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Genotype-specific responses of fluctuating asymmetry and of preadult survival to the effects of lead and temperature stress in *Drosophila melanogaster*

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"Capsule": *Caution should be used in using fluctuating asymmetry as a stress indicator.*

Abstract

Although fluctuating asymmetry (FA) increases with exposure to certain types of environmental stressors such as temperature extremes, relatively little is known about the effects of interaction (e.g., synergism) between known sources of environmental stress on FA. Knowledge of such interaction effects, and of the magnitude of genotype-by-environment interaction, are of fundamental importance toward predicting the usefulness of FA as a bioindicator of environmental pollution. We tested for synergistic effects on FA between elevated temperature and exposure to lead, and examined FA responses simultaneously in four genetic strains of *Drosophila melanogaster* known to differ in their degree of developmental instability, and presumably in their buffering capacity. In the absence of heavy metal, bristle FA increased with temperature, but in the presence of lead, FA at high temperature (30 °C) was reduced to levels similar, or below, that at lower temperature (25 °C). This temperature by lead interaction was statistically significant, but paradoxical in that the disruptive effects of temperature appeared to be attenuated in the presence of the heavy metal. In no case was there a significant effect of lead on bristle FAs, despite documented assimilation of heavy metal by flies, and in no case was the genotype by environment interaction significant. Whereas lead treatment did not influence survival, survival was reduced at the high temperature, but significantly so only in one genetic strain (Oregon-R). There was no relationship between survival and FA across stress treatments within lines. Thus, any disproportionate stress-induced mortality in developmentally unstable classes (developmental selection) was unlikely to bias the FA results. Our results underscore the need for independent replication of significant findings before FA-based biomonitoring can be responsibly and effectively implemented. The results call for caution in using FA as a biomarker of stress, because stress factors may interact in complex and unpredictable ways, which could result in erroneous conclusions about real levels of stress present in field populations, under the unduly simplistic assumption that stress factors will act additively to increase FA.

Keywords: Fluctuating asymmetry; Biomonitoring; Environmental stress; Temperature; Lead; Genotype by environment interaction; Developmental selection

1. Introduction

Fluctuating asymmetry (FA), the subtle deviations from perfect symmetry in otherwise bilaterally symmetrical morphological structures of organisms (Ludwig, 1932; Van Valen, 1962), has received a considerable amount of attention as a possible general (nonspecific) indicator of environmental stress (Hoffmann and Parsons, 1991; Sommer, 1996; Hoffmann and Woods, 2003; Leung et al., 2003). Environmental stress, by requiring organisms to divert energy to somatic maintenance at the expense of growth and reproduction, may lead to significant deterioration of fitness components, and therefore possibly to subsequent deterioration of population

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viability and community composition (Hoffmann and Parsons, 1991). Optimism favoring FA as a biomarker relates to the expectation that it might be sufficiently sensitive to serve as an "early warning" signal of populations suffering stress, alerting investigators to the presence of stress before life history traits of individuals themselves are damaged and ecological processes altered (Clarke, 1993; Graham et al., 1993a; Lens et al., 2001). Valentine et al. (1973, p. 368) suggest that analyses of asymmetry within populations may "increase the lead time between the detection of significant biological changes and community morbidity." Notable advantages of FA as a biomarker are that FA responses to stress may be examined in a wide range of habitats, organisms and traits, and that FA-based approaches are noninvasive and relatively straightforward and inexpensive to implement (Lens et al., 2001; Leung et al., 2003).

In support of the approach are several studies that have detected FA responses to stress in a variety of organisms. For example, Parsons (1962) showed that FA in sternopleural bristles of *Drosophila melanogaster* increased with temperature, from 25 to 30 °C, a finding confirmed by Imasheva et al. (1997) working with *D. melanogaster* and *D. buzzatii*. These latter authors studied FA responsiveness across a temperature range similar to that of Parsons (1962), and in three traits: sternopleural bristles, arista branches and wing length. Freebairn et al. (1996) and McKenzie and Yen (1995) found that developmental temperature, insecticide and larval density all influenced bristle asymmetry in the Australian sheep blowfly, *Lucilia cuprina*, and that these effects were genotype-specific. Valentine and Soulé (1973) found elevated FA in pectoral fins of grunion, *Leuresthes tenuis*, exposed to DDT, and Pankakoski et al. (1992), Graham et al. (1993b), and Eeva et al. (2000) found associations between elevated FA and exposure to heavy metals in skull traits in shrews (*Sorex araneus*), bristle number in *Drosophila melanogaster*, and femur length in the flycatcher, *Ficedula hypoleuca*, respectively.

In contrast, many studies have been unable to detect the expected relationship between stress and FA. For example, whereas some studies have reported a positive relationship between larval density and adult FA in some flies (Diptera) (Clarke and McKenzie, 1992), others have failed to do so (e.g., Mpho et al., 2000). Parsons (1961) showed that FA in sternopleural bristles in *D. melanogaster* was unaffected by phenylthiourea, despite this chemical having caused reductions in fly weight and delays in emergence time. Likewise, Polak et al. (2002) found that exposure to arsenic did not increase FA in sternopleural bristles, despite negative effects of the heavy metal on body size and bristle number. David et al. (1998) did not detect a significant effect of nutrition stress on ornament (eye span) asymmetry in stalk-eyed flies, *Cyrtodiopsis dalmani*. Woods et al. (1999) tested for FA responses in three metric traits and two meristic traits to cold-ethanol-nutrition stress in *D. melanogaster*, and found the predicted increase in FA with stress in only one trait (orbital bristles).

The available evidence, therefore, indicates that stress–FA relationships are inconsistent, and that when FA responses do occur, they often are trait-specific. Thus, attempts at developing FA as a biomarker will require experimental demonstration of a causal relationship between stress and FA in the species and trait(s) under examination. In the event of uncovering significant stress–FA associations, a prudent next step would be independent replication of the results (Palmer, 2000; Palmer and Strobeck, 2003). Only after independent confirmation of stress–FA relationships will FA in a set of traits be reasonably useful and appropriate to serve as a biomarker of environmental stress. For example, given the growing evidence derived from different species and laboratories of an effect of temperature on FA (Thoday, 1958; Parsons, 1961; Clarke and McKenzie, 1992; Freebairn et al., 1996; Woods et al., 1999; Chapman and Goulson, 2000), it seems increasingly likely that elevated FA in individual characters, or FA combined across a given set of characters *the FAs of which are known to be sensitive to temperature*, could be diagnostic of thermal stress in the environment. One explanation for the apparent greater responsiveness of FAs to temperature compared with other stress factors is that "temperature might be a generalized stress with multiple consequences for the basic physiology of the organism" (Hoffmann and Parsons, 1991, p. 106). Other stress factors, consequently, may be found to affect FA less often because they disrupt fewer metabolic pathways and developmental processes.

Because organisms in nature will be exposed to multiple stress factors, often simultaneously (Hoffmann and Parsons, 1991; Feder and Hofmann, 1999), and because most natural populations are genetically heterogeneous, another important step in the FA validation process will be to examine the extent to which environmental stressors might interact to influence FA, and the importance of any genotype by environment (G×E) interaction. For example, significant G×E interaction could attenuate the FA signal, especially if genotypes arising from variation segregating at one or a few loci differ in the direction of an FA response to stress.

In the present study, we examine the effects of temperature and exposure to lead, and effects of an interaction between these environmental stress factors, in sternopleural bristle FA of *D. melanogaster*. We chose to study these factors because previous work has demonstrated that both increase sternopleural bristle FA in this species (Parsons, 1962; Graham et al., 1993b; Imasheva et al., 1997). Moreover, exposure to lead is known to be related to a variety of reproductive and developmental deficits in animals (Apostoli et al., 1998;

De Gennari, 2002; Mendola et al., 2002; Morley et al., 2003). In Graham et al.'s (1993b) study sternopleural bristle asymmetry increased in dose-dependent manner with lead concentrations ranging from 0.001 to 10 ppb in the food medium on which flies were reared. Indeed, this study, which concluded that "fluctuating asymmetry is a valuable tool for studying stress in natural populations" (p. 193), is often cited as demonstrating effects of chemical stress on FA (e.g., Hallgrímsson, 1998; Chapman and Goulson, 2000; Graham et al., 2000; Hardersen, 2000; Polak et al., 2002).

In addition to seeking potential interaction between stress factors on sternopleural bristle FA, we incorporated into our study four genetic strains differing in their degree of developmental stability (determined on the basis of a preliminary survey of FA in eight fly strains). The role of positional FA (PFA) (Polak, 1997) is specifically evaluated, because both a developmental model (Starmer et al., 2002) and empirical data (Polak, 1997; Polak and Starmer, 2001; Polak et al., 2002) suggest it may be a sensitive indicator of underlying developmental stability. Because developmental stability is expected to reflect individual quality (Møller and Pomiankowski, 1993; Polak and Trivers, 1994), which subsumes ability to tolerate stress conditions, we predicted significant G×E interaction effects on FAs, such that high developmental instability strains would express greater FA under stress compared with relatively developmentally stable lines. Because we controlled larval densities across treatment categories, we were also able to measure egg-to-pupa survival rates, and hence address any potential confounding effects of differential mortality across developmental instability classes (Møller, 1997; Polak et al., 2002; Hoffmann and Woods, 2003).

2. Material and methods

2.1. Fly strains and rearing protocol

Eight wild type strains of *Drosophila melanogaster*, obtained from the *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA), were characterised in terms of FA in sternopleural bristles. Adult flies were allowed to lay eggs for 12 h on 1×1 cm squares of sterile Kleenex® tissue lightly coated with a yeast-molasses slurry. Under a stereomicroscope embryos on the tissue were counted. Embryo number on the tissue pieces was adjusted to exactly 50 by removing and discarding excess embryos with fine forceps; there was no contact with embryos from which experimental animals would be reared. Each tissue square with its complement of 50 embryos was transferred to a food bottle (squares were gently laid on the surface of the substrate), containing 5 g Instant Drosophila Medium™ (Carolina Biological), and 30 ml deionized water. Each line was reared from 2 to 4 replicate bottles (mean = 3 bottles). Adults emerging from bottles were harvested, anesthetized with ether, and under an Olympus stereomicroscope anterior and transverse sternopleural bristles on each fly were counted. Sternal bristles were not counted (Polak, 1997). All bristle counts throughout this study were done by one investigator (DEK).

2.2. Fluctuating asymmetry, size and measurement error

Fluctuating asymmetry (FA) was calculated as the absolute value of the difference in bristle number between right and left sides of the body (|R−L|). FA1 and FA2 refer to FA in the anterior sternopleural bristles and transverse sternopleural bristles, respectively. Each type of bristle [referred to as setulae in Polak (1997)] occurs in a distinct area on the sternopleuron (see Fig. 1 in Polak, 1997). Positional FA (PFA, Polak, 1997) was calculated as the absolute value of the difference in the ratio of these bristle types across the two sides of the body, and hence captures asymmetry in the placement of bristles on the sternopleuron:

$$\mathrm{PFA} = \Big|\big(\text{right number anterior bristles} / \text{right number transverse bristles}\big) - \big(\text{left number anterior bristles} / \text{left number transverse bristles}\big)\Big|.$$

Trait size was calculated as the mean number of bristles across the two sides of the body ((R + L)/2) for a given trait. Size1 and size2 correspond to trait size in anterior sternopleural bristles and transverse sternopleural bristles, respectively. Size1,2 is trait size summed over size1 and size2. Size1, size2, and size1,2 are corresponding measures of trait size for FA1, FA2 and PFA, respectively.

The magnitude of measurement error was assessed following the recommendations of Palmer and Strobeck (1986) which employs a two-way ANOVA where sides and individuals are entered as factors. Two replicate counts were made on each of 21 flies selected at random from a lab culture at the onset of the study. Counts were made 2 weeks apart and in different order; flies were stored frozen between counts. One counting error was made in the magnitude of one anterior bristle. No counting errors were made in the transverse sternopleural bristles. The resultant variance estimates due to measurement error, as a percentage of the between-sides variance representing FA (side×male interaction mean square), were 1.85 and 0.70% for FA in anterior bristles and for PFA, respectively. Thus, variability between sides due to measurement error was negligible relative to actual bristle asymmetry.

2.3. Incorporation of lead by flies

To test whether flies incorporate lead during development, we conducted an experiment in which flies were reared from bottles containing 100 mg Pb/kg Instant Drosophila Medium, and using inductive coupled plasma mass spectroscopy measured the level of Pb in adult flies; subsets of which were measured at 6 and 24 h post-eclosion. Flies were allowed to pupate in sterile Kleenex® tissue placed into bottles just before flies began to pupate. After all larvae had pupated, tissues were removed from contaminated bottles and the sections that had come into contact with the contaminated medium were cut away. Tissues were transferred to bottles containing uncontaminated food medium, and adult flies were harvested as they emerged (i.e., as tenerals). These procedural steps prevented the newly emerged adults from ingesting lead from contaminated medium.

Adult flies were held in vials with agar-molasses substrate until processing. At processing, flies were surface-washed in 5 ml mild (1%) nitric acid, dried in an oven at 65 °C and transferred to Teflon vessels previously cleaned with nitric acid and hydrogen peroxide in a microwave oven and dried. For the dissolution of flies, 100 mg of dried sample were added into a clean microwave vessel. Five milliliters of 50% pure nitric acid were added and a heating program shown in Table 1 was used for the mineralization. The digested solution was cooled in the microwave oven with an air stream, and diluted further to a final volume of 10 ml.

The Pb concentration was determined in the solutions obtained from the acid microwave mineralization by inductively coupled plasma mass spectrometry (ICP–MS) in quantitative mode. In this mode, internal standards were used to correct the reduction of intensity due to the acidity of the sample. A calibration curve with varying amounts of lead (in parts per billion, ppb) and with the same internal standard as the samples was made, yielding a regression coefficient of 0.998. Procedural blanks consisted of flies that had not been exposed to $PbCl_2$ but otherwise were treated identically. These blanks were also analyzed for their lead content to indicate background levels of the heavy metal.

Table 1
Conditions for microwave digestion

Stage	Power (w)	Pressure (psi)	Run time (min)	Temp (°C)
1	237	20	5	120
2	427	80	5	140
3	522	120	5	150
4	617	170	5	165
5	0	20	5	25

2.4. Genotype, lead and temperature treatments

An initial screen for PFA differences among the eight lines was conducted using analysis of covariance (ANCOVA), in which line and bottle (nested within line) were the factors, and trait size the covariate. On the basis of this analysis, four lines were chosen (see Section 3.2) for further study.

Tissue squares with embryos from each chosen line were transferred to experimental culture bottles (see Section 2.1). Treated bottles contained lead chloride ($PbCl_2$) at either of two concentrations, 1.0 and 100.0 mg Pb kg^{-1} dry Instant Drosophila Medium, and which encompass those used by Graham et al. (1993b). No $PbCl_2$ was added to control bottles. Bottles from each category (i.e., 0, 1.0 and 100.0 mg Pb kg^{-1}) were distributed across two temperature treatments, 25 °C day:23 °C night and 30 °C day:28 °C night cycles within a Percival incubator. Both temperature treatments were at a 12 h light:12 h dark photoperiod. Flies emerging from bottles were harvested, frozen and their bristles counted within 2 weeks of emergence. Three bottles for each line×concentration×temperature combination were used. A total of 2026 flies were scored in this part of the study.

Fixed effects of line, lead treatment and temperature, and interaction terms between them, on absolute PFA were tested using ANCOVA in which size1,2 was the covariate. Bottle, a random factor, was nested within lead treatment. *F*-statistics were calculated using appropriate mean squares (SAS, 1990).

FA1 and FA2 were first analyzed with multiple analysis of variance (MANOVA) using the same factors as in the above ANCOVA on PFA. The two FA measures were first each regressed on their corresponding measure of trait size (size1 and size2, respectively), and the residuals entered into the MANOVA. Roy's greatest root *F* statistic was employed to derive statistical conclusions (Scheiner, 1993).

In a second approach, FA1 and FA2 were each analyzed separately using univariate ANCOVA, with trait size as the covariate, as in the analysis of PFA described above. In addition, univariate ANOVAs on FA residuals were conducted and the consistency of the results compared to that from ANCOVA. Residuals were generated by regressing FA1 and FA2 each on its corresponding measure of trait size; the relationship between FA and size was linear in both cases and quadratic terms in separate tests were nonsignificant (*P* values >0.3). The numeric results of these ANOVAs were similar to those from ANCOVAs, and there were no qualitative differences in the statistical conclusions drawn, i.e., the factors that emerged significant or nonsignificant were the same in both analyses. Output from ANCOVA are presented in Section 3 because they are easier to interpret than residuals. Sex was a nonsignificant factor in all analyses and therefore was excluded from further consideration.

Survivorship was calculated as the number of flies emerging from each bottle divided by 50 (i.e., number of embryos that seeded each bottle, see above). Survivorship data were analyzed using ANOVA with line, temperature and lead concentration entered as factors. The relationship between mean survivorship and FA across bottles was examined using regression analysis. Because line effects on survivorship were significant (see Section 3), regression analyses were conducted for each line separately. All statistical procedures were conducted using SAS (1990).

3. Results

3.1. Descriptive statistics

Descriptive statistics for signed (R−L) asymmetry values are presented in Table 2. For each measure of asymmetry, the mean of the signed values did not differ significantly from zero. Thus no directional asymmetry was present, indicating that the asymmetry measures represent conventional FAs. Skewness and kurtosis values are also presented.

The correlation between absolute FA and trait size in the data set as a whole was positive and significant for FA1 ($r=0.233$, $P<0.0001$), FA2 ($r=0.229$, $P<0.0001$) and PFA ($r=117$, $P=0.0021$, all n values $=686$); all correlations remained significant in analyses conducted separately by sex. Although the FA1–FA2 ($r=0.00490$, $P=0.90$) correlation was not significant, both the FA1–PFA ($r=0.822$, $P<0.0001$), FA2–PFA ($r=0.157$, $P<0.0001$) correlations differed significantly from zero. SIZE1 and SIZE2 were strongly positively correlated ($r=0.347$, $P<0.0001$, $n=686$).

3.2. Inter-line differences in PFA

Nested ANCOVA on PFA with trait size entered as a covariate revealed significant effects of line ($F_{7,21.9}=3.19$, $P=0.0018$) but not of bottle ($F_{16,661}=1.12$, $P=0.33$). Mean PFA values ranged from 0.0535 (KSA2) to 0.113 (CT106), representing >2-fold difference across lines. To examine the effects of lead and temperature on PFA, we selected two lines at the low (KSA2 and Oregon-R) and high (CT106 and CT109) ends of the distribution of PFA means.

Table 2
Descriptive statistics for signed (R−L) values of FA1, FA2 and PFA

Trait	Mean (P[a])	n	VAR	Skewness	Kurtosis
FA1	−0.0117 (0.8)	686	1.057	0.306	3.097
FA2	0.0292 (0.6)	686	1.879	0.0565	0.0578
PFA	−0.00186 (0.7)	686	0.0131	0.401	3.37

[a] P testing H_0: mean = 0.

3.3. Lead uptake by flies

Adult flies reared from medium containing lead (100 ppm) were processed 6 and 24 h post-emergence. Mean detected lead concentration across samples was 94.3±5.9 ppm ($N=3$ samples) at 6 h and 18.3±0.67 ppm ($N=4$ samples) at 24 h post-emergence, a decrease that was statistically significant ($t=10.9$, df = 5, $P=0.0001$). Concentration of lead in the surface wash was negligible (mean = 0.045±0.020 at 6 h, and 0.020±0.0040 at 24 h, both Ns = 3 samples). Lead content in samples of control flies that had not been exposed to lead was also negligible (0.040±0.040, $N=4$ samples), representing less than 0.05% of that detected in samples of 6 h old flies reared on medium with lead.

Thus, flies reared in lead-contaminated medium assimilated the heavy metal, and metal content within their adult bodies decreased as they aged from 6 to 24 h post-emergence.

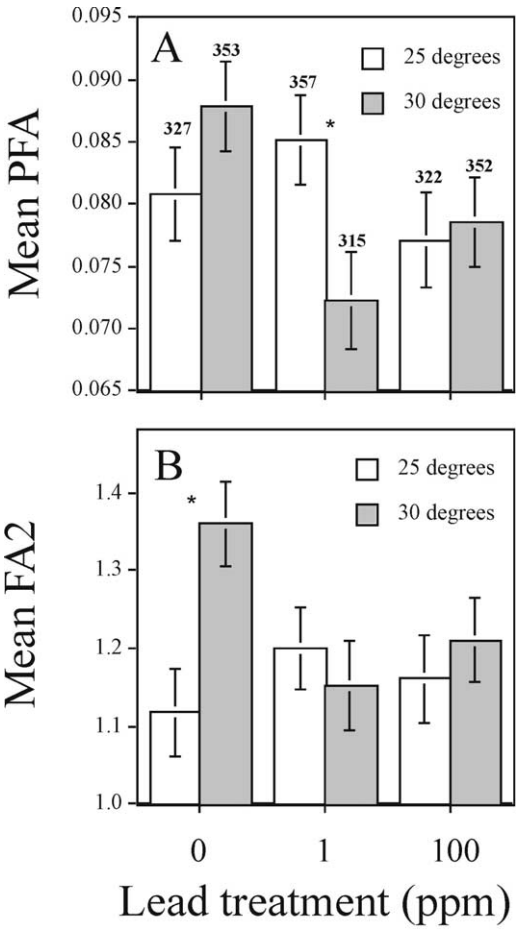

Fig. 1. Effect of lead and temperature treatments on absolute (unsigned) positional fluctuating asymmetry (PFA) (panel A), and absolute fluctuating asymmetry in transverse sternopleural bristles (FA2) (panel B). All four lines are represented. Error bars represent±1 standard error. Numerals above means in panel A represent sample sizes (number of flies), which apply also to means in panel B. The asterisks indicate the contrasts between means that were significant ($\alpha=0.05$) by the Tukey procedure.

3.4. Effects of Pb, temperature and genotype×environment interaction

We used four lines to test for responses to experimental factors in three FA measures: PFA, FA1 and FA2. For PFA, the effect of line was strong ($F_{3,\ 1995}=33.3$, $P<0.0001$), but the effects of lead treatment ($F_{2,\ 6.1}=1.12$, $P=0.39$), temperature ($F_{1,\ 1995}=0.23$, $P=0.63$) and bottle ($F_{6,\ 1995}=1.61$, $P=0.14$) were not significant. (When this analysis was performed without "bottle" as a factor, the effects of lead treatment remained nonsignificant, $F_{2,\ 2001}=1.32$, $P=0.27$). Interaction terms in the ANCOVA between line (genotype) and either lead ($F_{6,\ 1995}=1.14$, $P=0.34$) or temperature ($F_{3,\ 1995=}0.53$, $P=0.66$) were nonsignificant. The three-way interaction was likewise nonsignificant ($F_{6,\ 1995}=1.02$, $P=0.41$). There was, however, a significant interaction between lead concentration and temperature for PFA ($F_{2,\ 1995}=3.81$, $P=0.022$): whereas mean PFA tended to increase with temperature when no lead was present, PFA decreased sharply with temperature in the presence of 1 ppm lead (Fig. 1A). At highest lead concentration, PFA means across the two experimental temperatures were similar.

MANOVA on residuals in FA1 and FA2 revealed a significant overall effect of line ($F_{3,1996}=3.25$, $P=0.021$), but not of temperature or concentration (both Ps >0.05). Interaction terms were nonsignificant except again for the concentration×temperature interaction ($F_{2,\ 1996}=3.88$, $P=0.021$). For this interaction the standardized canonical coefficients for FA1 and FA2 were $+0.33$ and $+0.93$, respectively. The greater magnitude of the coefficient for FA2 indicates that interaction effects were strongest for this response variable. The fact that both coefficients are positive indicates that the direction of the response across treatments was similar for both FAs.

Results of univariate ANCOVAs confirmed these MANOVA-derived conclusions. For FA1, no factor reached statistical significance in the analysis (all Ps >0.13), and there was a marginally nonsignificant effect of line ($F_{3,\ 1995}=2.45$, $P=0.062$). For FA2, no factor emerged significant, with the exception of the interaction between lead and temperature treatments ($F_{2,\ 1995}=3.56$, $P=0.029$): in this case there was a significant increase in FA at 30 °C when no lead was present, but at 1 ppm the two temperature means converged (Fig. 1B). This pattern of variation is in general agreement with that for PFA.

Thus, FA in sternopleural bristles of *D. melanogaster* did not increase with lead concentration. Instead, FA in the transverse sternopleurals increased significantly with temperature when no lead was present, and temperature and lead interacted such that the effect of temperature was either lost (in the case of FA2) or reversed (in the case of PFA) in the presence of heavy metal. Because our results are inconsistent with the study of Graham et al. (1993b) that used the Oregon-R line to assess the effect of lead on sternopleural bristle FA, we analyzed the data for this line separately (Table 3), so that our results could be better compared to those of this previous report. ANCOVA on Oregon-R flies, with bristle number entered as a covariate, again revealed no effect of lead treatment on either FA1 ($F_{2,\ 6.21}=0.34$, $P=0.73$), FA2 ($F_{2,\ 6.08}=2.61$, $P=0.15$) or PFA ($F_{2,\ 6.27}=0.33$, $P=0.73$). The analysis likewise revealed no effect of either temperature or bottle on any FA measure (all Ps >0.1). However, the interaction term between temperature and lead treatment was again significant for FA2 ($F_{2,\ 520}=4.08$, $P=0.018$), but not for FA1 or PFA (both Ps >0.15). The nature of the significant interaction for FA2 is illustrated in Fig. 2A (means across lead concentrations are grouped together in this figure to emphasize changes in FA means across lead treatments at the two temperatures).

To help interpret these FA results for the Oregon-R strain in light of the developmental selection hypothesis, ANCOVA on survivorship data was also performed with temperature and lead treatment as factors. Mean FA2 (per bottle) was the covariate. Whereas the effect of temperature was significant ($F_{1,\ 11}=8.1$, $P=0.016$), the effects of lead treatment ($F_{2,\ 11}=0.56$, $P=0.6$), the

Table 3
Variance of signed asymmetry (R−L) and mean absolute (unsigned) asymmetry for three bristle asymmetry measures among Oregon-R flies exposed to different concentrations of lead chloride (mg PbCl/kg food medium)

Trait	Statistic	Concentration (sample size)		
		0 ($n=178$)	1.0 ($n=178$)	100.0 ($n=177$)
FA1	VAR (R−L)	0.672	0.779	0.669
	Mean |R−L|±S.E.	0.596±0.042	0.640±0.045	0.582±0.044
FA2	VAR (R−L)	2.308	1.886	1.996
	Mean |R−L|±S.E.	1.186±0.071	1.00±0.070	1.130±0.064
PFA	VAR (R−L)	0.0111	0.0119	0.0112
	Mean |R−L|±S.E.	0.0815±0.0051	0.0857±0.0050	0.0836±0.0050

interaction between temperature and lead ($F_{2,\ 11}=10$, $P=0.4$), and the FA2 covariate ($F_{1,\ 11}=0.0$, $P=0.9$) were not significant. Survivorship means across temperature and lead treatment are presented in Fig. 2B.

3.5. Effects on egg-to-pupa survivorship across lines

Effects of lead on preadult rates of survivorship among four strains of flies, reared at the two temperatures and three heavy metal treatments, were evaluated. Factorial ANOVA revealed significant effects of line ($F_{3,\ 45}=12.13$, $P<0.0001$) and temperature treatment ($F_{1,\ 45}=5.59$, $P=0.022$), but not of lead treatment ($F_{2,\ 45}=1.30$, $P=0.28$), on the dependent variable. Fig. 3 shows survivorship values across lines and temperature treatments. Across all lines, the trend was such that survivorship was reduced among flies reared at 30 °C, although the difference between temperatures was significant only in Oregon-R, indicating that this genotype exhibits enhanced sensitivity to temperature stress. No interaction term in the model was significant (Ps > 0.1).

Thus, exposure to lead at either concentration had no detectable effect on egg-to-pupa survivorship in *D. melanogaster*. In contrast, elevated temperature significantly reduced survivorship, although this effect was line-dependent, as revealed by post hoc tests.

Because line effects for both PFA (see Section 3.4) and survivorship were strong, the relationship between these variables was examined. This relationship was found to be pronounced and negative (Fig. 4), indicating that the most symmetrical genotypes exhibited highest preadult survival rates.

In contrast, the relationship between survivorship and PFA *within* any of the four lines was not significant (Table 4). Each PFA datum in these regressions represents a bottle mean, and survivorship for each corresponding bottle is expressed as the number of adults emerging divided by 50 (see Section 2). Data were pooled across lead and temperature treatments. Likewise, relationships between survivorship and either FA1 and FA2 were nonsignificant (Ps across lines ranged from 0.07 to 0.75). Multiple regressions were also performed on survivorship in which mean FA and mean trait size were simultaneously entered as covariates. In none of the 12 multiple regression analyses was either FA or size statistically significant (Ps > 0.05).

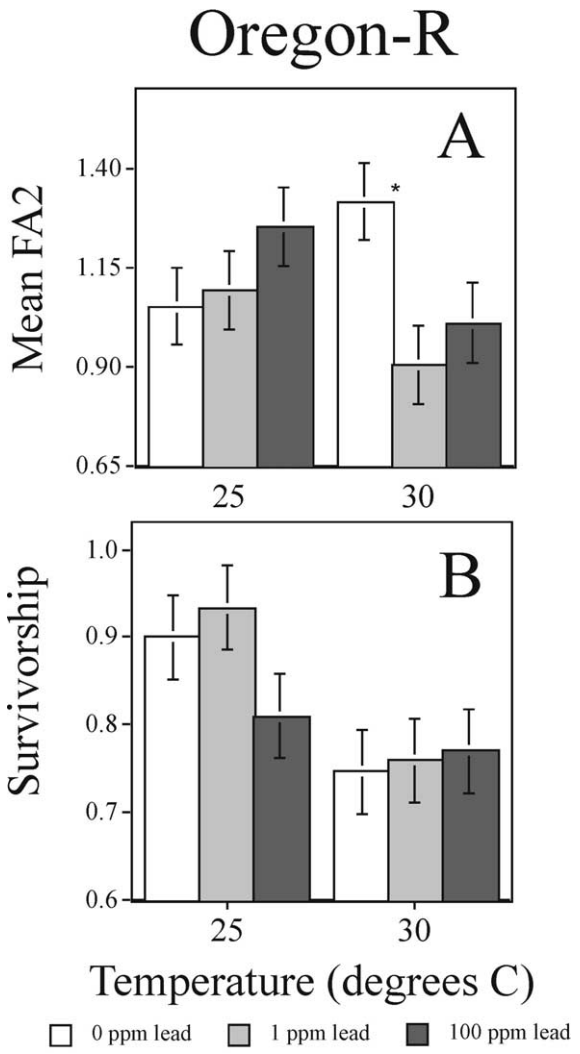

Fig. 2. Interaction between the effects of lead and temperature treatments on FA2 (panel A) and survivorship (panel B) in the Oregon-R strain of *Drosophila melanogaster*. Error bars represent ±1 standard error. Sample sizes range from 88 to 90 flies for each FA2 mean, and three bottles for each survivorship mean. The asterisk indicates the contrast between means that was significant ($\propto=0.05$) by the Tukey procedure.

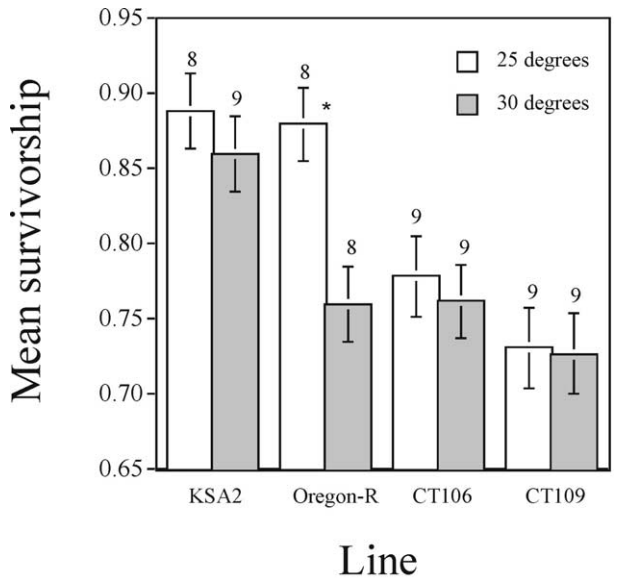

Fig. 3. Effect of temperature on survivorship across all lines. Error bars represent ±1 standard error, and numerals above means represent sample sizes (number of bottles). The asterisk indicates the within line contrast that was significant ($\propto=0.05$) by the Tukey procedure.

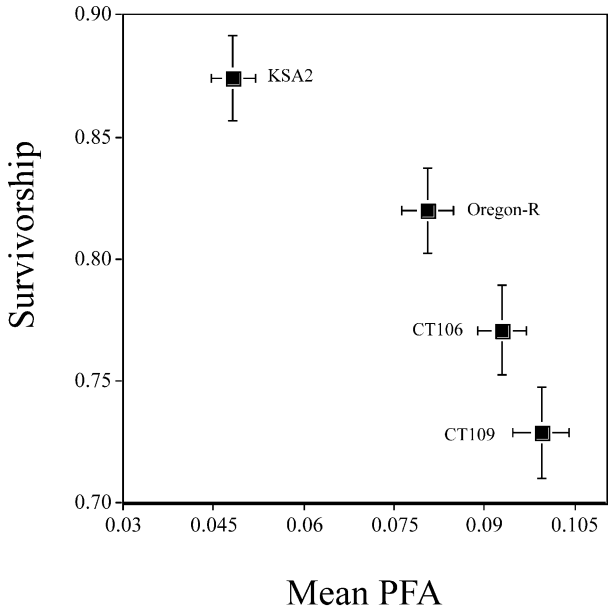

Fig. 4. Relationship between mean survivorship and mean positional FA (PFA) across genetic lines of *D. melanogaster* (Pearson product moment correlation, $r=-0.96$, $P<0.01$). Error bars represent ±1 standard error.

Table 4
Slopes of the regression of egg-to-pupa survivorship on PFA, by line

Line	Slope±S.E.	n[a]	t	P
CT106	−0.0088±0.067	17	0.13	0.90
CT109	−0.039±0.060	16	0.65	0.52
KSA2	0.023±0.022	18	1.0	0.33
Oregon-R	0.029±0.030	18	1.0	0.33

[a] Number of bottles.

4. Discussion

One main goal of this study was to test for synergistic effects between multiple putative stressors, and genotype by environment interaction, on FAs. Information on the importance of such interactions is needed to evaluate the usefulness of FA as indicator of environmental stress in natural populations. This is because free-ranging populations will usually be genetically heterogeneous, and exposed to a multitude of environmental challenges. For example, fly larvae undergoing development in field substrates may experience intense heat, U.V., and desiccation stress, as well as exposure to plant secondary compounds, parasites, and environmental toxins such as heavy metals, among other stressors (e.g., Heed and Mangan, 1986; R'Kha et a., 1991; Feder and Hofmann, 1997). Thus, realistic attempts at validating FA as a biomarker of environmental stress will require greater emphasis on studying FA responses to multiple stressors, and attention to any differential responses of genotypes (and see McKenzie and Yen, 1995, p. 186).

The present work examined FA responses to simultaneous exposure to lead and temperature across four genetic strains of *D. melanogaster*. These strains were chosen because they differed in their level of developmental instability, and therefore, presumably also in their ability to buffer development against stress. The suspected existence of genotype×environment interaction was not borne out by the data despite significant differences in developmental instability across genotypes (lines): there was no evidence for interaction between genotype and either temperature or lead treatment, indicating that FA responses were homogeneous across the four genetic backgrounds examined. In their study of asymmetry in *Lucilia cuprina*, Freebairn et al. (1996) similarly found nonsignificant genotype×environment interactions, but concluded that both genotype and environment can make important contributions to the asymmetry phenotype. They found that asymmetry was highest for temperatures and larval densities above and below standard culture conditions. Although asymmetry and pesticide concentration were positively correlated, there was a plateau level of asymmetry observed for all genotypes that could not be exceeded by exposure to pesticide or to any combination of environmental stressors.

We also tested for synergism between environmental sources of stress. Although overall neither temperature or lead treatment alone significantly increased FA, these factors interacted to influence PFA and FA2. This interaction was complex and not in accord with the straightforward prediction of additive effects of stress on FA, in which case we would have expected FAs to be greatest when both temperature and lead concentration were at a maximum. Instead, FAs increased with temperature only when heavy metal was *absent* from the system.

It therefore appears that temperature effects can be masked by the presence of lead, suggesting *ameliorating* synergism between these environmental factors. Although the mechanism of such an effect is unknown, it is conceivable that some sort of biochemical or physiological response to lead mitigated the deleterious effects of temperature on developmental stability. For example, exposure to lead may have induced expression of a specific class of stress proteins different from that induced by exposure to high temperature alone. Heat shock proteins (Hsps) are molecular chaperones that interact with other proteins in non-native conformations, serving to establish or maintain functionality of these proteins in a variety of scenarios, including when the organism is exposed to stress (Feder and Hofmann, 1999). Indeed, for *D. melanogaster* a possible role for Hsp90 in developmetal stability has recently been suggested (Rutherford and Lindquist, 1998; Klingenberg,

2003). In the present system, induction of greater quantities of a given such protein, or of different classes of stress proteins, may have had a greater positive effect on buffering against stress, offering a possible explanation for observed reductions in FAs when both forms of stress were present. Indeed, various forms of stress, including heavy metals, can result in distinctive patterns of stress protein induction (Stringham and Candido, 1994).

Our results do not agree with those of a previous study on the effects of lead and benzene on sternopleural FA of *D. melanogaster* (Graham et al., 1993b). This study, which used the Oregon-R strain only, showed dose-dependent increases in FA with increasing lead concentration from 0.001 to 10 ppm at 10-fold increments. Our study, in which flies were exposed to 1 and 100 ppm lead, revealed no such increase in FA. We failed to detect an effect despite using multiple genetic strains that differed in their level of developmental instability (which would tend to increase the sensitivity of the assay), and verification, using inductively coupled plasma mass spectrometry, of lead assimilation by the growing larvae. We also present results separately for Oregon-R, so that our data could be better compared with those of Graham et al. (1993b), although it should be emphasized that the flies used in our own work and in Graham et al.'s study undoubtedly differed genetically to some degree. Whereas there was a tendency for FA to increase with lead concentration at 25 °C (Fig. 2), FA differences across lead treatments were not significant.

One possible explanation for this disparity between studies relates to differences in the way in which flies were exposed to heavy metal. In our study, ovipositing females themselves were not exposed to the metal. Instead, eggs were laid by females onto a lead-free substrate (tissue squares), with the squares subsequently placed on the surface of lead-containing medium. In this way, eggs could be counted easily and their numbers manipulated so that larval densities within the food bottles could be standardized exactly across all treatments. The procedure also enabled separation of maternal effects from direct effects of lead on developing progeny (maternal investment ceases once eggs have been laid). In Graham et al.'s study, ovipositing females were placed in vials with lead-contaminated medium for 4 days, so that females had ample opportunity to feed and to provision their ovaries and oocytes with lead-contaminated resources. Hence, it is possible that the increased FA reported by Graham et al. (1993b) occurred by way of the maternal environment deteriorating as a function of ingested lead concentration. Lead sequestered by the ovaries could have interfered with normal developmental processes at the embryo stage, and carried over to later stages of development. Indeed, maternal effects on FA have been demonstrated previously in *D. nigrospiracula* (Polak, 1997), whereby ectoparasitism in mothers increased male offspring PFA in dose-dependent fashion, possibly because of impaired sequestration of specific nutrient constituents by the ovaries of infested females.

Graham et al.'s (1993b) study also did not experimentally control larval densities across treatment categories. However, the number of adult flies emerging per bottle did not differ significantly between treatment categories, suggesting that systematic differences in larval density did not confound their results. Notably though, larval densities were up to four times greater than in our own experiments, so that it is conceivable that FA sensitivity to lead is manifested only under conditions of high larval density—a potential synergistic stressor. Under such conditions there would have been greater larval competition and decreased substrate quality because of larval waste product accumulation. Alternatively, the use of 50 larvae per bottle in our study may itself have been unusually stressful, because a certain minimum larval density not reached in our study may be required for larval "facilitation" though substrate modification.

Another important consideration for studies of FA–stress relationships is the possibility of differential mortality occurring among developmental instability classes. If developmentally unstable genotypes experience reduced probability of survival, for example, under conditions of severe environmental stress, the surviving fraction of the population could exhibit greater developmental stability, on average, and hence reduced FA. For example, in a previous study of *D. melanogaster*, fly larvae surviving exposure to arsenite emerged significantly more *symmetrical* than control flies, suggesting the action of a "developmental filter" through which developmentally unstable individuals cannot pass (Polak et al., 2002). There was also a significant positive relationship between survival and FA in the presence of varying concentrations of arsenite. In the present study, lead did not affect survivorship (a finding consistent with that of Graham et al., 1993b), so that the lack of an effect of lead on FAs is unlikely to be the result of developmental selection (Hoffmann and Woods, 2003). Survivorship did, however, decrease with increasing temperature, although this effect was genotype-specific, being significant only in the Oregon-R strain. Despite this negative effect on survivorship, FA tended to *increase* with temperature, a pattern opposite that predicted by the developmental selection hypothesis. Moreover, there was no relationship between survivorship and mean FA across culture bottles within genetic strains (cf. Fig. 2 in Polak et al., 2002). These lines of evidence suggest that the results of the present study are not confounded by developmental selection.

The main results of the present study can be summarized as follows. Flies exposed to lead during development

(from embryo to pupa) showed no significant increase in adult sternopleural bristle FA. This result is in contrast to a previous study by Graham et al. (1993b). These authors reported dose-dependent increases in FA under exposure to this heavy metal in the same strain and bristle trait of *D. melanogaster* as used here. Thus, the effect of lead on sternopleural FA cannot be seen as a general phenomenon, meaning that perpetuation of the view that lead increases FA will no longer be appropriate. Although FA2 increased with temperature, this effect disappeared in the presence of heavy metal. More remarkable is the finding that PFA decreased significantly at high temperature in the presence of 1.0 ppb heavy metal, a reversal reflected in a significant interaction between temperature and lead treatments. This paradoxical reduction in FA cannot be accounted for by the developmental selection process.

Inconsistent findings of the effects of lead on FA across different laboratories, and evidence for complex, unpredictable interaction between putative sources of environmental stress, highlights the need for a better understanding of the mechanisms of developmental instability before robust predictions can be made regarding FA sensitivity to stress. Our results also call for caution in the use of FA in biomonitoring, because they suggest that attributing patterns of FA variation to particular forms of stress in natural populations will be complicated.

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