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HSP90 AND THE QUANTITATIVE VARIATION OF WING SHAPE IN *DROSOPHILA MELANOGASTER*

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Abstract.—The molecular chaperone protein Hsp90 has been widely discussed as a candidate gene for developmental buffering. We used the methods of geometric morphometrics to analyze its effects on the variation among individuals and fluctuating asymmetry of wing shape in *Drosophila melanogaster*. Three different experimental approaches were used to reduce Hsp90 activity. In the first experiment, developing larvae were reared in food containing a specific inhibitor of Hsp90, geldanamycin, but neither individual variation nor fluctuating asymmetry was altered. Two further experiments generated lines of genetically identical flies carrying mutations of *Hsp83,* the gene encoding the Hsp90 protein, in heterozygous condition in nine different genetic backgrounds. The first of these, introducing entire chromosomes carrying either of two *Hsp83* mutations, did not increase shape variation or asymmetry over a wild-type control in any of the nine genetic backgrounds. In contrast, the third experiment, in which one of these *Hsp83* alleles was introgressed into the wild-type background that served as the control, induced an increase in both individual variation and fluctuating asymmetry within each of the nine genetic backgrounds. No effect of Hsp90 on the difference among lines was detected, providing no evidence for cryptic genetic variation of wing shape. Overall, these results suggest that Hsp90 contributes to, but is not controlling, the buffering of phenotypic variation in wing shape.

Key words.—Canalization, cryptic genetic variation, developmental stability, evolutionary capacitor, geometric morphometrics, wing shape.

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Many phenotypic traits show a considerable degree of buffering against perturbations from internal and external causes. This is manifest in a constant phenotype of organisms that carry different alleles of genes relevant to the traits under study or that were reared under different environmental conditions. This phenomenon is known collectively as developmental buffering and includes the concepts of developmental stability and canalization. The processes that underlie it are still debated (Debat and David 2001; Meiklejohn and Hartl 2002; Klingenberg 2003; Dworkin 2005; Santos et al. 2005). Mechanisms that control buffering can influence the release of phenotypic variation upon which selection can act and they therefore may affect the rates of evolutionary change (Rutherford and Lindquist 1998; Rutherford 2000, 2003; Gibson and Dworkin 2004).

One of the few mechanisms that have been identified involves the heat shock protein 90 (Hsp90), which has been demonstrated to be part of a buffering mechanism in *Drosophila* and *Arabidopsis* (Rutherford and Lindquist 1998; Queitsch et al. 2002). The effects of Hsp90 on variation of phenotypic traits have been shown to depend on the genetic background (Rutherford and Lindquist 1998; Queitsch et al. 2002; Milton et al. 2003; Sollars et al. 2003). Inhibiting Hsp90 function reveals genetic variation that is otherwise hidden by buffering processes, and which may be a potential for evolutionary change under special circumstances, for instance, under stressful conditions. By storing genetic variation that is not exposed to selection under normal circumstances, Hsp90 may act as a capacitor for evolution (Rutherford and Lindquist 1998; Rutherford 2000, 2003; Ruden et al. 2003; Gibson and Dworkin 2004). Hsp90 has also been reported to be involved in the evolution of drug resistance in fungi (Cowen and Lindquist 2005).

Despite the potential importance of Hsp90 in evolutionary change, there is currently little empirical data concerning the effects of this protein on the expression of phenotypic variability. In *Drosophila melanogaster*, the effects of Hsp90 inhibition have been studied mostly for discrete qualitative traits such as various deformities, bristle phenotypes, and defects of the compound eyes (Rutherford and Lindquist 1998; Sollars et al. 2003). The few studies that have examined the effects of Hsp90 on quantitative traits, wing size, and bristle counts, did not find increased variation or asymmetry of these traits in response to pharmacological inhibition of Hsp90 or to mutations of the *Hsp83* gene (Rutherford et al. 2000; Milton et al. 2003; Milton et al. 2005). This raises the question whether Hsp90 effects on quantitative traits are fundamentally different from those on qualitative traits, and whether Hsp90 really is the basis of a general mechanism for phenotypic buffering (Milton et al. 2003, 2005).

Here we investigate the effect of experimental reduction of Hsp90 activity on variation in *Drosophila* wing shape, a type of trait that is different from those used in previous studies. In other studies of insect wings, shape has been shown to be a sensitive indicator of fitness and the effects

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of stress (e.g., Debat et al. 2003; Kölliker-Ott et al. 2003). Because wing shape can vary in many different ways, such as the shape of the wing margin and the relative arrangement of veins, it is potentially more informative than simple traits (larger or smaller size, more or fewer bristles). This makes shape a particularly suitable character for examining Hsp90 effects. We use the methods of geometric morphometrics, which combine the powerful and flexible tools of multivariate statistics with explicit consideration of the spatial organization of the wing (e.g., Dryden and Mardia 1998). We investigate the effects on wing shape for three different experiments previously analyzed in studies of bristle counts and wing size (Milton et al. 2003). The first experiment involved exposure of *Drosophila* larvae to a specific Hsp90 inhibitor, geldanamycin. In the second experiment, chromosomes carrying two different *Hsp83* mutations were combined with a set of nine distinct genetic backgrounds. The third experiment achieved a more complete genetic control by introgressing the *Hsp83* mutation into the wild-type background that served as a comparison. By quantifying variation of shape within individuals (fluctuating asymmetry), among individuals with the same genotype, and among lines with different genotypes, we examine the effects of Hsp90 on developmental instability as measured by fluctuating asymmetry and on environmental and genetic variance. Whereas our analyses of wing shape concur with the finding of Milton et al. (2003) that reducing Hsp90 activity does not generally increase asymmetry, they do reveal significant effects in one experiment and therefore indicate that there may be differences in how Hsp90 affects the developmental buffering of different quantitative traits.

Material and Methods

Experimental Designs and Strains

Hsp90 inhibition with geldanamycin.—Geldanamycin (GA) is an inhibitor of Hsp90, which binds to an ATP-binding pocket of the Hsp90 protein in a specific manner and blocks its signal transduction functions (Whitesell et al. 1994; Prodromou et al. 1997; Stebbins et al. 1997). Geldanamycin has been used for the pharmacological inhibition of Hsp90 in *Drosophila* and *Arabidopsis* (Rutherford and Lindquist 1998; Queitsch et al. 2002; Milton et al. 2003).

The flies used in the experiment were from a Canton-S stock that has been maintained at a large and stable population size in the laboratory for over 10 years and is expected to have accumulated significant genetic variation. The evolutionary capacitor hypothesis therefore predicts that the pharmacological treatment would induce an increase in phenotypic variation due to the uncovering of cryptic genetic variation. To test whether sensitivity to GA changes during development, staged larvae were collected (Milton et al. 2003). At the second and third instar, these larvae were transferred to vials consisting of fresh media containing 1 μg/ml GA (the highest dose tested that the flies could survive) or controls without GA. The larvae in both treatments were reared under the same conditions at a density of 50 larvae per vial in five replicates for each treatment. Ten females from each vial (a total of 50 per treatment) were collected and their wings were mounted and digitized.

Hsp83 mutant chromosomes.—The second experiment compared the effects of two chromosomes carrying different *Hsp83* mutations in crosses with a panel of nine wild-type backgrounds (Milton et al. 2003). This was done to test whether the potential effects on morphology were dependent on the nature of the mutation, or dependent only on the impairment of Hsp90, regardless of the kind of mutation inducing it. The two *Hsp83* mutant alleles were $Hsp83^{9J1}$, an allele with dominant negative effect due to an amino acid substitution (van der Straten et al. 1997, p. 1962), and $Hsp83^{P582}$, a loss-of-function allele induced by a P element insertion (van der Straten et al. 1997). Third chromosomes carrying these mutations were introduced into the genetic background of the Samarkand inbred stock (Milton et al. 2003).

The *Hsp83* mutations were combined with nine different strains of flies, here called Raleigh inbred (RI) lines, which had been derived from a wild population in Raleigh, North Carolina, and made nearly isogenic through 14 generations of full sibling inbreeding (Fry et al. 1998). Subsequently, the RI lines were cultured as small populations in vials, and therefore can be expected to have maintained nearly complete homozygosity. Males of the RI lines were mated to females with the *Hsp83* mutant chromosomes or, as a control, to females of the Samarkand stock. The resulting F_1 offspring differed in one of their third chromosomes, which either originated from the Samarkand inbred stock or were the chromosomes associated with one of the *Hsp83* mutations (Milton et al. 2003).

To control for density and vial effects, larvae were collected and placed at a constant density of 50 larvae per vial in five vials per genotype, and maintained at 25°C. From each vial, five males and five females (25 total of each sex) were selected at random and measured.

Introgressed allele.—To examine the effects of Hsp90 independent of other factors on the third chromosomes that carried the *Hsp83* mutations, a third experiment was conducted (Milton et al. 2003). For this purpose, the mutation $Hsp83^{P582}$ and the marker mutation $white^{1118}$ were introgressed into the Samarkand background to create the *P582* introgression strain (*P582i*). Multiple alternating generations of backcrossing and selection ensured that most of the genome, including the third chromosome, originated from the Samarkand strain (for details, see Milton et al. 2003).

Females of the *P582i* strain were crossed to males from all RI lines. The male offspring either carried the $Hsp83^{P582}$ mutation or a wild-type third chromosome from the Samarkand line, along with a third chromosome from the respective RI line, and were used for direct comparisons (distinguishable by the miniwhite construct carried in the P element). These flies differed only in terms of the $Hsp83^{P582}$ mutation and a small chromosome region associated with it. The background effects associated with the RI lines that were detected by the analyses had to be due to differences associated with the second or third chromosomes.

Larvae from each cross were placed at a constant temperature and density as for the previous experiment. Ten males per genotype were scored from each of five vials, for a total of 50 males for each genotype.

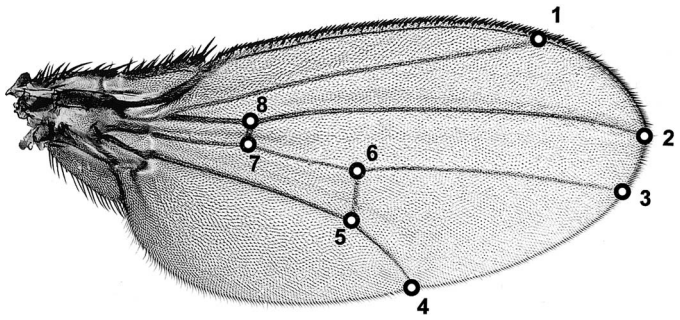

FIG. 1. Locations of the eight landmarks on the *Drosophila* wing.

Landmark Data and Statistical Analysis

The right and left wings were removed and mounted on glass slides with double-sided tape. Wing images were captured using a Panasonic WV-CP460 video camera. Eight landmarks located at the junctions of longitudinal veins with the wing margin or cross veins (Fig. 1) were used in this study. Their x and y coordinates were recorded using TPSdig program (freely available at http://life.bio.sunysb.edu/morph/).

To extract shape variation from the landmark data, we used a generalized least-squares Procrustes fit (e.g., Dryden and Mardia 1998; Klingenberg and McIntyre 1998). In a preliminary step, all left configurations were first reflected to their mirror images. The Procrustes superimposition consists of three successive steps: (1) All configurations are scaled to a unit centroid size (i.e., the square root of the sum of the squared distances from each landmark to the centroid of the configuration) by dividing all coordinates by the corresponding centroid size. (2) The centroids (centers of gravity) of the configurations are superimposed one onto another through translation. (3) The configurations are rotated around their centroids so as to minimize the sum of squared distances between homologous landmarks, that is, to optimize the superimposition. The new coordinates (Procrustes coordinates) are used as shape variables.

Because measurement error is of critical importance when analyzing phenotypic variation and particularly for studies of fluctuating asymmetry (Palmer and Strobeck 1986, 2003; Palmer 1994), replicate measurements were taken on a set of 30 individuals. The wings of these were each photographed twice to evaluate the variation in the image acquisition process. To assess the precision of the digitizing step, the landmarks were then digitized twice on all images. A Procrustes ANOVA (Klingenberg and McIntyre 1998; Klingenberg et al. 2002) was used to test for the effects of individuals, sides, the interaction (side $\times$ individual), imaging, and digitizing.

The effects of the experimental treatments on the mean wing shape were tested with MANOVA on Procrustes coordinates. The magnitudes of the components of shape variation (variation among individuals and fluctuating asymmetry) were quantified with a Procrustes ANOVA, as for the analysis of measurement error, but with the complete samples and including only a single measurement per side. The amounts of shape variation were computed as Procrustes variances, by summing variances over the landmark coordinates. These variances express the quantity of shape variance in units of squared Procrustes distance (Klingenberg and McIntyre 1998). Differences in the amounts of shape variation were tested using permutation tests (Good 2000) with 10,000 random permutations per test.

The covariance matrices for variation among individuals and for FA were computed for each group (e.g., treatment and control, wild-type and mutants). To compare pairs of covariance matrices, we used the method of matrix correlation, as modified for geometric morphometrics (Klingenberg and McIntyre 1998). The matrix correlation is the product-moment correlation between the corresponding elements of two matrices. Because both the variances and covariances can provide information about the similarity or difference of the covariance matrices, we included the diagonal elements of the matrices in the calculations. Matrix correlations were tested against the null hypothesis of complete dissimilarity of the covariance structures with a matrix permutation test (Mantel 1967; Cheverud et al. 1989). Because the x and y coordinates may be associated even under the null hypothesis of no relation of covariance structures among landmarks, we carried out the permutation procedures for the landmarks, keeping the x and y coordinates of each landmark together at all times (Klingenberg and McIntyre 1998).

The evolutionary capacitor hypothesis suggests that morphological variation that is normally hidden is expressed when Hsp90 activity is reduced. Accordingly, novel patterns of covariation among traits may appear in samples of flies carrying *Hsp83* mutations, depending on the genetic background. In comparisons of covariance matrices of *Hsp83* mutants and the wild-type controls across the different RI lines, we might therefore expect an increase in the diversity among mutant samples compared to the wild-type ones. To investigate this, we used an ordination analysis of the variation among matrices by metric multidimensional scaling (e.g., Mardia et al. 1979). This analysis, also known as principal coordinates analysis, is related to principal component analysis (Jolliffe 2002), but uses a matrix of distances between objects as the input data. We defined measure of distance between each pair of covariance matrices as one minus the matrix correlation between the two matrices. This measure was used to set up a matrix of distances between all possible pairs of covariance matrices, which was used as input for metric multidimensional scaling. The principal coordinates are axes that successively account for the maximum amount of the information contained in the distance matrix. Such an ordination allows one to visualize the relationship among covariance matrices. The evolutionary capacitor hypothesis predicts that the wild-type covariance matrices for the nine RI lines should cluster together and that the corresponding mutant matrices should be more widely dispersed.

We quantifed the effect of *Hsp83* mutations on genetic variation by computing the Procrustes variances among the mean shapes of the different RI lines, separately for the wild-type and *Hsp83* mutant samples. The statistical significance of the difference was tested with a permutation test (Good 2000), using 10,000 random permutations of the data.

RESULTS

Measurement error.—All main effects included in the Procrustes ANOVA, except for side, were statistically significant

TABLE 1. Analysis of measurement error using Procrustes ANOVA (Klingenberg and McIntyre 1998). Tabulated values are mean squares in units of squared Procrustes distance multiplied by 10^6 (MS), degrees of freedom (df), *F* statistics, and *P* values from permutation tests.

Source of variation	MS	df	*F*	*P*
Individual	217.5	348	5.88	< 0.0001
Side	62.9	12	1.70	0.12
Interaction (side × individual)	37.0	348	6.06	< 0.0001
Imaging	6.11	720	2.04	< 0.0001
Digitizing	3.00	1440	—	—

(Table 1). The error related to the data acquisition was about one sixth of the true FA component (mean square for the side × individual interaction). The main source of error was the imaging step, whereas digitizing accounted for about half as much of the error. Overall, measurement error was not strong enough to interfere with the interpretation of the results. The subsequent estimates of FA, which are based on a single image per wing, were nonetheless potentially slightly inflated by this amount of error.

Hsp90 inhibition with geldanamycin.—The treatment, time of larval transfer, as well as their interaction had significant effects on the mean shape (Table 2A, Fig. 2A). In contrast, the geldanamycin treatment did not increase wing shape variation (Table 3). To the contrary, in the experiment with larval transfer in the second instar, the estimated amounts of variation were slightly lower for the geldanamycin treatment than for the controls.

Introduction of Hsp83 mutant chromosomes.—The mean shape was affected significantly by both chromosomes carrying *Hsp83* mutations, by the RI line, and by sex (Table 2B, Fig. 2B). A strong interaction effect (chromosome × RI line) was detected, indicating that the observed effects of *Hsp83* on the shape means depended on the genetic background.

This experiment produced no consistent effect of *Hsp83* mutations on the variation of wing shape within samples (Table 4). In only one of 18 cases (nine RI lines × two sexes) an increase in shape variation remained significant after a sequential Bonferroni correction. Overall, no clear increase in phenotypic variation was thus detected either in terms of individual variation or fluctuating asymmetry.

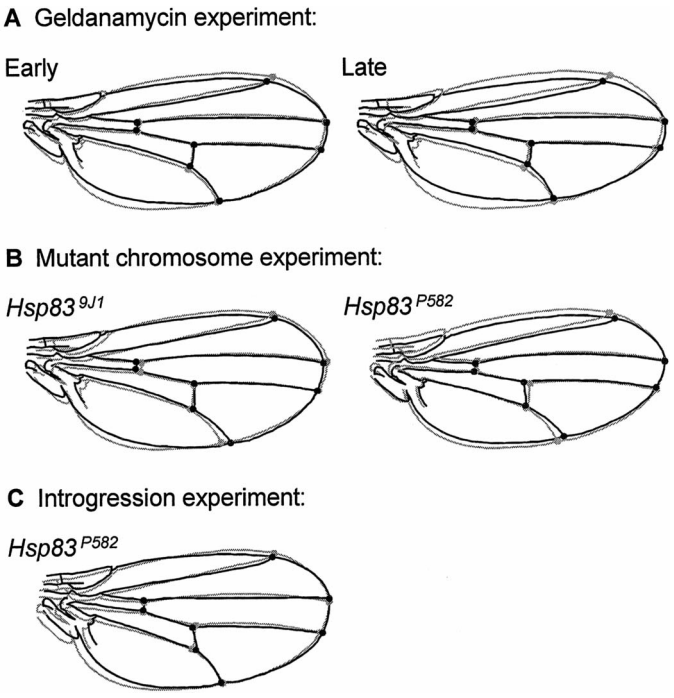

FIG. 2. Effects of reduced Hsp90 activity on the mean shape of the wing. The diagrams show the mean shape for the control or wild-type flies (hollow circles and gray outlines) and the shape transformation to the corresponding mean for the flies treated with geldanamycin or carrying Hsp83 mutations (solid dots and black outlines). The effects have been amplified three-fold for better visibility. The deformation of the outline diagrams used the thin-plate spline interpolation (Bookstein 1989); although the diagrams show the whole wing for ease of interpretation, the extrapolation outside of the region with landmarks is not reliable, and changes should not be interpreted.

Genetic variation, as measured by the amount of variation among the means of RI lines, was slightly lower in the two mutants than in the wild-type (Procrustes variance for $Hsp83^{9J1}$ = 0.0019; Procrustes variance for $Hsp83^{P582}$ = 0.0023; Procrustes variance for the wild type = 0.0025). This is contrary to the expectation of the capacitor hypothesis.

Introgressed allele.—In the introgression experiment, the

TABLE 2. Effects of Hsp90 deprivation on the mean wing shape. MANOVAs on Procrustes coordinates. ''Time'' corresponds to the developmental stage at which larvae were transferred to medium containing geldanamycin (i.e., second versus third instar). df, degrees of freedom.

	Experiment	Source of variation	Wilk's Lambda	*F*	df1	df2	*P*
A	Geldanamycin	Treatment	0.260	91.14	12	385	<0.0001
		Time	0.942	1.97	12	385	0.026
		Interaction (treatment × time)	0.857	5.34	12	385	<0.0001
B	Mutant chromosome	Chromosome	0.138	372.39	24	5270	<0.0001
		RI line	0.084	82.11	96	17761	<0.0001
		Sex	0.190	935.08	12	2635	<0.0001
		Interaction (chromosome × RI line)	0.579	7.71	192	25480	<0.0001
		Interaction (chromosome × sex)	0.944	6.36	24	5270	<0.0001
		Interaction (RI line × sex)	0.786	6.72	96	17761	<0.0001
		Interaction (chromosome × RI line × sex)	0.864	2.02	192	25480	<0.0001
C	Mutant allele	Allele	0.325	306.02	12	1771	<0.0001
		RI line	0.118	46.37	96	11940	<0.0001
		Interaction (allele × RI line)	0.662	7.84	96	11940	<0.0001

TABLE 3. Effect of geldanamycin on individual variation and FA of the wing shape. Variance components are in units of squared Procrustes distance multiplied by 10^6. GA, geldanamycin; FA, fluctuating asymmetry.

Component of variation	Time of transfer					
	Second instar treatment			Third instar treatment		
	GA	control	*P*	GA	control	*P*
Individual variation	7.7	10.2	0.310	6.4	4.9	0.540
FA	14.6	16.8	0.250	15.5	14.8	0.740

mutant *Hsp83*^{P582} allele significantly affected the mean shape (Table 2C, Fig. 2C). Again, a significant allele × RI line interaction showed that the effect of the mutation depended on the genetic background.

In contrast with the previous experiment, individual variation of flies carrying the mutation was higher than that of wild-type flies in eight of the nine RI lines (Table 5), and six of these differences remained significant after sequential Bonferroni correction. Similarly, shape FA appeared to be affected by the mutant allele. All lines containing the mutant allele had higher estimates of FA: seven of these differences were nominally significant and four remained significant after sequential Bonferroni adjustment. Overall these results suggest that, within genotypes, the mutant allele *Hsp83*^{P582} is associated with an increase in both individual variation and FA of wing shape.

To investigate the effect of the mutation on genetic variation, we compared the shape variation among RI lines in combination with wild-type or *Hsp83*^{P582} alleles. The difference was not statistically significant, although the variation among RI lines was slightly higher for mutant genotypes (Procrustes variance for wild type = 0.0013; Procrustes variance for mutants = 0.0019; Permutation test, $P = 0.11$).

Most matrix correlations between covariance matrices for wild-type and mutant samples were fairly high (Table 6). Note that all matrix correlations for FA were higher than

TABLE 4. Effects of mutant chromosomes on individual variation and FA of wing shape. Tabled values (except *P*-values) are in units of squared Procrustes distance multiplied by 10^6. *P*-values were obtained with permutation tests using 10,000 permutations per comparison. Values in bold remain significant after a sequential Bonferonni correction. WT, wild type; m, male; f, female, RI, Raleigh inbred, FA, fluctuating asymmetry.

RI	Sex	Components of variance			Differences			
		WT	*Hsp83*^{9J1}	*Hsp83*^{P582}	*Hsp83*^{9J1}-WT	*P*	*Hsp83*^{P582}-WT	*P*
Individual variation								
1	f	1.9	17.5	5.0	15.6	**0.0004**	3.1	0.71
1	m	4.4	6.2	5.8	1.8	0.68	1.4	0.16
7	f	3.6	7.3	2.8	3.6	0.37	−0.8	0.43
7	m	3.2	8.4	8.3	5.2	0.32	5.0	0.77
9	f	12.7	19.2	15.1	6.5	0.37	2.4	0.07
9	m	20.9	21.5	10.9	0.6	0.95	−10.0	0.71
11	f	11.9	10.4	17.0	−1.5	0.58	5.1	0.24
11	m	11.2	14.5	11.5	3.3	0.74	0.3	0.96
16	f	6.6	13.8	7.1	7.2	0.11	0.5	0.7
16	m	9.4	13.1	8.0	3.7	0.5	−1.3	0.9
25	f	11.2	9.4	16.5	−1.8	0.67	5.3	0.34
25	m	14.3	10.0	8.6	−4.3	0.37	−5.7	0.35
27	f	10.1	14.8	11.7	4.7	0.39	1.6	0.42
27	m	10.0	16.3	16.6	6.3	0.38	6.6	0.67
30	f	8.6	12.7	12.3	4.1	0.47	3.7	0.98
30	m	6.6	14.3	6.7	7.7	0.19	0.1	0.49
33	f	7.1	17.5	8.6	10.4	0.027	1.5	0.13
33	m	8.5	20.7	23.4	12.2	0.11	14.9	0.72
FA								
1	f	12.8	11.1	11.4	−1.7	0.31	−1.4	0.27
1	m	13.3	12.3	11.1	−1.0	0.67	−2.2	0.45
7	f	9.8	13.8	14.0	4.0	0.038	4.2	0.138
7	m	9.6	14.3	12.8	4.7	0.044	3.2	0.38
9	f	11.7	12.1	10.9	0.4	0.81	−0.8	0.33
9	m	10.9	12.1	12.9	1.2	0.51	2.0	0.72
11	f	10.9	11.7	9.9	0.8	0.13	−1.0	0.61
11	m	8.6	10.6	10.2	2.0	0.73	1.6	0.42
16	f	13.0	16.0	11.5	3.0	0.35	−1.5	0.41
16	m	12.7	17.0	11.2	4.3	0.07	−1.5	0.49
25	f	13.6	13.0	11.6	−0.6	0.78	−2.0	0.09
25	m	15.7	14.1	10.4	−1.6	0.81	−5.3	0.37
27	f	13.0	12.8	12.7	−0.2	0.92	−0.3	0.28
27	m	11.5	11.8	13.8	0.3	0.87	2.3	0.88
30	f	13.3	11.1	9.8	−2.2	0.19	−3.5	0.29
30	m	13.4	13.3	11.1	−0.1	0.96	−2.3	0.021
33	f	11.7	12.0	11.6	0.3	0.89	−0.1	0.67
33	m	11.7	14.9	12.7	3.2	0.072	1.0	0.96

TABLE 5. Effects of the introgression of the *Hsp83*^{P582} mutation on individual variation and fluctuating asymmetry of wing shape. Variance components in units of squared Procrustes distance, multiplied by 10^6, and the *P*-values from the permutation test for the difference between *Hsp83* genotypes. Boldface *P*-values remain significant after a sequential Bonferroni correction. WT, wild type.

	Individual variation			Fluctuating asymmetry		
Line	WT	*Hsp83*	*P*	WT	*Hsp83*	*P*
RI-1	4.6	12.6	**0.0014**	14.0	19.2	0.0169
RI-7	6.1	15.4	**0.0005**	11.1	17.5	**0.0003**
RI-9	18.8	31.7	**0.0010**	13.0	19.3	**0.0006**
RI-11	11.9	22.6	**0.0020**	11.2	20.1	**<0.0001**
RI-16	8.8	16.1	0.0243	15.6	28.5	**0.0003**
RI-25	11.2	23.4	**0.0004**	16.4	18.0	0.3165
RI-27	16.4	26.2	0.0321	14.2	18.9	0.0184
RI-30	9.1	35.4	**0.0001**	14.6	16.5	0.1732
RI-33	23.4	21.5	0.6091	13.5	16.2	0.0475

those for individual variation, suggesting that covariance matrices for wild type and mutant samples tended to be more similar for FA than for individual variation. The matrix correlations between covariance matrices for FA and individual variation were weaker, suggesting that the level of variation (FA vs. individual variation) made a greater difference than the *Hsp83* allele. The matrix correlations between covariance matrices for FA and individual variation tended to be slightly higher for the mutant than for the wild-type samples. A comparison of principal components from each of these covariance matrices did not reveal any consistent patterns of shape variation associated with the *Hsp83*^{P582} mutants.

The ordinations of the covariance matrices of the nine RI lines for each kind of variation (individual variation and FA for both the wild type and the mutants) did not suggest any consistent structure (Fig. 3). There appeared to be no clustering among backgrounds and no recurrent pattern in the relative positions of the RI lines. This suggests that each line responded to the introduction of the mutation in a specific way, both for FA and individual variation (note, however, the slight separation of mutant and wild type FA matrices along the second axis). Overall, the mutation appeared to alter the structure of the shape variation, but no consistent pattern was detected in this alteration.

A different result emerged from the simultaneous ordination of all covariance matrices for individual variation and FA (Fig. 4). The covariance matrices for FA clustered together fairly tightly, whereas those for individual variation were dispersed more widely. This pattern indicates a greater similarity among the covariance matrices for FA than among those for individual variation. Because this analysis was based on distances computed from matrix correlations, a simple scaling effect (greater variances and covariances among individuals than for FA) cannot account for this pattern. As in the previous ordination analyses (Fig. 3), there appeared to be no clustering of the covariance matrices for wild-type versus mutants, regardless of the level of variation considered.

DISCUSSION

The three experiments of this study did not reveal consistent phenotypic effects of a reduction of functional Hsp90. Neither pharmacological inhibition of Hsp90 by geldanamycin nor the two *Hsp83* mutations in comparisons of whole chromosomes induced the predicted increase in phenotypic variation of wing shape. In contrast, in the introgression experiment, the mutation *Hsp83*^{P582} induced an increase in phenotypic variation. Even in this experiment, however, no detectable effect on the amount of genetic variation was found. Such mixed results do not support the idea that Hsp90 is a general capacitor of phenotypic variation and suggest that its role in phenotypic buffering should be reconsidered. These results reinforce and complement earlier findings from wing size and bristle counts (Milton et al. 2003, 2005), and contribute to a more differentiated picture of the role of Hsp90 in developmental buffering.

Negative results.—The many negative results in this study raise the question of whether they may be due to problems with the experiments or lack of statistical power or whether the expected effects are really absent. The pharmacological inhibition of Hsp90 and the experiments with third chromosomes carrying two different *Hsp83* mutations did not match the expectations under a hypothesis that Hsp90 is a general capacitor of phenotypic variation because no increase in the amount of variation was recorded (Tables 3 and 4). There was even a slight decrease in wing shape variation in some lines for both mutant chromosomes and within lines in some cases (Table 4). This indicates that the result is not just due to a lack of statistical power, but suggests the absence of the pattern expected under the hypothesis that the level of Hsp90 activity is critical for phenotypic buffering. It is also

TABLE 6. Comparisons of covariance component matrices in the *Hsp83*^{P582} introgression experiment. Tabled values are the matrix correlations between the covariance component matrices and the *P*-values from the corresponding matrix permutation tests (in parentheses). WT, wild type; FA, fluctuating asymmetry.

	Wild-type versus *Hsp83*		Individual variation versus FA	
Line	Individual variation	FA	WT	Hsp83
RI-1	0.60 (0.0002)	0.86 (< 0.0001)	0.47 (0.043)	0.68 (0.0001)
RI-7	0.67 (0.0007)	0.94 (< 0.0001)	0.60 (0.014)	0.71 (0.0004)
RI-9	0.90 (< 0.0001)	0.93 (< 0.0001)	0.51 (0.0055)	0.72 (< 0.0001)
RI-11	0.82 (< 0.0001)	0.93 (< 0.0001)	0.73 (< 0.0001)	0.81 (< 0.0001)
RI-16	0.52 (0.012)	0.81 (0.0005)	0.54 (0.027)	0.45 (0.10)
RI-25	0.64 (0.0002)	0.84 (< 0.0001)	0.72 (0.0002)	0.67 (0.0015)
RI-27	0.78 (< 0.0001)	0.91 (< 0.0001)	0.64 (0.0005)	0.83 (< 0.0001)
RI-30	0.83 (< 0.0001)	0.90 (0.0002)	0.52 (0.0002)	0.72 (< 0.0001)
RI-33	0.40 (0.12)	0.83 (< 0.0001)	0.59 (0.0054)	0.65 (0.0014)

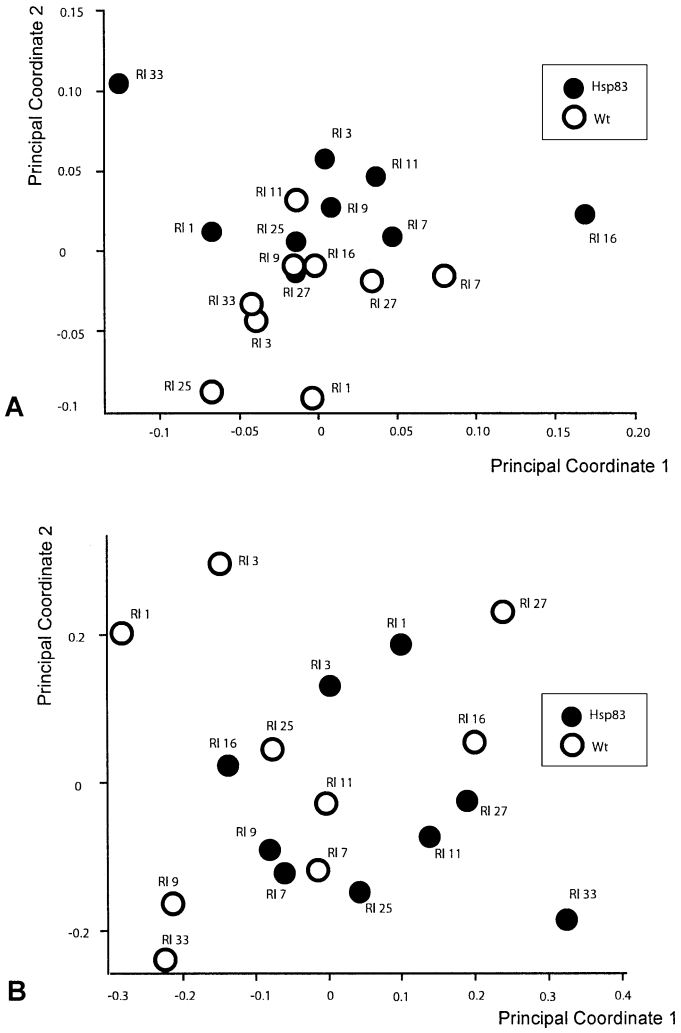

FIG. 3. Ordination of the covariance matrices corresponding to the nine genetic backgrounds. The metric multidimensional scaling was applied independently to the covariance matrices of fluctuating asymmetry (A) and of individual variation (B). Hsp83, *Hsp83*^{P582} mutants; Wt, wild type; RI, Raleigh inbred lines.

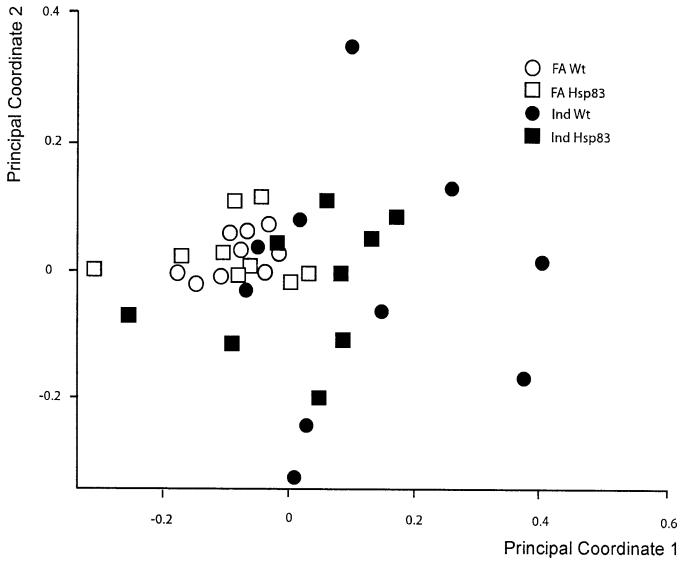

FIG. 4. Ordination of the covariance matrices of individual variation and fluctuating asymmetry (FA) for the nine genetic backgrounds. The metric multidimensional scaling was applied to the individual variation and FA matrices of the wild type (Ind wt and FA wt) and mutant lines (Ind Hsp83 and FA Hsp83) simultaneously. FA matrices are displayed as open symbols, individual variation matrices as black symbols. Note the clustering of FA matrices relative to individual variation matrices.

noteworthy that all these experiments did have statistically significant effects on the average wing shape (Fig. 2). Similar negative results were also reported for the centroid size of the wing (Milton et al. 2003) and for bristle counts (Milton et al. 2003, 2005).

Hsp90 effects on the buffering of traditional quantitative traits.—The introgression experiment, which introduced the *Hsp83*^{P582} allele into an entirely new genetic background, produced greater amounts of shape variation in flies carrying the mutation than in the wild type flies (Table 5). This shows that Hsp90 can contribute to the buffering of traditional quantitative traits as well as threshold traits (Rutherford and Lindquist 1998; Rutherford 2000; Queitsch et al. 2002; Milton et al. 2003; Sollars et al. 2003; Gibson and Dworkin 2004). Because the experimental design ensured that all individuals in each line had identical genotypes, this increase of phenotypic variation within lines reflects an increased sensitivity of the developing wings to nongenetic influences, that is, to developmental and environmental variation. The observed effect therefore suggests that Hsp90 is involved in environmental canalization and developmental stability.

The ordination analyses of covariance matrices for samples of flies with and without the *Hsp83* mutation did not form distinct clusters (Figs. 3, 4). Moreover, the variation among covariance matrices for the different RI lines in crosses with mutant or wild-type lines showed no consistent pattern. This suggests that the activity of Hsp90 per se does not determine the nature of the induced variation, but depends on the genetic background. Moreover, the additional variation produced by inhibiting Hsp90 is not a simple amplification of the variation present in the genetic background. Overall, therefore, the patterns of variation are not primarily determined by either Hsp90 activity or by the genetic background on their own, but by their interaction.

Our experiments produced either no increase or a nonsignificant increase in variation among the means of RI lines when they were crossed to *Hsp83*mutations. This means that our study did not find increased genetic variation due to reduced Hsp90 activity, as would be expected under the capacitor hypothesis. This is not surprising given the small number of genotypes considered (the nine RI lines), which implies a weak statistical power for addressing this question. This experiment therefore cannot provide conclusive evidence whether a reduction of Hsp90 activity uncovers cryptic genetic variation (Rutherford and Lindquist 1998; Rutherford 2000, 2003; Gibson and Dworkin 2004).

Hsp90 and developmental stability of wing shape.—In the introgression experiment, the lines carrying the *Hsp83*^{P582} mutation had significantly higher levels of FA of wing shape (Table 5). In addition to the inconsistency with the other experiments in our study, this result is in contrast to the

results of earlier studies on wing size (Milton et al. 2003) and on bristle traits (Milton et al. 2003, 2005), which did not find an increase of FA associated with Hsp90. The difference may be due in part to the nature of the characters investigated. It is likely that wing shape is more sensitive to Hsp90 deprivation than wing size and bristle numbers. Shape, as a multidimensional feature, is inherently able to convey more information than scalar quantities such as size and bristle number. Accordingly, studies of shape tend to be more sensitive for recording differences in variation. For instance, QTL studies of shape in mandibles and teeth of mice have documented a similar contrast between scalar and multidimensional data: approximately twice as many QTLs were detected for shape than for size in mandibles (Klingenberg et al. 2001b) and six times for teeth (Workman et al. 2002). Similarly, shape variation has proved to be a more sensitive indicator of fitness and stress than traditional scalar measures (e.g., Hoffmann et al. 2002; Hoffmann and Shirriffs 2002; Kölliker-Ott et al. 2003), and mutations of many genes in *Drosophila* have effects on wing shape (Dworkin and Gibson 2006).

The observed increase in FA associated with the $Hsp83^{P582}$ mutation is consistent with the hypothesis that Hsp90 is a candidate for controlling developmental stability (Klingenberg 2003; McKenzie 2003; Hoffmann and McKenzie 2005). Although Hsp90 may only be one of many molecular factors contributing to developmental stability, this nonetheless remains an interesting result, particularly when considering the paucity of data concerning the molecular basis of developmental instability (Klingenberg 2003; McKenzie 2003; Willmore and Hallgrímsson 2005). The best examples of identified genes involved in the control of FA so far remain *Rop1* and its modifier, a homologue of *Notch*, in the sheep blowfly (e.g., Davies et al. 1996; Clarke 1997; McKenzie 2003), and *Notch* in *Drosophila* (Indrasamy et al. 2000). In addition, experiments manipulating the expression patterns of a range of genes in wing imaginal discs produced increased asymmetry in the wings (Trotta et al. 2005).

The fact that the variation among individuals within line (environmental variation) and the variation within individuals (FA; Table 5) are altered simultaneously suggests that these components of phenotypic variation can be sensitive to similar conditions, in this case, the presence of $Hsp83^{P582}$ mutant. This is of particular interest because the relationship between processes buffering against genetic, environmental and random variation are mostly unknown (e.g., Debat and David 2001).

The relationship between canalization and developmental stability can be investigated by comparing the patterns of FA and individual variation. Our analyses indicated significant similarities for most but not all genotypes (Table 6). In some previous studies, these patterns were found to be different, suggesting that canalization and developmental stability are distinct processes (Woods et al. 1999; Debat et al. 2000; Hoffmann and Woods 2001; Klingenberg et al. 2003; Réale and Roff 2003; Pélabon et al. 2004; Santos et al. 2005). Other studies reported highly similar patterns, leading to the conclusion that there is no evidence for more than one mechanism (Clarke 1998; Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000; Klingenberg et al. 2001a; Hallgrímsson et al. 2002). Resolution of this question requires further experiments with larger sample sizes.

In this context, the tight clustering of covariance matrices for FA, by comparison with the broader dispersion among covariance matrices for individual variation in the same samples, is particularly intriguing (Fig. 4). The relative constancy of covariance patterns for FA appears to contrast with greater differences in patterns of environmental variation among individuals (recall that the flies of each sample have identical genotypes). This difference in the behavior of covariance matrices at the two levels of variation may reflect the differences in the developmental origins of covariation (e.g., Klingenberg 2005). Clearly, there is much scope for further experiments exploring the genetic basis of the control of phenotypic variation.

The mechanisms involved in the control of variation have been explicitly addressed by theoretical models of developmental processes (e.g., Gavrilets and Hastings 1994; Klingenberg and Nijhout 1999). These studies have typically concluded that a single mechanism is sufficient to explain the buffering of phenotypic variation regardless of the forces generating it (reviews by Meiklejohn and Hartl 2002; Klingenberg 2003). Furthermore, models of genetic networks have examined the hypothesis that robustness might emerge as an inherent property of a network's structure and complexity (e.g., von Dassow et al. 2000; Meir et al. 2002; Siegal and Bergman 2002; Proulx and Phillips 2005). These approaches themselves have sparked controversy (e.g., Kerszberg 2004), and additional mechanisms for buffering phenotypic variation have been suggested (Hornstein and Shomron 2006). Whatever the origins of developmental buffering are, however, the apparent lack of an effect of the *Hsp83* mutation on the clustering of covariance matrices suggests that Hsp90 plays a relatively minor role in determining the patterns of variation in the wing.

Overall, the mix of positive and negative results in this study suggests that Hsp90, rather than controlling shape variation, is one of multiple factors that participate in the developmental buffering of shape. In contrast to the analyses for bristle counts and wing size (Milton et al. 2003), our analyses of wing shape did find that reduction of Hsp90 activity led to significant increases of individual variation and FA in one of the experiments. Nevertheless, the many negative results suggest that Hsp90 has a contributory rather than a controlling role in developmental buffering.

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