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Phenotypic and genetic variability of sternopleural bristle number in *Drosophila melanogaster* under daily thermal stress: developmental instability and anti-asymmetry

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ABSTRACT

Question: Daily thermal cycles are the rule under natural conditions, and may be very stressful. Are there specific effects of such cycling regimes upon a phenotypic bilateral trait, sternopleural bristle number?

Organism: Experiments were performed on two temperate natural populations of *Drosophila melanogaster*, collected in the vicinity of Paris (France).

Methods: Altogether, 33 isofemale lines were investigated in their second laboratory generation. Four thermal regimes were used: two constant non-stressful temperatures (17 and 25°C) and two stressful, alternating regimes, one applying a daily cold stress (8–25°C, average 17.5°C), the other applying a daily heat stress (18–33°C, average 25.5°C).

Conclusion: Both alternating regimes increased the within-line (environmental) variance but not the genetic variance. These results are at odds with what was observed, on the same set of lines, for body size traits. Fluctuating asymmetry of bristle number was increased by stress. This was accompanied by the induction of a bimodal shape of the right minus left difference. Such anti-symmetry implies a negative, random interaction between sides of the thorax.

Keywords: bilateral symmetry, bimodality, canalization, cold stress, fluctuating asymmetry, heat stress, intraclass correlation, isofemale lines.

INTRODUCTION

Among the many abiotic factors, temperature plays a major role in explaining the distribution and abundance of animals (Andrewartha and Birch, 1954). The thermal environment is highly variable both according to season and time of day, and stressful conditions are often encountered. The capacity to tolerate extreme (heat or cold) conditions defines the thermal

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range of any species (Precht *et al.*, 1973; Cossins and Bowler, 1987; Leather *et al.*, 1993). Most comparative laboratory investigations have used constant temperatures, whereas in nature daily rhythms are the rule, with an amplitude that is often greater than 15°C, and which may exceed 30°C (Gibbs *et al.*, 2003).

The consequences of stress may be analysed not only by considering viability (survival capacity), but also the mean value of various traits and their phenotypic variance. A consensus appears to exist that any stress will increase the phenotypic variance of most quantitative traits (Bijlsma and Loeschcke, 1997; Hoffmann and Parsons, 1997; Hoffmann and Hercus, 2000).

In *Drosophila*, it is well established that the phenotypic variance of wild-collected individuals is greater than that of laboratory-grown flies (Coyne and Beecham, 1987; Imasheva *et al.*, 1994; David *et al.*, 1997; Gibert *et al.*, 1998). Such a phenotypic variability has a double origin: genetic and non-genetic. The higher variability in wild-living flies is generally assumed to be due to an increase of the environmental, non-genetic component, resulting in lower heritability (Coyne and Beecham, 1987; Gibert *et al.*, 1998; Hoffmann and Hercus, 2000). It has long been suggested that several mechanisms might alter the components of phenotypic variation (Waddington, 1942; Schmalhausen, 1949) and recent studies have tried to identify their respective roles in an evolutionary context (e.g. Zakharov, 1992; Debat and David, 2001; Meiklejohn and Hartl, 2002): genetic canalization reduces genetic variation, environmental canalization reduces environmental variation, and developmental stability reduces the effect of random errors affecting development. The latter is particularly identifiable when looking at the amount of small deviations from bilateral symmetry (i.e. the so-called fluctuating asymmetry) in an otherwise perfectly symmetrical trait (e.g. Palmer, 1994; Palmer and Strobeck, 2003).

We investigated the effect of a periodic 12-hour, daily stress of either cold (8°C) or heat (33°C) on *Drosophila melanogaster* isofemale lines. When applied continuously, temperatures of 8°C or 33°C induce a complete mortality (Pétavy *et al.*, 2001). With our periodic design, the deleterious effects were prevented by a daily return to an intermediate temperature (Renault *et al.*, 2004) so that a fairly high overall viability (over 50%) was obtained. The use of an isofemale line design (Hoffmann and Parsons, 1988; David *et al.*, 2005) permitted a precise comparison of the experimental conditions in which the various lines are considered as experimental repeats, and also provided an estimate of two components of the phenotypic variance.

In a previous paper (Pétavy *et al.*, 2004), we investigated two size-related traits, wing and thorax length, and found that both alternating stressful regimes resulted in an increase of the within-line, environmental variance, compared with controls. Divergent responses, however, were obtained for the genetic variance: a significant increase under cold stress, but a reduction under heat stress. In the present paper, using the same set of 33 isofemale lines, we focus on another trait, widely investigated in quantitative genetics (Mackay, 1996, 2001, 2004), the number of sternopleural bristles (SB) on both sides of the thorax. The variability in developmental stability of SB number under daily fluctuating temperature (20–30°C) has previously been compared to that observed at 25°C by Beardmore (1960). Variations of fluctuating asymmetry however were small, and the results were variable according to the genotypes investigated. Our experiments are characterized by much stronger thermal stresses, with lethal temperatures during part of the day. The results on SB number did not corroborate the body size results, because both kinds of stresses had similar effects and mainly increased the within-line variability. Since SB number is a symmetrical trait, the variability between the right and left sides was also investigated. The developmental noise (fluctuating asymmetry) was significantly increased by both daily stresses, but was also accompanied by an unexpected anti-symmetric response, most pronounced with the cold stress, and which implies negative developmental interactions between the left and right sides of the thorax.

MATERIALS AND METHODS

Populations and experiments

Wild *D. melanogaster* adults were collected with banana traps in autumn in two localities 60 km apart, in the south (Draveil) and the southwest (Prunay) of Paris. Pairs of flies (one female and one male) were isolated in vials containing a corn-meal sugar medium seeded with live yeast, and reared at 20–22°C. After offspring emergence, 10 pairs were randomly taken from each line and used as parents of the second laboratory experimental generation. A few days later, each parental group was allowed to lay eggs at 21°C for a few hours in a vial containing a high-nutrient, killed yeast food (David, 1962), which practically eliminates larval density effects (Karan *et al.*, 1999). For each parental group, four such vials were used successively. After removal of parents, vials were transferred to incubators regulated either at constant temperatures, 17°C or 25°C, or at alternating temperatures with two 12-hour phases, either 8–25°C or 18–33°C.

Measurement of sternopleural bristle number

After emergence, adults of each line were transferred to vials containing fresh food and kept at 21°C. A few days later, flies were anaesthetized, and 10 females and 10 males randomly chosen. Two body size traits were measured (Pétavy *et al.*, 2004), the thorax and the left wing length. The sternopleural bristles were counted twice, first on the right (R) side, then on the left (L) side. For such a meristic trait, there is no measurement error. As in most other previous investigations, all bristles were counted, short, long and medium-sized.

Data analyses

The Draveil and Prunay populations were represented by 15 and 18 lines respectively. With four thermal conditions, 10 flies of each sex and 33 lines, the whole data set comprised 5280 measures. For genetic analyses, the sum of the right and left sides (R + L) was used as in most previous papers on the same trait.

The data were analysed at various levels of variability: individuals within lines, lines within populations, sexes and thermal regimes. The within-line variation was calculated as a coefficient of variation, since in a previous paper (Moreteau *et al.*, 2003) we found a slight positive correlation between the standard deviation and mean, which has been confirmed in the present work. Moreover, the coefficient of variation is a convenient means for comparing different traits [e.g. wing length or thorax length (Pétavy *et al.*, 2004)] or different species having clearly different average bristle numbers (Capy *et al.*, 1993). The within-line variability represents mostly a measure of the environmental part of the phenotypic variation (David *et al.*, 2005). The between-line variation estimates a genetic component, and two parameters were calculated, the intraclass correlation coefficient and the genetic coefficient of variation, also called evolvability (Houle, 1992). Evolvability gives a more direct estimate of the ability of the trait to respond to selection, as it is standardized by the trait mean and thus does not depend on the magnitude of the total variance. Sexual dimorphism was expressed as a female/male ratio at the line level (David *et al.*, 2003).

Bilateral symmetry was analysed by comparing the SB number on both sides. A diversity of techniques can be used for such a purpose (Palmer and Strobeck, 1986; Palmer, 1994). We considered the distribution of the signed asymmetry difference (R – L), calculating: (i) two indices of fluctuating asymmetry (see legend of Table 6); (ii) the R × L correlation; and (iii) two indices characterizing the shape of the (R – L) distribution, revealing an anti-symmetric process under alternating temperatures (see Results section). The data were analysed using standard statistical methods included in the Statistica package (Statsoft, 1999), with either parametric or non-parametric tests.

RESULTS

The results have been subdivided into two sections. First, we counted SB number on the right (R) and left (L) sides of the thorax. Then, in line with most investigations on the same trait (Falconer and Mackay, 1996; Mackay, 2001, 2004; Dilda and Mackay, 2002), their sum (R + L) was analysed for how this trait reacts to daily stress. Second, fluctuating asymmetry was considered and the effects of the two alternating temperature regimes on the symmetry described: an increased developmental instability is accompanied by an anti-symmetrical response. For the sake of simplicity, values for the right and left sides and for their sum are generally given in the same tables.

Total bristle number

Variation of mean number according to thermal regime

Mean values of SB number, according to sex, population and thermal regime, are given in Table 1. Analysis of variance (ANOVA) of the total data set revealed a significant effect of each factor. The sex effect is well known: females have more bristles than males. In all cases, there was a slight but significant (on average about 4%) difference between the two populations: flies had generally more bristles in Prunay than in Draveil.

Table 1. Influence of thermal regime on the number of sternopleural bristles on the right (R) and left (L) sides of the thorax, and the sum R + L

Regime	Sex	R	L	R + L
17°C	female	10.3 ± 0.2	10.3 ± 0.2	20.6 ± 0.3
	male	10.1 ± 0.2	10.1 ± 0.2	20.2 ± 0.3
25°C	female	9.9 ± 0.2	9.9 ± 0.2	19.8 ± 0.3
	male	9.3 ± 0.2	9.3 ± 0.2	18.6 ± 0.3
8–25°C	female	9.4 ± 0.2	9.4 ± 0.2	18.9 ± 0.3
	male	9.1 ± 0.2	9.1 ± 0.2	18.1 ± 0.3
18–33°C	female	8.7 ± 0.2	8.9 ± 0.2	17.6 ± 0.3
	male	8.1 ± 0.2	8.2 ± 0.2	16.3 ± 0.3

Note: Values (mean ± standard error) are calculated from the number ($n = 33$) of isofemale lines.

The effect of thermal regimes was examined further. A comparison of the two constant temperatures revealed a significantly greater number of bristles at 17 than at 25°C, due to phenotypic plasticity. As shown by Moreteau *et al.* (2003) for another French population, the reaction norm of SB number in females is a concave curve with a maximum at about 19.8°C. Our mean values conform to this curve: SB number at 17°C is closer to the maximum than at 25°C.

A comparison of the data under the two stressful regimes with the corresponding constant temperatures revealed fewer bristles in each case. Compared with 17°C, the reduction observed at 8–25°C is about 9%. This reduction is slightly higher (12%) when the 18–33°C data are compared with those at 25°C.

Within-line variability

For each line, within population and thermal regime, 10 flies of each sex were measured, and the variability was analysed by calculating for each family a coefficient of variation. The whole data set for the sum of the right and left sides (Table 2) was submitted to an ANOVA (data not shown). Significant differences were found between the sexes, with males being slightly more variable than females, and between thermal regimes, but not between populations or between lines.

In a previous paper, Moreteau *et al.* (2003) showed that the within-line coefficient of variation changed according to constant growth temperatures, with a minimum (9% in females) in the middle of the thermal range, and maxima (12–13%) at extreme temperatures, 12 and 31°C respectively. The present data are in fair agreement with those results. Variability was slightly but significantly higher at 25 than at 17°C, the mean values for males and females combined being 10.1 ± 0.3 and 8.9 ± 0.3 respectively. Both alternating regimes increased significantly the variability when compared with the constant temperatures, and the effect of a periodic heat stress (average 13.2 ± 0.4) was significantly stronger than that of a cold one (average 11.2 ± 0.4).

It is noteworthy that the coefficients of variation for each side, left and right, are slightly but consistently higher than for their sum (Table 2). This is because the left–right correlation, although positive, is less than unity (see below).

Table 2. Influence of thermal regime on the within-line variability (CV) of sternopleural bristle number

Regime	Sex	R	L	R + L
17°C	female	9.8 ± 0.4	10.0 ± 0.5	8.5 ± 0.5
	male	10.9 ± 0.5	10.3 ± 0.5	9.3 ± 0.5
25°C	female	10.9 ± 0.4	11.1 ± 0.5	9.4 ± 0.5
	male	12.1 ± 0.5	12.5 ± 0.5	10.7 ± 0.4
8–25°C	female	13.2 ± 0.6	12.8 ± 0.6	10.5 ± 0.5
	male	15.3 ± 0.6	14.4 ± 0.5	11.9 ± 0.5
18–33°C	female	15.4 ± 0.7	14.5 ± 0.7	12.5 ± 0.6
	male	15.7 ± 0.6	16.8 ± 0.5	14.0 ± 0.5

Note: Values (mean ± standard error) are given for the right (R) and left (L) sides and for the two sides combined (R + L).

Between-line variability

We consider here both the values on each side and their sum. All analyses failed to reveal any differences between the sexes, populations and thermal regimes, and we present, for the complete data set (Table 3), the genetic variance, the intraclass correlation coefficient and the genetic coefficient of variation. As expected, the genetic variance for the sum (R + L) was about four times that of each side. The intraclass correlation was also slightly higher for the sum than for the individual sides, due to a positive correlation between left and right on each fly. When the genetic coefficient of variation (evolvability) was considered, however, the results were identical for the individual sides and for their sum.

The overall means and standard errors of the intraclass correlation coefficient and of the genetic coefficient of variation for the sum are 0.292 ± 0.021 and 7.06 ± 0.28 respectively ($n = 16$ in each case).

Sexual dimorphism of bristle number

As noted earlier, males have a smaller number of bristles than females (Table 1). The sexual dimorphism was analysed at the line level by considering the correlation between the sexes and the female/male (F/M) ratio. Results are shown in Table 4.

Table 3. Genetic parameters of sternopleural bristle number for the right (R) and left (L) sides and both sides combined (R + L)

	Sex	R	L	R + L
Genetic variance	female	0.40 ± 0.06	0.49 ± 0.06	1.79 ± 0.20
	male	0.41 ± 0.05	0.46 ± 0.04	1.77 ± 0.16
Intraclass correlation	female	0.22 ± 0.03	0.26 ± 0.03	0.31 ± 0.03
	male	0.21 ± 0.03	0.23 ± 0.02	0.28 ± 0.03
Evolvability (%)	female	6.47 ± 0.48	7.22 ± 0.53	6.91 ± 0.45
	male	6.91 ± 0.37	7.41 ± 0.44	7.21 ± 0.37

Note: Evolvability is the genetic coefficient of variation. Each value (± standard error) is the mean of 8 (2 populations × 4 thermal regimes).

Table 4. Influence of thermal regime on the female–male (F–M) correlation and the female/male (F/M) ratio of total SB number

Regime	F–M correlation	F/M ratio (mean ± S.E.)
17°C	0.768	1.023 ± 0.009^{a}
25°C	0.704	1.063 ± 0.012^{b}
8–25°C	0.741	$1.042 \pm 0.011^{a,b}$
18–33°C	0.755	$1.081 \pm 0.012^{b,c}$

Note: Female–male correlation coefficients are all positive and significantly greater than zero ($n = 33$). Sexual dimorphism (F/M ratio) is variable among thermal treatments. Results of ANOVA are summarized by letters (Tukey *post-hoc* test): means with different letters are significantly different.

In all cases female and male values were significantly correlated, with no difference according to thermal regime: the overall value is $r = 0.73 \pm 0.02$ ($n = 4$). The F/M ratio was never different between Prunay and Draveil. *Post-hoc* tests (ANOVA) revealed that dimorphism was more pronounced at 25 than at 17°C (1.063 vs. 1.023). An intermediate value (1.042) was found after a periodic cold stress (8–25°C) while the most extreme dimorphism (1.081) was observed after a heat stress (18–33°C).

Correlation between bristle number and body size

A sexual dimorphism (F/M ratio) greater than unity could be related to the larger size of females. The dimorphism for SB number is, however, much less than for the size traits: in the same set of lines, the average F/M ratio varied between 1.11 and 1.14 for thorax length, and between 1.11 and 1.15 for wing length (Pétavy *et al.*, 2004).

We further explored the possible relationship between SB number and body size (wing or thorax length) by calculating correlation coefficients. All values were very low and many were negative. There was no trend distinguishing the thermal regimes. An average correlation was thus calculated for each treatment, and the overall mean was considered [the calculations were made on z-transformed values, and the results expressed by the inverse function, $r = \tanh z$ (Sokal and Rohlf, 1995)]. At the within-line level, the average correlation coefficient was low but significantly positive: $r = 0.21 \pm 0.03$ ($n = 8$). A slightly but significantly higher value was observed at the between-line level: $r = 0.33 \pm 0.04$ ($n = 8$). If we consider the coefficient of determination (R^2), we may conclude that variability in body size explains only 4% of SB variability at the within-line level and 10% at the between-line level.

Bilateral symmetry

Absence of directional asymmetry

Results for mean values on the right and left sides (Table 1) are in all cases very similar and never significantly different. Thus, with respect to average SB number, both sides are equivalent, without any directional asymmetry. The same conclusion applies to the within-line variability (Table 2) and to the genetic variability (Table 3).

Correlation between the right and left sides

Variations between individuals from the same line arise from developmental instability, which is responsible for a lack of correlation, but also from genetic differences and common garden effects, which should induce a positive correlation between sides (Kristensen *et al.*, 2003). We calculated the correlation between sides both at the within- and between-line level (Table 5).

Within-line coefficients were submitted to ANOVA after a z-transformation. There was no difference between the sexes, which could be pooled, but strong differences between alternating and constant temperatures. The coefficients were almost identical at 17 and 25°C (0.53 ± 0.05 and 0.54 ± 0.05 respectively), significantly lower at 18–33°C (0.46 ± 0.04), and even lower at 8–25°C (0.37 ± 0.04).

The between-line correlation was in all cases much higher than the within-line value (Table 5). The coefficients were slightly higher under constant temperatures than under alternating regimes but the differences were not significant, and the overall mean was 0.88 ± 0.02 ($n = 8$). This higher correlation may be explained by the fact that, at the mean

Table 5. Correlation coefficients (*r*) between the number of bristles on the left and right sides at the within-line and the between-line level, and for the total set of 330 or 660 individuals

		Within-line		Between-line		Total set	
Regime	Sex	*N*	*r*	*N*	*r*	*n*	*r*
17°C	female	33	0.50 ± 0.07	33	0.92	330	0.633
	male	33	0.57 ± 0.07	33	0.88	330	0.664
	total	*66*	0.54 ± 0.05^a	66	0.90	660	0.651
25°C	female	33	0.50 ± 0.06	33	0.89	330	0.625
	male	33	0.57 ± 0.07	33	0.94	330	0.663
	total	66	0.53 ± 0.05^a	66	0.93	660	0.659
8–25°C	female	33	0.36 ± 0.05	33	0.83	330	0.454
	male	33	0.39 ± 0.07	33	0.78	330	0.442
	total	66	0.37 ± 0.04^b	66	0.82	660	0.455
18–33°C	female	33	0.42 ± 0.05	33	0.86	330	0.532
	male	33	0.50 ± 0.05	33	0.90	330	0.598
	total	66	0.46 ± 0.04^c	66	0.90	660	0.582

Note: Values in column 4 are calculated from the *z*-transformed *r*, then from the inverse function tan*hz* (Sokal and Rohlf, 1995, p. 575). Data from Draveil and Prunay were pooled. N = number of lines; n = number of individuals. For the within-line correlation, means with different letters are significantly different.

line level, the magnitude of the developmental instability component (the error term) is reduced with respect to the genetic component.

Overall distributions of signed asymmetry (R – L difference)

Symmetry analyses are notoriously imprecise (Palmer and Strobeck, 1986; Houle, 1992; Palmer, 1994) and require a large number of observations. As a first step we considered the overall distributions of signed asymmetry of males and females in the four thermal regimes (Fig. 1). The results confirm the lack of directional asymmetry, since the means are close to zero in all cases. A visual inspection, however, reveals a general difference between constant and alternating temperatures. Under constant conditions, the median, zero class is the most frequent, whereas it is deficient under alternating temperatures, and less than classes −1 or +1. In other words, there is a trend towards bimodal distributions, which suggests an anti-symmetrical response to periodic stress. Another general trend is that the variability (standard deviations are given in Fig. 1) is greater under alternating conditions than constant ones: a Bartlett test for homoscedasticity revealed significant differences among variances of the thermal regimes ($P < 0.001$).

The Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilks tests of normality gave inconsistent results for the eight histograms of Fig. 1, probably due to the small number of classes. Skewness was low and generally not significantly different from zero. Bimodality under alternating conditions may be considered an extreme case of platykurtosis (Palmer and Strobeck, 1986; Palmer, 1994) producing highly negative values of this parameter. All distributions produced negative kurtoses, but without clear-cut differences between constant and alternating regimes. From these calculations, we draw two conclusions. First, all distributions

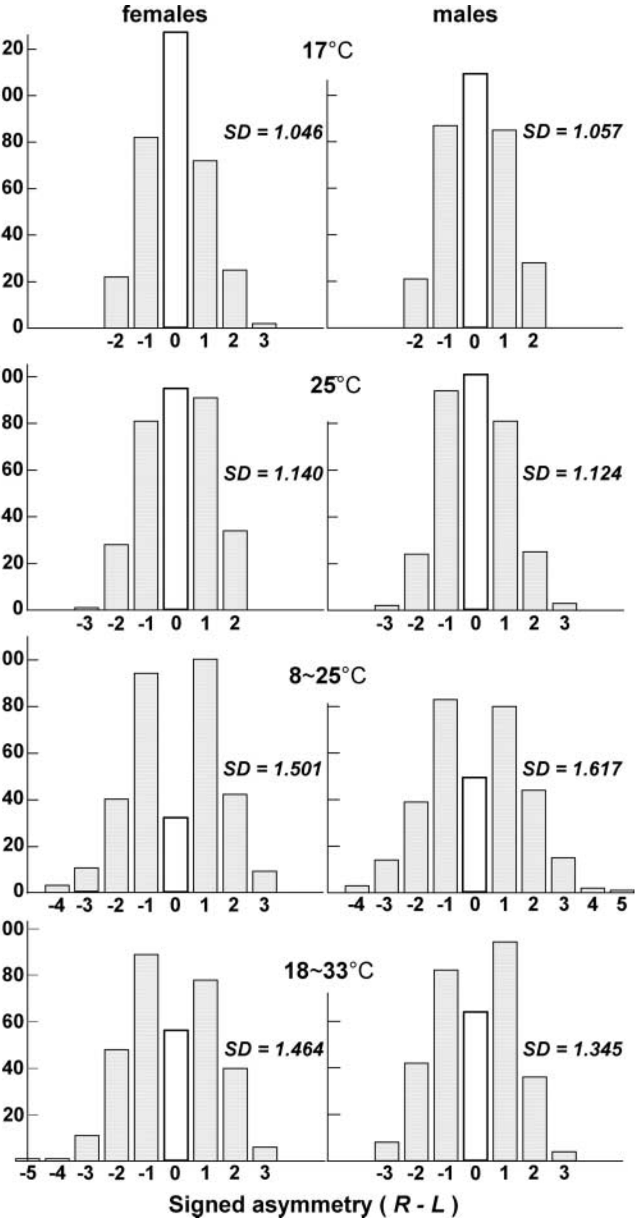

Fig. 1. Frequency distribution of the signed asymmetry (R – L) in bristle number on the right (R) and the left (L) sternopleura. For each thermal regime, data from both populations are pooled (no significant difference), thus the sample size is 330. The standard deviation (SD) is indicated for each distribution.

are somehow platykurtic, suggesting some anti-symmetry to occur in all cases (Palmer, 1994). Second, if we want to distinguish the shapes of the distributions under constant and alternating conditions, we need more precise analytical techniques. These results are presented in the following sections.

Fluctuating asymmetry

Two indices were used to estimate fluctuating asymmetry: (1) the absolute value of the difference between sides |R – L|; and (2) the variance (or standard deviation) of R – L (Palmer and Strobeck, 1986, 2003; Palmer, 1994). The first index may be calculated at the individual level, but we consider here the line level only (i.e. the mean for 10 flies).

We first explored the relationship between the variance and the mean bristle number, using non-parametric tests. For the four thermal regimes, we found an average positive correlation ($r = 0.195 \pm 0.068$, $P < 0.05$) and maximum values were observed at 17°C ($r = 0.28$) and at 18–33°C ($r = 0.33$) ($n = 66$ in each case). Because of this relationship, both fluctuating asymmetry indices were scaled to the mean. For the absolute asymmetry index, the absolute |R – L| average difference in each line was divided by the mean and multiplied by 100, as done previously by Moreteau *et al.* (2003). For the second index, the standard deviation was also divided by the mean and multiplied by 100, and we call this index the fluctuating asymmetry coefficient of variation.

Values of the two indices are given in Table 6. Classical ANOVA and non-parametric tests gave similar results. For each index, there was no significant effect due to sex, population or line, but a strong effect of thermal regime. For the absolute value index, means were much higher after alternating conditions than after constant ones and the difference between 17 and 25°C was also significant. The coefficient of variation of R – L gave similar conclusions – that is, higher average values under alternating conditions than under constant ones. Although the two indices had different mean values, we believed that they would provide similar information and would be correlated. Such was the case (Table 6): Spearman correlation coefficients were always highly significant (average 0.88 ± 0.02, $n = 4$). Figure 2 illustrates the dispersal among isofemale lines, the major difference between the effects of alternating regimes and those of constant ones, and a similarity between the two constant temperatures and between the two alternating temperature regimes.

Table 6. Influence of thermal regime upon two indices of fluctuating asymmetry (FA) and two indices of modality ($n = 66$ in each case)

	17°C	25°C	8–25°C	18–33°C
FA indices				
Absolute asymmetry	7.8 ± 0.3^a	9.3 ± 0.3^b	14.3 ± 0.4^c	13.6 ± 0.4^c
Coefficient of variation	10.1 ± 0.3^a	11.7 ± 0.4^b	16.6 ± 0.5^c	16.4 ± 0.4^c
Correlation	0.81	0.91	0.92	0.83
Modality indices				
Perfect symmetry	3.58 ± 0.19^a	2.97 ± 0.19^b	1.21 ± 0.10^c	1.88 ± 0.16^d
Modal index	1.80 ± 0.18^a	1.49 ± 0.20^a	0.54 ± 0.05^b	0.94 ± 0.12^c
Correlation	0.97	0.95	0.88	0.90

Note: The absolute asymmetry is the mean of |R – L| divided by the mean and multiplied by 100. The coefficient of variation is the standard deviation of signed asymmetry divided by the mean and multiplied by 100. Both indices are calculated for each isofemale line, and the correlation between the two indices is given. Modality indices are: perfect symmetry, the average number of perfectly symmetric flies in each line; modal index, the ratio $2N_0/N_{-1} + N_{+1}$ (see text). The correlation between the two indices is also given. Comparisons between thermal regimes were made with the Tukey *post-hoc* test: significant differences among temperatures are indicated by different letters.

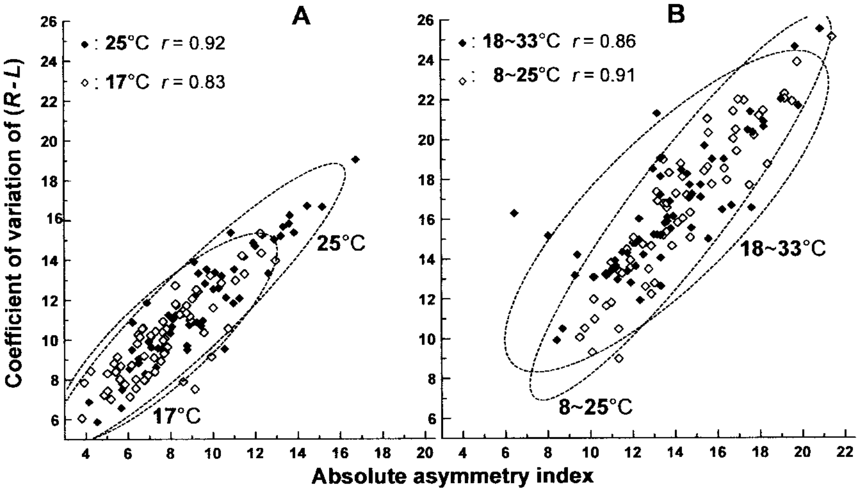

Fig. 2. Relationship between the standard deviation of signed asymmetry (R – L) and mean absolute asymmetry |R – L|. (A) constant temperatures; (B) alternating temperatures. For each thermal regime, data from both sexes and populations are pooled (no significant difference). Each symbol: value for 10 females or 10 males of each line. The Spearman correlation coefficient (*r*) is given for each regime (****P* < 0.001). The ellipses help to compare the distributions.

Relationship between the variance of R – L and the R*L correlation

Based on statistical considerations, we expect, at the line level, a relationship between the R – L variance and the correlation coefficient between SB number on the right and left sides. The variance of the R – L difference may be expressed by its components:

$$\mathrm{Var}_{(R-L)} = \mathrm{Var}_R + \mathrm{Var}_L - 2\,\mathrm{Cov}_{(RL)}$$

where $\mathrm{Cov}_{(RL)}$ is the covariance between the right and left sides. We may also write:

$$\mathrm{Cov}_{(RL)} = r \times S_R \times S_L$$

where S_R and S_L are the standard deviations of the right and left sides respectively. Thus:

$$\mathrm{Var}_{(R-L)} = \mathrm{Var}_R + \mathrm{Var}_L - 2rS_RS_L$$

From this equation, a negative correlation between the coefficient *r* and the variance of R – L is expected. This relationship will also hold for the scaled variance – that is, the variance divided by (R + L)/2, which is also a good estimator of fluctuating asymmetry (Palmer and Strobeck, 1986). Such was the case for the four thermal regimes, and the average Spearman correlation coefficient *r* is –0.58 ± 0.05 ($n = 4$).

Proportion of symmetrical flies: perfect symmetry index

From a visual inspection of the histograms in Fig. 1, a prominent effect of periodic stresses is to decrease the proportion of flies with the same numbers of sternopleural bristles on the left and right sides so that the distributions become bimodal under alternating regimes.

For a better analysis of this phenomenon, we first consider the proportion of perfectly symmetrical flies – that is, the magnitude of class zero in the R – L distribution. Since 10 flies of each sex were measured in each line, the number N_0 of symmetrical individuals may range between 0 and 10 but, in practice, experimental values fluctuated between 0 and 7. These values were used for statistical analyses. Non-parametric tests failed to detect any significant difference due to sex, population or line, but there was a highly significant effect of thermal regime. Average values are given in Table 6. The mean number per line of purely symmetrical flies decreases from 3.58 at 17°C down to 1.21 at 8–25°C, with intermediate and significantly different means at 25°C and 18–33°C.

A reduction in the number of symmetrical flies may be expected if the shape of the distribution is conserved while SB number increases and is thus accompanied by a greater number of phenotypic classes. We explored this problem by considering the relationship between the symmetry index and mean SB number (Fig, 3). In all cases, and as expected, a negative correlation was observed, but the average Spearman coefficient was very low ($r = -0.18 \pm 0.06$, $n = 4$), although significantly negative ($P < 0.05$). Considering the square of *r*, we may conclude that variation in SB number explains only 3% of the variability of the symmetry index. Moreover, under alternating regimes, there was a parallel reduction in the SB number and in the number of symmetrical flies (i.e. a positive correlation). We can safely conclude that alternating regimes had the specific effect of decreasing the bilateral symmetry.

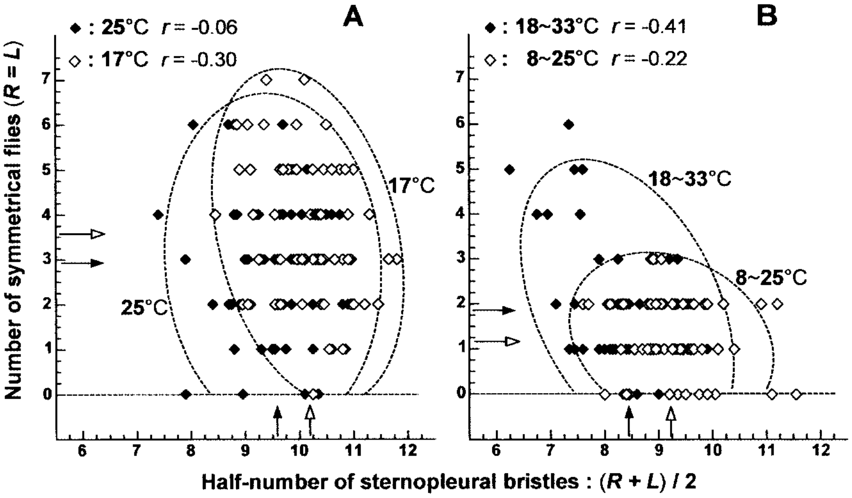

Fig. 3. Relationship between the number of symmetrical flies (R = L) and the mean half-number of bristles [(R + L)/2] at the line level. (A) constant temperatures; (B) alternating temperatures. For each thermal regime, data from both sexes and populations are pooled (no significant difference). Each symbol: value for 10 females or 10 males of each line. The Pearson correlation coefficient (*r*) is given for each regime. Ellipses of 95% confidence are shown. Open arrowheads indicate the means at 17 or 8–25°C; solid arrowheads indicate means at 25 or 18–33°C.

Modal index

The unimodal or bimodal shape of the R – L distribution may also be appreciated by comparing class zero with the two adjacent classes. More precisely, we define a modal index (MI) as

$$\mathrm{MI} = 2N_o/(N_{-1} + N_{+1})$$

where N_o is the number of symmetrical flies, and N_{-1} and N_{+1} are the numbers in classes −1 and +1 respectively.

By definition, for a distribution centred on zero, the value of the modal index will tend to decrease when a bimodal trend appears. More precisely, the modal index equals 1 when the three classes of R – L (−1, 0 and 1) are identical. A clear, unimodal distribution is characterized by a value significantly greater than 1. A bimodal curve will be demonstrated by a mean significantly less than 1.

Values for the modal index are given in Table 6. A Mann-Whitney *U*-test failed to detect any significant effect due to sex, population or line. Thermal regime, however, had a strong effect. Under both alternating temperature regimes, the modal index was significantly greater than 1, confirming the unimodal shape of the distributions for each isofemale line. A significant decrease in the modal index (0.94 ± 0.12) was observed under the heat stress regime, but in that case the bimodal shape was not confirmed, since the index was close to 1. A clear bimodality was however evidenced for the 8–25°C regime ($\mathrm{MI} = 0.54 \pm 0.05$). As might be expected, values of the modal and symmetry indices were positively correlated (Table 6). Spearman correlation coefficients (r) ranged between 0.88 and 0.97. There was a trend for this correlation to decrease under alternating regimes, but the variation was not significant. Both indices may be considered as convenient descriptors of the shape of the distribution of signed asymmetry, and of the possible occurrence of anti-symmetry.

Relationship between fluctuating asymmetry and modality indices

Indices of fluctuating asymmetry measure the breadth of the R – L distribution or, in other words, its size, and a clear conclusion is that fluctuating asymmetry increases under stressful conditions. The other two indices consider the shape of the distribution for a possible trend towards bimodality. We now ask the question, are indices of size and shape correlated?

The non-parametric correlation coefficients are presented in Table 7. Under non-stressful, constant conditions, we find negative and in most cases highly significant correlations, with an average value of -0.62 ± 0.05 ($n = 16$; $P < 0.001$). A broader distribution is accompanied by a decrease in the number of symmetrical flies and in the value of the modal index. This may be considered as a direct consequence of the increase in the number of phenotypic classes and of the flattening of the distribution.

Under both alternating temperature stresses, the negative correlations either decreased or disappeared (Table 7). The phenomenon is more pronounced with the modal index, for which the average r is -0.55 ± 0.08 ($P < 0.01$) under constant conditions and -0.06 ± 0.05 (non-significant) under alternating conditions ($n = 8$ in each case). Such a difference is another argument for specific effects of alternating temperature stresses.

Table 7. Correlations between indices of fluctuating asymmetry, and indices of symmetry and modality at the line level

			Absolute asymmetry index		Coefficient of variation of (R – L)	
Regime	Sex	*N*	vs. symmetry index	vs. modal index	vs. symmetry index	vs. modal index
17°C	female	33	−0.92 ***	−0.85 ***	−0.66 ***	−0.53 **
	male	33	−0.74 ***	−0.59 ***	−0.36 *	−0.22
	total	66	−0.86 ***	−0.74 ***	−0.51 ***	−0.37 *
25°C	female	33	−0.76 ***	−0.56 ***	−0.46 **	−0.26
	male	33	−0.89 ***	−0.77 ***	−0.75 ***	−0.60 ***
	total	66	−0.85 ***	−0.69 ***	−0.62 ***	−0.44 ***
8–25°C	female	33	−0.43 *	−0.08	−0.21	+0.07
	male	33	−0.49 **	−0.11	−0.28	+0.05
	total	66	−0.37 **	−0.02	−0.14	+0.18
18–33°C	female	33	−0.65 ***	−0.32	−0.24	+0.07
	male	33	−0.52 **	−0.17	−0.11	+0.02
	total	66	−0.59 ***	−0.24	−0.17	+0.14

Note: The correlation between indices is given by the Spearman correlation coefficient. Data from Draveil and Prunay are pooled (no significant difference). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION AND CONCLUSIONS

We used two alternating temperature regimes, each consisting of 12 hours at a lethal (low or high) temperature, followed by 12 hours at an intermediate temperature which allowed a daily functional recovery of the harmful effects (Renault *et al.*, 2004). An isofemale line design, which permits the comparison of similar genotype flies in different environments (David *et al.*, 2005), proved to be very useful for statistical comparisons. Sternopleural bristle number, which is classically investigated as a quantitative trait (Mackay, 2001, 2004), was first analysed by considering the sum of the right and left sides. We compared the results under the two alternating temperature regimes to those obtained under the two constant temperature regimes.

Both alternating temperature regimes produced smaller adult flies. This result may be considered the consequence of the non-linearity of the sternopleural bristle number reaction norm and of its concave shape (Moreteau *et al.*, 2003). When the values of two different temperatures located on both sides of the maximum are averaged, the mean is significantly less than that observed at the average temperature.

Our results support a well-known fact (Reeve and Robertson, 1954) that on average females have more bristles than males. A simple interpretation of this sexual dimorphism is that, since females are bigger than males, there is more space for more bristles on the sternopleura. Indeed, we observed for the complete data set a slight but positive average correlation between size and bristle number. A more detailed analysis, however, leads us to reject this simple, mechanistic interpretation. If we consider, for example, thorax length at 25°C, we know that the female/male ratio is quite stable at about 1.15 (Reeve and Fairbairn, 1999; David *et al.*, 2003; Pétavy *et al.*, 2004). For a surface, we expect that the increase will be proportional to the square (1.32) of the lengths ratio. In other words, we might expect 32% more bristles in females than in males. Experimental observations are far from meeting this expectation, since we found on average a ratio of only 1.07. Even smaller ratios were observed on inbred laboratory strains, for example 1.03 (Shakarad *et al.*, 2001) and 1.01 (Mather, 1953), presumably corresponding to a genetic drift. A relative independence between size and sternopleural bristle number is also suggested by species comparisons. For example, *D. simulans*, which is slightly smaller than *D. melanogaster*, has more sternopleural bristles (Capy *et al.*, 1993; Gibert *et al.*, 2004).

We found that sexual dimorphism of SB number was a plastic trait, which depended on developmental temperature. More precisely, there is a tendency for the F/M ratio to decrease at a lower temperature. A similar phenomenon exists for sexual dimorphism of body size (David *et al.*, 1994; Pétavy *et al.*, 2004). Whether other sexually dimorphic traits follow this trend deserves further investigations.

Both alternating temperature stresses increased the within-line variability, which mainly expresses an environmental component (David *et al.*, 2005). A similar increase among full sibs was also observed at extreme constant temperatures for SB number (Moreteau *et al.*, 2003). This phenomenon might be quite general for quantitative traits, since it is observed for body size (Imasheva *et al.*, 1998; Karan *et al.*, 1999; Pétavy *et al.*, 2004) and ovariole number (Delpuech *et al.*, 1995). The general interpretation is that unfavourable conditions tend to decrease developmental canalization, thus rendering the individuals more sensitive to uncontrolled, small fluctuations of their environment (Zakharov, 1981; Bijlsma and Loeschcke, 1997; Hoffmann and Parsons, 1997; Debat and David, 2001).

Things were more complicated concerning the between-line, genetic variance. For SB number, we did not find any significant effect of the stressful alternating temperature conditions. This result contrasts with that observed, in the same set of lines, for two size-related traits, thorax and wing length (Pétavy *et al.*, 2004). For body size, we did find a significant increase in variance under cold stress (8–25°C) but a decrease under heat stress (18–33°C). This discrepancy points to the difficulty of finding general evolutionary rules under adverse conditions (McKenzie and Clarke, 1988; McKenzie and O'Farrell, 1993; Bijlsma and Loeschcke, 1997; Hoffmann and Parsons, 1997; Hoffmann and Merila, 1999) when either different stress-inducing conditions are applied or different traits are compared.

In line with many other investigations of fluctuating asymmetry (Møller and Swaddle, 1997), we found that developmental instability (represented by fluctuating asymmetry) also increased under stress. However, this was complicated by an unexpected phenomenon, namely the induction of an anti-symmetric response. Anti-symmetry is notoriously difficult to analyse and quantify (Palmer, 1994; Rowe *et al.*, 1997; Polak, 2003). We found that our experimental design, with 33 isofemale lines in four thermal environments, was convenient for a quantification of the shape of the R – L distribution.

First, we considered the proportion of perfectly symmetrical flies, in class zero of the R – L distribution, as done previously by others (Shakarad *et al.*, 2001). This proportion was 36% at 17°C, 30% at 25°C and decreased to 19% under the 18–33°C and to 12% under the 8–25°C alternating temperature regime. For this index, we may expect a positive relationship between the mean of the trait and the number of phenotypic classes. Increasing the number of phenotypic classes will decrease the probability of finding identical values on the right and left sides. In the present study, however, we found that alternating temperature stresses decreased both the mean number of bristles and the frequency of the R – L zero class. The applied stresses, therefore, appear to decrease specifically the proportion of symmetrical flies. In another study of the sternopleural bristles of *D. melanogaster*, the proportion of symmetrical flies was observed to be 31% at 25°C (Shakarad *et al.*, 2001).

To investigate the possible bimodality of the R − L distribution, we also defined a modal index (MI). This index is the ratio of class 0 to the mean of adjacent classes +1 and −1. If the three classes are identical, the index will be 1, an indication of a fairly flat, platykurtic distribution. An index significantly higher than 1 suggests a unimodal distribution centred on zero. In constrast, a value significantly less than 1 demonstrates a bimodal distribution. With this index, we clearly discriminated the four thermal regimes, observing a significant bimodal pattern ($\mathrm{MI} = 0.54 \pm 0.05$) in the 8–25°C alternating regime. Such a bimodality is probably the consequence of an anti-symmetry process, which is a negative interaction between the right and left sides that may also occur with a milder stress.

Finally, the occurrence of anti-symmetry can also be inferred from the analysis of the right–left correlation. As a general case, we expect that any increase in the variance of two related traits, either due to environmental heterogeneity or to an increase in genetic variability, is likely to increase their correlation (Kristensen *et al.*, 2003). For example, in wild-collected flies, the variability of size traits is very great with a coefficient of variation of about 7%, against 2.5% in laboratory progeny (Gibert *et al.*, 1998). As a consequence, the wing–thorax correlation, which is close to 0.7 in laboratory-grown flies, becomes greater than 0.95 in wild flies (J.R. David, unpublished). We found the opposite result in our experiments. The alternating temperature regimes did increase the variability between individuals, but also decreased the right–left correlation. We consider this is due to the occurrence of a novel, negative component in the covariance between the right and left sides, induced by the daily thermal stresses. If, for an unknown reason, the right side of an individual is favoured, developmental feedback will induce a specific reduction on the left side (Graham *et al.*, 1993, 2003; Palmer *et al.*, 1993). Such a functional interaction, resulting in a phenotypic anti-symmetry, has long been known in the development of claws in Decapoda Crustacea (Zeleny, 1905; Rensch, 1959). It is surprising that such a phenomenon might also be at work in a trait, sternopleural bristle number, which is a paradigm for quantitative genetic studies and QTL analyses (Mackay, 2001, 2004).

In our analyses, we generally found a small but significant variability in fluctuating asymmetry among lines, suggesting a genetic component. That fluctuating asymmetry can be genetically variable was clearly demonstrated by the pioneering work of Mather (1953). In the case of SB number of *D. melanogaster*, Mather (1953) observed not only increased fluctuating asymmetry under directional selection, but also the appearance of an anti-symmetric response in the line selected for high fluctuating asymmetry. Similar transitions from fluctuating asymmetry to anti-asymmetry and vice versa have been observed in other species, for example in the sheep blowfly (McKenzie and Clarke, 1988) and in various vertebrate species (see Graham *et al.*, 2003, for further examples). Finally, and as discussed by Palmer (2004), there can be various evolutionary transitions between fluctuating asymmetry, anti-asymmetry and directional asymmetry. Extreme cases of directional asymmetry are interesting since they are more easily amenable to genetic analysis. In this respect, exciting new results have recently been reported for vertebrate embryos (Kawakami *et al.*, 2005; Tanaka *et al.*, 2005; Vermot and Pourquié, 2005).

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