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Effect of *E(sev)* and *Su(Raf) Hsp83* Mutants and *Trans*-heterozygotes on Bristle Trait Means and Variation in *Drosophila melanogaster*

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ABSTRACT

The Hsp90 protein encoded by the *Hsp83* gene is required for the development of many traits in Drosophila. *Hsp83* is also thought to play a role in the expression of phenotypic and genetic variability for subsequent selection and evolutionary change. Here we examine the impact of different *E(sev)* and *Su(Raf) Hsp83* mutants on means and phenotypic variances of invariant and variable bristle traits. One of the mutants influenced the normally invariant thoracic bristle number, while none affected invariant scutellar bristle number. *E(sev)* alleles consistently influenced variable bristle traits while there were fewer effects of the *Su(Raf)* alleles. For the variable traits, none of the *Hsp83* alleles had any effect on phenotypic variance, environmental variance, or developmental stability of the bristle traits. When alleles were combined in *trans*-heterozygotes, there were both cumulative and complementary effects on thoracic and variable bristle trait numbers, depending on the allelic combination. Overall, the results suggest that *Hsp83* mutants do not have detectable effects on the phenotypic or environmental variance of bristle traits and that complementation of *E(sev)* and *Su(Raf) Hsp83* mutants can extend to thoracic bristles as well as previously reported effects on viability. Some allelic combinations lead to more severe effects on variable bristle trait means than do single *Hsp83* mutations.

THE Hsp90 chaperone protein is required for the development of many different morphological structures in *Drosophila melanogaster*, such as bristles, eyes, and wings (Rutherford and Lindquist 1998). Apart from being of interest because of its central role in development, Hsp90 has also been investigated because of its potential role in facilitating evolutionary change. In particular, Rutherford and Lindquist (1998) used both Hsp90 inhibitors and mutations in the *Hsp83* gene, which encodes the Hsp90 protein in Drosophila, to demonstrate that lowered levels of Hsp90 can lead to the expression of abnormal phenotypes. Moreover, these phenotypes could then be selected to increase in frequency. This "evolutionary capacitor" model involves two components: the increased expression of phenotypic variation and a subsequent genetic change based on the utilization of this variation.

In conjunction with its proposed evolutionary capacitance, *Hsp83* has also been regarded as a candidate gene for "canalization." Waddington was the first to use this term, defining it as the ability of an organism to produce a consistent phenotype despite variation in the genotype or environment (Waddington 1957). He later widened this definition to include the ability of developmental networks to endure genetic or environmental disruptions. Schmalhausen (1949) also independently considered the concept of canalization, using the term "autonomization" instead of canalization, although he and Waddington were essentially describing the same process.

Rutherford and Lindquist's (1998) hypothesis that Hsp90 acts as an important evolutionary capacitor and canalization gene has recently been questioned. Bergman and Siegal (2003) simulated a complex gene network model and used yeast expression data to show that almost any knockout mutation in a gene can result in the expression of previously hidden variation. They suggested that Hsp90 is just one of a large class of evolutionary capacitors. Hermisson and Wagner (2004) argued that hidden genetic variation is a by-product of complex genetic networks and that hidden genetic variation could exist for any trait. They argued that no specific molecular mechanism was necessarily needed for the release of hidden genetic variance. There is also limited evidence that Hsp90 influences canalization and the expression of phenotypic variation: the Hsp90 inhibitor geldanamycin had no impact on variance in non-canalized bristle traits, and there was also no impact on the developmental instability of these traits as measured by fluctuating asymmetry (Milton *et al.* 2003).

Hsp90's chaperoning activity is known to particularly target signal transduction proteins. This role in the maintenance of signaling networks was confirmed by the identification of *Hsp83* mutations in *D. melanogaster*

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during screens for suppressors of *Raf* and enhancers of *sevenless* (Simon *et al.* 1991; Cutforth and Rubin 1994; Dickson *et al.* 1996; van der Straten *et al.* 1997). Both of these genes are the central components of signaling pathways. The Raf signaling cascade allows the nuclear translocation of mitogen-activated protein kinase to take place (van der Straten *et al.* 1997). The sevenless signaling cascade enables differentiation of the R7 photoreceptor neuron to proceed in the developing eye of *D. melanogaster* (Cutforth and Rubin 1994).

Five *Hsp83* mutations were identified as *Enhancers of sevenless* [*E(sev)*] alleles (Simon *et al.* 1991; Cutforth and Rubin 1994), while two *Hsp83* mutations were identified as *Suppressors of Raf* [*Su(Raf)*] alleles (van der Straten *et al.* 1997). While each of these *Hsp83* mutations is homozygous lethal, several are viable as *trans*-heterozygotes; that is, they are able to complement one another for viability (van der Straten *et al.* 1997). Generally, those *Hsp83* alleles isolated as *E(sev)* mutations complement those isolated as *Su(Raf)* mutations. It is still unclear why complementation occurs. Two explanations have been proposed in the literature on the basis of the understanding of the structure of the Hsp90 protein, which contains three highly conserved domains that are found in all Hsp90 family members and that are connected by a "charged linker" of variable length and composition (Scheibel and Buchner 1998; Pearl and Prodromou 2000). The ~25-kDa N-terminal domain contains the binding site for ATP or geldanamycin, an ansamycin antibiotic that specifically inhibits Hsp90 (Young *et al.* 2001). The ~33-kDa middle segment of Hsp90 contains a binding site for the protein kinase PKB/Akt (Sato *et al.* 2000). The ~22-kDa C-terminal domain is involved in dimerization, which is essential for the *in vivo* function of Hsp90 (reviewed in Minami *et al.* 2001). Thus, the first explanation is that these complementation patterns may result from Hsp90 proteins acting as dimers. A dimer containing proteins with different defects may still be able to perform the normal role of Hsp90 (van der Straten *et al.* 1997; Yue *et al.* 1999). Alternatively, complementation of the different *Hsp83* alleles may be reliant on the nature of a mutation, that is, whether the mutation is antimorphic or hypomorphic (van der Straten *et al.* 1997). For example, none of the antimorphic *Su(Raf)* alleles map to the C-terminal region of Hsp90, which is necessary for dimerization of the protein. All but one of the hypomorphic *E(sev)* alleles map to this region (Minami *et al.* 1994; van der Straten *et al.* 1997).

Yue *et al.* (1999) noted no abnormalities in the bristles of these *trans*-heterozygotes, although they did not examine phenotypic variation in detail or measure the variance of traits. However, Rutherford and Lindquist (1998) detected abnormalities in bristle traits, as well as in eye and leg defects in a $Hsp83^{9J1}/Hsp83^{e1D}$ *trans*-heterozygote. Other Hsp90 mutant strains have also been shown to influence the means of bristle traits (Milton *et al.* 2003). Considering that the *Hsp83 trans*-heterozygotes contain two defective copies of *Hsp83* (compared to the *Hsp83/+* flies previously analyzed, which possessed one defective and one wild-type copy of *Hsp83*), large influences on bristle traits might be expected in *trans*-heterozygotes, depending on the pattern of complementation of the mutants.

In this study we test one prediction of the evolutionary capacitor hypothesis, namely that Hsp90 increases some components of phenotypic variance in bristle traits, using the suppressors of *Raf* and enhancers of *sevenless Hsp83* mutants. Two canalized ("invariant") bristle traits and four variable traits were considered to test if the mutants influenced the bristle traits (*cf.* Milton *et al.* 2003). As well as bristle variances, the impact on bristle abnormalities of invariant bristle traits was also considered. The phenotypic variances were separated into within-strain and developmental instability components. We also test the hypothesis that complementation occurs among some *Hsp83* mutants when in *trans*-heterozygote form. The impact of mutant *trans*-heterozygotes of *Hsp83* on bristle traits was analyzed to determine whether flies carrying two defective, but complementary, copies of *Hsp83* exhibited complementary or cumulative effects on bristle trait variation. If there are complementary effects, then *trans*-heterozygotes should exhibit lower levels of bristle variation than single heterozygotes. Alternatively, if there is a cumulative effect, higher levels of phenotypic variation should be observed in *trans*-heterozygotes compared with single heterozygotes.

MATERIALS AND METHODS

Trait measurements: The number of scutellar, thoracic, sternopleural, orbital, ocellar, and vibrissa and carina bristles were counted on the left and right sides of each of 50 flies. Two of these traits, the scutellar and thoracic bristles, are regarded as largely invariant or canalized between two thresholds (Rendel 1967) because almost all individuals show the same number of bristles while a few rare individuals show higher or lower scores. The remaining four characters were variable traits. These six bristle traits are shown in Figure 1.

Strains: The strains used in this study are shown in Table 1. $Hsp83^{13F3}$ and $Hsp83^{19F2}$ are independent isolates of the same amino acid replacement. The strains were kindly provided by Suzanne Rutherford (w^{1118}, $w^{1118}sev^{d2}$, $Hsp83^{9J1}$, $Hsp83^{13F3}$, $Hsp83^{19F2}$, $Hsp83^{e1D}$, and $Hsp83^{e3A}$) and the Bloomington Stock Center ($Hsp83^{e6A}$ and $Hsp83^{e6D}$). The *Hsp83* alleles were initially isolated by Simon *et al.* (1991), Cutforth and Rubin (1994), Dickson *et al.* (1996), and van der Straten *et al.* (1997). $Hsp83^{9J1}$ is viable with all of the alleles except $Hsp83^{13F3}$ and $Hsp83^{19F2}$. $Hsp83^{13F3}$ and $Hsp83^{19F2}$ complement $Hsp83^{e1D}$, $Hsp83^{e3A}$, and $Hsp83^{e6A}$ but are lethal over $Hsp83^{e6D}$. Marker strains w^{1118} and $w^{1118}sev^{d2}$ were used as the controls. These were the respective genetic backgrounds used in the initial screens in which the *Su(Raf)* and *E(sev)* alleles were recovered. Therefore, the genetic variation contained in the *Hsp83* alleles originated from these backgrounds. Previous results (Milton *et al.* 2003) indicated that genetic background plays a significant role in bristle variation when a mutation is present in *Hsp83*.

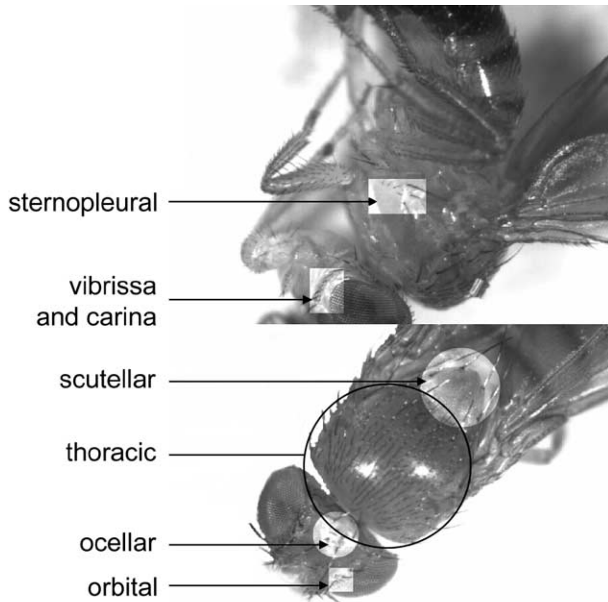

FIGURE 1.—The six bristle characters analyzed for trait variation. The position of the invariant traits [thoracic (circled) and scutellar bristles] and the four variable traits (sternopleural, ocellar, orbital, and vibrissa and carina bristles) are marked.

Crosses: To obtain *Hsp83 trans*-heterozygotes (flies carrying two different *Hsp83* alleles), crosses were performed between the lines in Table 1 that complement each other to form viable *trans*-heterozygotes. Virgin females were crossed to males in population cages at 25° and allowed to lay on standard Drosophila medium plates spread with yeast paste. To control for density and vial effects, first instar larvae were collected from the plates and transferred to vials containing standard medium at a constant density of 50 larvae per vial. Five vials were used per genotype. The balancer marker *Tubby* can be scored in the larvae, so larvae that did not carry the balancer could be selected. The strains were kept at a constant temperature of 25°. From each vial, 10 females (50 total) were selected at random and the six bristle characters were scored.

To obtain *Hsp83* heterozygotes (intermediates) for comparisons with the *trans*-heterozygotes and controls, the *Hsp83* alleles were crossed back into the genetic background in which they were initially isolated. Therefore, *Su(Raf)* alleles ($Hsp83^{9J1}$, $Hsp83^{13F3}$, and $Hsp83^{19F2}$) were crossed into w^{1118} and *E(sev)* alleles were crossed into $w^{1118}sev^{d2}$. Crosses were performed in population cages as described above. *Hsp83*/+ larvae from each cross were placed at constant temperature and density as before, and 10 female F_1 *Hsp83* heterozygotes were selected from each vial and scored for bristle characters as described previously. A total of 50 females were scored for each genotype.

The two control lines (w^{1118} and $w^{1118}sev^{d2}$) were placed in population cages and larvae were collected and seeded at a constant density as described above. Ten females from each vial were selected and scored for bristle characters as described above. Fifty females were scored for each control strain.

Data analysis: A different approach was used in the analysis of the invariant and variable traits. For the invariant traits, to examine effects of the genetic background and the *Hsp83* allele on invariable bristle numbers, each genotype was divided into two groups: the number of flies that exhibited the canalized phenotype and the number of flies that had more or less bristles than the canalized phenotype. The canalized wild-type phenotype for thoracic bristles is 11 bristles on either side of the thorax. To determine the effect of the genetic background ($w^{1118}/w^{1118}sev^{d2}$) on the invariable bristle traits, chi-square contingency tests were performed comparing the number of flies in these two classes for w^{1118} and $w^{1118}sev^{d2}$. Chi-square contingency tests were also performed to determine the effect of each of the seven *Hsp83* alleles on invariable bristle number, with the single *Hsp83* heterozygotes compared to the genetic background in which the *Hsp83* allele was recovered. Because low numbers of individuals in cells meant that probabilities based on the chi-square distribution were not valid, significance levels were determined by permutation using SPSS version 11.5. In all analyses, probability values were corrected for multiple comparisons with the Dunn-Sidak method due to the number of traits compared or number of comparisons made with the same background strain (SOKAL and ROHLF 1995).

To compare *trans*-heterozygotes to the mutant strains for the invariant thoracic bristle trait, contingency tests were used. If complementation occurred, bristle abnormalities were expected to decline relative to either of the parental strains. However, under combinatory effects, bristle numbers were expected to increase in frequency relative to the parentals. Because the two genetic backgrounds from which the mutants were derived did not influence thoracic bristle abnormalities, background effects were ignored when making these comparisons. Similar procedures were used for the scutellar bristle system, but these turned out to exhibit a low level of variability and were not further considered.

For the variable traits, the effect of genetic background ($w^{1118}/w^{1118}sev^{d2}$) on variable trait means was examined using one-way ANOVAs. To test for effects of individual *Hsp83* alleles, one-way ANOVAs were performed comparing each *Hsp83*

TABLE 1

D. melanogaster **strains used in this study and their viability with other mutants when in *trans*-heterozygote form**

Category	Strain	Description	Viability in *trans*-heterozygotes
Screening stocks	w^{1118}	w^{1118} used in original screen	—
	w^{1118}, sev^{d2}	sev^{d2} used in original screen	—
Hsp83 strains	*w*; $Hsp83^{9J1}$/TM3, *Tb*	E377K	$Hsp83^{e1D}$, $Hsp83^{e3A}$, $Hsp83^{e6A}$, $Hsp83^{e6D}$
	w; $Hsp83^{13F3}$/TM6B, *Tb*	R48C	$Hsp83^{e1D}$, $Hsp83^{e3A}$
	w; $Hsp83^{19F2}$/TM6B, *Tb*	R48C	$Hsp83^{e1D}$, $Hsp83^{e3A}$
	w; $Hsp83^{e1D}$/TM6B, *Tb*	S38L	$Hsp83^{9J1}$, $Hsp83^{13F3}$ $Hsp83^{19F2}$
	$Hsp83^{e3A}$/TM6B, *Tb*	S574C	$Hsp83^{9J1}$, $Hsp83^{13F3}$ $Hsp83^{19F2}$
	$Hsp83^{e6A}$/TM6B, *Tb*	S592F	$Hsp83^{9J1}$
	$Hsp83^{e6D}$/TM6B, *Tb*	E317K	$Hsp83^{9J1}$

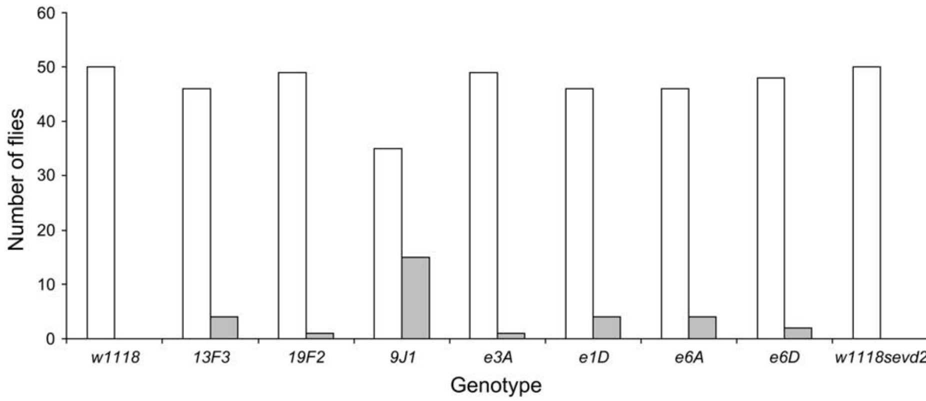

FIGURE 2.—Number of flies with canalized and noncanalized thoracic phenotypes for genetic background ($w^{1118}/w^{1118}sev^{d2}$) and single *Hsp83* heterozygotes. The canalized phenotype (☐) for the thoracic bristles is 11 bristles on either side of the thorax, with the noncanalized phenotype (▒) having either less than or more than 11 bristles on either side of the thorax. A total of 50 flies were scored for each genotype.

allele to the genetic background in which it was recovered. Variances of the single *Hsp83* heterozygotes and the two *w* backgrounds were analyzed following the method outlined by PERTOLDI *et al.* (2001). The different components of phenotypic variance (V_P) for the single *Hsp83* heterozygotes and the two *w* backgrounds were separated. Developmental instability (DI) was defined as the variance of the difference between bristle counts on the left and right sides of the fly and the measured variation within an individual. We also estimated variation among individuals from a particular mutant/background. This variation is likely to be mainly due to environmental variance (V_e) but also includes a component of genetic variance due to minor genetic differences within the mutant strains. We therefore refer to this as the variance within strains (V_{WS}). Confidence intervals for these parameters were estimated by bootstrapping, while permutation was used to compare each mutant to its *w* background for each of the four variable traits, using the PopTools add-in for Microsoft Excel.

The effects of interactions in the *trans*-heterozygotes on variable trait means was complicated by the fact that there were differences due to background between the strains from which the mutants had been derived (see below). This meant that background as well as the effect of the mutants had to be taken into account. When the *trans*-heterozygotes differ from the parental strains by having higher or lower trait values than those of either parent, this might indicate more severe effects of *Hsp83* on the trait than those exerted by either mutant in the heterozygous state (*i.e.*, a lack of complementation), at least if changes are in the same direction as any differences between the parental and mutant strains. Any tendency of *trans*-heterozygotes to show changes in means that made trait values more similar to those of the parental strains might indicate complementation, but it was not possible to interpret results in terms of complementation because of the large differences among the parental strains. MANOVAs and ANOVAs were carried out to compare *trans*-heterozygotes to the midvalue of two heterozygous mutant strains. Differences between the *trans*-heterozygotes and the parental strains were also assessed to test for evidence that *trans*-heterozygotes exhibited extreme phenotypes that could arise from combinations of the mutant alleles.

RESULTS

Genetic background and single *Hsp83* heterozygotes

Invariant traits: To determine what effect the various *Hsp83* alleles have on invariant traits (traits in which little variation from the wild-type phenotype is observed), the thoracic and scutellar bristles were analyzed. The number of flies with canalized/noncanalized thoracic bristle phenotypes are shown in Figure 2. Results of chi-square tests comparing thoracic bristle phenotypes of the two different *w* backgrounds, and each *Hsp83* allele and its particular *w* background, are shown in Table 2. Genetic background had no effect on the number of thoracic bristles, as all 50 flies of both w^{1118} and $w^{1118}sev^{d2}$ exhibited the canalized thoracic phenotype. Of the seven *Hsp83* alleles examined, only $Hsp83^{9J1}$ exhibited a significantly different thoracic bristle phenotype. Of the 50 $Hsp83^{9J1}$ flies, 15 exhibited a changed thoracic bristle phenotype, 12 had an extra bristle, and 3 were missing bristles. The $Hsp83^{9J1}$ allele affects three thoracic bristles: the aDC, pDC, and H bristles.

For the scutellar bristles, very few individuals (3%) exhibited an aberrant phenotype and there was no evidence of differences between the *Hsp83* alleles and the *w* backgrounds. Therefore, scutellar bristle data were not analyzed further.

Variable traits: To investigate the effect of *Hsp83* alleles on variable traits (traits in which there is no stereotyped or canalized phenotype), the sternopleural, orbital, ocellar, and vibrissa and carina bristles were analyzed. ANOVAs indicated that genetic background ($w^{1118}/w^{1118}sev^{d2}$) had a highly significant effect

TABLE 2

Contingency tests on effect of *Hsp83* allele and *w* background on canalized *vs.* noncanalized thoracic bristle numbers

Allele	Chi-square (d.f. = 1)	*P*
$Hsp83^{13F3}$	4.17	0.117
$Hsp83^{19F2}$	1.01	1
$Hsp83^{9J1}$	17.65	<0.001[a]
$Hsp83^{e3A}$	1.01	1
$Hsp83^{e1D}$	4.17	0.117
$Hsp83^{e6A}$	4.17	0.117
$Hsp83^{e6D}$	2.04	0.495
w background	0	—

Mutants were compared to their originating background strain. Probabilities are based on permutation tests.

[a] Significant after correcting probabilities for multiple comparisons made with the same background strain.

TABLE 3

MANOVAs comparing effects of *Hsp83* alleles on variable bristle trait means relative to the *w* background in which they were recovered

Allele	Wilks λ	F (d.f. = 4, 95)	P
$Hsp83^{13F3}$	0.909	2.37	0.058
$Hsp83^{19F2}$	0.914	2.24	0.070
$Hsp83^{9J1}$	0.978	0.54	0.710
$Hsp83^{e3A}$	0.358	42.59	<0.001[a]
$Hsp83^{e1D}$	0.556	18.96	<0.001[a]
$Hsp83^{e6A}$	0.285	59.64	<0.001[a]
$Hsp83^{e6D}$	0.407	34.54	<0.001[a]

[a] Significant after correcting probabilities for the number of comparisons made with the same background strain.

($P < 0.001$) on the means of all four variable bristle traits (results not shown). A MANOVA on all four variable bristle traits also indicated a significant effect of genetic background (Wilks $\lambda = 0.362$, $F = 41.93$, d.f. $= 4, 95$, $P < 0.001$). Results of MANOVAs for each of the seven *Hsp83* alleles compared to their originating *w* background are shown in Table 3. These indicated that the *Hsp83* alleles had varying effects on bristle trait means, with the *E(sev)* alleles having significant effects on variable bristle trait means compared to the $w^{1118}sev^{d2}$ background, while there was no significant difference between the *Su(Raf)* alleles and the w^{1118} background.

Means are shown in Figure 3. Most of the significant differences in trait mean were exhibited by the *E(sev)* alleles. ANOVAs indicated that all four *E(sev)* alleles had a significant effect on vibrissa and carina means ($Hsp83^{e3A}$: $F = 37.86$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e1D}$: $F = 98.12$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e6A}$: $F = 147.95$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e6D}$: $F = 52.37$, d.f. $= 1, 98$, $P < 0.001$). In each case bristle numbers decreased in the mutants (Figure 3). The *E(sev)* alleles, with the exception of $Hsp83^{e6D}$, also had a significant effect on ocellar bristle trait mean ($Hsp83^{e3A}$: $F = 22.42$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e1D}$: $F = 30.18$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e6A}$: $F = 55.08$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e6D}$: $F = 5.68$, d.f. $= 1, 98$, $P = 0.019$). This involved an increase in bristle number (Figure 3). $Hsp83^{e6D}$ and $Hsp83^{e1D}$ also caused a significant decrease in sternopleural bristle trait when compared to the $w^{1118}sev^{d2}$ background ($Hsp83^{e6D}$: $F = 104.37$, d.f. $= 1, 98$, $P < 0.001$; $Hsp83^{e1D}$: $F = 29.01$, d.f. $= 1, 98$, $P < 0.001$). The only *Su(Raf)* allele to exhibit a difference in variable bristle trait mean compared to the w^{1118} background was $Hsp83^{13F3}$, which had a significant effect on ocellar bristle trait mean compared to the w^{1118} background ($F = 9.73$, d.f. $= 1, 98$, $P = 0.002$) involving a decrease in bristle number.

While the *Hsp83* mutants influenced bristle trait means, there were no consistent effects on trait variability. Figure 4 shows the V_P, V_{WS} and DI values and the 95% confidence limits for each variable trait. Generally, the confidence intervals overlapped and there were few significant differences between the mutants and their background stocks when compared by permutation. Overall only 6 comparisons were significant—in each case at the 5% level and not the 1% level—which is similar to chance expectations, given that there were 96 comparisons. Moreover, in cases where differences between background strains and mutants were significant, the mutants did not necessarily have higher levels of V_P, V_{WS}, or DI than the background strains (Figure 4). Therefore, the results indicate that the *Hsp83* mutants do not lead to detectable changes in the phenotypic variance or environmental variance of these bristle traits. Furthermore, they do not increase developmental instability.

Hsp83 trans-heterozygotes

Invariant traits: The number of flies from the *trans*-heterozygote and single heterozygote categories that had abnormal or canalized numbers of thoracic bristles are shown in Figure 5. As there was no difference among the genetic backgrounds for this trait, differences between the *trans*-heterozygotes and heterozygous mutant strains were interpreted in terms of the effects of the combined mutants on bristle abnormalities. *Trans*-heterozygotes had an increase in the number of abnormal bristles compared to parental heterozygotes in four cases (Figure 5: $Hsp83^{13F3} \times Hsp83^{e1D}$, $Hsp83^{19F2} \times Hsp83^{e1D}$, $Hsp83^{13F3} \times Hsp83^{e3A}$, and $Hsp83^{19F2} \times Hsp83^{e3A}$). The difference between the *trans*-heterozygote and either parental strain was significant in three of the four cases by permutation tests on contingency tables (the comparison involving the $Hsp83^{19F2} \times Hsp83^{e3A}$ cross was nonsignificant). Some combinations of alleles therefore seem to lead to cumulative effects on abnormal bristle frequency rather than to complementation.

For the remaining crosses involving the $Hsp83^{9J1}$ mutant that markedly increased the incidence of abnormality, there was no change in the incidence of abnormalities in one cross ($Hsp83^{9J1} \times Hsp83^{e1D}$) or a decrease in the incidence of abnormalities when compared to $Hsp83^{9J1}$. The decrease was nonsignificant in one cross ($Hsp83^{9J1} \times Hsp83^{e3A}$). However, it was significant in two other cases ($Hsp83^{9J1} \times Hsp83^{e6A}$ and $Hsp83^{9J1} \times Hsp83^{e6D}$) when the incidence of abnormalities was similar to that observed in the other mutant strain involved in the cross (Figure 5). Therefore, there seems to have been complementation reducing the incidence of abnormality toward that of the wild-type background strain. When combined with the *E(sev)* alleles, the same three thoracic bristles affected by the single $Hsp83^{9J1}$ heterozygote were usually affected, as well as two additional bristles, aPA and pSA.

Variable traits: As the differences in the genetic background of the *Su(Raf)* and *E(sev)* alleles resulted

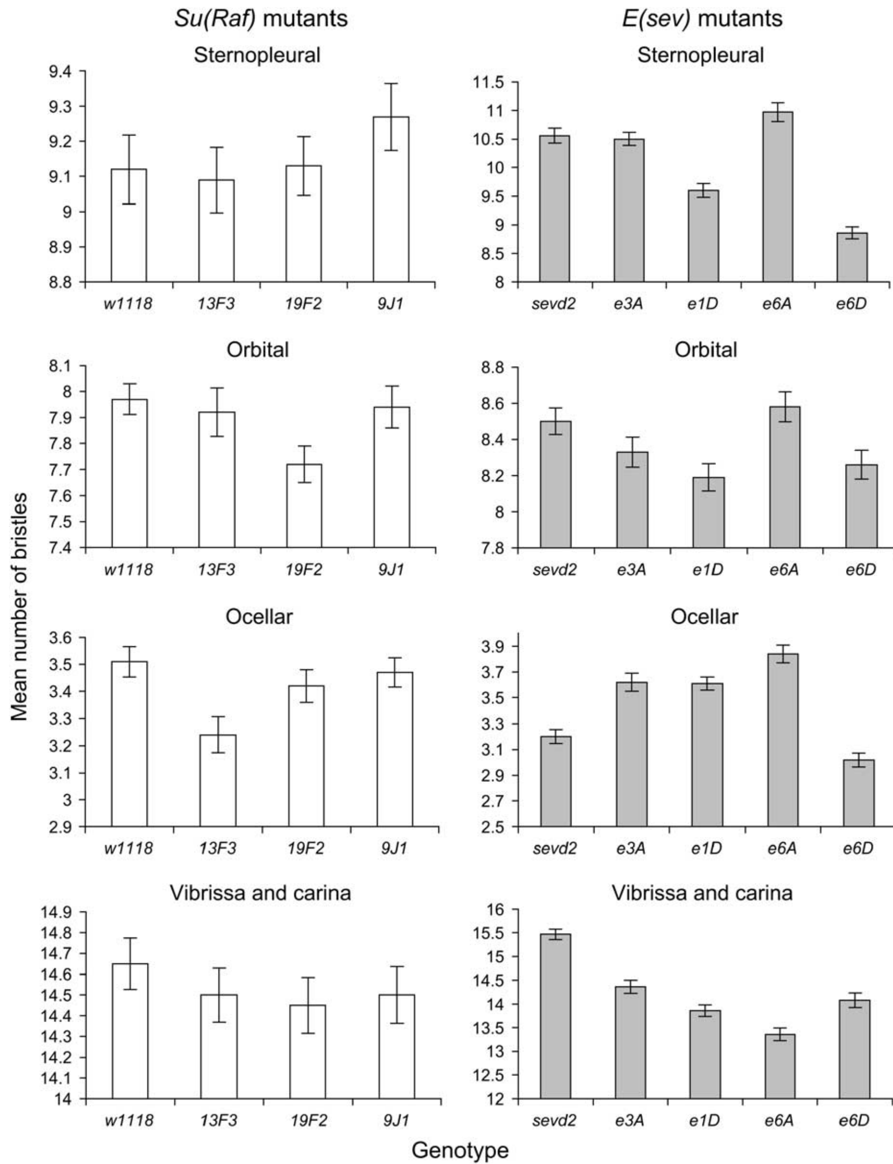

FIGURE 3.—Means for variable bristle traits for single *Hsp83* heterozygotes (± standard error). The two different genetic backgrounds are indicated by open and shaded bars.

in significant changes in variable bristle trait means, data from the *trans*-heterozygotes need to be interpreted cautiously and compared to parental as well as the mutant heterozygote strains. MANOVAs were initially undertaken to test if *trans*-heterozygotes differed from the mean value of the two mutant heterozygotes. In all cases, MANOVAs indicated significant ($P < 0.001$) deviations from expectations (analyses not shown). Planned contrasts in ANOVAs were then undertaken to examine patterns for bristle traits (Table 4). In addition, mean values for the *trans*-heterozygote crosses as well as the mutant heterozygotes and original parental strains were plotted (Figure 6) to interpret patterns and assess potential effects of background as well as mutant combinations on the bristle scores.

For sternopleural bristle number, the *trans*-heterozygote means were similar to one of the mutant heterozygotes or were intermediate between the mutant heterozygotes (Figure 6). Differences from the midmutant value were significant in two cases ($Hsp83^{19F2} \times Hsp83^{e3A}$ and $Hsp83^{9J1} \times Hsp83^{e6A}$) when the *trans*-heterozygote was more similar to the mutant with higher bristle values but nevertheless still intermediate between the two values (Figure 6). The results suggest that the defective *Hsp83* in the *trans*-heterozygotes never influenced bristle number sufficiently to extend outside the effects of the mutants expressed as heterozygotes or outside values of the parental strains.

For orbital bristle number, the *trans*-heterozygotes differed significantly from the mean values of the mutant heterozygotes in four cases (Table 4). In each case, the *trans*-heterozygote had bristle numbers that were greater than those of both the mutants, and in one case ($Hsp83^{9J1} \times Hsp83^{e6A}$) the value of both parents was also exceeded (Figure 6). These results suggest more severe effects associated with the combination of mutants. In the three cases where bristle numbers in the *trans*-heterozygote reverted to parental numbers, there

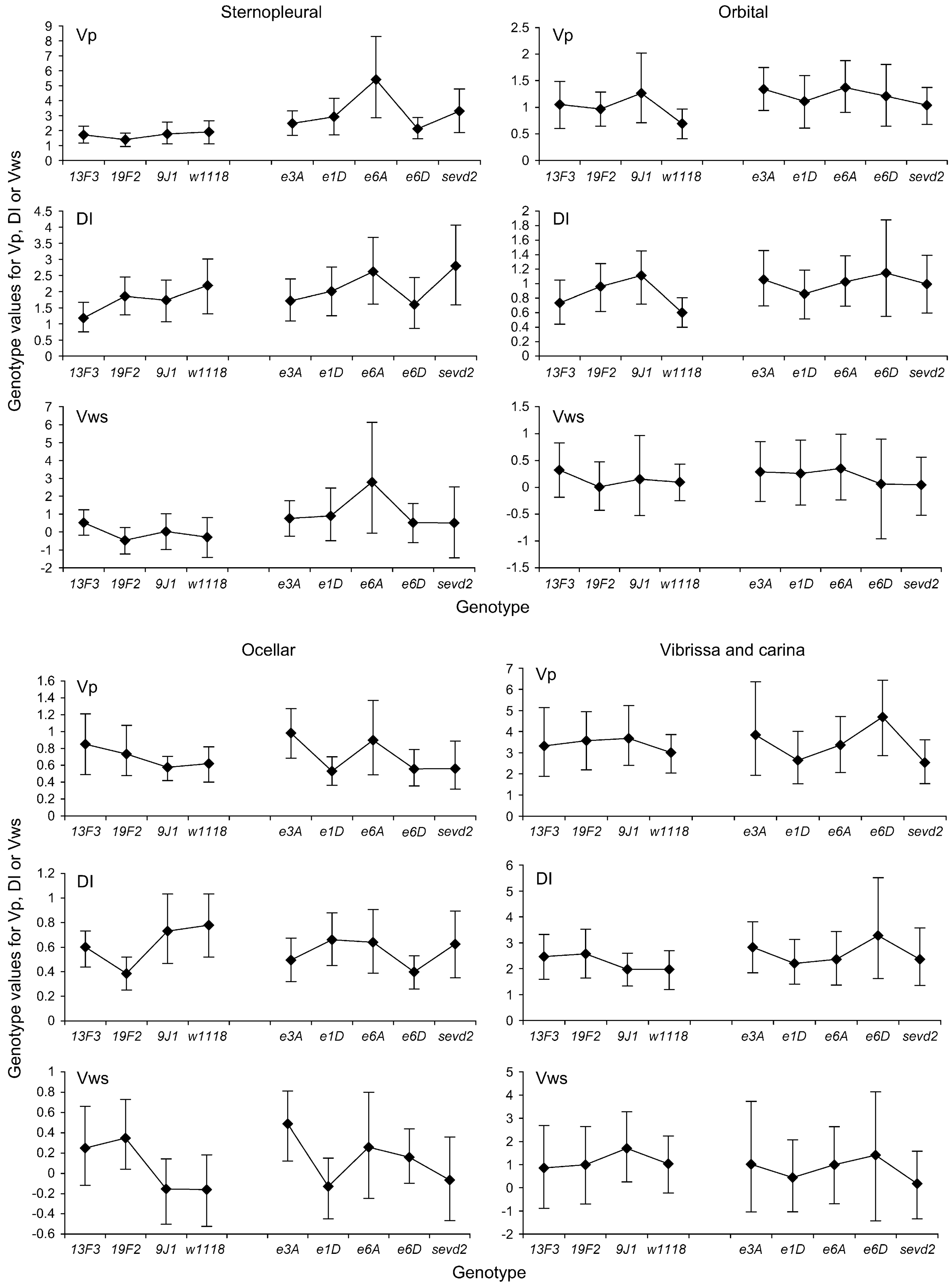

FIGURE 4.—Comparison of the phenotypic variance (V_P), variance within strains (V_{WS}), and DI values for each single *Hsp83* heterozygote to its originating *w* background for each variable trait (±95% confidence limits).

may have been compensation where the combination of the two different alleles complements each other's defects in the Hsp90 protein.

Ocellar bristle means in the *trans*-heterozygotes form the midmutant value in four crosses (Table 4). The *trans*-heterozygote mean was lower than that of both mutant

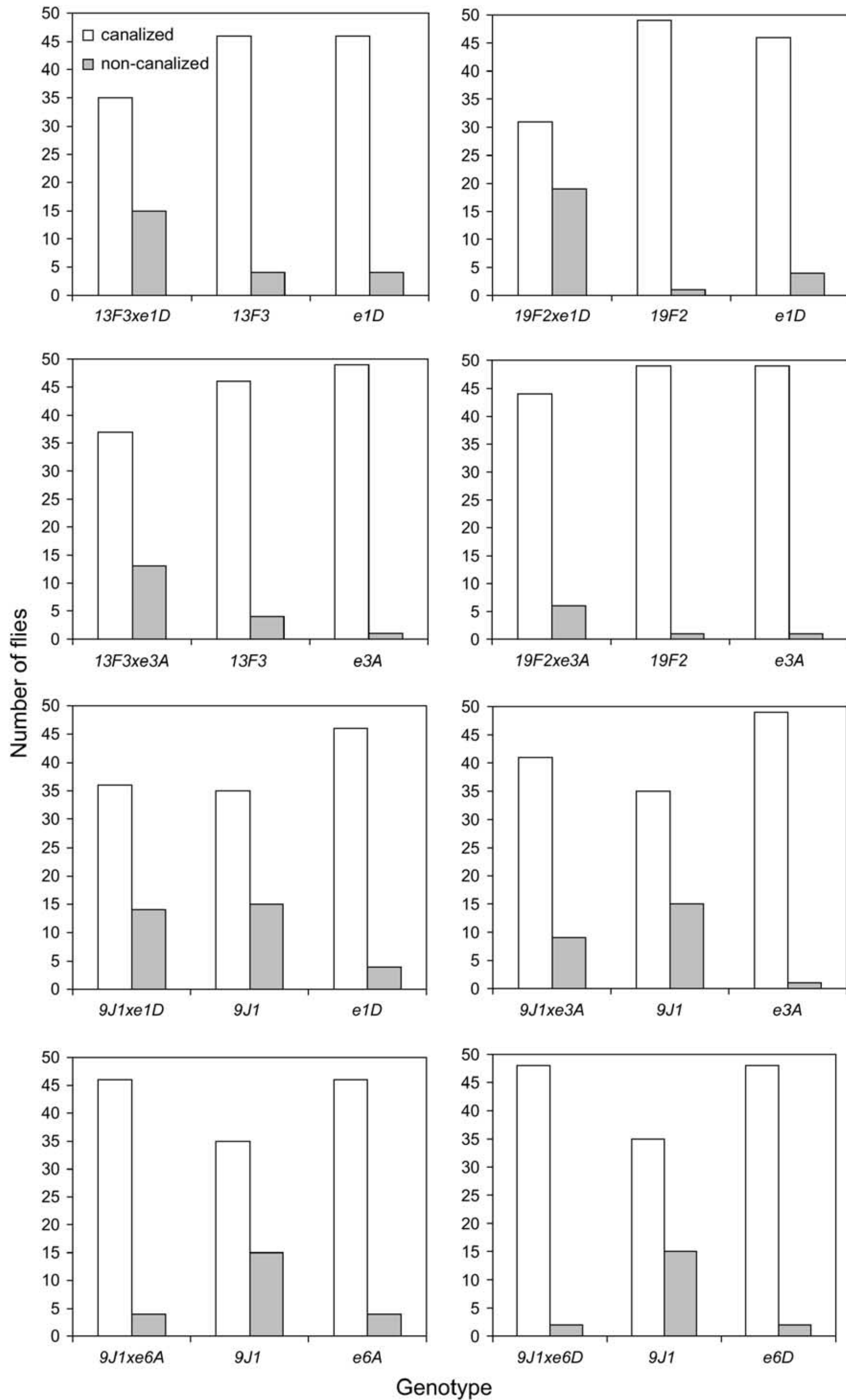

FIGURE 5.—Number of flies with canalized and noncanalized thoracic phenotypes for *Hsp83 trans*-heterozygotes and single *Hsp83* heterozygotes. The canalized phenotype for the thoracic bristles is 11 bristles on either side of the thorax, with the noncanalized phenotype having either less than or more than 11 bristles on either side of the thorax. Fifty flies were scored for each genotype.

heterozygotes in three crosses (*Hsp83*^{13F3} × *Hsp83*^{e3A}, *Hsp83*^{19F2} × *Hsp83*^{e3A}, and *Hsp83*^{9J1} × *Hsp83*^{e6D}) and was similar to the lower mutant heterozygote in one of the other crosses (*Hsp83*^{13F3} × *Hsp83*^{e1D}) as evident in Figure 6. In one case (*Hsp83*^{13F3} × *Hsp83*^{e3A}) the *trans*-heterozygote mean fell outside that of the parentals, suggesting that additional effects result from combinations of the two mutants.

Finally, for the vibrissa and carina bristles, the *trans*-heterozygote differed from the mean of the mutant heterozygotes in only one case (Table 4). This involved an increase in mean bristle number in the *Hsp83*^{19F2} × *Hsp83*^{e3A} cross, but not outside the mean of both the parental strains (Figure 6).

DISCUSSION

***Hsp83* alleles and genetic background affected bristle trait means but not variances or developmental instability:** Consistent with our previously reported results (Milton *et al.* 2003), the seven *Hsp83* alleles examined in this study have an influence on bristle numbers, whether as single heterozygotes or as

TABLE 4

Planned contrasts comparing the mean of the *trans*-heterozygotes to the midvalue of the single heterozygotes for the variable bristle traits

Trait	T (d.f. = 147)	P
$Hsp83^{13F3}/Hsp83^{e1D}$ *vs.* $Hsp83^{13F3}/+$ and $Hsp83^{e1D}/+$		
Sternopleural	2.02	0.045
Orbital	3.09	0.002[a]
Ocellar	−2.29	0.023
Vibrissa and carina	0.86	0.389
$Hsp83^{13F3}/Hsp83^{e3A}$ *vs.* $Hsp83^{13F3}/+$ and $Hsp83^{e3A}/+$		
Sternopleural	1.97	0.051
Orbital	1.04	0.301
Ocellar	−5.92	<0.001[a]
Vibrissa and carina	2.11	0.036
$Hsp83^{19F2}/Hsp83^{e1D}$ *vs.* $Hsp83^{19F2}/+$ and $Hsp83^{e1D}/+$		
Sternopleural	0.11	0.909
Orbital	−0.16	0.872
Ocellar	−3.40	0.001[a]
Vibrissa and carina	−1.43	0.155
$Hsp83^{19F2}/Hsp83^{e3A}$ *vs.* $Hsp83^{19F2}/+$ and $Hsp83^{e3A}/+$		
Sternopleural	3.23	0.002[a]
Orbital	−0.48	0.631
Ocellar	−3.60	<0.001[a]
Vibrissa and carina	3.39	0.001[a]
$Hsp83^{9J1}/Hsp83^{e1D}$ *vs.* $Hsp83^{9J1}/+$ and $Hsp83^{e1D}/+$		
Sternopleural	1.25	0.215
Orbital	3.41	0.001[a]
Ocellar	−1.21	0.227
Vibrissa and carina	−0.82	0.412
$Hsp83^{9J1}/Hsp83^{e3A}$ *vs.* $Hsp83^{9J1}/+$ and $Hsp83^{e3A}/+$		
Sternopleural	0.97	0.334
Orbital	1.32	0.190
Ocellar	0.19	0.846
Vibrissa and carina	0.06	0.951
$Hsp83^{9J1}/Hsp83^{e6A}$ *vs.* $Hsp83^{9J1}/+$ and $Hsp83^{e6A}/+$		
Sternopleural	2.78	0.006
Orbital	5.25	<0.00[a]
Ocellar	−0.07	0.947
Vibrissa and carina	0.41	0.680
$Hsp83^{9J1}/Hsp83^{e6D}$ *vs.* $Hsp83^{9J1}/+$ and $Hsp83^{e6D}/+$		
Sternopleural	2.52	0.013
Orbital	3.35	0.001[a]
Ocellar	−3.78	<0.001[a]
Vibrissa and carina	−0.73	0.467

[a] Significant after correcting probabilities for the number of traits being compared.

trans-heterozygotes. These effects had not been detected in previous studies (Rutherford and Lindquist 1998; Yue *et al.* 1999) in which *Hsp83 trans*-heterozygotes were analyzed. However, the significant changes in bristle means observed in this study are not as wide ranging or across the board as seen for the alleles analyzed previously. The majority of changes are decreases in bristle number, which is consistent with previous results. However, unlike the previous results, there is an increase in ocellar bristle numbers in all of the *E(sev)* alleles except for $Hsp83^{e6D}$. In the single heterozygotes, the orbital bristles appeared to be the best buffered against changes in trait mean, as no significant differences were detected.

When in single heterozygote form, the *E(sev)* allelic class tended to influence variable traits more than the *Su(Raf)* allelic class did. Nine differences were detected for the *E(sev)* heterozygotes, while only one change in mean was observed for the *Su(Raf)* heterozygotes. The ocellar and vibrissa and carina traits were particularly affected by the *E(sev)* alleles. It seems unlikely that these different effects of the two allelic types result from the locations of the amino acid replacements within *Hsp83*, as the *E(sev)* mutations are located throughout the three conserved regions of the gene.

While the *Hsp83* alleles had an effect on bristle numbers, they generally had no effect on the V_P, V_{WS}, and DI values for any of the four variable traits. Therefore, one component of the evolutionary capacitance hypothesis of Hsp90—the increased expression of phenotypic variability—was not supported. In previous research on bristle trait variability, *Hsp83* mutants were shown to influence the response to selection for various eye and wing deformities, due to the fact that inhibited Hsp90 levels increase variability in canalized traits, including some bristle traits, in specific genetic backgrounds (Rutherford and Lindquist 1998). In contrast, the present results suggest that the expression of variation in bristle traits that are variable in populations is unaffected by variation in Hsp90 levels. Moreover, variation within an individual, as measured by DI, was also not affected. The DI result is consistent with Milton *et al.* (2003) who found that bristle DI, as measured by fluctuating asymmetry, was not affected by the Hsp90 inhibitor geldanamycin or by a different set of mutants that decreased Hsp90 activity. Moreover, Milton *et al.* (2003) also showed that geldanamycin did not influence phenotypic variability in bristle traits.

Rutherford and Lindquist (1998) have suggested that Hsp90 may act as a capacitor for evolutionary change. They showed that Hsp90 mutant strains led to the expression of morphological abnormalities in several traits and that those new variants could be selected for several generations and expressed without the mutant background. They argued that a similar process could occur under stress if levels of Hsp90 were suppressed and that Hsp90 could therefore facilitate some types of evolutionary change. The expression of variation in thoracic bristle numbers in some of the Hsp90 mutants is consistent with the notion that Hsp90 expression levels could have some effect on the evolution of morphological change. In contrast, the absence

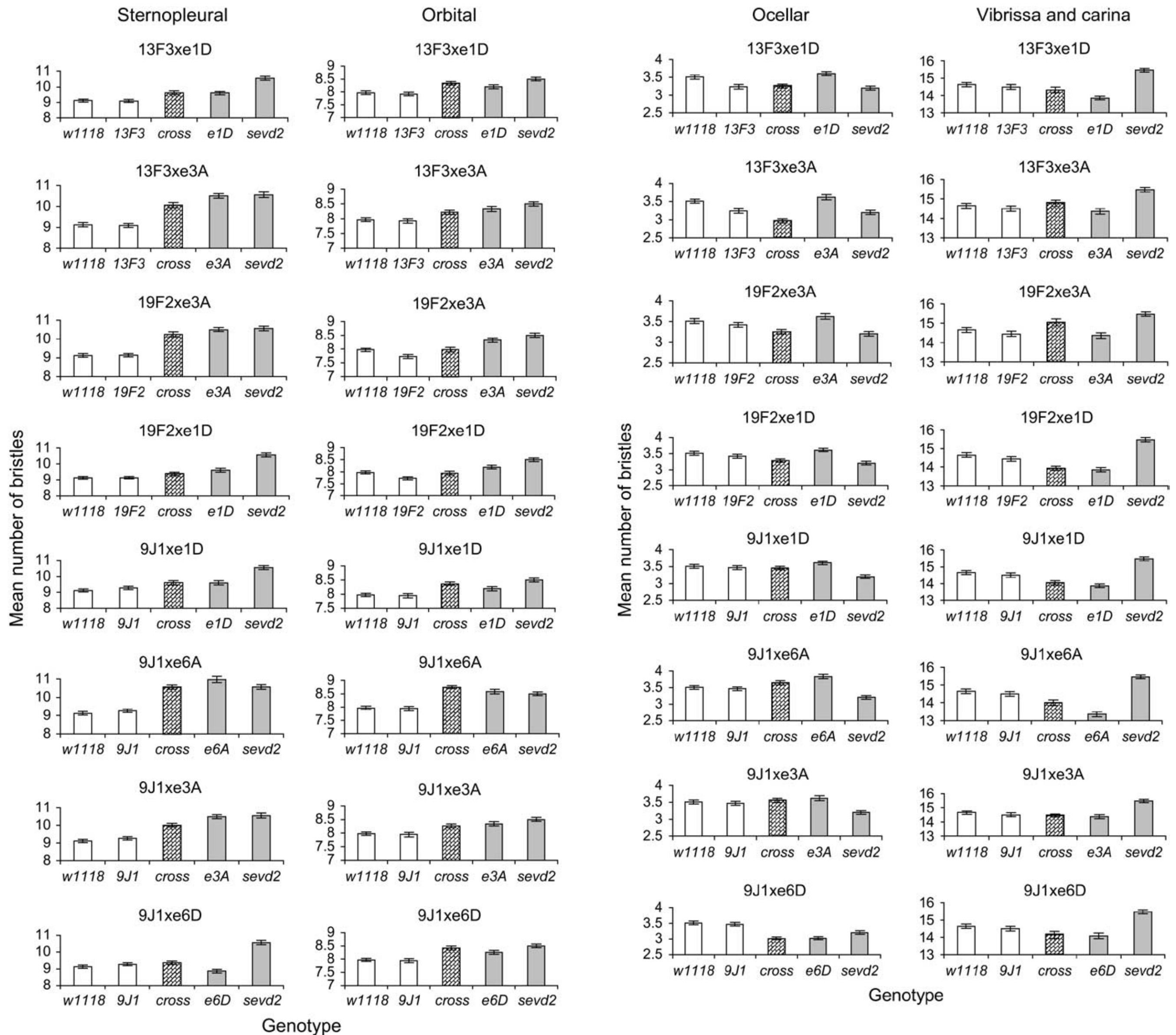

Figure 6.—Means of *Hsp83 trans*-heterozygotes compared to single *Hsp83* heterozygotes and background values (± standard error). The two different genetic backgrounds are indicated by open and shaded bars, while the *trans*-heterozygote cross is indicated by a patterned bar.

of Hsp90 mutant effects on the variance of the other bristle traits suggests that the Hsp90 capacitor model does not apply to these traits.

However, our experiments have limitations for testing the capacitor hypothesis. One issue is whether small effects on trait variance could have been detected due to Hsp90-associated abnormalities. Power tests indicate that we would have detected a difference in variance only between strains of 30% or more. Thus abnormalities at a low frequency may not have been detected, given our sample sizes. Another issue is that the lines were not ideal for testing for specific effects of Hsp90 alleles. Given that cryptic genetic variation may be released whenever genetic or environmental change occurs (Hermisson and Wagner 2004), there is the potential for strain differences other than those associated with Hsp90 to obscure effects of this gene on trait variability. Ideally, strains should have the same background and differ only for Hsp90 alleles. Finally, we did not specifically test for changes in genetic variance (V_G). While DI reflected variation within individuals, genetic variation may have contributed to differences among individuals within strains. To examine V_G specifically, the effects of the same allele across different backgrounds should have been examined.

***Hsp83^{9J1}* results in decanalization of thoracic bristle phenotype:** The allele *Hsp83^{9J1}* leads to a marked change in the thoracic bristle phenotype. Essentially, the presence

of $Hsp83^{9J1}$ appears to result in decanalization of the processes underlying thoracic bristle development. The majority of flies with a noncanalized thoracic phenotype had an extra bristle. The remaining six *Hsp83* alleles did not have a significant impact on thoracic bristle numbers.

On its own, the $Hsp83^{9J1}$ allele affects two main regions of the thorax. The aDC and pDC bristles are situated toward the middle of the thorax, while the H bristles are located on the very top or shoulder region of the thorax. Why the $Hsp83^{9J1}$ allele has an effect on these regions only is unclear.

Cumulative or complementary effects of *Hsp83 trans*-heterozygotes? By examining *trans*-heterozygotes, it can be determined whether combining two different *Hsp83* alleles results in cumulative or complementary effects on bristle trait means. Effects in the *trans*-heterozygotes were dependent on which alleles were present. The *Su(Raf)* alleles in particular behaved differently when in *trans*-heterozygote form. Specifically, $Hsp83^{13F3}$ and $Hsp83^{19F2}$ exhibited cumulative effects while $Hsp83^{9J1}$ exhibited complementary effects.

Why do these alleles, all isolated as *Su(Raf)* alleles, behave in such different ways when in *trans*-heterozygote form? The difference may relate partly to the fact that $Hsp83^{9J1}$ has a much stronger effect than either $Hsp83^{13F3}$ or $Hsp83^{19F2}$. $Hsp83^{9J1}$ on its own altered the canalization of the thoracic bristles, whereas the other mutants did not have this effect. Perhaps there is a limit to the incidence of abnormal bristle phenotypes that has been reached in the case of $Hsp83^{9J1}$ heterozygotes, which might explain why *trans*-heterozygotes with $Hsp83^{e1D}$ and $Hsp83^{9J1}$ heterozygotes had a similar incidence of abnormalities. However, there are new phenotypic changes in the *trans*-heterozygotes that are not evident in the single-mutant heterozygotes. When the $Hsp83^{13F3}$ and $Hsp83^{19F2}$ alleles are combined with $Hsp83^{e3A}$, only the aDC and H bristles are affected. Changes in these bristles are also observed when $Hsp83^{13F3}$ and $Hsp83^{19F2}$ are as single heterozygotes. But when the $Hsp83^{13F3}$ and $Hsp83^{19F2}$ alleles are combined with $Hsp83^{e1D}$, a wider range of thoracic bristles are affected, with pDC, aSA, aPA, and aNP also affected. These results suggest that *Hsp83* allele-specific effects are dependent on *trans*-heterozygote combinations. The different locations of the point mutations may be another reason for the different *trans*-heterozygote behavior, with $Hsp83^{13F3}$ and $Hsp83^{19F2}$ located in the N-terminal domain and $Hsp83^{9J1}$ located in the middle region of the protein.

However, despite $Hsp83^{13F3}$ and $Hsp83^{19F2}$ being independent isolates of the same allele, some differences are observed in the interactions that include $Hsp83^{13F3}$ and $Hsp83^{19F2}$. For example, when $Hsp83^{13F3}$ is combined with $Hsp83^{e1D}$, the orbital bristles are significantly affected; when $Hsp83^{19F2}$ is combined with $Hsp83^{e1D}$, the orbital bristle mean does not change while the ocellar bristle mean is significantly different. This may be due to slight variation in the original backgrounds of these alleles.

The thoracic data also suggest that there is compensation in combinations of the $Hsp83^{9J1}$ allele with $Hsp83^{e6A}$ and $Hsp83^{e6D}$. As seen in Figure 5, the highly significant effect of the $Hsp83^{9J1}$ allele on the thoracic bristle phenotype disappears in the *trans*-heterozygotes; the phenotype reverts to the thoracic phenotype of the $Hsp83^{e6A}$ and $Hsp83^{e6D}$ single heterozygotes. Therefore, there is an underlying mechanism that prevents the $Hsp83^{9J1}$ allele from exerting its usual effects on the thoracic bristles. Given their location, it is not clear why there is compensation with these alleles and not the other *E(sev)* alleles.

The interpretation of changes in the thoracic bristle phenotypes of the *trans*-heterozygotes is relatively simple due to the lack of differences in the two *w* backgrounds for the thoracic bristles. In comparison, the analysis of the four variable bristle traits was complicated by highly significant differences between these two *w* backgrounds. Despite this, the results suggest that some combinations of the *Hsp83* alleles have more severe effects on the variable traits than single mutants do. This applied particularly to the ocellar and orbital bristles where the *trans*-heterozygote means fell outside those for the single-mutant heterozygote and parental means. Cumulative effects were specific to particular types of bristles, suggesting that specific types of Hsp90 impairment influenced the traits differently.

Conclusions: The results of this study support previous findings indicating that impaired Hsp90 has an impact on bristle numbers in *D. melanogaster*, but not on the expression of variability among or within individuals as predicted by the evolutionary capacitor hypothesis. In these *Hsp83* mutants there is a tendency toward a reduction of bristle numbers, with the exception of the ocellar bristles, which increase for most of the *E(sev)* alleles. The different alleles of *Hsp83* have diverse effects on bristle numbers, which may be the result of the point mutations being located in different regions of the protein. Viable combinations of the *Hsp83* alleles also have an impact upon bristle numbers, but these effects are allele and trait specific. Decanalization of the thoracic bristles is observed for $Hsp83^{9J1}$ but not for the other alleles, and this decanalization can be complemented by some *E(sev)* alleles. The absence of mutant effects on bristle trait variance within strains and developmental instability in the noncanalized bristle traits suggests that Hsp90 is unlikely to act as a capacitor for the evolution of these traits.

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