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Specific effects of cycling stressful temperatures upon phenotypic and genetic variability of size traits in *Drosophila melanogaster*

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ABSTRACT

In previous studies, the relationship between developmental temperature and phenotypic or genetic variability has in the main been investigated using different constant temperatures. Natural conditions, however, are characterized by daily thermal cycles, sometimes resulting in a periodic daily stress. Using the isofemale line technique, we examined the effects of daily cycles on body size in two French populations of *Drosophila melanogaster*. We used either cold stress (daily cycle 8–25°C, average 16.5°C) or heat stress (cycle 18–33°C, average 25.5°C), and the results were compared to those obtained at two constant temperatures, 17 and 25°C. In all cases, stressful regimes produced specific effects, the mean trait values being smaller than those observed under constant conditions. Significant differences were also found for the variance parameters. For the within-line variance, which mostly expresses an environmental component, the two cycling regimes produced similar outcomes – that is, a significant increase in individual variability. For the between-line, genetic variance, however, contrasting results were obtained: an increase with cold stress but a decrease with heat stress. With respect to constant-temperature conditions, evolvability (genetic CV) was increased by daily cold stress, but decreased by daily heat stress. Within-line correlations, between wing and thorax length, were stable and not affected by stress, whereas the between-line, genetic correlation was maximum under cold stress and minimum under heat stress. These results demonstrate that a periodic stress may induce specific effects with respect to permanent stress, and that heat and cold are not equivalent. A possible adaptive interpretation, related to the fact that temperate populations are certainly submitted to cold stress but not to heat stress, is discussed.

Keywords: daily cycles, evolvability, genetic correlation, intraclass correlation, isofemale lines, phenotypic correlation, thorax length, wing length.

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INTRODUCTION

The occurrence of significant genetic variability is a prerequisite for the evolution of a natural population under natural selection. Because environmental conditions are variable among habitats, days, seasons or years, genetic parameters are difficult to estimate in nature (Weigensberg and Roff, 1996; Hoffmann and Merilä, 1999; Hoffmann, 2000). Evolutionary theory predicts that, for fitness traits, natural selection should exhaust the amount of additive genetic variation (Fisher, 1930). Indeed, it has been shown that the heritability of fitness traits is, on average, significantly less than that of other traits (Mousseau and Roff, 1987; Roff and Mousseau, 1987; Weigensberg and Roff, 1996). This expectation, however, is valid only if the environment is fairly stable and predictable. It has been repeatedly argued that environmental variations are so variable that they result in frequent physiological stresses, and that selection under stress might be more efficient for a population to adapt to a new environment (Hoffmann and Parsons, 1991, 1997; Bijlsma and Loeschcke, 1997; Feder and Hoffmann, 1999; Hoffmann and Hercus, 2000; Hoffmann *et al.*, 2003). To be valid, this hypothesis implies a strong genotype × environment interaction, sometimes accompanied by an increased heritability in extreme, although uncommon, conditions.

Temperature is a major abiotic factor for explaining the distribution and abundance of ectotherm species (Andrewartha and Birch, 1954). It offers optimum conditions in the middle of the thermal range of a given species, but stressful conditions at low and high temperatures (Precht *et al.*, 1973; Cossins and Bowler, 1987; Leather *et al.*, 1993). In *Drosophila melanogaster*, the selection imposed by environmental temperature is demonstrated by the occurrence of clear-cut latitudinal clines for many quantitative traits (David and Capy, 1988; James *et al.*, 1997; Azevedo *et al.*, 1998; David *et al.*, 2004a; Gibert *et al.*, 2004) and also by the evolution of laboratory populations kept for years at different temperatures (Cavicchi *et al.*, 1985). Latitudinal clinal variations are always strongly correlated with climate, and more precisely with the average yearly temperature or the mean temperature in winter. In a given locality, temperatures may, however, be very variable among seasons. Thus, periodic heat stresses are expected in warm climates and cold stresses in cold ones. Stressful conditions occur during the warm or the cold season, but also, due to daily cycles, during the warm or the cold part of the day. Indeed, daily variations surpassing an amplitude of 15°C are common in nature (McKenzie and Parsons, 1974; Cossins and Bowler, 1987; Gibbs *et al.*, 2003).

Many studies have examined phenotypic and genetic variation of body size in *Drosophila* under mild and stressful constant temperatures (Parsons, 1983; David *et al.*, 1994; Barker and Krebs, 1995; Noach *et al.*, 1996; Bijlsma and Loeschcke, 1997; De Moed *et al.*, 1997; Imasheva *et al.*, 1997, 2000; Karan *et al.*, 1999; Bubliy and Loeschcke, 2000, 2002). In most cases, a significant increase in phenotypic variability has been observed under stressful conditions, whether cold or heat. Whether the environmental and genetic variances are increased simultaneously is, however, less well documented. As stated, for example, by Hoffmann and Merilä (1999), an increase in heritability will be observed only if the genetic component of variance reacts more strongly than the environmental component. Compared with constant thermal conditions, daily periodic cycles have received far less attention (Kelty and Lee, 1999), although they are a rule in nature (McKenzie and Parsons, 1974; Feder, 1997; Gibbs *et al.*, 2003). Working with mass laboratory populations, Pétavy *et al.* (2001b) observed an increase in phenotypic variance for size traits, broadly

proportional to the amplitude of the daily period. Such a protocol, similar to that of Imasheva *et al.* (1997), does not provide, however, an estimate of genetic variability.

In this study, we addressed two related questions: What are the effects of a daily stress upon the genetic and environmental components of variance? Do cold and heat stress have symmetrical effects? We used the widespread isofemale line technique (Hoffmann and Parsons, 1988; David *et al.*, in press) to measure within- and between-line variability on the same set of strains submitted to different environments, and two periodic conditions were compared. We used a daily cold stress of 8°C for 12 h. Such a low temperature is lethal when applied permanently, since the lower constant temperature compatible with the emergence of a few adults is 11°C (Pétavy *et al.*, 2001a). So the harmful effects of cold were offset by spending 12 h each day at a normal temperature of 25°C, with an average of 16.5°C. We also used a heat stress, 12 h at 33°C, a temperature which is also lethal when applied permanently (Pétavy *et al.*, 2001a). This lethal effect was offset by spending 12 h at 18°C, with an average of 25.5°C. As controls, we used two constant temperatures, 17 and 25°C. Both stressful regimes had similar effects on the within-line variance, which was significantly increased. In contrast, the between-line genetic variability was increased by cold stress but decreased by heat stress. The possible significance of such a divergence for the adaptation of a natural population is discussed.

MATERIALS AND METHODS

Populations and experiments

We investigated two French populations in the vicinity of Paris: Draveil in the southeast suburbs and Prunay near Rambouillet, about 60 km southwest of Paris. Wild-living flies were collected in autumn. Pairs (one female one male) were isolated in culture vials and reared at 21°C under a 16 : 8 light : dark photocycle, each pair initiating an isofemale line. Thirty-three of these lines were investigated: 18 from Draveil and 15 from Prunay. From the first laboratory generation, ten pairs were randomly taken from each line as parents of the experimental flies. Three days later, parental groups oviposited directly for a few hours at 21°C in a culture vial containing a high-nutrient, killed yeast food (David and Clavel, 1965). This operation was repeated four times for each line. Larval density was not precisely controlled but remained less than 150 adult flies per vial. The use of a high nutrient food makes morphometrical traits practically insensitive to larval density fluctuations (see Karan *et al.*, 1999), and common environment effects are not significant (David *et al.*, in press).

After removal of the parents, the experimental vials were transferred to incubators regulated at ±0.2°C. We used four regimes for each line: two constant mild temperatures, 17 and 25°C, and two alternating temperatures with a daily cycle of 12 h. We consider that one regime, 8–25°C, imposed a cold stress, while the other, 18–33°C, imposed a heat stress. In each cycling regime, embryonic development began at the start of the mild phase (25 or 18°C). After emergence, adults were transferred to fresh food and maintained for a few days at 21°C before morphometrical measurements.

Traits measured

Measured flies belonged to the second generation in the laboratory. Ten males and ten females were randomly taken from each line in each environment and slightly anaesthetized

before measurement, for a total sample size of 2640 flies. The length of the thorax was measured on a left side view from the neck to the tip of the scutellum; the length of the left wing was measured from the thoracic articulation to the tip. Measurements were made with an ocular micrometer in a binocular microscope with an accuracy of 5 μm (thorax) or 10 μm (wing), and each length was then expressed in millimetres $\times$ 100. The wing/thorax ratio, which is considered a specific trait (David *et al.*, 1994) and is negatively correlated with wing loading (Pétavy *et al.*, 1997), was calculated for each fly.

Data analyses

The data were analysed using standard statistical methods included in the Statistica package (StatSoft, 1999). As thorax and wing length are metric traits, their variability was expressed either by the variance, the standard deviation or the coefficient of variation (CV). For each sex, population and thermal regime, we calculated the variance within and between lines. Genetic variance was estimated from the between-line variance, after a correction for family size (see Capy *et al.*, 1994; David *et al.*, in press). The within-line variance, for full-sib families, harbours both an environmental and a genetic component (Falconer and Mackay, 1996); in many cases, however, the environmental component is preponderant (Bell, 1997; Imasheva *et al.*, 2000). Both variance components were used to calculate an intraclass correlation, which is proportional to the amount of genetic variability harboured in the origin population and may be considered as a special kind of heritability (Capy *et al.*, 1994). Since wing and thorax length have very different means, their variances are also very different. The scaling effect disappears when a coefficient of variation is used. Within-line variability was analysed using the within-line coefficient of variation. We also considered the genetic coefficient of variation, also called 'evolvability' (Houle, 1992). The covariation between wing and thorax length was analysed by calculating for each line a within-line correlation. Genetic correlations were estimated by calculating the coefficients among family means (see Gibert *et al.*, 1998). Variations among correlation coefficients were analysed after z-transformation.

RESULTS

Viability and fertility

Under constant temperatures ranging between 14 and 28°C, viability (percentage of adults from laid eggs) is about 80% (Pétavy *et al.*, 2001a). Constant temperatures of 10 or 33°C are, however, 100% lethal. The viability was not measured in each line, but all of them produced a sufficient number of adults in all conditions. In other words, the harmful effects of 8 or 33°C were offset by spending 12 h each day at a milder temperature of 25 or 18°C. Viability under both cycling conditions was measured on mass populations (Draveil and Prunay), and ranged between 50 and 60%. After emergence, adults from each line were transferred to fresh food vials and kept at 21°C before being measured. As expected, all lines grown at 17 or 25°C rapidly produced progeny after 2 or 3 days. Adults from cycling regimes also produced viable progeny but with a significant delay, the first larvae being observed after about 7 and 10 days for the 8–25°C and 18–33°C regimes, respectively.

Basic morphometric data

Means of the two measured traits and of the wing/thorax ratio are given in Table 1. A scatterplot of isofemale line values is illustrated in Fig. 1, as a correlation between wing and thorax length.

Considering the two constant temperature regimes, our results support what is already well established for *D. melanogaster* (David *et al.*, 1994; Pétavy *et al.*, 2001b): females are larger than males at each temperature, but the wing/thorax ratio is almost the same in both sexes. Size is greater at 17°C than at 25°C as evidenced by wing length, but there was only a small difference in thorax length, especially in females. This arises from the fact that the wing and thorax have different reaction norms (David *et al.*, 1994, 2004b). As a consequence, the wing/thorax ratio is much higher at 17°C than at 25°C (2.59 *vs* 2.41, $P < 0.001$). A comparison of the two populations (ANOVA, not shown) failed to find any significant difference for any trait.

For the alternating temperature regimes, the adult size traits were always much less at 8–25°C than at 17°C, and at 18–33°C than at 25°C (Fig. 1, Table 1). These results confirm previous data on the effects of cycling temperatures (Pétavy *et al.*, 2001b): any stressful condition decreases body size. There were significant differences between the two alternating temperature regimes (ANOVA, not shown), which were more pronounced for wing length and wing/thorax ratio than for thorax length. A comparison of the two populations

Table 1. Basic quantitative data for thorax and wing length (millimetres × 100) and wing/thorax ratio under the four thermal regimes

Thermal regime	Population	Sex	*n*	Thorax length	Wing length	Wing/thorax ratio
25°C	Draveil	female	18	114.1 ± 0.5	276.6 ± 1.1	2.424 ± 0.009
		male	18	100.7 ± 0.4	240.6 ± 1.1	2.391 ± 0.008
	Prunay	female	15	114.7 ± 0.4	277.7 ± 1.4	2.421 ± 0.009
		male	15	100.5 ± 0.5	240.0 ± 1.4	2.388 ± 0.012
17°C	Draveil	female	18	115.3 ± 0.5	297.7 ± 1.2	2.583 ± 0.008
		male	18	103.4 ± 0.4	268.3 ± 0.9	2.596 ± 0.006
	Prunay	female	15	115.6 ± 0.6	300.4 ± 1.9	2.598 ± 0.010
		male	15	103.8 ± 0.6	269.7 ± 1.8	2.598 ± 0.012
18–33°C	Draveil	female	18	105.7 ± 0.4	253.8 ± 1.2	2.402 ± 0.011
		male	18	93.2 ± 0.4	223.9 ± 0.8	2.402 ± 0.009
	Prunay	female	15	106.0 ± 0.4	255.8 ± 1.0	2.415 ± 0.010
		male	15	93.7 ± 0.4	225.5 ± 0.7	2.409 ± 0.009
8–25°C	Draveil	female	18	107.2 ± 0.7	267.5 ± 1.9	2.496 ± 0.009
		male	18	95.3 ± 0.6	237.8 ± 1.6	2.496 ± 0.011
	Prunay	female	15	109.2 ± 0.8	272.1 ± 1.6	2.494 ± 0.011
		male	15	96.0 ± 0.7	238.6 ± 1.6	2.487 ± 0.011

Note: Mean values (± standard errors) are calculated from the number (*n*) of isofemale lines. Ten males and ten females were measured in each line, on the second line laboratory generation.

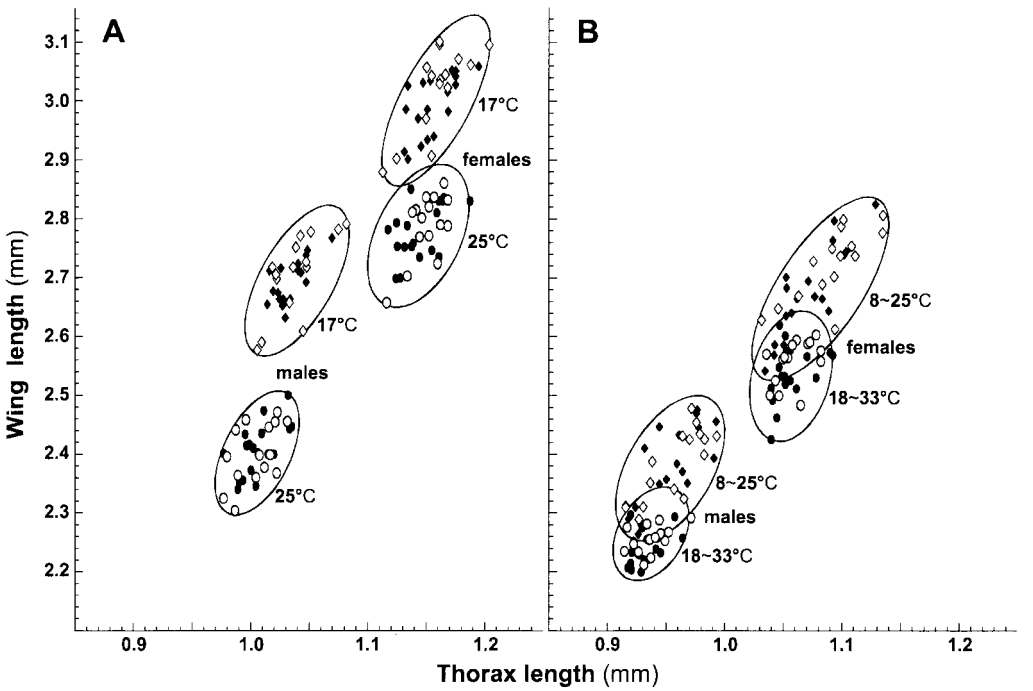

Fig. 1. Scatterplots of wing and thorax length: (A) constant temperatures; (B) cycling thermal regimes. Each symbol is the mean of 10 flies of each line. Solid symbols: Draveil; open symbols: Prunay. For each set, ellipses of 95% confidence limits are drawn on principal axes according to Sokal and Rohlf (1995, pp. 586–593).

found just one slightly significant difference for female thorax length at 8–25°C ($P = 0.047$, *t*-test). However, the significance disappeared when the data were corrected for multiple comparisons (Bonferroni correction). A practical conclusion is that it will be possible, when required, to pool the data of Draveil and Prunay, providing a single set of 33 isofemale lines.

A general question arises when comparing the alternating and constant temperature conditions: What is the constant temperature that is equivalent to – or, in other words, would provide the same phenotypic value as – the alternating temperature? This is possible when reaction norms under various constant temperatures are available, such as in Pétavy *et al.* (2001b). In a previous paper (Pétavy *et al.*, 2001a), we calculated an 'equivalent developmental temperature' – that is, the constant temperature that would produce the same average duration. This kind of reasoning may be extended to other traits, resulting in 'equivalent temperatures' for each of them. In Table 2, equivalent temperatures for various traits are given and compared to the average daily temperature of the two alternating regimes. Equivalent temperatures appear very variable among traits: for duration of development, they are fairly close to the average daily temperatures; differences, however, are larger for size traits, especially for the low temperature cycling regime of 8–25°C.

Table 2. Estimation of 'equivalent temperatures' (ET, see text) for different traits under the two alternating regimes

		8–25°C		18–33°C	
Trait	Sex	ET	*D*	ET	*D*
Duration of development		17.5	+1.0	23.0	−2.5
Thorax length	female	29.0	+12.5	27.0	+1.5
	male	28.5	+12.0	26.5	+1.0
Wing length	female	30.0	+13.5	29.0	+3.5
	male	29.5	+13.0	28.5	+3.0
Wing/thorax ratio	both sexes	20.5	+4.0	25.0	−0.5

Note: Equivalent temperatures are obtained by comparison with the reaction norms at various constant temperatures for French populations (David *et al.*, 1994; Pétavy *et al.*, 2001b). *D* is the difference from the average daily temperature at each cycling regime, namely 16.5°C and 25.5°C for 8–25°C and 18–33°C, respectively. Values are expressed in °C.

Within-line variability

For each line, trait, sex and thermal treatment, the variability among flies was estimated by calculating a coefficient of variation (CV), for a total of 264 values.

Under constant temperatures, we did not find any significant effect of sex or population, and the CV distributions are shown in Fig. 2A. The CVs for thorax length were on average 2.15 ± 0.08 at 17°C and 2.04 ± 0.08 at 25°C, slightly but significantly larger than those for

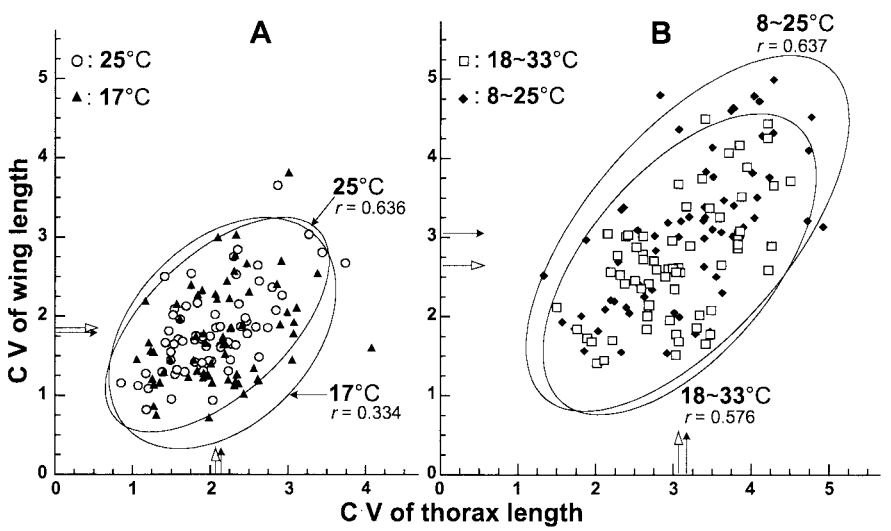

Fig. 2. Relationship between the within-line coefficients of variation (CVs) of wing and thorax length: (A) constant temperatures; (B) cycling thermal regimes. Each symbol is the mean of 10 flies (either males or females) of each line. For each set of CVs, ellipses of 95% confidence limits are drawn as in Fig. 1. Arrowheads on coordinate axes indicate average CVs: solid symbols for CVs at 17 or 8–25°C; open symbols for CVs at 25 or 18–33°C.

wing length: 1.77 ± 0.08 and 1.83 ± 0.07 at 17 and 25°C, respectively. This difference could be a consequence of the smaller length of the thorax and of a relatively greater impact of measurement errors (see Imasheva *et al.*, 2000). The average CV was significantly much less for the wing/thorax ratio (1.44 ± 0.06 and 1.38 ± 0.05 at 17 and 25°C, respectively), because wing and thorax length are positively correlated (see David *et al.*, 1994).

Under cycling regimes, there was a highly significant increase in the CVs (see Fig. 2B), with overall mean values about 1.5 times greater than those observed at constant temperatures: 3.17 ± 0.11 at 8–25°C and 3.05 ± 0.09 at 18–33°C for thorax length, and 3.05 ± 0.12 at 8–25°C and 2.69 ± 0.10 at 18–33°C for wing length. All the differences were highly significant ($P < 0.001$, *t*-test). A similar increase was found for the wing/thorax ratio: 2.08 ± 0.07 and 2.07 ± 0.08 at 8–25°C and at 18–33°C, respectively.

The two cycling regimes were also compared and a significant difference was found for wing length only, indicating less variability at 18–33°C.

Finally, the correlation between wing and thorax length was analysed at the individual level, calculating for each line and sex a coefficient among the 10 flies measured. The total set of 264 coefficients was submitted to ANOVA (not shown) after a *z*-transformation. There was no significant effect due to thermal regime, population or sex, but there was highly significant variability between the 33 lines ($P = 0.005$). On average, the within-line correlation between wing and thorax length appears fairly stable: 0.74 ± 0.04, when pooling all thermal conditions, in close agreement with previously published data on other French populations (Karan *et al.*, 1999).

Between-line variability

For each thermal regime, ANOVA (not shown) revealed a highly significant line effect. Differences among lines have a genetic basis (Hoffman and Parsons, 1988; Capy *et al.*, 1994; Gibert *et al.*, 1998), and their magnitude is usually estimated by calculating an intraclass correlation (ICC) which is assumed to be related to heritability. After a correction for sample size, the between-line variance estimates a genetic variance that depends on the mean of the trait measured. For that reason, the genetic coefficient of variation (CVg), also called evolvability (Houle, 1992), was considered. Although the ICC and CVg are both a function of genetic variance, they are generally not correlated (Karan *et al.*, 1999) and provide different information (David *et al.*, in press); their values are given in Table 3 for each population and sex.

For each trait and thermal regime, the genetic parameters were compared by ANOVA (not shown). Highly significant effects due to temperature conditions were found in all cases, except for the CVg of the wing/thorax ratio. Differences between populations were found for CVg of the wing and wing/thorax ratio, and a significant temperature × population interaction for both parameters of wing length. The two kinds of regimes were then analysed separately.

For the two constant temperatures, ANOVA failed to find any difference between temperatures but there was a significant population effect for wing length (i.e. greater genetic variability in Prunay). The values found for the genetic parameters (Table 3) are in close agreement with previously published data on the same species (Capy *et al.*, 1994; David *et al.*, 1994; Karan *et al.*, 1999). For example, the ICC for wing length is greater than that for thorax length (0.48 ± 0.05 *vs* 0.30 ± 0.03, $n = 8$), and the ICC for the wing/thorax ratio (0.49 ± 0.04) is similar to that of wing length. The genetic CV is similar for thorax

Table 3. Genetic parameters under various thermal regimes of thorax length, wing length and wing/thorax ratio

				Thorax length		Wing length		Wing/thorax ratio	
Regime	Population	Sex	*n*	CVg	ICC	CVg	ICC	CVg	ICC
25°C	Draveil	female	18	1.49	0.366	1.56	0.443	1.55	0.535
		male	18	1.39	0.280	1.66	0.389	1.39	0.446
	Prunay	female	15	1.01	0.182	1.87	0.499	1.47	0.584
		male	15	1.55	0.348	2.02	0.529	1.84	0.621
	Mean ± SE			1.36 ± 0.13	0.29 ± 0.05	1.78 ± 0.11	0.46 ± 0.04	1.56 ± 0.10	0.55 ± 0.04
17°C	Draveil	female	18	1.37	0.274	1.59	0.393	1.28	0.411
		male	18	1.14	0.232	1.24	0.302	0.93	0.289
	Prunay	female	15	1.71	0.359	2.30	0.603	1.45	0.473
		male	15	1.88	0.390	2.44	0.644	1.70	0.558
	Mean ± SE			1.53 ± 0.17	0.31 ± 0.04	1.89 ± 0.29	0.49 ± 0.09	1.34 ± 0.17	0.43 ± 0.06
18–33°C	Draveil	female	18	1.12	0.105	1.63	0.225	1.76	0.361
		male	18	1.01	0.085	0.94	0.089	1.34	0.285
	Prunay	female	15	1.11	0.121	1.22	0.178	1.42	0.353
		male	15	1.30	0.172	0.77	0.099	1.21	0.237
	Mean ± SE			1.14 ± 0.07	0.12 ± 0.02	1.14 ± 0.19	0.15 ± 0.04	1.43 ± 0.12	0.31 ± 0.03
8–25°C	Draveil	female	18	2.20	0.320	2.74	0.432	1.32	0.272
		male	18	2.37	0.321	2.68	0.424	1.79	0.347
	Prunay	female	15	2.46	0.413	2.06	0.335	1.65	0.421
		male	15	2.25	0.304	2.27	0.302	1.53	0.378
	Mean ± SE			2.32 ± 0.06	0.34 ± 0.03	2.44 ± 0.17	0.37 ± 0.04	1.57 ± 0.10	0.36 ± 0.04

Note: CVg = genetic coefficient of variation (evolvability); ICC = coefficient of intraclass correlation (isofemale line heritability). For each trait and thermal regime, the mean ± the standard error (SE) are indicated. *n* = number of lines.

length and wing/thorax ratio (1.44 ± 0.10 and 1.45 ± 0.10, respectively); it is, however, slightly larger for wing length (1.84 ± 0.15).

Things were more complex under cycling conditions. First, the two genetic parameters were compared with ANOVA for each trait. Highly significant differences were found between the two regimes for CVg and ICC of wing and thorax length, but not for wing/thorax ratio. Clearly, the genetic variability was much higher at 8–25°C than at 18–33°C (Table 3), a result which is also visible in Fig. 1B.

The parameters under the cycling regimes were compared with those under constant temperatures. Since there was no significant difference between 17 and 25°C, an average value of the genetic CV was calculated for each of the four samples (females and males of Draveil and Prunay). Then, for each sample, the difference between values of the CVg at 8–25 or 18–33°C and the mean under constant temperatures ($n = 4$ in each case) was calculated. For thorax length, all differences were significantly different from zero, and positive at 8–25°C (0.88 ± 0.14, $P = 0.008$, t-test) but negative at 18–33°C (-0.31 ± 0.04, $P = 0.004$). For wing length, differences among CVg had the same signs but were non-significant, due to a broader dispersal of values: 0.60 ± 0.35 at 8–25°C ($P = 0.18$); -0.72 ± 0.30 at 18–33°C ($P = 0.12$). In other words, a periodic cold stress produced a significant increase of the genetic variability of thorax length among lines, while a periodic heat stress had the opposite effect. A similar, but non-significant trend was found for wing length. This phenomenon is illustrated in Fig. 3.

Another way to describe the same phenomenon is to compare the between-line variance of thorax and wing length under different conditions (Table 4). The variances did not

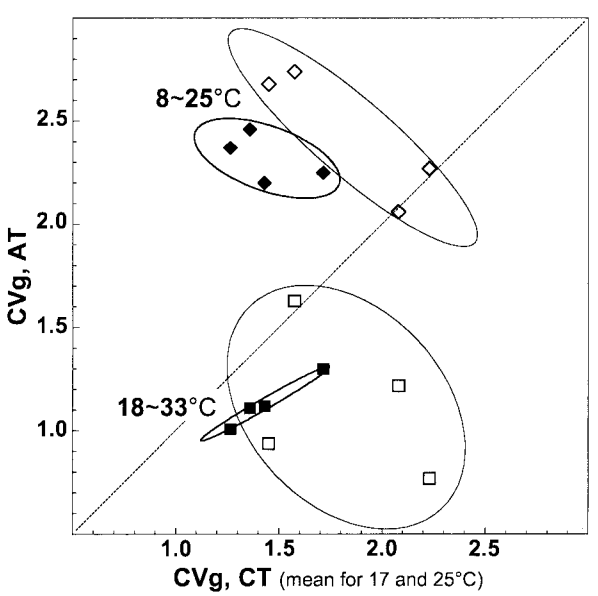

Fig. 3. Relationship between genetic coefficients of variation (CVg) under constant (CT) and alternating (AT) temperatures for wing length (open symbols) and thorax length (solid symbols). When compared with CT values (abscissa), there is an increase in CVg with cold stress (8–25°C) but a decrease in CVg with heat stress (18–33°C). The phenomenon is, however, significant for thorax length only. For each set, ellipses of 70% confidence limits are drawn.

Table 4. Comparison of between-line variances in the four thermal experimental regimes (both sexes averaged)

	Thorax length		Wing length	
Regime	Variance	*F*	Variance	*F*
25°C	2.67		22.76	
17°C	3.35	1.25	32.00	1.41
25 + 17°C	3.01		27.38	
18–33°C	2.21	1.36	13.76	1.99*
8–25°C	7.05	2.34**	47.47	1.73*

Note: *F* parameters compare either the two constant temperature regimes, or the average constant temperature with each cycling regime. $*P < 0.05$; $**P < 0.01$. The variances under 18–33 and 8–25°C are highly different ($P < 0.001$ for each trait).

differ significantly between the sexes and thus were averaged for all cases. There was also no significant difference between the two constant temperature regimes. A highly significant difference, however, was observed in the comparisons between the two alternating temperature regimes, with a much larger variance under cold stress (8–25°C). The variance under stress 8–25°C was also larger, for both traits, than that observed under constant temperature conditions. For the 18–33°C alternating temperature regime, the reduction in variance, compared with constant temperatures, was significant for wing length only.

For the wing/thorax ratio, we failed to find any significant variation in the genetic coefficient of variation between constant temperatures and cycling regimes (see Table 3). In other words, the genetic variance did not change significantly. Since, however, the within-line variance was increased under cycling conditions, the intraclass correlations were significantly less than at constant temperatures (0.33 ± 0.03 *vs* 0.49 ± 0.04; $P = 0.01$, *t*-test, $n = 8$).

Genetic correlations between traits or environments

Correlating isofemale line means provides an estimate of genetic correlation (Via, 1984; Gibert *et al.*, 1998). Such correlations can be calculated either between different traits under the same environment or between different environments for the same trait.

Wing–thorax correlation coefficients were calculated in the different experimental conditions and submitted to ANOVA (not shown) after a *z*-transformation. Significant differences between thermal regimes were observed. Coefficients were highest at 17 and 8–25°C (0.71 ± 0.05 and 0.79 ± 0.08, respectively; difference not significant), slightly lower at 25°C (0.58 ± 0.05; comparison with 17°C: $P = 0.017$) and lowest at 18–33°C (0.44 ± 0.03). There was no effect of sex or population. The major difference observed is between the two cycling regimes, which is highly significant ($P = 0.008$, $n = 4$). This phenomenon is also visible in Fig. 1B. Consequently, the increase in the genetic variance due to cold stress is accompanied by an increase in the covariance and vice versa.

Correlations across thermal environments were also analysed, and values for the 33 lines are given in Table 5. Analysis of variance on the *z*-transformed values (not shown) led to the conclusion that there were significant differences between traits and thermal regimes.

Table 5. Correlation coefficients between line means of the same trait under different thermal conditions

	Thorax length		Wing length		
Comparison	Female	Male	Female	Male	Mean ± SE
17 *vs* 25°C	0.64***	0.51**	0.86***	0.67***	0.67 ± 0.08
8–25 *vs* 25°C	0.50**	0.64***	0.50**	0.58***	0.56 ± 0.04
18–33 *vs* 25°C	0.48**	0.41*	0.43*	0.45**	0.44 ± 0.02
8–25 *vs* 17°C	0.41*	0.42*	0.53**	0.53**	0.47 ± 0.04
18–33 *vs* 17°C	0.12	0.54**	0.42*	0.51**	0.40 ± 0.10
8–25 *vs* 18–33°C	0.28	0.47**	0.49**	0.57***	0.45 ± 0.07
Mean ± SE	0.40 ± 0.08	0.50 ± 0.04	0.54 ± 0.07	0.55 ± 0.04	0.50 ± 0.04

Note: Means from Draveil and Prunay are pooled, thus $n = 33$. Means and standard errors (SE) are calculated for each line and column. Significance of coefficients of correlation: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Correlations were greater for wing than for thorax length, and also highest between the two constant temperatures. Both alternating temperature regimes revealed a tendency to decrease the genetic correlations.

DISCUSSION AND CONCLUSION

In this study, we examined the effects of cold and heat stress during development, using daily cycling thermal regimes and the widespread experimental technique of isofemale lines. Each day, a stressful, lethal temperature (either 8 or 33°C) was followed by a recovery phase at a physiological temperature (either 25 or 18°C). The efficiency of the recovery process was demonstrated in that all lines produced many adult flies. Some deleterious effects on reproduction were observed, however, and the late production of progeny after a return to an optimum temperature was probably due to transient male sterility (Chakir *et al.*, 2002; Vollmer *et al.*, 2003; Araripe *et al.*, 2004). The thermal daily cycles resulted in a diversity of effects on trait values, within- and between-line variability and various kinds of correlations. These different aspects will be discussed in turn.

Variation of mean trait values

Comparing the values obtained under alternating temperatures with the values obtained at the average constant temperature in general reveals significant differences (Table 1). Using reaction norms under constant temperatures (Pétavy *et al.*, 2001a,b; David *et al.*, in press), it is possible to calculate an equivalent temperature that would provide the same phenotype. As seen in Table 2, the results are quite diverse according to traits. This phenomenon is explained, at least in part, by the non-linearity of the reaction norms. Consider, for example, thorax length. It has a concave reaction norm (a negative curvature) with a maximum around 19°C. If we average the phenotypes obtained at 8°C (by extrapolating the reaction norm) and at 25°C, the mean is much less than at 17.5°C. This kind of reasoning is also valid for the 18–33°C cycle. Within this range, thorax length decreases almost monotonically but

still with a significant negative curvature. Averaging the values obtained at 18 and 33°C gives a mean less than that obtained at 25°C. But in that case, the difference (the distance to the reaction norm) is much less, in agreement with Table 2. The same reasoning is valid for wing length, which has a concave reaction norm with a maximum around 15°C, and for the wing/thorax ratio, which exhibits a monotonically decreasing sigmoid norm. Besides these geometric effects, it is possible that alternating temperatures produce specific deleterious effects, resulting in smaller phenotypes. This point, however, requires specific investigation within the thermal viability range.

Within-line variability

Considering the within-line variance, the two stressful regimes had similar effects – that is, a very significant increase in the variability among individuals. An increase in phenotypic variance under stress is a fairly general observation in *D. melanogaster*, whatever the kind of stress, either environmental (Imasheva *et al.*, 1994; Pétavy *et al.*, 2001b) or genetic (inbreeding depression: Hoffmann and Parsons, 1991, 1997; outbreeding depression: David *et al.*, 2002). For comparative purposes, it is better to consider the coefficients of variation (CVs). Under non-stressful conditions, we found for wing and thorax length average CVs of 1.80 and 2.10 respectively, in close agreement with other studies (David *et al.*, 1994; Karan *et al.*, 1999). Under stressful conditions, the mean CVs rose to 2.80 and 3.10, respectively. This corresponds to more than a doubling of the within-line variance.

The biological significance of the within-line (full-sib family) variance is not clear. According to classical genetic models (Falconer and Mackay, 1996), this variance harbours not only the environmental component (V_E) but also a part of the genetic variance (V_G). In many cases, investigators of natural populations have considered that the within-family variance was an approximation of V_E (Bell, 1997). Our results clearly show that within- and between-family variances can change in opposite directions. This is an argument for considering that the V_E part is preponderant in the within-line variance (Hoffmann and Schiffer, 1998).

Between-line variability

The variability among isofemale lines estimates a genetic component (Hoffmann and Parsons, 1988; Capy *et al.*, 1994; David *et al.*, in press). Under constant thermal conditions, within- and between-line variance exhibit generally parallel variations (Noach *et al.*, 1996; Karan *et al.*, 1999) so that heritability may remain stable (Hoffmann and Merilä, 1999). However, because of the founder effects that occur when lines are isolated, it is likely that V_G is not the additive variance but harbours significant dominance and epistatic components (see Wolf *et al.*, 2000). Using two alternating temperature regimes, we found significant variations in genetic variance, but in opposite directions: an increase under cold stress and a decrease under heat stress. Since in all cases the within-line component was increased, this results in a stability of heritability in cold conditions, but a decrease in hot conditions. An increase in the genetic variance of size traits under extreme low or high constant temperatures has often been observed (Noach *et al.*, 1996; Imasheva *et al.*, 1998, 2000; Karan *et al.*, 1999), although not always (Hoffmann and Merilä, 1999). It is worth mentioning that what might be a rule in *D. melanogaster* may not be valid in its sibling *D. simulans* (Imasheva *et al.*, 2000).

Evolvability (here estimated by the genetic CV) measures the amount of change that might be observed under selection. It takes into account the genetic variance and the mean value of the trait. Since changes in trait means remained relatively small, variations in evolvability were mostly related to changes in the genetic variance. Evolvability was slightly greater for wing than for thorax length, but significant differences were observed between treatments: an increase under cold stress and a decrease under heat stress. In other words, the evolutionary rate of the investigated populations would be much faster under cold alternating temperatures.

Correlations

The isofemale line design permits an analysis of correlations, either within or between lines (Via, 1984; Gibert *et al.*, 1998). Within lines, the wing–thorax correlation was found to be fairly stable and slightly higher than 0.70, in line with previous studies (David *et al.*, 1994; Karan *et al.*, 1999). This might be a fairly stable property of the species, in relation to developmental constraints. Our results are interesting in two ways. First, when we compared the four experimental treatments, we found a significant line effect, which suggests that some lines express a stronger correlation than others. In other words, the correlation appears to be genetically variable among lines. Second, we did not find any change in the average correlation among treatments. Based on theoretical considerations (Kristensen *et al.*, 2003) it would seem that an increase in the phenotypic variance of two correlated variables should increase the observed correlation. This was not the case in our study; this result is difficult to interpret and deserves further investigation.

Genetic correlations between wing and thorax length among family means did, however, change among environments. The major difference was found between the two alternating temperatures: $r = 0.79 \pm 0.08$ under 8–25°C and $r = 0.44 \pm 0.03$ under 18–33°C. This difference parallels that observed between the genetic variances and is probably due to parallel changes in genetic covariance.

The isofemale line technique also allows an investigation of the correlations between the same trait in different environments (Falconer and MacKay, 1996; Karan *et al.*, 2000). Correlations (Table 5) were greater between constant temperature conditions (0.67 ± 0.08) and decreased when the alternating temperature regimes were involved (average $r = 0.46 \pm 0.03$, $n = 5$). This result suggests that different size trait genes are involved under constant and alternating temperatures.

Ecological and evolutionary consequences

As stated in the Introduction, daily thermal cycles are a rule in nature, and we may ask the question: What are the consequences of such cycles for the phenotypes of wild-living flies?

It is well known that the size of wild-living *Drosophila* is both very variable and on average smaller than that of flies raised at the same constant temperature in the laboratory (David *et al.*, 1980, 1997; Coyne and Beecham, 1987; Moreteau *et al.*, 1995; Imasheva *et al.*, 1997; James *et al.*, 1997; Gibert *et al.*, 1998). Significant differences among populations may reflect latitudinal clinal patterns (James *et al.*, 1997) or seasonal temperature variation (Kari and Huey, 2000). It is generally assumed that, in a given place, the huge phenotypic variability arises mostly from nutritional variations and also from thermal fluctuations. Nutritional variations arise because some larvae do not find sufficient resources and must

pupate prematurely. The effects of thermal fluctuations are more difficult to analyse. As a whole, a local population is submitted to the same average temperature. However, different resources may be available in divergent microclimates (Feder, 1997). Also, since the adult ages are not identical, some may have experienced different conditions during their development (Chakir *et al.*, 2002). Finally, besides the mean local temperature, the daily amplitude may vary, resulting on average in smaller and more variable adults. All these observations show how it will be difficult, from a phenotypic analysis, to understand the breeding environment (resources and temperature) of a given fly. These effects mostly contribute to the environmental component of the variance, explaining why, in nature, heritability is much less than in the laboratory, at least for morphometric traits (Coyne and Beecham, 1987; Gibert *et al.*, 1998; Orengo and Prevosti, 1999; Hoffmann, 2000). Our results demonstrate that the genetic variance may also be affected, but in a complex way, highlighting the need for more extensive studies.

Finally, the significant difference between the two cycling regimes raises the question of its possible adaptive value. In this respect, we wish to point out that we used two French populations in the vicinity of Paris, living under a cool temperate climate where cold stress is common, either during the night or during the intermediate seasons, while heat stress is much less frequent. There are only a few days in each year during which day temperature reaches 33°C, and such stress lasts only for a few hours a day. It is also known that larval breeding sites may remain at a much lower temperature than the air, due to evaporation (Feder, 1997), thus reducing the heat stress. It is thus possible to argue that, near Paris, cold stresses are a rule but heat stresses are exceptional, and that the increase in genetic variance specifically concerns the conditions encountered in nature. On the other hand, heat stresses are expected to be the rule for tropical populations of the same species. In this respect, Noach *et al.* (1996) described a clear difference between a European and an Afrotropical population in terms of their change in genetic variance under various constant temperatures. De Moed *et al.* (1997) found different responses of the genetic variance under low and high temperature stress, when the thermal effects were combined with a nutritional stress. Clearly, more extensive investigations are needed in two directions: first, a comparison of temperate and tropical populations; second, a comparison of the effects of different kinds of stress, and especially of interactions among stresses.

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