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A STUDY OF CANALIZATION AND DEVELOPMENTAL STABILITY IN THE STERNOPLEURAL BRISTLE SYSTEM OF *DROSOPHILA MELANOGASTER*

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Abstract.—Among the explanations for minimizing the effects of extraneous variation has been canalization and developmental stability. However, there is little agreement as to whether these two processes reflect a common set of mechanisms. This confusion is inflated due to the lack of consensus as to a precise definition of canalization. In this study, canalization in the sternopleural bristle system is used to investigate the relationships between measures of canalization and developmental stability by comparing how a panel of naturally derived lines responds to both genetic and environmental perturbations. No evidence for a common mechanism between the different measures of canalization was observed. Furthermore, a hypothesis regarding a common mechanism for environmental and genetic canalization was tested, and no evidence was found to support it. However, there is evidence for a relationship between at least one form of canalization and developmental stability.

Key words.—Canalization, cryptic genetic variation, developmental stability, fluctuating asymmetry, genotype-environment interaction.

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The role of developmental buffering of trait variation in the evolutionary process has recently become a topic of increased interest. However, there is little agreement as to the appropriate target measures of buffering, or what fundamentally defines it. In a number of recent reviews, different definitions of buffering have been explored, especially with regard to the concepts of canalization, developmental stability, and phenotypic plasticity (Gibson and Wagner 2000; Debat and David 2001; Nijhout and Davidowitz 2003). Canalization was intended to describe the underlying mechanism that organisms used to modulate the effects of environmental or genetic perturbations, reducing overall phenotypic variance (V_P) of the trait (Waddington 1952, 1956). However, different components of variation can be distinguished, and there is disagreement as to which aspects of variation should be the focus of studies on canalization (Table 1). Some have argued that a relatively low degree of within-individual variation (V_{Es}) reflects trait canalization (Mather 1953; Reeve 1960; Eshel and Matessi 1998; Gibson and Wagner 2000). However, others suggest that V_{Es} reflects an individual's ability to buffer the effects of random developmental noise (Reeve 1960), with canalization referring to a homeostatic mechanism that buffers against specific and recurrent perturbations (Waddington 1960; Palmer 1994; Debat et al. 2000). This form of macroenvironmental canalization can be considered the opposite of phenotypic plasticity (Nijhout and Davidowitz 2003), where a canalized trait shows no evidence for plasticity across environments ($V_{Em} = 0$).

Wagner et al. (1997) made a further distinction between environmental and genetic canalization, referring to the respective reduction of the effects of external environmental variation or mutational perturbations on trait expression. Genetic canalization refers to the buffering of the effects of genetic variation (V_G). Many classic studies inferred genetic canalization from the observation that otherwise invariant traits demonstrate considerable genetic variation revealed after environmental or genetic perturbation (Waddington 1953, 1956; Dun and Fraser 1958; Rendel 1959; Gibson and Hogness 1996). The logic of the inference is as follows: given that genetic variation for invariant traits is not normally expressed phenotypically, it follows that there is some mechanism that suppresses the effects of the genetic variation. The analysis of cryptic genetic variation has since been extended using quantitative genetics (Gibson and van Helden 1997; Polaczyk et al. 1998; Gibson and Dworkin 2004; Dworkin 2005b). Wagner et al. (1997) developed theory that suggests environmental canalization can evolve, but the conditions for the evolution of genetic canalization are limited. Given the ample evidence for genetic canalization from empirical studies (Dun and Fraser 1958; Rendel 1959; Polaczyk et al. 1998; Atallah et al. 2004; Dworkin 2005b; but see Hermisson and Wagner 2004), Wagner et al. (1997) hypothesized that selection for environmental canalization may facilitate the evolution of genetic canalization. However, the ability to test this is obscured by what components of variation are liable to be reduced by canalization (Table 1).

Here I employ the sternopleural bristle system of *Drosophila melanogaster* as a model for the study of canalization. This is a particularly useful system because inbreeding appears to have no significant impact upon levels of V_{Es} (Imasheva et al. 1998; Gilligan et al. 2000), V_P (Fowler and Whitlock 1994; Imasheva et al. 1998), or the trait mean (Lynch and Walsh 1998), allowing the study of individual genotypes without a concern for experimental artifact. In addition, perturbations such as mutations and rearing *D. melanogaster* under ecologically relevant temperature stress increases V_P for sternopleural bristle number (Beardmore 1960; Mackay 1980; Imasheva et al. 1998, 1999; Bubliy et al. 2000).

To test whether a set of genotypes showed similar response to mutational and environmental stress, I simultaneously used temperature and genetic perturbations on a series of naturally derived lines and examined changes in components of variance (Table 1). The mutation *Sternopleural*[1] (*Sp*[1]) was introgressed into a panel of lines to examine how they respond

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TABLE 1. Definitions of canalization and their inference in this study. $\mu_{Li,G2}$, mean for line *i* in genotypic background G2 (perturbation); $\mu_{Li,E2}$, mean for line *i* in environment E2 (perturbation), where E1 or G1 are the control (nonperturbed) states; V_G, genetic variance; V_{Em}, macroenvironmental variance; V_{Eg}, within-line variance; V_{Es}, within-individual variance, or fluctuating asymmetry; LS, Levene's statistic.

Homeostatic mechanism	Variance component	Line measure
Genetic canalization	V_G	$\lvert\mu_{Li,G2} - \mu_{Li,G1}\rvert$
Macroenvironmental canalization	V_{Em}	$\lvert\mu_{Li,E2} - \mu_{Li,E1}\rvert$
Microenvironmental canalization	V_{Eg}	$\lvert LS_{Li,E2} - LS_{Li,E1}\rvert$
Developmental stability	V_{Es}	$\lvert FA_{Li,E2} - FA_{Li,E1}\rvert$

to mutational stress (genetic canalization). Concurrently, wild-type individuals from each of the lines was subjected to an ecologically relevant temperature stress. By comparing how each line responded to the genetic and environmental perturbations relative to the control, I addressed several questions. Did lines show a similar response to environmental and genetic perturbations? Did the perturbations have different effects on distinct components of variance? What was the relationship between measures of canalization? Was cryptic genetic variation revealed by environmental and genetic perturbations? If so, for what aspects of the variation? Based on the observations of how lines responded to the genetic and environmental perturbation, there was no evidence for an association between genetic and environmental canalization. Furthermore, there was little evidence for an association between different measures of canalization, however, there does appear to be evidence for a relationship between V_{Eg} and V_{Es}. The results are consistent with a release of cryptic genetic variation for different components of variation due to both mutational and environmental perturbation. The results will be discussed within the framework of the evolutionary study of canalization and buffering.

MATERIALS AND METHODS

Sternopleural[1].—*Sp*[1] is a homozygous lethal allele that alters number and positioning of sternopleural bristles as a heterozygote (''*Sp*[1]'' refers to the mutation, and ''sternopleural'' the phenotype). *Sp*[1] displays a classic form of temperature sensitivity. At 18°C, the distribution of bristle number for *Sp*[1]/+ individuals overlap those of wild type, while at 25°C there is little overlap. At 30°C many *Sp*[1]/+ individuals have no sternopleural bristles, although bristle sockets were present (pers. obs.). While the exact mechanism responsible for this is unknown, the phenotype was consistent with necrosis. Due to these effects, I did not study the phenotype for *Sp*[1] at 30°C. While the molecular polymorphism responsible for the *Sp*[1] phenotype is unknown, previous studies suggest that it is either allelic to the wingless (*wg*) locus (Neumann and Cohen 1996) or in close physical proximity and interacts epistatically with *wg* (Buratovich et al. 1997).

Lines.—Single females were collected from Toronto, Ontario (lines 01–10), and Algonquin Park, Ontario (lines 11–18, collected by R. Bonduriansky), to establish isofemale lines. These lines were further inbred by full-sib mating for two generations. In addition, 10 lines from the worldwide wild-type collection were used, available from the Bloomington *Drosophila* stock center (Gibson and van Helden 1997), as well as several traditional laboratory wild-type stocks, Oregon-R, Canton-S, and *white*[1118] (*w*[1118]), commonly used as control lines for developmental genetics. I refer to all of these lines as the ''inbred lines'' (IL), given that to a first approximation each line can be considered a distinct genotype, as opposed to a population of genotypes. Analyses including only the wild-collected lines showed similar results to the analyses including all lines (not shown).

Introgression procedure.—Males of the double balancer stock *w*/Y; *Cy*O/*Sp*[1]; *TM6*, *D*/*TM3*, *Sb* were crossed to females of each of the lines. F_1 females of the genotype *w*/+; *Sp*[1]/+; *TM3 Sb*/+ from each line-cross were backcrossed to males from their respective IL. To replace the entire third chromosome with that of the IL, females with *Sp*[1], but no *Sb* were used for backcrosses. Backcrosses were continued (using three to five *Sp*[1] virgin females, and three males from each of the ILs) for eight additional generations. Large numbers of *Sp*[1]/+ virgin females and +/+ males were collected from each line and population cages were set up. Flies measured for the experiment were of the 13th generation of introgression, which should result in about 92% of the genome being replaced by that of the respective IL (Gibson and van Helden 1997). Unless otherwise stated, all crosses were at 25°C, in a moderate (60–70%) humidity incubator.

Experimental setup.—For each line, two population cages (VWR 100-mL disposable plastic beakers), were set up with about 40 pairs of flies on apple-juice agar media (3.5% agar in a solution of 1:1 apple juice and distilled water, supplemented with live yeast paste). Flies were allowed to deposit eggs, changing the plates every 12 h. Between 8 and 16 h after hatch, 35 first-instar larvae were transferred to each vial. Previous tests at this density did not reveal any significant effects on body size (pers. obs.). This procedure was done for two successive 12-h collection periods. For each line and each collection period, up to 15 vials (525 flies) were collected; however, for some lines this was not possible (minimum number of vials for a given line was eight for any collection period). Vials from the first collection period were placed in a 25°C incubator, while vials from the second were all placed in a 30°C incubator.

Vials were randomized within trays in the incubators, and the trays were rotated each day to minimize the effects of any incubator temperature gradients (edge effects). No temperature gradient greater than ±1°C was detected during daily examinations. No evidence for differential viability or rate of eclosion of *Sp*[1]/+ and +/+ individuals from a number of lines was observed. After eclosion flies were stored in 70% ethanol. Given that *Sp*[1]/+ and wild-type (wt) siblings were raised in common vials, artifacts due to environment-by-genotype correlations are unlikely.

Counting bristles and thorax measurements.—For each

line, 20 males and 20 females for $Sp^1/+$ (25°C only) and +/+, were randomly selected (within and between vials) and scored for number of sternopleural bristles on both the left and right side. Bristles were counted on each side several times, until there was certainty as to the number.

Images of the thorax were captured with a Hitachi (Tokyo) KP-250 digital camera attached to a Leica (Wetzlar, Germany) MZ-8 microscope and measured using the digital caliper in the Image-Pro software package (MediaCybernetics, Silver Spring, MD). After determining that any correlated effects of body size on bristle number were minor and did not change the general conclusions for flies raised at 25°C (see Results), I counted bristle number, but did not measure thorax length for the remaining treatment (wt 30°C).

Analysis

Analysis of variance framework.—All analyses were performed using SAS V8.2e (SAS Institute, Cary, NC). For mixed models, the MIXED procedure in SAS using Type III sum of squares (METHOD = TYPE3) was employed. In addition for the random components (terms including line), a likelihood-ratio test (LRT) was also performed using the restricted maximum likelihood estimates (METHOD = REML) sequentially removing one parameter at a time. Given the truncated normal distributions of Levene's statistic (and fluctuating asymmetry) the parametric critical values can be overly conservative (Schultz 1985). Thus, 1000 permutations were performed on the data to empirically derive critical values for the LRT (random) and *F*-statistics (fixed). The results of the permutations and parametric estimates were consistent (not shown). The general model to be fit was

$$Y = \mu + L + T + S + (S \times L) + (S \times T) + (T \times L) + (S \times L \times T) + \varepsilon, \quad (1)$$

where μ corresponds to the grand mean; L represents line, which is an estimate of the genetic contribution (random); sex (S) and treatment (T) were both fixed effects. Treatment refers to the genetic and external environmental perturbations used: control (wild-type raised at 25°C), *Sp* ($Sp^1/+$ individuals raised at 25°C), and high temperature (wild-type individuals raised at 30°C). Vial terms were not included as individuals (across vials) were randomized. When the third-order term was not significant for the model, it was excluded from the analysis, and the significance was adjusted to control for multiple tests. Reduced models were also examined removing one or two treatment factors. Given that a subset of the lines were not derived recently from natural populations and the exact degree of inbreeding is unknown, analyses were performed excluding these lines as well, with similar results to the entire dataset (not shown).

Measuring within line variation.—While the coefficient of variation (CV) is a traditional measure for relative variation, statistical inferences using it within an *F*-test framework result in false positives if distributions are not normal (Van Valen 1978; Schultz 1985). Therefore, the CV is shown as a standardized measure of variation for comparative purposes, and Levene's statistic is employed for formal analysis of within-line variation. Levene's statistic is:

$$Y_{ijkl} = |\log(x_{ijkl}) - Md[\log((x_{ikl})]|, \quad (2)$$

where Y_{ijkl} is the new variable for individual i from line j, sex k, and treatment l. $Md(\log((x_{jkl}))$ is the median of the log sample, that is, the median of the log of sternopleural bristles for all wt (l) males (k) from line 01 (j)).

Measuring fluctuating asymmetry.—The framework laid out by Palmer (1994) and Palmer and Strobeck (1986, 2003), was used for this study. Plotting left (L) versus right (R) sides for total number of bristles followed by Grubb's test suggested two samples were nominal outliers (both $Sp^1/+$); however, their inclusion had no effect on the analysis (not shown). Spearman's rank correlation was used to determine if there was any dependence of fluctuating asymmetry (FA) on trait size, examining number of bristles versus indices FA1, ($|L - R|$), and FA8 ($|\ln L - \ln R|$). Log transformation was used, as it can often remove any dependence of size on the level of asymmetry found in the observations (Palmer and Strobeck 2003).

Examination of the distributions for L − R for both $Sp^1/+$ and wt individuals were normally distribution with mean = 0, consistent with FA, but not directional symmetry or antisymmetry. A mixed-model ANOVA was performed on the variate of LN L − LN R, (signed measure of asymmetry), as a more quantitative test for patterns of asymmetry inconsistent with FA. Both unsigned measures (FA1 and FA8) were employed for the full and reduced ANOVA models.

Measuring Canalization

Between-line measures.—Canalization of a given line can be measured by:

$$C_j = |\mu_{j,E1} - \mu_{j,E2}|, \quad (3)$$

where $\mu_{j,E1}$ is the estimate for the line mean under treatment E1, and E2 represents the control. This measure is commonly used in studies of phenotypic plasticity and is an estimate of the effects of the environmental change for a given line, where canalization decreases as C increases.

Testing for relationships between genetic canalization and environmental canalization and between canalization and fluctuating asymmetry.—I employed statistical associations to test the various hypotheses of relationships between variables. For each line, mean FA and measures of line-specific canalization were used (Table 1). Correlations between measures were performed using Spearman rank correlations.

Heritability and co-efficient of genetic variance.—Broad-sense heritability was calculated according to standard measures (Falconer and Mackay 1996):

$$H^2 = \sigma^2_G/\sigma^2_P = \sigma^2_G/(\sigma^2_G + \sigma^2_E), \quad (4)$$

where

$$\sigma^2_G = \sigma^2_L/(2F), \quad (5a)$$

$$\sigma^2_L = (MS_L - MS_E)/40, \quad \text{and} \quad (5b)$$

$$\sigma^2_E = MS_E - (1 - F)\sigma^2_G, \quad (5c)$$

where MS_L is the between-line mean square, MS_E is the error mean square for the model, and the denominator of 40 is the number of individuals per line (20 males + 20 females) calculated separately for each environment. Similar estimates

TABLE 2. Summary statistics based on (A) overall phenotypic variation or (B) line means. LS, Levene's statistic; *N*, number of individuals (A) or lines (B) per treatment; SD, standard deviation; CV, coefficient of variation. All *F*-ratios were computed using the value for the control treatment in the denominator. LS were compared using paired one-way *t*-tests. wt, wild-type treatment (25°C); HT, high temperature (30°C); *Sp*, Sp^1 mutation (25°C).

(A) Treatment	*N*	Mean	SD	CV (%)	*F* (*P*)
HT	1118	17.1	2.8	16.4	1.3 (9×10^{-7})
Sp	1108	31.6	7.3	23.2	2.6 (1×10^{-57})
wt	1114	18.3	2.6	14.2	

(B) Treatment	*N*	Mean	SD	CV (%)	*F* (*P*)	LS (*P*)
HT	28	17.1	2.2	12.6	1.6 (0.1)	0.04 (0.03)
Sp	28	31.6	5.9	18.6	3.5 (8×10^{-4})	0.06 (4×10^{-4})
wt	28	18.3	1.8	10.0		0.034

(for σ_L^2 and σ_E^2) were obtained from the covariance parameters from the mixed-model analysis. The exact amount of inbreeding for the lines was not known, therefore two estimates of inbreeding ($F = 1$ for completely inbred and 0.69 estimated from a similar study) provide an upper and lower bound for heritability estimates of bristle number. As an alternative measure of evolvability, the coefficient of genetic variation is also provided (Houle 1992):

$$CV_G = \sqrt{\sigma_G^2}/\mu, \quad (6)$$

where σ_G^2 is the genetic variance and μ is the mean for the trait.

Cryptic genetic variation.—If there is a systematic breakdown of trait canalization due to a perturbation (either Sp^1/+ 25°C or +/+ 30°C), then the subsequent release of cryptic genetic variation can be detected by examining the change in the between-line variance across environments. Although there are several methods to examine such patterns of between-line variation (Dworkin 2005a), here an *F*-test (Sokal and Braumann 1980) on the coefficient of variation (CV), and Levene's test are used. For this measure Levene's test is constructed as follows:

$$LS_{jk} = |\log(\mu_{jk}) - Md[\log(\mu_k)]|, \quad (7)$$

where $\log(\mu_{jk})$ is the log-transformed mean for line *j* in treatment *k*, $Md[\log(\mu_k)]$ is the median across all log-transformed line means for treatment *k*. Mean deviates for each line are derived and used for paired *t*-tests across treatments (Sp^1/+ vs. +/+ 25°C, and +/+ at 30° vs. 25°C). This approach is preferable to casting the variances in an *F*-ratio (Schultz 1985), as it is more robust to violations of the assumption of normality. In addition, the genetic correlation is used (Falconer and Mackay 1996) to partition the interaction effects into crossing of line means and increases in between-line variation (Robertson 1959; Fry 1992). Specifically, tests were performed against the null hypothesis of $r_G = 0$, and $r_G = 1$. Rejecting the hypothesis of $r_G = 0$ is consistent with interaction variance being in part due to a change in the line variance between environments, while no significant difference from $r_G = 1$ suggests that virtually all of the interaction variance is due to such an increase.

RESULTS

Distribution of effects and general trends.—As a check on the efficacy of the experiment, the observed means and variances for sternopleural bristle number in this study were examined and appear comparable to previous studies (Mackay and Langley 1990; Houle 1992; Gurganus et al. 1998; Imasheva et al. 1998; Lyman and Mackay 1998). Mutant Sp^1/+ individuals show an increase in mean and variance for bristle number compared with +/+ while a decrease in the mean, and increase in variance for bristle number, was observed under high temperatures stress (Table 2). These results are generally consistent with previous observations (Gurganus et al. 1998; Imasheva et al. 1998; Lyman and Mackay 1998).

Change in genetic variance with environment.—Several criteria must be met to be confident in the inference of canalization in the sternopleural bristle system. When inferring canalization by its breakdown, it is requisite to demonstrate a significant treatment × line (T × L) interaction, which is in part due to an increase in between-line variance in the stressful environments (wt 30°C or Sp^1/+ 25°C) as compared to the benign environment (wt 25°C). Given these results, we can then proceed with tests of the relationships between the various measures.

For sternopleural bristle number, there is a marginally significant S × T × L and a highly significant T × L interaction (Table 3), suggesting a dependence of treatment effect on genetic background (Fig. 1). An ANOVA on log-transformed variates produced similar results (T × L, $P \ll 0.0001$), consistent with the results being robust to the violation of the assumption of homogeneity of variance. A second concern is that the effects of Sp^1 are mediated through some correlated trait, such as body size. Using thorax length as a proxy for overall body size, the Pearson correlation was computed. For the Sp^1 (25°C) and wild-type (25°C) samples, $r = 0.02$, although the result was highly significant ($P < 0.00001$, $N = 1978$). Including body size as a covariate in the model (ANCOVA) did not notably alter the results (not shown). Thus, it appears that body size is not an important covariate for variation in sternopleural bristle number and was not measured further for the +/+ individuals at 30°C.

Reduced models were examined to determine if the T × L interaction was due to Sp^1, high temperature stress, or both (Fig. 1). There is a highly significant interaction between treatment and line when Sp^1 is compared to wild-type (LR = 671.6, $P \ll 0.0001$). Similarly, the effects of high temperature stress compared to the control also show a significant T × L effect (LR = 42.2, $P \ll 0.0001$). These results are

TABLE 3. Summary of the ANOVA for the complete model across all environments for bristle number. df, degrees of freedom; dfe, df error, calculated using the Satterthwaite method; MS(E), mean square (error); LR, likelihood ratio statistic, tested from a χ^2 distribution with 1 df. Regardless of measure (raw or log-transformed variates) the T × L interaction was highly significant.

Factor	df/dfe	MS	MSE	*F* (*P*)	LR (*P*)
S	1/27.0	502.5	22.0	22.9 (5.5×10^{-5})	
T	2/54.0	71,925.2	271.2	265.2 (1.2×10^{-28})	
L	27/56.5	1134.4	279.6	4.1 (4.6×10^{-6})	1580.2 ($\ll$0.0001)
S × T	2/54.0	39.4	13.6	2.9 (0.06)	
S × L	27/54.0	22.0	13.6	1.6 (0.07)	4.5 (0.03)
T × L	54/54.0	271.3	13.6	19.9 (6.3×10^{-22})	1076.5 ($\ll$0.0001)
S × T × L	54/3179	13.6	9.2	1.5 (0.01)	4.5 (0.03)

consistent with the effects of the *Sp*[1] mutation and temperature stress being dependent on genetic background (line).

In addition, the *T* × L interaction may in part be due to a release of cryptic genetic variation due to the perturbation. Both the temperature and genetic perturbations result in an increase in the amount of genetic variation (Tables 2, 4). The estimates of H^2 and the coefficient of genetic variation (CV_G) for bristle number of the wild-type flies raised at 25°C is similar to previous estimates (Houle 1992), while the estimates are much greater for the *Sp*[1] treatment. Indeed, the genetic correlation across environments are high (*Sp*[1]–wt 25°C, $r = 0.81 \pm 0.11$, wt 30°C–wt 25°C $r = 0.98 \pm 0.03$) and *t*-tests against a null correlation of $r = 0$ are highly significant for both, but do not significantly differ from $r_G = 1$. These results are consistent with the T × L interaction effect being due to an increase in between-line variance across treatments, suggesting that perturbations reveal cryptic genetic variation, consistent with a breakdown of canalization of the sternopleural bristle system. Similar results have been reported for a number of characters across environments such as nutritional (Imasheva et al. 1999), temperature (Imasheva et al. 1998; Bubliy et al. 2000), and chemical stressors (Mackay 1980). One other study has demonstrated a similar increase in genetic variation for sternopleural bristle number using a mutational perturbation (Lyman and Mackay 1998). Thus, not only are the treatment effects dependent upon line, but the perturbations may be sufficient to reduce developmental buffering, revealing cryptic genetic variation.

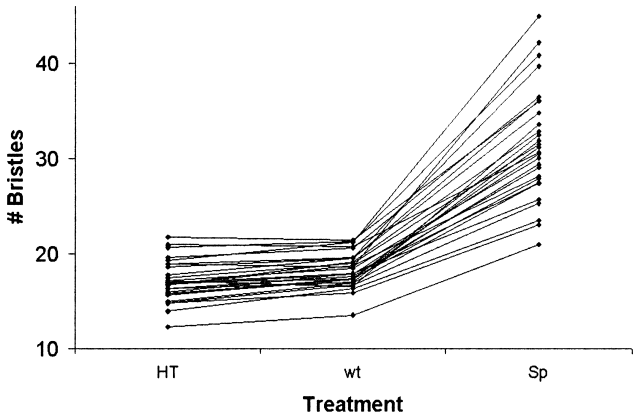

FIG. 1. Plot of the reaction norms of line means for bristle number across environment. wt, wild-type treatment (25°C); HT, high temperature (30°C); Sp, *Sp*[1] mutation (25°C). The between-line variance increases (relative to wt) somewhat in the HT treatment and considerably with *Sp*[1].

Change in within-line variance (V_{Eg}) with environment.—An alternative definition of canalization is based on the reduction of phenotypic variation for a given genotype (V_{Eg}). To study changes in the levels of phenotypic variation within a line, Levene's statistic was employed (Dworkin 2005a). For the full model, the S × T × L interaction was not significant and was therefore excluded and the data reanalyzed. The T × L interaction is highly significant for this model (Table 5), as are the T and T × S terms. This suggests a number of important features. The treatment effect is consistent with earlier results demonstrating an increase in phenotypic variation due to perturbation (Table 2). The T × L effect is consistent with there being genetic variance for within-line variation. To determine if this effect was due to one or both of the treatment effects, reduced models were examined for each treatment. Most of the genetic variation for treatment effects on within-line variation are due to the genetic perturbation (LR = 5, $P = 0.01$), with only marginal evidence for an independent contribution for high temperature stress (LR = 1.3, $P = 0.1$) It is worth noting that when each treatment is considered separately, there is still strong evidence for line effects, consistent with the presence of genetic variation for within-line variation (not shown). Within-line variation appears to show more evidence of line crossing rather than scaling effects (Fig. 2), unlike what was observed for the means of bristle number. This is quantified by examining the genetic correlation, r_G, across treatments (Robertson 1959). Consistent with the graphical interpretation for *Sp*[1] versus +/+ 25°C, $r_G = 0.29 \pm 0.19$ (SE) is not significantly different from $r_G = 0$, but is from $r_G = 1$ ($t = 3.8$, df = 26, $P = 0.0004$), suggesting that most of T × L effect for Levene's statistic is due to crossing of line means. How-

TABLE 4. Genetic and environmental perturbations increase trait evolvability. Heritability, (H^2) was estimated under the assumption that each line represented a true genetic clone or a partial amount of inbreeding ($F = 0.69$, H^{2*}). For bristle number, both measures of H^2 show a substantial increase due to both the mutational (*Sp*[1]) and high-temperature (HT) treatments. Heritability estimates for both V_{Eg} (measured by Levene's statistic) and V_{Es} (FA8) also increased due to perturbations. An alternative measure, the coefficient of genetic variation (CV_G), also demonstrated that the perturbations can increase the evolvability for bristle number and variance.

Treatment	H^2	Bristle number H^{2*}/CV_G (%)	V_{Eg} H^2/CV_G (%)	V_{Es} H^2/CV_G (%)
wt	0.31	0.45/8.4	0.021/9.3	0.001/3.3
Sp[1]	0.45	0.64/15.7	0.027/9.8	0.046/17.3
HT	0.40	0.58/10.7	0.062/15.9	0.013/9.9

TABLE 5. ANOVA summaries for within-line variation using Levene's statistic. Terminology as per Table 3.

Factor	df/dfe	MS	F (P)	LR (P)
S	1/27.0	0.009	1.7 (0.2)	
T	2/54.0	0.38	41.2 $\ll$ 0.0001	
S × T	2/3233	0.03	5.8 (0.003)	
L	27/29.7	0.023	2.6 (0.006)	44 (3.3×10^{-11})
L × S	27/3233	0.005	0.94 (0.5)	0 (1)
L × T	54/3233	0.009	1.6 (0.003)	7.46 (0.006)
Residual	3233	0.006		

ever, there is an increase in both heritability and the coefficient of genetic variation due to the genetic and environmental perturbations (Table 4). It is also worth noting that the sex-specific effects observed for the full models are only found for the (+/+) raised at 25°C when the environments are analyzed separately.

Analysis of the Fluctuating Asymmetry of Sternopleural Bristle Number

Sternopleural bristles fluctuating asymmetry.—FA1 ($|L - R|$), showed a strong positive correlation between asymmetry and number of bristles (Spearman $r = 0.36$, $P < 0.0001$), consistent with an effect of trait size on asymmetry. Log transformation of the data using FA8 ($|\ln L - \ln R|$), removes this association ($r = -0.034$, ns). Using a mixed-model ANOVA on signed measures of asymmetry ($L - R$ and $\ln[L] - \ln[R]$), no term showed any significance, allowing us to reject directional effects as the cause for the asymmetry (not shown). Finally, a histogram of the signed data suggested a normally distributed trait, with a mean of zero. Together, these results suggest that sternopleural bristle asymmetries were due to FA (Palmer and Strobeck 2003). All additional analysis were performed using FA8.

Patterns of variation for fluctuating asymmetry.—There was no evidence for a significant three-way S × T × L interaction, and this term was excluded from further analyses. The T × L interaction is highly significant (Table 6) and the data exhibited considerable crossing of line means (Fig. 3). The T × L effects were also evident in the reduced models, comparing the control to either the mutational (LR = 15.4, $P < 0.0001$) or high temperature effects (LR = 4.4, $P = 0.02$) each also showed a significant T × L interaction. These results are consistent with the presence of genetic variation for the effects of both the mutation and temperature perturbation on levels of FA.

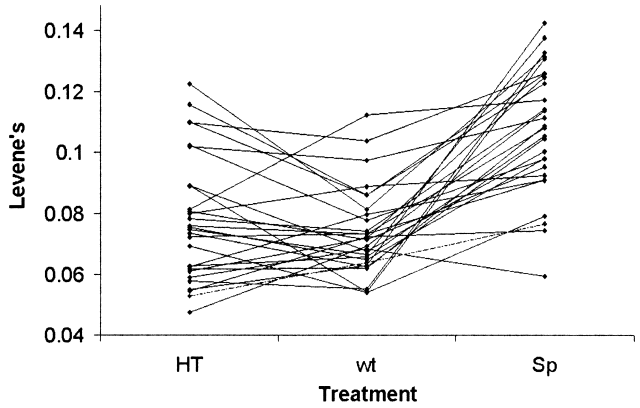

FIG. 2. Reaction norm plot of Levene's statistic for line means across the three treatments in this experiment. For the most part, the line × treatment interaction is due to crossing of line means, not an increase in between-line variance, as shown for bristle number.

Perturbations increase heritability of fluctuating asymmetry.—While there is no evidence for a significant line term for the wild-type 25°C treatment, there is a significant effect when measured under both the genetic and temperature perturbations (Table 4). The heritability estimates are low, typical of estimates for FA (Fuller and Houle 2003); however, H^2 and CV_G are increased due to the perturbations (Table 4). This suggests that stressors that affect expression of FA can potentially reveal cryptic genetic variation for FA, as well as for trait size itself.

The Relationship between Measures of Canalization and Developmental Stability

No significant correlation between measures of canalization.—Canalization has been viewed as a mechanism that either reduces among-environment (V_{Em}) or within-line variance (V_{Eg}). However, it is not clear if there is any relationship between these measures. Using a Spearman rank correlation, the line-specific measures based on changes in line means or variances were compared. No evidence was found for a positive correlation for measures of canalization in the data for either the environmental (+/+ 25°C vs. 30°C, Spearman $r = 0.014$, ns) or genetic (+/+25°C vs. $Sp^1/+$, Spearman $r = -0.07$, ns) perturbation. Thus, lines that show low V_{Em} do not necessarily show low V_{Eg}, suggesting independent mechanisms for these two aspects of canalization.

No evidence for a positive correlation between measures of environmental and genetic canalization.—Wagner et al. (1997) suggested a mechanism whereby genetic canalization evolves as a correlated response to the evolution of environmental canalization. While this idea has garnered some proponents (Ancel and Fontana 2000; Meiklejohn and Hartl 2002), to date there has been little empirical assessment of this idea. To test this, I compared how lines responded to the mutational perturbation of Sp^1 and the high temperature stress. Using Spearman rank correlations, no association was observed between measures of genetic and microenvironmental canalization (Spearman $r = 0.02$, ns) or between macro- and microenvironmental canalization (Spearman $r = 0.03$, ns). There was a significant negative correlation observed between genetic and macroenvironmental canalization (Spearman $r = -0.46$, $P = 0.0125$). However, given that a

TABLE 6. ANOVA summary for within-individual variation using FA8. For notation, see Table 3.

Factor	df/dfe	MS	F (P)	LR (P)
S	1/27.0	0.018	2.8 (0.1)	
T	2/54.0	0.26	10.85 (1×10^{-4})	
T × S	2/3231	0.0009	0.087 (0.9)	
L	27/32.3	0.041	2.08 (0.02)	38.1 (6.7×10^{-10})
L × S	27/3231	0.0064	0.60 (0.9)	0 (1)
T × L	54/3231	0.024	2.28 (3.7×10^{-7})	23.6 (1.2×10^{-6})
Residual	3231	0.011		

positive association is predicted, this evidence does not support the original hypothesis of Wagner et al. (1997).

Another recent study (Dworkin 2005b) examined a set of related questions using an independent genetic perturbation and traits. I combined the data from that study and the current one to address the questions of a common mechanism between genetic and environmental canalization, as discussed above. To appropriately combine the data I examined standardized measures of canalization across traits and employed a stratified permutation test (permuting measures within but not between traits). In no case was there a significant positive correlation between measures of environmental and genetic canalization (not shown).

Relationship between measures of within-individual variation (V_{Es}) and between-individual, within-genotype variation (V_{Eg}).—Previous work has produced somewhat mixed results, with some evidence suggesting a strong positive correlation between V_{Es} and V_{Eg} (Clarke 1998a,b). For sternopleural bristles, there is a strong positive correlation between V_{Es} and V_{Eg}, measured using either CV (Spearman $r = 0.63$, $P < 0.0001$) or Levene's statistic (Spearman $r = 0.66$, $P < 0.0001$) with FA1 as shown in Figure 4. However, there are two potential issues with treating the environmental effects as independent. The first concern is that there may be some dependence upon the mean (i.e., as the mean increases, so do variances and FA). To consider this, the correlations for CV and Levene' statistic were examined against FA8 (Table 7). This does decrease the magnitude of the correlation (although it remains highly significant), suggesting that some of the correlation between V_{Es} and V_{Eg} is due to the effects of trait size, and this must be taken into account.

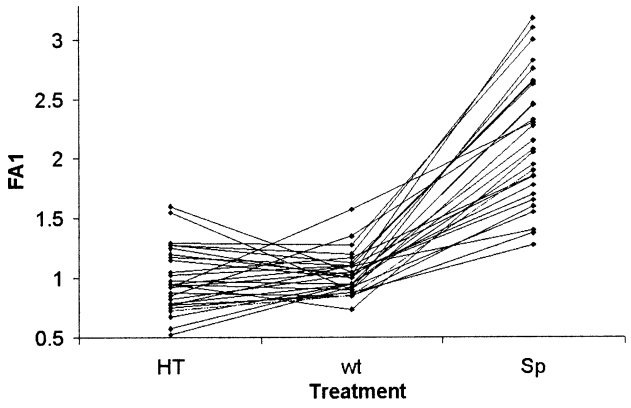

FIG. 3. Reaction norm plot for lines means for FA1 across treatments. Mean (FA1) is the sample mean for each line for $|L - R|$. *X*-axis as in Figure 1.

The second concern is the possibility of pseudoreplication via the repeated use of a set of lines, under several environments (if the line variation or FA is itself correlated across environment). While this is not the case for the CVs, there are marginally significant correlations between FA1 across environments. Therefore, there may be some effect of pseudoreplication on this result. One method to get around this is to analyze the correlations between FA and CV (or Levene's) separately in each environment. While this effectively reduces sample size (and power), it is independent for each test. Table 7 provides the summary statistics for these correlations. The results are equivocal. While the correlations persist under the HT treatment and (for Levene's) for Sp^1, there is no evidence for any correlation for the wild-type controls. However in all cases (regardless of significance) the relationship remains positive (and similar in magnitude to the correlation across lines), consistent with a loss of power. Thus, it appears that there is indeed a moderate, positive correlation between V_{Es} and V_{Eg}.

Relationship between within-individual variation (fluctuating asymmetry) and measures of canalization.—While previous studies have used the correlation of within-versus between-individual variation to address the relationship between canalization and developmental stability, in this study I use the measures of canalization employed earlier (Table 1). What is not clear, however, is what we should use to measure FA. Specifically, do we use the FA1 for lines in the wt 25°C, wt 30°C, or Sp^1 environments? Perhaps the most

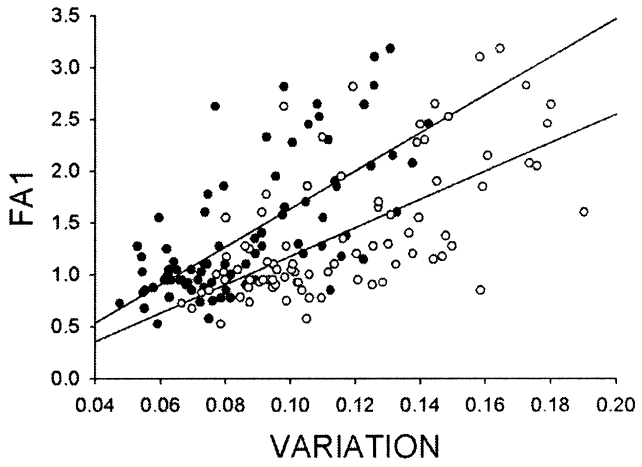

FIG. 4. Positive correlation for within-individual variation (FA1) and between-individual variation (CV, filled circles; and Levene's statistic, open circles). For either measure of V_{Eg}, there is a strong positive correlation with fluctuating asymmetry (V_{Es}).

TABLE 7. Summary of correlations for V_{Es} (FA8) versus V_{Eg}. LS, Levene's statistic; CV, coefficient of variation. Significance was determined by empirical permutation.

Grouping	*N*	Spearman *r* (CV vs. FA8)	Spearman *r* (LS vs. FA8)
wt, *Sp*, HT	84	0.38 ($P < 0.0001$)	0.39 ($P < 0.0001$)
wt	28	0.19 ($P = 0.16$)	0.22 ($P = 0.15$)
Sp	28	0.30 ($P = 0.064$)	0.34 ($P = 0.026$)
HT	28	0.32 ($P = 0.052$)	0.41 ($P = 0.024$)

TABLE 8. Relationship between measures of canalization and measures of developmental stability using FA1 as a proxy. Spearman rank correlation is shown with *P*-values in parentheses. (A) Measures of environmental canalization; (B) measure of genetic canalization. See Table 1 for description of measures of canalization.

(A)	Macroenvironmental canalization
FA1 (HT)	−0.48 (0.01)
FA1 (wt)	−0.21 (0.27)
FA1 (wt + HT)/2	−0.49 (0.009)
(B)	Genetic canalization
FA1 (Sp^1)	0.48 (0.010)
FA1 (wt)	0.39 (0.04)
FA1 (wt + Sp^1)/2	0.57 (0.0016)

interesting comparison is that of the measures of canalization with FA1 in the wild-type environment, given that if there is a significant correlation, it would suggest that FA alone is sufficient to examine patterns of canalization, without requiring genetic or environmental perturbations. However, there is no biological rationale that suggests that this comparison is superior to any other. In general, there is evidence for a significant correlation between measures of both macroenvironmental and genetic canalization with FA (Table 8). While there is a positive correlation associated with genetic canalization and FA, there is a negative one with respect to macroenvironmental canalization. It is worth noting that only two of the three tests show a significant relationship, although they all share the same sign. Changes in within line variance (V_{Eg}) due to either the genetic or high temperature perturbation showed no evidence for any type of relationship with FA (not shown). This counter-intuitive result suggests that genotype specific levels of FA (a variance) are correlated with how a perturbation changes mean trait expression for a genotype, not changes in within-genotype phenotypic variance.

DISCUSSION

While canalization is expected to reduce phenotypic variance (V_P) of a trait, there is still no agreement as to what components of variation should be affected in particular (Gibson and Wagner 2000; Debat and David 2001; Nijhout and Davidowitz 2003; Dworkin 2005a). Previous studies have demonstrated the effects that either genetic (Reeve 1960; Moreno 1994; Lyman and Mackay 1998) or environmental stress (Imasheva et al. 1998) can have on phenotypic and genetic variation, but not both simultaneously. In this study, I concurrently examined the consequence of genetic and environmental stress on different components of variation in a set of inbred lines of *D. melanogaster*. Furthermore, I tested a hypothesis for the coevolution of genetic and environmental canalization (Wagner et al. 1997). While the effects of both genetic and environmental perturbations can have profound effects on trait variance, the response to such perturbations appear to be independent of one another, with respect to both the nature of the perturbation and the aspect of variation examined.

In addition, I observed that the genetic (Sp^1) and temperature perturbations increased V_{Es} (using FA) for bristle number. Results from previous studies have been mixed, showing an increase in some (Reeve 1960; Bourguet 2000) but not all cases (Indrasamy et al. 2000; Milton et al. 2003). It is not clear why results have been inconsistent; however, in at least some instances where no increase was observed for FA, it was unclear if the genetic or environmental stress was affecting trait expression at all (Woods et al. 1999; Hoffmann and Woods 2001). The perturbations appear to increase heritability of all the variance components (Table 4), suggesting a release of cryptic genetic variation (CGV) for the variance of bristles as well as their total number. Studies focusing on mapping the variants responsible for the underlying CGV (Dworkin et al. 2003) may facilitate understanding the evolutionary history of this hidden variation, although it is likely that the polymorphisms affecting number and trait variance differ (Dworkin 2005a; Mackay and Lyman 2005), and the genetic architecture of trait variance is complex.

While the release of CGV has been a traditional approach to inferring the breakdown of trait canalization (Waddington 1952; Rendel 1959; Atallah et al. 2004), a recent theoretical study has argued for caution with respect to this interpretation (Hermisson and Wagner 2004). In particular, Hermisson and Wagner suggested that CGV can be maintained without the evolution of genetic canalization, and the expression of CGV only reflects decanalization under limited circumstances. However, based on the arguments presented in Hermisson and Wagner (2004), the design of the present study is suitable for inferring decanalization by the release of CGV. First, the lines used in this study did not originate from a single population experiencing a common selection regime. Second, the high temperature perturbation used in this study is a common, ecologically relevant stressor for flies (Feder 1996) that would be a likely target for the evolution of environmental canalization. Finally, while the specific genetic variant, Sp^1, used in this study is likely an evolutionarily rare perturbation, the mutational target size for bristles is relatively large (Houle et al. 1996; Norga et al. 2003). Thus, new mutations affecting bristle number are likely to be common, increasing the likelihood for selection for genetic canalization. The optimal approach to address this particular question requires the examination of line-specific mutational variances with and without perturbation (Gibson and Wagner 2000; Hermisson and Wagner 2004) and correlating this with the response to other perturbations. However, such a study may not be feasible in most multicellular organisms.

No relationship between environmental and genetic canalization.—Wagner et al. (1997) proposed that the existence of a correlation between genetic and environmental variance can facilitate the evolution of genetic canalization. I therefore tested for such correlations, using both mutational and en-

vironmental perturbations known to alter sternopleural bristle number. Both of these perturbations were applied to a set of common, naturally derived inbred lines via introgression of the mutation (Sp^1), and rearing of their wild-type congenics under a stressful high-temperature regime. No evidence for any type of positive associations between these measures of genetic and macroenvironmental (or microenvironmental) canalization was observed. A related study examining the effects of a different mutational perturbation on the prothoracic leg of *D. melanogaster* also failed to observe a significant association between genetic and microenvironmental canalization (Dworkin 2005a). There was a weak positive correlation between genetic and macroenvironmental canalization in the previous study, while in the current study there was a weak negative correlation. This difference may be due to false positive relationships or to inherent differences in the systems of study. However, the results of both studies consistently found no association based on the original prediction with respect to the relationship genetic and microenvironmental canalization. Therefore, we can tentatively reject the idea of a common mechanism with regard to canalization of the sternopleural bristle system.

There are several alternative explanations for the lack of associations of the results with the conjecture of Wagner et al. (1997). The effect of the Sp^1 mutation on trait expression is so strong that the observed effects are outside of the normal zone of canalization where the model may be operating. The fact that no correlation is observed for this or other traits (Dworkin 2005b) suggests that it may be a more general observation. Second, it is plausible that the high-temperature stressor employed in this study is not the one under which canalization evolved. However, high-temperature stress is a known ecological variable effecting *Drosophila* in natural populations (Feder et al. 1996); thus, it is a likely target for the evolution of environmental canalization.

Finally, I tested whether developmental stability and canalization may reflect a common set of buffering mechanisms. While not all results were significant, the evidence is consistent with an association between the proposed measures of canalization and FA. Nevertheless, some results suggest that there are differences between the measures of V_{Eg} and V_{Es} as examined here. A review of the literature by Clarke (1998a, b) provided strong correlational evidence for a relationship for within-(V_{Es}), and among-individual variation as measured across traits within organisms. In contrast, some studies have not found such associations (Debat et al. 2000; Hoffmann and Woods 2001; Reale and Roff 2003). These differences may be due to different methodologies, as well as different measures of canalization used. Milton et al. (2003) examined the effects of the chaperone HSP83 on FA, which has been proposed to be part of a general buffering mechanism. However, they found neither evidence for an increase in FA nor for a change in variance for the traits under study. Thus, future studies should specifically examine this question, using great care with regard to the sensitivities of these measures. This was made clear in the present study by the degree the correlation coefficient was sensitive to different measures of FA.

This study has shown that canalization can be studied successfully in a quantitative genetics framework (Stearns and Kawecki 1994; Gibson and van Helden 1997), and that a method based considerations of power, genetic background, and methodology can be used to address questions about the evolution and development of canalization. While the techniques of quantitative genetics and statistics are valuable tools for addressing such questions, there is no doubt that the approaches of developmental genetics and genomics can have a great impact on this field.

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