Møller and Swaddle, 1997). Developmental instability results when developmental noise (e.g. noise at the molecular level or random variation in rates of physiological processes among cells) or stress affects the buffering capacity of the processes that provide stability (Palmer, 1996).

In a sexually reproducing population, the phenotypic variability (σ^2_p) is given by

$$\sigma^2_p = \sigma^2_g + \sigma^2_e + (G \cdot E) + \text{cov}(ge) + DI$$

where σ^2_g and σ^2_e are the genetic and environmental variance, respectively, (G · E) is the genotype–environment interaction, cov(ge) is the genotype–environment covariance and DI is developmental instability (Pertoldi *et al.*, 2001a).

In an asexual strain, phenotypic variability is the sum of two components: (1) the direct effect of differences between the environments of different individuals (environmental variance) and (2) the effect of local 'accidents' of development that prevent the perfect replication of the same phenotype, even under identical environmental conditions (developmental instability) (Waddington, 1960). To use the phenotypic variability of a quantitative character as a measure of developmental instability, there must be no genetic variance and no environmental variance among individuals, reducing the above equation to $\sigma^2_p = DI$ (Pertoldi *et al.*, 2001b).

The parthenogenetic strain of *Drosophila mercatorum* reproduces by pronuclear duplication (Templeton *et al.*, 1976). This results in total homozygosity, meaning that all offspring from the strain will be identical, barring mutation, and can be considered as one clone (Templeton, 1983). When all individuals have an identical genotype, phenotypic differences are entirely due to environmental effects and developmental instability.

Two methods are commonly used to measure developmental instability. Most studies have used fluctuating asymmetry, which is the random non-directional difference between sides of a bilateral trait (Van Valen, 1962; Bjorksten *et al.*, 2001; see Møller and Swaddle, 1997, and references therein). Others have used phenotypic variability instead or both fluctuating asymmetry and phenotypic variability (Pankakoski *et al.*, 1992; David *et al.*, 1994; Woods *et al.*, 1999), even though the estimate of phenotypic variability not only reflects developmental instability, but is also influenced by both genetic and environmental variance in sexually reproducing individuals. Recently, it has been advocated that studies of developmental instability should include comparisons of more than just two environments, and that a combination of several indices of fluctuating asymmetry and trait variability should be used when evaluating the impact of stress on trait developmental instability (Woods *et al.*, 1999; Hoffmann and Woods, 2001). When estimating developmental instability using phenotypic variability, it is important to minimize the genotypic and environmental differences among individuals. It is generally assumed that fluctuating asymmetry has the advantage that dissimilarity at the individual level in expression of a given character on the left and on the right side cannot be explained by either genotypic or environmental differences, since its development is ensured by the same genotype under identical environmental conditions (Mather, 1953; Palmer and Strobeck, 1986). However, phenotypic variability and fluctuating asymmetry are not necessarily independent, especially when considered in the context of stressful conditions. In the literature, the two measures of developmental instability are often assumed to be closely related or even

synonymous (Rasmuson, 1960; Hoffmann and Parsons, 1997; Clarke, 1998). Waddington (1960), however, has suggested that separate genetic mechanisms are responsible for the effects on phenotypic variability and fluctuating asymmetry caused by stress; some studies have provided support for this hypothesis (see Scheiner *et al.*, 1991; Tarasjev, 1995; Woods *et al.*, 1999; Hoffmann and Woods, 2001).

In this study, we compared the fluctuating asymmetry and phenotypic variability of sternopleural bristle number and two wing length traits of a parthenogenetic strain of *Drosophila mercatorum*. We examined how short-term high-temperature stress imposed on eggs impacts on the phenotypic variability, environmental variance and fluctuating asymmetry of morphological traits in adult flies. Most previous studies of developmental instability have used continuous stresses. However, as organisms may adjust their development so that the impact of continuous stress is reduced, short-term exposure to stress is expected to have a greater impact on developmental instability (Bjorksten *et al.*, 2001). The innovative aspect of this study was the attempt to estimate environmental variance using a method that has been applied to clonal strains (Pertoldi *et al.*, 2001b). Environmental variance may be unpredictable even under controlled laboratory conditions, thus explaining some of the conflicting evidence from previous studies of developmental instability.

MATERIALS AND METHODS

Experimental design

The parthenogenetic strain of *Drosophila mercatorum* originated from a single stock (Iv-23-0-Im). This strain was established in 1990 from one female after selection for parthenogenetic reproduction in wild-caught flies from Hawaii (Kramer and Templeton, 2001). The strain has frequently been established from only one individual to account for spontaneous mutations, and the total homozygosity in the strain has recently been documented by molecular investigations (data not presented). The parthenogenetic rate, defined as the number of viable larvae divided by the number of eggs laid, has been reported to be approximately 1% (Templeton *et al.*, 1976; see Templeton, 1983, for a review on the genetics of parthenogenetic *Drosophila mercatorum*). The flies used in this study were kept at 25°C on instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC) until the experiment was performed.

Adult flies aged 4–8 days, controlled in terms of density during development, were allowed to lay eggs for 7 h in vials on plastic spoons containing standard laboratory *Drosophila* medium (sugar, yeast, oats and agar) covered with a solution of live yeast dissolved in apple juice (approximately 15 flies per vial). The number of eggs laid on the spoons was not counted. Therefore, we were unable to calculate mortality from the egg to larva stage. The eggs were treated in water baths (Holm & Halby, HMT200) at 12 different temperatures up to 8 h after being laid: 25°C and from 35 to 40°C at steps of 0.5°C. The temperature stress applied did not coincide with the development of the traits being investigated (Bainbridge and Bownes, 1981). Thirty vials with eggs on plastic spoons were treated at each temperature for 30 min. The accuracy of the temperature of the water in the baths was 0.1°C. After the heat treatment, the vials were kept at 25°C. First-instar larvae were collected from the spoons after 16 h. Ten vials with 20 larvae from each temperature were set up.

Adults began to emerge after about 12 days. In each vial and for all temperatures, the first emerging flies were used in the analysis. The temperatures at which the eggs were treated did not affect development time or the number of emerging flies in the vials (data not shown).

The number of sternopleural bristles was counted 'blind' by one of the researchers (T.N.K.) on the right and left sides of 50 ± 5 flies (sampled across vials using 4–6 flies per vial) at each temperature. The wings were removed and mounted on a glass slide in a drop of lactic acid and a cover slip was placed over them. The wings were measured blind by another of the researchers (C.P.) using a camera attached to a dissecting microscope, a Macintosh computer and the computer package Object Image 1.62p2 (Visher, 2000). Three landmarks (A, B and C) were used for each wing. From these three landmarks, two linear measures were recorded. The distal length of the third longitudinal vein from its intersection with the anterior cross-vein to the tip of the wing was estimated from the point coordinates A and B. Wing width was estimated from point coordinates B and C, the distance from the tip of the second longitudinal vein to the tip of the fifth longitudinal vein (Fig. 1). For all three traits, we tested for differences in mean size among vials (within-treatment). We found that the mean sizes of the three traits for flies taken from the same vial were not significantly different from those of flies sampled from different vials (one-way analysis of variance: $0.06 < P < 0.87$).

Statistical analysis

Statistical properties of fluctuating asymmetry, phenotypic variability and environmental variance

Measurement error can cause extreme bias in studies of fluctuating asymmetry; accurate estimates of measurement error are, therefore, essential (Palmer, 1994; Merilä and Björklund, 1995). When sample sizes are large, repeat measures of the whole sample may not be practical, in which case the effect of measurement error should be calculated from repeat measures of a sub-sample of at least 30 individuals (Palmer, 1994). Using 30 individuals, we calculated the measurement error involved in counting bristles (the difference between two independent estimates of fluctuating asymmetry). In line with previous

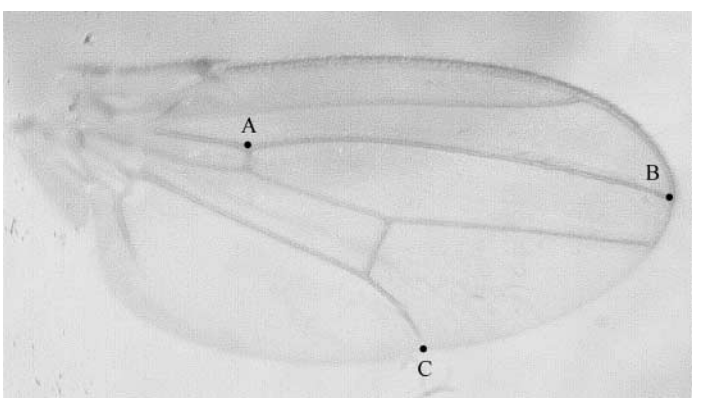

Fig. 1. *Drosophila* wing showing the three points whose coordinates were recorded.

studies (Woods *et al.*, 1999; Jenkins and Hoffmann, 2000), we found no measurement error for sternopleural bristle counts.

To determine measurement error for the wing traits, a random sub-sample of 50 flies was measured twice. The second set of measurements was made without reference to the first set. Furthermore, repeat measurements were conducted one or more days apart to reduce subjective decision-making. A two-way (side × individuals) analysis of variance (ANOVA), in which side (right *vs* left) was a fixed factor and individuals was a random factor, was conducted to test for the significance of fluctuating asymmetry in relation to measurement error (following Palmer and Strobeck, 1986). We found that the wing traits were measured with high repeatability. The interaction mean square containing information about fluctuating asymmetry was tested against error mean square (reflecting measurement error); we found that fluctuating asymmetry was significantly larger than measurement error in all cases ($0.33 <$ interaction mean square < 1.16, $0.0001 <$ error mean square < 0.04; d.f. = 49, $P < 0.0001$). The overall repeatability of measuring fluctuating asymmetry was estimated to be between 96 and 98%.

Three indices were calculated to describe developmental instability at the treatment level. The fluctuating asymmetry (FA) for each treatment group and trait was estimated as the sum (Σ) of the absolute value of the difference between sides divided by the number of individuals (n) (the indices of fluctuating asymmetry follow Palmer and Strobeck, 1986):

$$FA_1 = \Sigma \mid r - l \mid / n \text{ (at the individual level, } FA_1 = \mid r - l \mid) \quad (1)$$

Fluctuating asymmetry was also calculated as a variance estimate:

$$FA_4 = \sigma^2(r - l) \quad (2)$$

where σ^2 is the variance and r and l are the bristle numbers on the right and left sides, or the length of the wing traits measured on the right and left sides, respectively. The third estimate of developmental instability was calculated as the total phenotypic variance:

$$\sigma^2_p = \sigma^2(r + l) \quad (3)$$

Statistically, the value of the variance of the sum (equation 3) is equal to the value of the variance of the difference (equation 2) if the correlation between right and left is zero (Sokal and Rohlf, 1995). Correspondingly, if the correlation is positive, the variance of the sum becomes higher than the variance of the difference. If right and left are negatively correlated, then the variance of the difference will be higher than the variance of the sum. Hence, if there is only one source of variation in a group of individuals (chance disturbances during individual development), then the total σ^2_p (equation 3) will be equal to the chance developmental variance (FA_4):

$$\sigma^2(r + l) = \sigma^2(r - l) \quad (4)$$

The individuals studied here were clonal and so a potential correlation between the right and left sides can only be due to environmental factors. Hence, if environmental variance is introduced, $\sigma^2(r + l)$ will be affected by both developmental instability (estimated by FA_4) and environmental variance. Equation (4) then becomes

$$\sigma^2(r + l) = \sigma^2(r - l) + \sigma^2_e \quad (5)$$

because the variance of (r + l) is equal to

$$\sigma^2(r + l) = \sigma^2_r + \sigma^2_l + 2\mathrm{cov}(r, l) \quad (6)$$

where σ^2_r and σ^2_l are the variances of the right and left sides, respectively. The variance of (r – l) is equal to:

$$\sigma^2(r - l) = \sigma^2_r + \sigma^2_l - 2\mathrm{cov}(r, l) \quad (7)$$

From equations (6) and (7), we can see that in the presence of positive covariance, phenotypic variability will be overestimated and FA_4 will be underestimated, at the population level. In the text, we will refer to the effect produced by the cov on fluctuating asymmetry and phenotypic variability as the environmental cov effect.

To determine whether the data we obtained display the statistical properties of fluctuating asymmetry (i.e. an approximate normal distribution of signed asymmetry scores around a mean of zero), we tested the hypothesis that the mean of right minus left character equals zero in a one-sample *t*-test (Sokal and Rohlf, 1995). Skewness and kurtosis were assessed for deviations following D'Agostino (Zar, 1999). Furthermore, we checked for the presence of anti-symmetrical distributions by inspecting the distributions in graphical form. We checked for the dependence of test treatment at the individual level of the right side of the wing traits or of the number of sternopleural bristles using a linear and a polynomial regression analysis. Because of the many tests performed, we applied the sequential Bonferroni correction (Rice, 1989).

Effects of temperature on measures of developmental instability, environmental variance and mean trait sizes

For each treatment group, we calculated the phenotypic variability and FA_4 of the traits investigated. An *F*-test (Sokal and Rohlf, 1995) was used for pairwise comparisons of phenotypic variability and FA_4 between groups exposed to temperatures above normal and the control group. A Spearman rank correlation test and a linear and a polynomial regression analysis were used to determine whether FA_1 of bristle number and the two wing traits were correlated with the temperature at which the eggs were treated (temperature = the independent variable and FA_1 = the dependent variable).

To examine the influence of the environmental cov effect on phenotypic variability and FA_4 (either an increase or a reduction), the environmental variance and 2cov(r, l) were calculated for each temperature treatment according to equations (6) and (7).

An unpaired *t*-test (Zar, 1999) was used for pairwise comparisons of FA_1 between groups exposed to temperatures above normal and the control group for all three traits. An unpaired two-tailed *t*-test was used for pairwise comparisons of mean number of bristles and mean length of the two wing traits of (r + l) for each group exposed to temperatures above normal and the control group.

Correlations between measures of developmental instability within and between traits

We performed a correlation analysis for each trait (pooling the data points from the different temperature conditions) to determine whether phenotypic variability and FA_4 were correlated with both measures transformed into standard deviations for the graphical representation: σ_p and $\sigma(r - l)$. Correlation analysis was also performed across traits to determine whether phenotypic variability and FA_4 (transformed into standard deviations) were correlated among traits.

RESULTS

Statistical analysis of fluctuating asymmetry and size dependency

None of the traits studied deviated from a distribution with a mean (r – l) of zero. We found no significant deviations from the normal distribution of FA_4 (kurtosis and skewness) for any of the traits. Graphical inspection of FA_4 did not show any sign of a leptokurtic or platykurtic distribution or of anti-symmetry.

We found no significant linear correlation between bristle number (independent variable) and FA_1 (dependent variable), whereas a significant U-shaped relationship was noted between bristle number and FA_1 for nine of the 12 temperatures. For three of the 12 temperatures, we found a significant negative linear relationship between the distal length of the third longitudinal vein (independent variable) and FA_1 (dependent variable); for another three temperatures, there was a significant U-shaped relationship. For wing width, we found a significant negative linear relationship for 11 of the 12 temperatures.

The number of sternopleural bristles did not change in a consistent way with increasing heat stress. However, for the groups stressed at 35.5°C ($t = 2.05$, $P < 0.05$) and 38°C ($t = 2.76$, $P < 0.01$), mean bristle number was significantly higher than for the control group. The magnitudes of the two wing traits in flies developed from eggs exposed to temperatures above 25°C did not differ from those of flies from the control group (results not shown).

Effects of temperature on measures of developmental instability and environmental variance

The results based on both measures of fluctuating asymmetry showed that fluctuating asymmetry in sternopleural bristle number did not differ significantly between the control group and any of the experimental groups, even though there was a trend towards greater fluctuating asymmetry at both high and very high temperatures (Table 1, Fig. 2). In most comparisons, the groups stressed at temperatures above normal had higher FA_1 and FA_4 values than the control group (Table 1, Fig. 2).

For both wing traits, there was a clear tendency for the fluctuating asymmetry indices to be higher at temperatures of 37–39°C than at 39.5–40°C (Table 1, Fig. 2). Also, the regression analysis and the Spearman rank test showed a highly significant positive relationship between the FA_1 of the two wing traits and the temperature at which the eggs were stressed. A polynomial relationship between FA_1 and temperature for the distal length of the third longitudinal vein (trait AB) revealed the best fit (AB polynomial regression: $r = 0.39$, $P < 0.0001$; Spearman test: $r_s = 0.19$, $P < 0.0001$), whereas a linear relationship between FA_1 and temperature for wing width (trait BC) revealed the best fit (BC linear regression: $r = 0.32$, $P < 0.0001$; Spearman test: $r_s = 0.28$, $P < 0.0001$). We found no significant relationship between the FA_1 of bristle number and temperature at which the eggs were stressed (linear regression: $r = 0.05$, $P = 0.21$; Spearman test: $r_s = 0.04$, $P = 0.356$).

Comparisons of the phenotypic variability of sternopleural bristle number (data points for the different temperatures were pooled) in the control group with that of the 11 high-temperature groups yielded no significant results and we observed no trend in either direction (Table 1). In contrast, both wing traits displayed greater phenotypic variability in the experimental groups than in the control group (Table 1).

Table 1. $FA_4 = \sigma^2(r - l)$ and $\sigma^2_p = \sigma^2(r + l)$ values are shown for each temperature for the three traits (bristle number, wing length (AB) and wing width (BC))

Temp. (°C)	FA_4	*P*	FA_1 (mean ± standard error)	*P*	$\sigma^2_p = \sigma^2(r + l)$	*P*	$\sigma^2_e = 2\text{cov}(r, l)$	*P*	FA_4 (env. cov effect) (%)	σ^2_p (env. cov effect) (%)
Bristles										
25	2.737		1.328 ± 0.125		3.861		0.281	N.S.	20.533	14.556
35	2.622	N.S.	1.290 ± 0.123	N.S.	4.490	N.S.	0.467	N.S.	35.622	20.802
35.5	2.601	N.S.	1.236 ± 0.142	N.S.	3.625	N.S.	0.256	N.S.	19.685	14.124
36	2.962	N.S.	1.367 ± 0.136	N.S.	4.256	N.S.	0.323	N.S.	21.81	15.179
36.5	2.899	N.S.	1.271 ± 0.147	N.S.	3.838	N.S.	0.234	N.S.	16.143	12.194
37	2.270	N.S.	1.173 ± 0.134	N.S.	4.884	N.S.	0.653	**	57.533	26.74
37.5	3.914	N.S.	1.471 ± 0.187	N.S.	2.534	N.S.	−0.345	N.S.	−17.629	−27.23
38	3.198	N.S.	1.400 ± 0.156	N.S.	3.538	N.S.	0.084	N.S.	5.253	4.748
38.5	3.638	N.S.	1.452 ± 0.155	N.S.	5.103	N.S.	0.366	N.S.	20.121	14.345
39	3.342	N.S.	1.500 ± 0.155	N.S.	3.045	N.S.	−0.074	N.S.	−4.428	−4.86
39.5	3.327	N.S.	1.423 ± 0.156	N.S.	4.592	N.S.	0.316	N.S.	18.996	13.763
40	3.216	N.S.	1.353 ± 0.163	N.S.	4.097	N.S.	0.220	N.S.	13.682	10.74
Wing AB										
25	4.58E-05		0.011 ± 1.00E-03		3.60E-05		−1.00E-06	N.S.	−4.367	−5.556
35	6.84E-05	N.S.	0.007 ± 1.00E-03	**	1.00E-04	***	9.00E-04	N.S.	26.316	18.0
35.5	3.74E-04	***	0.017 ± 1.00E-03	***	3.24E-04	***	−1.10E-05	N.S.	−5.882	−6.79
36	1.47E-04	***	0.022 ± 2.00E-03	***	3.61E-04	***	5.00E-05	***	68.027	27.701
36.5	1.00E-03	***	0.017 ± 2.00E-03	N.S.	9.00E-04	***	8.50E-05	N.S.	17.0	18.889
37	1.00E-03	***	0.021 ± 2.00E-03	***	8.41E-04	***	2.40E-05	N.S.	4.8	5.707
37.5	1.00E-03	***	0.023 ± 3.00E-03	***	7.29E-04	***	1.10E-05	N.S.	2.2	3.018
38	2.00E-03	***	0.051 ± 4.00E-03	***	1.52E-04	***	−1.20E-04	N.S.	−12.0	−157.895
38.5	1.00E-03	***	0.035 ± 4.00E-03	***	2.30E-03	***	1.80E-04	N.S.	18.0	15.652
39	1.00E-03	***	0.019 ± 2.00E-03	***	6.25E-04	***	1.20E-05	N.S.	2.4	3.84
39.5	4.26E-04	***	0.017 ± 2.00E-03	***	1.09E-03	***	1.66E-04	***	77.934	30.459
40	1.58E-04	***	0.013 ± 1.00E-03	N.S.	9.61E-04	***	1.97E-04	***	249.367	40.999
Wing BC										
25	1.29E-06		0.100 ± 1.46E-04		1.00E-06		1.50E-07	N.S.	23.256	30.0
35	2.39E-06	N.S.	0.100 ± 2.03E-04	N.S.	4.00E-06	N.S.	−3.00E-08	N.S.	−2.51	−1.5
35.5	3.00E-06	N.S.	0.101 ± 2.38E-04	**	4.00E-06	N.S.	−4.00E-08	N.S.	−2.667	−2.0
36	1.86E-06	N.S.	0.101 ± 1.76E-04	N.S.	1.00E-06	N.S.	−1.00E-08	N.S.	−1.075	−2.0
36.5	7.86E-06	***	0.101 ± 3.65E-04	N.S.	4.00E-06	N.S.	−6.00E-07	N.S.	−15.267	−30.0
37	1.29E-06	N.S.	0.103 ± 4.69E-04	N.S.	1.60E-05	***	9.70E-07	N.S.	150.388	12.125
37.5	3.23E-05	***	0.098 ± 1.00E-03	N.S.	1.60E-05	N.S.	−4.10E-06	N.S.	−25.387	−51.25
38	6.26E-05	***	0.106 ± 1.00E-03	***	6.50E-05	***	−1.10E-06	N.S.	−3.514	−3.385
38.5	4.58E-05	***	0.107 ± 1.00E-03	***	2.50E-05	***	−5.10E-06	*	−22.271	−40.8
39	3.41E-05	***	0.107 ± 1.00E-03	***	3.60E-05	***	−5.40E-07	N.S.	−3.167	−30.0
39.5	4.21E-05	***	0.103 ± 1.00E-03	**	6.50E-05	**	4.19E-06	N.S.	19.905	12.892
40	1.11E-05	***	0.102 ± 4.71E-04	**	1.60E-05	**	1.44E-06	N.S.	25.946	18.0

Note: For each trait, variances for the high temperatures are tested (*F*-test) against variances for the control group (25°C). FA_1 ($\Sigma \mid r - l \mid /n$) values are listed. For each trait, FA_1 for groups stressed at high temperatures are compared with that for the control group (unpaired two-tailed *t*-tests). The relative environmental cov effect due to σ^2_e (producing an increase or a decrease depending on whether the value is positive or negative) on FA_4 and σ^2_p are estimated for each temperature as $(2\text{cov}(r, l)/FA_4) \times 100$ and as $(2\text{cov}(r, l)/\sigma^2_p) \times 100$. Significance is corrected by the sequential Bonferroni technique individually for each index.
*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

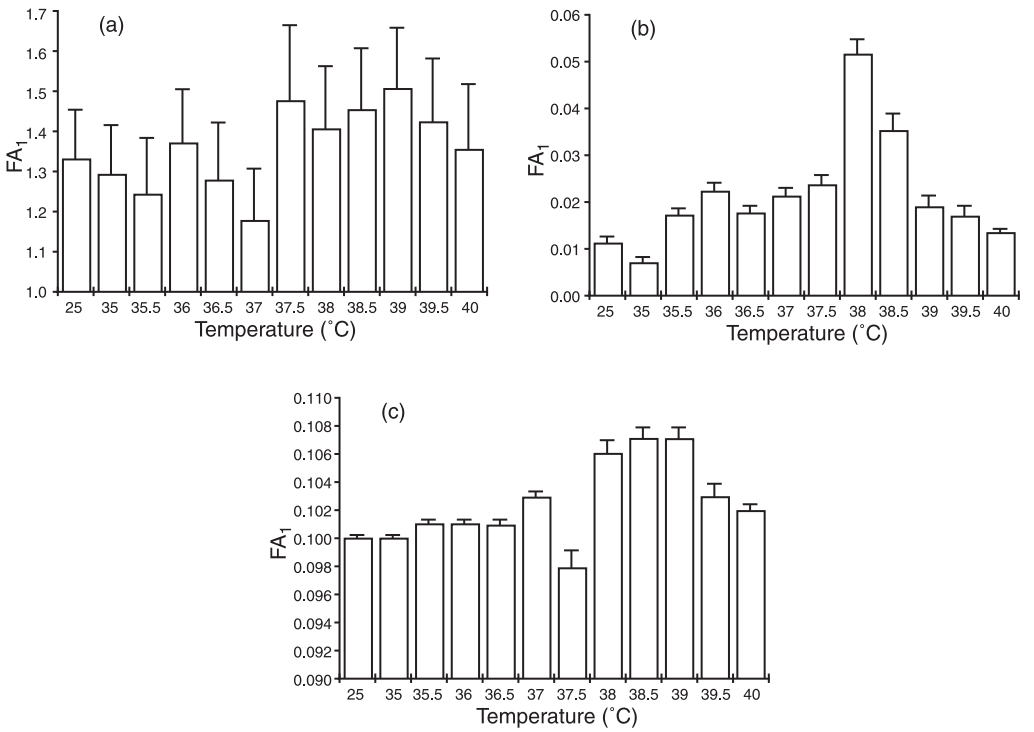

Fig. 2. The FA_1 in three traits for adult flies developed from eggs exposed to non-stressful or stressful temperatures (25°C and 35–40°C in 0.5°C steps) (mean ± standard error). (a) Sternopleural bristles; (b) distal length of the third longitudinal vein (trait AB); (c) wing width (trait BC).

Calculation of environmental variance was performed as described on pp. 56–58. Significant environmental variance was observed at only a few temperatures (Table 1) and was trait-specific, and no clear directional change was seen with increasing temperature stress. The environmental cov effect due to environmental variance on FA_4 ranged from –25.387% to 249.367%, whereas the effect on phenotypic variability ranged from –157.895% to 40.999% (see Table 1).

Correlations between measures of developmental instability within and between traits

A correlation analysis including data points for all temperatures between FA_4 and phenotypic variability in sternopleural bristle number revealed a very weak and non-significant association between the two measures of developmental instability ($r^2 = 0.083$, N.S.). In contrast, the correlation analysis between FA_4 and phenotypic variability in the two wing traits (data points for the different temperatures were pooled) showed significant relationships (trait AB: $r^2 = 0.723$, $P = 0.0005$; trait BC: $r^2 = 0.828$, $P < 0.0001$; Fig. 3).

Correlation analysis between traits showed that FA_4 values between wing width (trait BC) and sternopleural bristle number ($r^2 = 0.67$, $P = 0.018$) and between wing width and the distal length of the third longitudinal vein (trait AB) ($r^2 = 0.831$, $P = 0.0008$) were significantly correlated, whereas that between sternopleural bristle number and the distal length of the third longitudinal vein was not ($r^2 = 0.40$, $P = 0.19$).

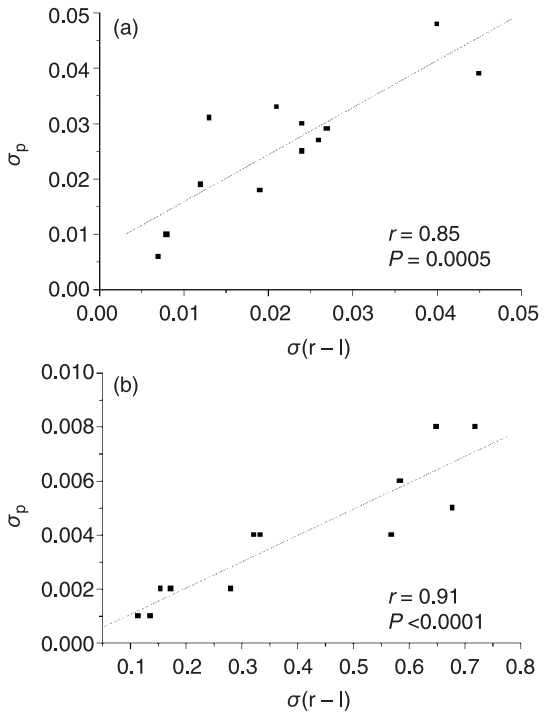

Fig. 3. Relationship between FA_4 and phenotypic variability (σ^2_p) (both measures transformed into standard deviations) for (a) distal length of the third longitudinal vein (trait AB) and (b) wing width (trait BC). The correlation coefficient (r) and the significance of the correlation (P) are given for both traits.

The correlations between phenotypic variability in sternopleural bristle number and phenotypic variability in the two wing traits were non-significant (trait BC: $r^2 = 0.076$, $P = 0.81$; trait AB: $r^2 = 0.18$, $P = 0.57$), whereas that between phenotypic variability in the two wing traits was significant ($r^2 = 0.69$, $P = 0.012$).

DISCUSSION

Statistical properties of fluctuating asymmetry

Palmer *et al.* (1993) described two kinds of covariances between the left and right sides in sexually reproducing populations. The first is the positive covariance, which reflects environmental or genetic factors that affect the development of both sides equally in either direction. A positive covariance between left and right decreases FA_4 but increases phenotypic variability, as shown by equations (6) and (7). The second type of covariance is the negative covariance or offset variation, which reflects environmental or genetic factors that influence the extent of bilateral offset in a given individual. A negative covariance between left and right increases FA_4, reduces phenotypic variability and is typically associated with a platykurtic distribution or, in more extreme cases, with anti-symmetry (Palmer *et al.*, 1993).

However, despite the fact that we did not find any deviations from the normal distribution of FA_4, we found that the covariance played an important role (Table 1). In this study, neither the positive nor the negative covariance between left and right reflected genetic factors, since $\sigma^2_g = 0$. Hence, we conclude that the covariances can only be due to environmental factors. We observed a positive covariance in most test treatments. The FA_4 was, therefore, underestimated and phenotypic variability was overestimated (see Table 1).

Correlations between fluctuating asymmetry and stress

In other *Drosophila* experiments, fluctuating asymmetry in sternopleural bristle number has also been shown to be influenced by heat stress (Imasheva *et al.*, 1997, 1999). That we found fluctuating asymmetry in metric morphological traits (e.g. wing width and the distal length of the third longitudinal vein) to be more sensitive to heat stress than that in meristic traits is consistent with the results of Imasheva *et al.* (1997) for *Drosophila buzzatii*. Wing measures are positively correlated to several life-history traits (Partridge and Fowler, 1993). Such traits are generally thought to be highly canalized and, therefore, fluctuating asymmetry and phenotypic variability are expected to be relatively unaffected by environmental and genetic variation (Waddington, 1960). However, empirical data (including ours) have not always supported this view; therefore, the suggestion that studies of fluctuating asymmetry should be performed on traits that are distinctly related to fitness (Clarke, 1995) does not appear to hold. A distinction between metric and meristic traits may be more important in choosing traits for studies of fluctuating asymmetry. Meristic characters may be thought of as threshold traits (Falconer and Mackay, 1996). Such characters have an underlying continuity with thresholds, which impose discontinuity on their visible expression. The underlying continuous variable, termed the 'liability', is both genetic and environmental in origin and could, in theory, be measured and studied as a metric character. In the absence of genetic and environmental variation among individuals, variance in liability (like morphometric variance) depends only on the precision of the underlying developmental processes, whereas variance in meristic counts (unlike morphometric variance) also depends on the position of the mean liability between thresholds of liability (i.e. the position of the mean counts between whole values; Swain, 1987).

One explanation for the general lack of linear relationships between indices of fluctuating asymmetry and the temperature at which eggs are stressed may be the higher exploitation of variation in the amount of non-genetic adaptation (i.e. variation in maternal effects) at specific temperatures. The strength of phenotypic selection is expected to be more intensive the higher the stress. We observed a tendency for both indices of fluctuating asymmetry to begin to decrease in groups stressed at 38.5–39°C (see Table 1). Therefore, we suspect that phenotypic selection acts primarily on eggs stressed at temperatures higher than 38.5°C. As shown by Campbell and co-workers (Campbell and Emlen, 1996; Campbell *et al.*, 1998), this may cause selective mortality of highly asymmetrical individuals during development and, as a result, measures of fluctuating asymmetry on surviving individuals may not detect the true relationship between fluctuating asymmetry and stress.

Another reason for the lack of an association between stress and fluctuating asymmetry in general could arise from uncontrolled environmental variance, which, in studies of sexually reproducing individuals, is impossible to estimate (see 'Correlations between environmental variance and stress', below). For the number of sternopleural bristles, the environmental cov effect was, on average, much lower than that for the wing traits (3.4 and

2.3 times lower than for the distal length of the third longitudinal vein and wing width, respectively). Despite this, we did detect an increase in FA_4 for the wing traits with increasing heat stress. For the distal length of the third longitudinal vein, the environmental variance at 36, 39.5 and 40°C was significantly higher than at 25°C (control group).

Correlations between phenotypic variation and stress

That we did not find a significant increase in phenotypic variability for sternopleural bristle number with increasing heat stress could be due to the uncontrolled effect of environmental variance (see next sub-section). Another reason could be that the range of phenotypic variability in bristle number is too narrow. Such a narrow range could be due to the absence of genetic variance, which, if present, would introduce an additional source of variability, thereby increasing the phenotypic variability. That there was no association between phenotypic variability in bristle number and stress is in line with the results of other studies of different species of the genus *Drosophila* (Imasheva *et al.*, 1997; Hoffmann and Schiffer, 1998; Woods *et al.*, 1999), whereas they are at odds with those of Bubliy *et al.* (2000). However, for both wing traits we found strong evidence for an increase in phenotypic variability in groups exposed to high temperature stress at the egg stage compared with the control group. This was presumably due to the same factors as discussed in the previous sub-section.

Disruption of the environmental canalization processes may also contribute to the observed increase in phenotypic variability in the two wing traits under stressful conditions. Environmental canalization can be interpreted as: $\sigma^2_p = \sigma^2_a + \sigma^2_{an} + \sigma^2_e$, where σ^2_a is the additive genetic variance and σ^2_{an} is non-additive genetic variance (Debat and David, 2001). Under non-stressful conditions, environmental canalization processes decrease environmental variance through environment-canalizing genes (Debat and David, 2001). In our study, neither additive nor non-additive genetic variance was present; however, phenotypic variability could increase at higher temperatures because of the disruption of environmental canalization, thus reducing the environmental variance. It is generally believed that characters of greater functional significance to an organism should be more canalized (Palmer and Strobeck, 1986) and are expected to be better buffered against developmental disturbances (Palmer, 1996). The wings are of great functional significance and are, therefore, expected to be a canalized trait; the bristles, on the other hand, are not believed to be closely related to fitness and, therefore, are not expected to be influenced by environmental canalization genes. The disruption of environmental canalization could also partly explain the higher environmental cov effect on the phenotypic variability of the two wing traits compared with that of the sternopleural bristles.

Correlations between environmental variance and stress

We found evidence for the presence of environmental variance at several temperatures and for the different traits (see Table 1). This is interesting because we tried to reduce the environmental variance as much as possible. The absence of a clear pattern between increasing stress and environmental variance can only be explained by the unpredictability and complexity of the latter. Maternal effects and eggs being stressed at different developmental stages (due to eggs having a physiological age spanning from newly laid to 8 h at the time when they were treated in the water baths) could have affected the environmental

variance. The maternal environmental cues can stimulate phenotypic plasticity in progeny through mechanisms other than transmission of nuclear genes. It has been suggested that growth and survival of progeny are influenced by the amount and quality of resources allocated to eggs by the mother (for a review, see Hercus and Hoffmann, 2000).

We suspect that uncontrolled fluctuations in environmental variance could also have been present in other studies. This may be one reason why many authors have failed to show the expected correlations between fluctuating asymmetry and stress and between phenotypic variability and stress.

Table 1 shows that environmental variance in the heat-stressed groups was trait-specific. The trait specificity of environmental variance is probably related to the functionality of the traits and to the different developing 'windows' of traits, which make them more or less susceptible to environmental changes during the development process. If exposure to stress coincides with the development of the traits in question – which was not the case in the present study – environmental sensitivity could be even higher (Brakefield, 1997).

Correlations between phenotypic variability and fluctuating asymmetry

Conflicting results regarding the correlation between phenotypic variability and fluctuating asymmetry can be found in the literature (Woods *et al.*, 1999; Hoffmann and Woods, 2001). Most studies have not reported a significant association (Rabitsch, 1997; Siikamäki and Lammi, 1998; Woods *et al.*, 1999). This has led some researchers to suggest that the processes affecting phenotypic variability and fluctuating asymmetry across environments are independent, representing canalization and developmental stability, respectively (Waddington, 1960; Hoffmann and Woods, 2001). This view may be correct, but studies of sexually reproducing individuals do not allow the estimation of environmental variance. The presence of environmental variance in our study might also be expected in others. It is clear from Table 1 that every time the covariance is positive, the environmental cov effect on phenotypic variability is less pronounced than that on fluctuating asymmetry. In the case of negative covariance, the reverse is true. Therefore, the environmental cov effect could explain why an association between the two estimates of developmental instability is often not observed. Furthermore, genotype × environment interactions may affect the two estimates in different ways in sexually reproducing populations. The only way to avoid this problem is to use specific clonal strains in studies of fluctuating asymmetry and phenotypic variability. In sexually reproducing organisms, the absence of an association between fluctuating asymmetry and phenotypic variability is, therefore, likely because of the presence of additive genetic variance, epistatic interactions, uncontrolled directional selective forces and environmental variance, which, in this study, has been shown to be difficult to control.

Future directions

It is well known from quantitative genetics how biased the estimates of environmental and genetic variance may be. As a consequence, heritability (broad and narrow sense and realized heritability) estimates are also biased. Furthermore, genetic variance can never be considered to be zero in sexually reproducing populations, even when working with inbred isofemale lines. It has also been hypothesized that the genetic variance may change with

environmental conditions (Hoffmann and Parsons, 1997). Together with the amount of epistatic interactions, this may add to the complexity.

Based on the results of the present study, it is clear that the amount of developmental instability can only be estimated precisely for traits considered individually. Furthermore, we have to be aware that the method used here to measure the environmental cov effect on the two estimates of developmental instability operates on the mean developmental instability of individuals. This is important, as differences in developmental instability among individuals result from them having experienced different micro-environments during their development.

CONCLUSION

The method proposed here for partitioning out the different components of phenotypic variability is expected to increase the value of fluctuating asymmetry and phenotypic variability as indicators of environmental stress. However, further studies are required to understand the differences in response between metric and meristic traits, and to test the applicability of the method for different clonal organisms, both from the plant and animal kingdoms.

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