# Quantitative trait symmetry independent of Hsp90 buffering: Distinct modes of genetic canalization and developmental stability

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**The Hsp90 chaperone buffers development against a wide range of morphological changes in many organisms and in *Drosophila* masks the effects of hidden genetic variation. Theory predicts that genetic and nongenetic buffering will share common mechanisms. For example, it is argued that Hsp90 genetic buffering evolved solely as a by-product of environmental buffering, and that Hsp90 should mask morphological deviations from any source. To test this idea, we examined the effect of Hsp90 on purely nongenetic variation in phenotype, measured as differences between the left and right sides of several bilaterally symmetrical bristle and wing traits in individual flies. Consistent with previous reports, Hsp90 buffered the expression of rare morphogenic variants specific to particular genetic backgrounds. However, neither trait-by-trait nor global asymmetry was affected in outbred flies treated with an Hsp90 inhibitor or across a series of inbred genetic backgrounds from a wild population tested in isogenic $F_1$ heterozygotes carrying either (*i*) a dominant negative Hsp90 allele on a mutant 3rd chromosome or (*ii*) a null *P*-insertion mutation, which was introgressed into the control genetic background on all chromosomes. By contrast, Hsp90-regulated trait means and significant effects of sex, temperature, and genetic background on trait symmetry were clearly detected. We conclude that, by maintaining the function of signaling proteins, Hsp90 masks variation affecting target pathways and traits in populations independent of purely nongenetic sources of variation, refuting the idea that a single Hsp90-dependent process generally controls genetic canalization and developmental stability.**

Despite surprisingly large amounts of molecular variation among individuals, development is robust to perturbation by genetic and environmental influences, allowing the expression of characteristic morphological differences between species and constancy of phenotype within species (1). The suggestion that mechanisms of phenotype buffering evolved through stabilizing selection for robust developmental processes (canalization) (2–4) raises the possibility of specific "canalizing genes," whose primary adaptive function is to maintain a constant phenotype across different genetic backgrounds and environments (5, 6). The strongest candidate to date for an evolved mechanism of canalization is the Hsp90 chaperone system (10–12), which buffers the expression of dramatic morphogenic variants in *Drosophila* and other species (7–9). For example, when *Drosophila* Hsp90 is impaired, either in mutant heterozygotes (*Hsp83*/+) or through pharmacological inhibition, depending on genetic background, virtually any structure in the adult fly can be affected (8).

It is argued that mechanisms that maintain the phenotypic stability of a trait against one type of genetic or environmental perturbation will protect the trait against all other types of perturbation (2, 4, 5, 22). For example, in a recent perspective Meiklejohn and Hartl (5) discuss the types of biological processes that might promote canalization and the circumstances under which these mechanisms would evolve. Selection is thought to be strongest for the evolution of environmental canalization (2, 23, 24), and weakest for the evolution of genetic canalization, suggesting that genetic buffering by Hsp90 could have evolved only as a by-product of selection for its buffering against environmental disturbance. They conclude that Hsp90 buffering should protect against all sources of phenotypic variation (5).

The nearly ubiquitous expression of Hsp90, its participation in environmental stress responses, and its ability to mask a wide range of morphogenic deviations make Hsp90 an ideal candidate for a general canalizing gene. To test whether Hsp90 also generally regulates the developmental stability of normal quantitative traits, we measured left–right symmetry within individual flies. Symmetry differences between the left and right sides of bilateral quantitative traits within a particular genotype are a sensitive measure of nongenetic variation in phenotype ($V_e$), reflecting individual organism's ability to buffer stochastic and microenvironmental perturbations of the same developmental processes as they occur simultaneously on the left and right sides of the animal (25, 26).

## Materials and Methods

**Trait Measurements.** In most experiments (except experiment 2), the numbers of sternopleural, orbital, ocellar, and vibrissae and carnia bristles were counted on the left and right sides of each fly. To score wing-size variation, the wings of the flies were removed with forceps and mounted on glass slides with double-sided tape. Wing images were captured by using a Panasonic WV-CP460 video camera. The eight junctions of longitudinal veins with the wing margin or crossveins were used as landmarks, and their exact positions were determined with TPSDIG Version 1.31 (written by F. J. Rohlf, Department of Ecology and Evolution, State University of New York, Stony Brook). From the 28 interlandmark distances, one centroid size (the sum of squared deviations between the eight landmarks) was calculated for each wing. Centroid size is an overall measure of wing size.

**Generation of Replicated Hsp90 Mutant and Control Genotypes.** Table 1 contains the sources and descriptions of the *Drosophila* strains used in this study. To control the genetic backgrounds of the Hsp90 mutants, single chromosomes, carrying either a dominant-negative allele created by an amino acid substitution in the *Drosophila* Hsp90 (*Hsp83*) gene [*9J1* (21)] or a protein null (27) created by a *P* element insertion 29 bp downstream of the transcription start site (P582) (28), were crossed into the highly inbred *Samarkand I-236* (*Sam*) background by six generations of backcrossing to *Sam 1; Sam 2; TM3/TM6B* (see Table 1). The *Sam* stock was derived from a wild-type strain that had been made isogenic by >236 generations of full sibling inbreeding (T. Mackay, personal communication). *Sam* was tested for heterozygosity by denaturing HPLC (29), and was completely homozygous over 30,000 bp of 300- to 400-bp PCR

Abbreviations: FA, fluctuating asymmetry; GA, geldanamycin; LLM, log-linear models; RI, Raleigh inbred.

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**Table 1. *Drosophila melanogaster* strains used in this study**

| Strain | Source | Description |
|---|---|---|
| *Canton-S* | Bloomington | Population cage stock |
| Raleigh inbred (RI) lines | T. Mackay | Inbred wild type |
| *Samarkand 1–236* | T. Mackay | Isogenic wild type |
| *Sam1; Sam2; TM3/TM6B* | T. Mackay | Double balancer in *Sam* background |
| *w; Hsp83[9J1]/TM6B* | E. Hafen | Hsp90 mutant (E377K) |
| *yw; P582/TM3,Ser* | P. Deak | Hsp90 mutant (*P* element insertion) |
| *Sam1; Sam2; 9J1/TM6B* | S.L.R. | *9J1* derivative (see *Materials and Methods*) |
| *Sam1; Sam2; P582/TM6B* | S.L.R. | *P582* derivative (see *Materials and Methods*) |
| *wi; Sam2; P582i/Sami* | S.L.R. | *w,P582* introgression (see *Materials and Methods*) |

products, sampled across the 2nd and 3rd chromosomes (S.L.R., unpublished data). The RI lines represent a random sample of genotypes isolated from a wild Raleigh, North Carolina population in 1994 and isogenized through 14 generations of full sibling inbreeding (30), which is expected to have decreased the heterozygosity of these strains by >95% (31). The wild-type *Sam*, and the two Hsp90 mutant 3rd chromosomes are from three different sources, and these fly strains are genetically distinct from one another and from each of the RI lines.

To examine the effect of Hsp90 mutants independent of other factors on the unique 3rd chromosomes associated with each allele, we created the *P582i* line (Table 1). The *P* element mutation, *P582*, carrying the miniwhite gene (resulting in orange eyes), was introgressed into the *Sam* strain background along with the $w^{1118}$ mutation from the *P582* stock, which allowed the miniwhite marker to be scored. Initially, a single *yw; P582/TM3, Ser* male was crossed to *Sam* (*Sam1; Sam2; Sam3*) virgins. The resulting completely heterozygous $F_1$ females were crossed back to *Sam* males. In the $F_2$, *yw* or *w/Y; P582* orange-eyed males were selected and backcrossed to create heterozygous (*w*/+), red-eyed and therefore unselected, $F_3$ females of indeterminate genotype (*P582* or +). These flies were backcrossed to *Sam* males, and orange-eyed *yw* or *w/Y; P582* $F_4$ males were selected. Alternate cycles of backcrossing and selection of *w* and *P582* were repeated for 14 generations. The *P582i* chromosome was then carried over *Sam3* for an additional year (≈24 generations) with selection for *P582*, allowing further homogenization of the *Sami* and *P582i* chromosomes before testing. By selecting against *y*, which is just 1.5 centimorgans (cM) from *w* at the tip of the X chromosome, we ensured that the distal tip of the *P582i* X chromosome had been replaced. The residual *P582* background remaining on the other side of *w* on the X chromosome, and around the *P* element insertion site on the 3rd chromosome, is expected to have been <7 cM at each position (31).

**Experiment 1: Hsp90 Inhibitor Studies.** The effect of geldanamycin (GA), a specific inhibitor of Hsp90, was tested on *Canton-S* flies from an outbred population cage stock, which had been maintained at a large and stable population size for >10 years and is expected to have accumulated and carry significant genetic variation (31). To stage larvae, *Canton-S* eggs were collected for 6 h, after which the egg-collection plates were removed and maintained at constant temperature (25°C). At 20 h, larvae were cleared from the plates, and larvae that hatched during the subsequent 2 h were collected as a group. This process was repeated the next day to provide larvae 24 h younger. Three days later both sets of timed 2nd or 3rd instar larvae were transferred to vials of fly food containing 1 $\mu$g/ml GA (the highest dose tested that the flies could survive) or controls without GA, at a density of 50 larvae per vial in five replicates for each treatment. Flies were reared at 25°C, and 10 females from each vial (50 total) were collected and scored for symmetry as described above.

**Experiment 2: Multifactorial Study.** We initially surveyed the effects of Hsp90 on sternopleural bristles and wing traits alone and in combination with sex and temperature. To create replicated, genetically matched groups of flies, three females from the wild-type control background (*Sam1; Sam2; Sam3*) or the backcrossed Hsp90 mutants (*Sam1; Sam2; Hsp83/TM6B*) were separately crossed to males from each of 10 RI lines at either 18°C or 25°C. After 3 days, flies were transferred to a new vial (replicate) and allowed to lay for another 3 days, after which parents were removed. The resulting groups of genetically replicated $F_1$ progeny were completely heterozygous for *Sam* and each RI line background, and they differed only in carrying either the *Sam* or one of the two Hsp90 mutant 3rd chromosomes.

Ten males and 10 females from each vial were scored on the right and left sides for sternopleural bristle number, and the flies were stored in ethanol for the later analysis of wings. To measure the wing traits, right and left wings were mounted on glass slides and photographed with a micrometer by using a SPOT 2.2 digital video camera (Diagnostic Instruments, Sterling Heights, MI). Image processing and analysis software (NIH IMAGE, http://rsb.info.nih.gov/nih-image/Default.html) was used for measurements of the L4 wing vein and a trapezoidal measure of the wing area between four wing vein–margin junctions.

**Experiment 3: Multiple Traits at Constant Temperature and Density.** In a separate experiment, we used the same strains as in experiment 2 but controlled for density (replicate vial) effects by seeding larvae at a constant density of 50 larvae per vial in five vials per genotype, as described above. Five males and five females (25 total of each sex) were selected at random from each vial, and characters were scored for trait symmetry.

**Experiment 4: Allelic Effects.** To examine the allelic effects of the Hsp90 mutation independent of other factors on the 3rd chromosome we used the *P582i* strain, described above, which was highly introgressed into the *Sam* background on all chromosomes. *P582i* females (*wi; Sam2; P582i/Sam3i*) were crossed to males from each of nine RI lines to make isogenic *Sam*-RI line hybrid backgrounds as before. However, in this experiment, the white-eyed $F_1$ males (*wi/Y; Sam2/RI-2; Sam3i/RI-3*) were compared with orange-eyed (*wi/Y; Sam2/RI-2; P582i/RI-3*) male siblings from the same cross. The mutant and control males had nearly isogenic 3rd chromosomes in addition to being isogenic for all other chromosomes as before. Timed larvae from each cross were placed at constant temperature and density as before, and 10 Hsp90 mutants (*P582i*) and 10 controls (*Sami*) were scored from each vial, for a total of 50 males for each genotype.

**Data Analysis.** Most trait means differed significantly depending on Hsp90 genotype (e.g., Table 2). Therefore, to determine the effects on trait symmetry of GA treatment, *Hsp83* mutant chromosome, or *P582i* allele by ANOVA (SPSS, Chicago), |left ($L$) − right ($R$)| symmetry data were normalized to the mean trait size within each individual, $|L - R|/[(L + R)/2]$; mean relative fluctuating asymmetry (FA, as in ref. 25). These corrected values were averaged over all traits to compute composite asymmetry scores (34). Analyses by ANOVA of mean relative FA for the continuous wing traits and composite asymmetry are reported; however, analyses performed on uncorrected symmetry scores or size effects corrected by multiple regression (33) led to the same conclusions.

Much of the asymmetry data for the discontinuous bristle traits fell into just a few classes, making the analysis of bristle-trait FA by continuous linear models (e.g., ANOVA) invalid to various degrees, depending on trait and data set. For example, most of the individuals in experiment 1 were perfectly symmetric for sternopleural bristles (33%), or differed from perfect symmetry by only one bristle (45%). Therefore, although standard ANOVAs were used initially to examine all main effects and their interactions, to

**Table 2. Effect of GA treatment on quantitative traits in outbred flies in experiment 1 (ANOVA)**

| Trait | Source | df | MS | *P* | Variance component, % |
|---|---|---|---|---|---|
| Sternopleural bristles | GA treatment | 1 | 15.68 | <0.001 | 96.58 |
| | Larval stage | 1 | 0.02 | 0.844 | 0.12 |
| | GA × Stage | 1 | 0.02 | 0.844 | 0.12 |
| | Error | 196 | 0.51 | | 3.17 |
| Ocellar bristles | GA treatment | 1 | 0.01 | 0.914 | 0.69 |
| | Larval stage | 1 | 0.25 | 0.452 | 33.76 |
| | GA × Stage | 1 | 0.05 | 0.747 | 6.20 |
| | Error | 196 | 0.43 | | 59.43 |
| Orbital bristles | GA treatment | 1 | 2.42 | <0.001 | 76.95 |
| | Larval stage | 1 | 0.50 | 0.120 | 15.90 |
| | GA × Stage | 1 | 0.02 | 0.755 | 0.64 |
| | Error | 196 | 0.21 | | 6.52 |
| Vibrissae and carnia | GA treatment | 1 | 137.78 | <0.001 | 98.22 |
| | Larval stage | 1 | 0.25 | 0.581 | 0.17 |
| | GA × Stage | 1 | 1.45 | 0.181 | 1.03 |
| | Error | 196 | 0.80 | | 0.57 |
| Wing centroid | GA treatment | 1 | 5,051.25 | <0.001 | 90.03 |
| | Larval stage | 1 | 27.00 | 0.679 | 0.48 |
| | GA × Stage | 1 | 375.43 | 0.124 | 6.69 |
| | Error | 196 | 156.99 | | 2.80 |

MS, mean square is the sum of squares $[\Sigma(x_i - \bar{x})^2]$ divided by its associated df.

examine the most important interactions in each experiment, we subsequently used log-linear models (LLMs) comparing the number of individuals that showed perfect symmetry as opposed to differing by one, two, or three bristles. Either method of analysis provided the same (generally negative) conclusion regarding the effect of Hsp90 on trait symmetry, and only the more valid LLM results are presented.

**Data Transformation.** The absolute value of Gaussian *L–R* distributions ($|L - R|$) are not expected to be normal, making it necessary to transform the wing symmetry variables for ANOVA. The following transformations were identified by the Box–Cox procedure and confirmed according to the Kolmogorov–Smirnov test of normality: wing centroid size ($\lambda = 0$ log transformation), wing vein length ($1/2$; square root transformation), and wing area ($1/2.35$) (37). RI lines, sampled at random from a wild population, were treated as a random effect in the models. The full-model ANOVA used in experiment 2 had Hsp90 chromosome, RI line, sex, replicate vial, and temperature as effects with replicate vial nested within genotype, line, and temperature. Subsequent experiments controlled for vial effects by seeding larvae at a constant density and considered fewer factors to focus progressively more powerfully on Hsp90. Experiment 3 examined the effects of Hsp90 chromosome, RI line, and sex, whereas experiment 4 examined only the effects of the introgressed *P582i* allele and RI line and their interaction.

**Correction for Multiple Comparisons and Measurement Error.** Because of the large number of traits examined, probability values for all analyses were adjusted to account for multiple comparisons by the Dunn–Sidak method (37). Measurement error corrections were unnecessary for the bristle traits, because repeated bristle counts were scored with perfect accuracy. A repeatability analysis was performed on the wing characters by measuring 30 flies at all landmarks on two occasions 4 days apart (25). As expected, measurement error had a small, but significant, effect on FA, and there was no directional symmetry (36). Because measurement error was small and constant across all treatments, it did not affect the comparisons made here.

## Results

We showed previously that Hsp90 buffers morphology against both additive and epistatic sources of genetic variation and variation due to genotype–environment interactions (8). However, purely environmental buffering and buffering against stochastic perturbations were not specifically addressed. As a buffer for genetic variation, Hsp90 protects flies against the expression of extreme morphogenic phenotypes specific to particular genetic backgrounds and environments. By contrast, the effects of Hsp90 as a general buffer for nongenetic sources of variation ($V_e$) would not depend on trait and genetic background. We ask here whether Hsp90 buffers purely stochastic perturbations of normal (Gaussian) wing and bristle traits using FA. Differences in left–right symmetry within individuals are appropriately measured only for standard quantitative traits (36).

**Table 3. Effect of GA treatment (experiment 1) on quantitative trait distributions in outbred flies**

| | Control | | Inhibitor (GA) | |
|---|---|---|---|---|
| Trait | Mean (*L* + *R*) | Variance | Mean (*L* + *R*) | Variance |
| Sternopleural bristles | 19.32 | 2.46 | 18.20 | 1.62 |
| Orbital bristles | 18.20 | 2.02 | 18.18 | 1.40 |
| Ocellar bristles | 7.46 | 0.80 | 7.02 | 0.85 |
| Vibrissae and carnia | 29.28 | 3.78 | 25.96 | 2.64 |
| Wing centroid | 3,130.0 | 6,008.4 | 3,073.2 | 4,067.6 |

Many studies of FA rely on single estimates of developmental stability based on just two measures (left and right) of each individual taken across uncontrolled genotypes and environments, leaving much of the FA literature indeterminate (38, 39). However, our experiments used several different approaches and populations in highly controlled and extensively replicated designs, enabling us to systematically and unambiguously determine the effect of Hsp90 on the FA of several different wing and bristle traits and their composite measure. Almost identical groups of flies differed only in Hsp90 status in these studies, including (*i*) an outbred population of wild-type flies with or without Hsp90 inhibitor treatment during development, (*ii*) heterozygous but nearly isogenic flies that differed only in whether they received Hsp90 mutant or wild-type control 3rd chromosomes, and (*iii*) flies that differed only in whether they carried an Hsp90 mutation or a wild-type allele highly introgressed into an isogenic background on all chromosomes.

**Morphogenic Variation.** As reported (8), Hsp90 reproducibly buffered strain-specific morphogenic defects in a small number of flies in 2 of 10 RI line backgrounds tested. The Hsp90 mutant heterozygotes, but not the wild-type controls, had a low frequency of flies with bristle defects ("split" scutellar bristles). This trait was specific to one RI line at 18°C, but not 25°C, and it was again seen at low frequency in an independent replicate of the same cross at the same temperature. In another case, a particular cuticle defect was specific to the Hsp90 mutants and a different RI line at 25°C. A similar frequency of isofemale lines having Hsp90-buffered defects was observed in populations of freshly caught wild flies (8).

**Wing and Bristle Traits Require Hsp90.** Of five traits examined, all but orbital bristle number were consistently affected by Hsp90. Sternopleural bristles, ocellar bristles, and wing size were significantly reduced in the Hsp90 mutants and when flies were treated with the Hsp90 inhibitor (Tables 2 and 3). Vibrissae and carnia were also significantly decreased in the Canton-S population cage stock treated with GA, and in isogenic flies carrying Hsp90 mutant or control 3rd chromosomes. The variance of most traits decreased with their means when Hsp90 was impaired, either by mutations or by the inhibitor (Table 3). Thus, although Hsp90 buffers widespread morphogenic variation for threshold traits in particular genetic backgrounds, the phenotypic variation of normal quantitative traits is not generally affected by the chaperone.

**Table 4. Effect of GA treatment (experiment 1) on wing asymmetry and composite asymmetry in outbred flies (ANOVA)**

| Trait | Source | df | MS | *F* | *P* | Variance component, % |
|---|---|---|---|---|---|---|
| Wing centroid | GA treatment | 1 | 0.153 | 0.09 | 0.762 | 0 |
| | Larval stage | 1 | 3.370 | 2.02 | 0.156 | 0.40 |
| | GA × Stage | 1 | 2.694 | 1.62 | 0.205 | 1.22 |
| | Error | 196 | 1.665 | | | 98.38 |
| Composite | GA treatment | 1 | 5.099 | 1.44 | 0.231 | 1.17 |
| | Larval stage | 1 | 3.140 | 0.89 | 0.347 | 0.62 |
| | GA × Stage | 1 | 0.900 | 0.25 | 0.615 | 0 |
| | Error | 196 | 3.529 | | | 98.21 |

**Hsp90 Inhibitor Studies.** Despite the highly significant effects of GA on the mean phenotype of most traits, drug treatment during either second or third instar larval development in outbred Canton-S flies had no effect on the symmetry of most traits. ANOVA on individual flies of their mean relative FA, left/right variance $|L - R|$ data that were log-transformed for normality ($\lambda = 0$), indicated that GA treatment had no significant effect on any trait. The wing and composite asymmetry analysis shown in Table 4 provided no evidence for an effect of GA treatment, larval stage at time of treatment, or an interaction between these factors.

The wing and composite asymmetry measures are continuous variables that can be transformed to normal distributions suitable for ANOVA. However, bristle numbers fell into just a few discrete classes and were more appropriately tested by using discontinuous models. Flies were grouped into bristle-asymmetry classes based on whether they were asymmetric for zero, one, two, or three bristles, and the data were analyzed by using LLMs (Table 5), where a significant interaction between asymmetry class and GA treatment indicates an effect of the inhibitor on trait asymmetry. Orbital, ocellar, and vibrissae and carnia bristles were clearly unaffected by GA. GA had a marginally significant effect on sternopleural bristle asymmetry in this analysis ($P = 0.02$), reflecting an increase in asymmetry in individuals exposed to GA, but this effect was not significant after correction for multiple comparisons. Moreover, as shown in Table 4, treated flies had no overall tendency to be more asymmetric because composite asymmetry was unaffected by the drug.

**Hsp90 Mutant Versus Control Chromosomes.** Although we used nearly lethal concentrations of GA, it is active in fly food for only about 24 h. We reasoned that Hsp90 mutations, which impair Hsp90 function throughout development, might have stronger effects than the inhibitor. Therefore, to more carefully examine the relationship between Hsp90 and sternopleural bristle asymmetry, we tested the effect of chromosomes carrying Hsp90 mutations in otherwise controlled genetic backgrounds. We first examined sternopleural bristle and wing trait FA for the effects of dominant-negative (*9J1*) and null (*P582*) Hsp90-mutant chromosomes alone and in combination with other factors previously shown to influence trait symmetry. The marginally significant effect of GA on sternopleural bristle asymmetry in outbred flies was not evident in isogenic groups of flies carrying Hsp90 mutant versus control 3rd chromosomes. As shown in Table 6, two independent experiments (experiments 2 and 3), using the same crossing design and isogenic strains, showed that Hsp90 mutant chromosomes, either alone or in combination with other factors, had no effect on the FA of any trait.

Highly significant sex and temperature effects on wing-trait asymmetry were detected in these experiments (Table 7). For example, L4 wing vein length was more asymmetric at 18°C than at 25°C, particularly in females. RI line backgrounds also affected wing vein FA depending on both temperature and sex. Wing area FA was influenced by an interaction between sex and vial and probably reflected a difference in larval density between the first and second replicate vials. However, a significant interaction between temperature and Hsp90 for wing vein FA reflected that the *P582* (null) mutant chromosome had *decreased* FA relative to the wild-type chromosome at 18°C. Because the stronger (dominant-negative) *9J1* mutant was indistinguishable from the control at either temperature, the *P582* effect is most likely due to a temperature-dependent interaction of unrelated alleles carried on the *P582* and control chromosomes.

In experiment 3 we used the same Hsp90 mutant and control fly strains, but controlled for the effects of larval density, replicate vials, and temperature by seeding larvae at a constant density across five different vials and by performing the experiment at a single temperature. At 25°C, in the absence of confounding variation due to possible differences in larval density, females were again more asymmetric than males; however, neither Hsp90 mutant chromo-

**Table 5. Effect of GA treatment (experiment 1) on bristle-trait asymmetry classes in outbred flies (LLM)**

| Trait | Source | df | *G** | *P*† |
|---|---|---|---|---|
| Sternopleural bristles | GA treatment × Class | 3 | 9.49 | 0.024 |
| | Larval stage × Class | 3 | 3.01 | 0.391 |
| | GA × Stage × Class | 3 | 4.03 | 0.259 |
| Orbital bristles | GA treatment × Class | 3 | 5.44 | 0.142 |
| | Larval stage × Class | 3 | 2.99 | 0.393 |
| | GA × Stage × Class | 3 | 3.09 | 0.378 |
| Ocellar bristles | GA treatment × Class | 3 | 2.28 | 0.517 |
| | Larval stage × Class | 3 | 1.53 | 0.675 |
| | GA × Stage × Class | 3 | 2.45 | 0.484 |
| Vibrissae and carnia | GA treatment × Class | 3 | 0.60 | 0.897 |
| | Larval stage × Class | 3 | 1.30 | 0.730 |
| | GA × Stage × Class | 3 | 1.79 | 0.617 |

*A LLM with the three-way effect (Treatment × Stage × Class) was first fitted to the data testing for the significance of the interaction between Stage and Treatment. Treatment (Treatment × Class) and Stage (Stage × Class) were then added separately to this model to determine whether the addition of these effects improved the model.

†No probabilities were significant after correction for multiple comparisons.

**Table 6. Effect of Hsp90 genotype and RI line background on bristle-trait asymmetry classes in experiments 2 and 3 (LLM)**

| Trait | Source | df | *G** | *P*† |
|---|---|---|---|---|
| Sternopleurals (Exp. 2) | Hsp90 × Class | 18 | 8.87 | 0.963 |
| | RI line × Class | 27 | 23.12 | 0.679 |
| | Hsp90 × RI line × Class | 54 | 36.09 | 0.971 |
| | Hsp90 × Temperature × Class | 6 | 6.93 | 0.327 |
| | Temperature × Class | 3 | 0.957 | 0.812 |
| | RI line × Temperature × Class | 27 | 26.59 | 0.486 |
| | Hsp90 × RI line × Temperature | 54 | 32.74 | 0.990 |
| Sternopleurals (Exp. 3) | Hsp90 × Class | 6 | 10.62 | 0.101 |
| | RI line × Class | 24 | 26.58 | 0.324 |
| | Hsp90 × RI line × Class | 48 | 46.21 | 0.546 |
| Orbital bristles (Exp. 3) | Hsp90 × Class | 6 | 3.49 | 0.481 |
| | RI line × Class | 24 | 23.95 | 0.091 |
| | Hsp90 × RI line × Class | 48 | 39.80 | 0.161 |
| Ocellar bristles (Exp. 3) | Hsp90 × Class | 6 | 9.09 | 0.169 |
| | RI line × Class | 24 | 38.01 | 0.035 |
| | Hsp90 × RI line × Class | 48 | 43.72 | 0.649 |
| Vibrissae and carnia (Exp. 3) | Hsp90 × Class | 6 | 12.83 | 0.046 |
| | RI line × Class | 24 | 22.15 | 0.571 |
| | Hsp90 × RI line × Class | 48 | 61.07 | 0.098 |

*A LLM with a four-way effect (experiment 1, RI line × Hsp90 × Temperature × Class) or three-way effect (experiment 2, RI line × Hsp90 × Class) was first fitted to the data before successively adding lower-order effects, including the effects of RI line (RI line × Class), Temperature (Temperature × Class), and Hsp90 (Hsp90 × Class).

†No probabilities were significant after correction for multiple comparisons.

EVOLUTION

**Table 7. Effect of Hsp90 chromosome, RI line, sex, temperature, and vial on wing asymmetry in experiment 2 (ANOVA)**

| Trait | Source | df | MS × $10^{-5}$ | $P$* | Variance component, % |
|---|---|---|---|---|---|
| L4 vein | Hsp90 chromosome | 2 | 420.38 | 0.236 | 0.09 |
| | RI line (random) | 9 | 616.66 | 0.037 | 0.71 |
| | Sex | 1 | 3322.85 | <0.001† | 1.00 |
| | Temperature | 1 | 3873.92 | 0.001† | 1.15 |
| | Vial (Hsp, Line, Temperature) | 59 | 284.21 | 0.093 | 1.11 |
| | Hsp90 × RI line | 18 | 198.45 | 0.799 | 0.00 |
| | Hsp90 × Sex | 2 | 20.28 | 0.911 | 0.00 |
| | Hsp90 × Temperature | 2 | 1644.21 | 0.005† | 1.31 |
| | RI line × Sex | 9 | 125.63 | 0.807 | 0.00 |
| | RI line × Temperature | 9 | 347.26 | 0.299 | 0.20 |
| | Sex × Temperature | 1 | 1196.52 | 0.022 | 0.63 |
| | Sex × Vial (Hsp, Line, Temperature) | 59 | 216.16 | 0.580 | 0.00 |
| | Hsp90 × RI line × Sex | 18 | 359.31 | 0.073 | 1.38 |
| | Hsp90 × RI line × Temperature | 18 | 293.96 | 0.438 | 0.09 |
| | Hsp90 × Sex × Temperature | 2 | 15.02 | 0.933 | 0.00 |
| | RI line × Sex × Temperature | 9 | 868.09 | 0.001† | 4.19 |
| | Hsp90 × RI line × Sex × Temperature | 18 | 246.18 | 0.340 | 0.58 |
| | Error | 2,350 | 226.91 | | 87.55 |
| Wing area | Hsp90 chromosome | 2 | 529.19 | 0.339 | 0.02 |
| | RI line (random) | 9 | 731.54 | 0.161 | 0.33 |
| | Sex | 1 | 14.09 | 0.876 | 0.00 |
| | Temperature | 1 | 2217.54 | 0.036 | 0.35 |
| | Vial (Hsp, Line, Temperature) | 59 | 480.28 | 0.077 | 1.25 |
| | Hsp90 × RI line | 18 | 626.04 | 0.219 | 0.58 |
| | Hsp90 × Sex | 2 | 817.54 | 0.247 | 0.15 |
| | Hsp90 × Temperature | 2 | 793.99 | 0.200 | 0.19 |
| | RI Line × Sex | 9 | 793.63 | 0.214 | 0.44 |
| | RI Line × Temperature | 9 | 204.57 | 0.916 | 0.00 |
| | Sex × Temperature | 1 | 2035.83 | 0.064 | 0.59 |
| | Sex × Vial (Hsp, Line, Temperature) | 59 | 571.86 | 0.007† | 4.69 |
| | Hsp90 × RI line × Sex | 18 | 480.61 | 0.647 | 0.00 |
| | Hsp90 × RI line × Temperature | 18 | 659.34 | 0.179 | 1.07 |
| | Hsp90 × Sex × Temperature | 2 | 172.10 | 0.741 | 0.00 |
| | RI Line × Sex × Temperature | 9 | 467.39 | 0.602 | 0.00 |
| | Hsp90 × RI line × Sex × Temperature | 18 | 570.36 | 0.475 | 0.00 |
| | Error | 2,245 | 376.47 | | 90.34 |

*For single-trait asymmetry, not for composite asymmetry (which was unadjusted).
†Probabilities significant after correction for multiple comparisons.

some or RI line background influenced the symmetry of any trait or their composite measure (Table 8).

**Allele-Specific Effects.** In experiment 4, we used the *P582i* introgression stock to separate the allelic effects of the Hsp90 mutation from the effects of background alleles unique to the mutant and control 3rd chromosomes. The *P582i* versus *Sami* male progeny of crosses between *wi/Sam1; Sam2; P582i/Sam3* virgins and males from each RI line were distinguished by their eye color. Because these flies were otherwise isogenic on all chromosomes, the specific effects of the Hsp90 alleles and RI line backgrounds were examined in isolation, apart from other confounding factors.

RI line background had a highly significant effect on composite (global) symmetry (Table 9), consistent with the idea that developmental stability is influenced by alleles specific to the different RI line backgrounds. The effect of Hsp90 on trait means was still highly significant. However, as shown in Tables 9 and 10, even when Hsp90 effects were isolated from other factors, Hsp90 did not affect the symmetry of any trait, either individually or in composite. Based on the results of the four independent experiments reported here,

**Table 8. Effect of Hsp90 chromosome, RI line, and sex, on wing and composite asymmetry in experiment 3 (ANOVA)**

| Trait | Source | df | MS × $10^{-5}$ | $P$* | Variance component, % |
|---|---|---|---|---|---|
| Wing centroid | Hsp90 chromosome | 2 | 1.11 | 0.598 | 0.21 |
| | RI line (random) | 8 | 0.98 | 0.887 | 0.15 |
| | Sex | 1 | 7.06 | 0.071 | 0.48 |
| | Hsp90 × RI line (random) | 16 | 0.97 | 0.969 | 0.00 |
| | Hsp90 × Sex | 2 | 0.41 | 0.827 | 0.00 |
| | RI line × Sex (random) | 8 | 1.81 | 0.565 | 0.00 |
| | Hsp90 × RI line × Sex (random) | 16 | 2.28 | 0.390 | 0.24 |
| | Error | 1,296 | 2.15 | | 98.92 |
| Composite FA | Hsp90 chromosome | 2 | 6.24 | 0.234 | 0 |
| | RI line (random) | 8 | 4.80 | 0.348 | 0.20 |
| | Sex | 1 | 25.31 | 0.015 | 1.66 |
| | Hsp90 × RI line (random) | 16 | 5.56 | 0.191 | 0.64 |
| | Hsp90 × Sex | 2 | 3.84 | 0.409 | 0 |
| | RI line × Sex (random) | 8 | 2.77 | 0.738 | 0 |
| | Hsp90 × RI line × Sex (random) | 16 | 4.18 | 0.484 | 0 |
| | Error | 1,296 | 4.29 | | 97.49 |

*No probabilities were significant after correction for multiple comparisons.

we conclude that, although Hsp90 is the most general buffer known against genetic perturbations of development, it cannot be a similarly general buffer for nongenetic perturbations, and is no longer a candidate gene for developmental stability.

## Discussion

Our failure to find an effect of Hsp90 on developmental stability was not due to a lack of statistical power to detect FA. First, our experiments were sensitive enough to detect highly significant effects of sex, temperature, and genetic background. Second, a power analysis (13) indicated that significant differences in asymmetry much smaller than reported for other *Drosophila* studies (36) would have been detected. For example, we would have detected at the 5% significance level asymmetry differences of 0.33–0.23 bristle in the GA experiment, and differences as low as 0.11–0.18 bristle in the other experiments. Further, the lack of an Hsp90 effect was not simply due to the wrong choice of traits; the highly significant effect of Hsp90 on the mean values of nearly every trait highlights its importance for many developmental processes, including the wing and bristle traits examined here. Finally, Hsp90 did not have an effect on composite asymmetry, which is expected to be more powerful than single-trait asymmetry for examining the developmental stability of whole organisms (33) and can differ among treatments even when single-trait asymmetries are generally not significant (35, 36).

**Table 9. Effect of Hsp90 allele, RI line, and sex on wing and composite asymmetry in experiment 4 (ANOVA)**

| Trait | Source | df | MS × $10^{-5}$ | $F$ | $P$* | Variance component, % |
|---|---|---|---|---|---|---|
| Wing centroid | Hsp90 allele | 1 | 7.20 | 5.70 | 0.017 | 1.05 |
| | RI line | 8 | 1.18 | 0.94 | 0.486 | 0 |
| | Hsp90 × RI line | 8 | 1.19 | 0.94 | 0.482 | 0 |
| | Error | 882 | 1.26 | | | 98.95 |
| Composite FA | Hsp90 chromosome | 2 | 1.34 | 0.36 | 0.547 | 0 |
| | RI line | 8 | 10.64 | 2.88 | 0.004† | 1.72 |
| | Hsp90 × RI line | 8 | 4.15 | 1.12 | 0.344 | 0.24 |
| | Error | 882 | 3.69 | | | 98.04 |

*No probabilities were significant after correction for multiple comparisons, except as marked by †.

**Table 10. Effect of Hsp90 allele and RI line on bristle-asymmetry classes in experiment 4 (LLM)**

| Trait | LLM | df | $G$* | $P$† |
|---|---|---|---|---|
| Sternopleural bristles | Hsp90 × Class | 4 | 9.79 | 0.044 |
| | RI line × Class | 32 | 31.78 | 0.478 |
| | Hsp90 × RI line × Class | 32 | 40.81 | 0.137 |
| Orbital bristles | Hsp90 × Class | 3 | 4.32 | 0.229 |
| | RI line × Class | 24 | 35.64 | 0.059 |
| | Hsp90 × RI line × Class | 24 | 18.13 | 0.797 |
| Ocellar bristles | Hsp90 × Class | 3 | 1.20 | 0.753 |
| | RI line × Class | 24 | 41.95 | 0.013 |
| | Hsp90 × RI line × Class | 24 | 26.93 | 0.308 |
| Vibrissa and carnia | Hsp90 × Class | 4 | 6.52 | 0.164 |
| | RI line × Class | 32 | 42.59 | 0.100 |
| | Hsp90 × RI line × Class | 32 | 23.80 | 0.852 |

*See Table 6.
†No probabilities were significant after correction for multiple comparisons.

**Trait-Specific Buffers.** Certain developmental genes and pathways do influence the symmetry of particular traits. The best known of these is the modifier mutation in sheep blowflies, which is a homologue of the *Notch* gene of *Drosophila* (14). *Notch* regulates bristle development (15) and specifically buffers bristle-trait symmetry, but it does not affect the symmetry of wing traits and is not associated with changes in trait means or variances (ref. 16; J. McKenzie, personal communication). Indeed, many mutations are highly asymmetric, including most known developmental mutations and the polygenic abnormalities buffered by Hsp90.

It is difficult to imagine how genes would directly regulate stochastic sources of variation in development, such as the unequal left–right partitioning of small numbers of critical regulatory molecules. However, development is made up of a complex series of binary decisions with discrete outcomes governed by thresholds. Any mutation or environment that brings an individual closer to such a threshold could increase the sensitivity of that particular developmental outcome to stochastic perturbations. As Hsp90 buffers thresholds for the expression of many different traits (1), Hsp90-dependent processes or pathways likely do regulate the developmental stability of some trait(s) in certain genetic backgrounds or environments. It is proposed that Hsp90 genetic buffering arose as a by-product of its adaptive evolution as an environmental buffer (5), but perhaps its critical function is its demonstrated ability to buffer $V_{G\times E}$, protecting optimal phenotypes from the interaction of genetic variation with coarse environmental disturbances.

**Many Modes of Canalization?** Whether Hsp90 buffering evolved as an adaptive trait, or whether its properties as a genetic buffer are an unselected by-product of some other function, e.g., regulating $V_{G\times E}$ or physiological stability (17), is still undecided. However, before this work, Hsp90 was the strongest, and indeed the only, plausible candidate for a general canalizing gene. The separation of Hsp90 genetic buffering and buffering against random perturbations of developmental symmetry refutes the idea that Hsp90 generally influences all sources of phenotypic variation (5) and calls for a reevaluation, both of our expectations about the generality of mechanisms of canalization and of theoretical models for their evolution.

The existence of genes that globally regulate developmental stability within individuals seems unlikely in light of our current results and the previous work showing a poor correlation between the FA of different traits in individual flies (25, 36, 39). Rather, evidence suggests that developmental processes that participate in the production of particular traits easily evolve independent and idiosyncratic buffering mechanisms for developmental noise, unique to each particular trait under selection. Increasing numbers of biological processes are found to be stabilized by feedback loops, parallel pathways, genetic redundancy, and other aspects of network architecture (1, 17–20). Features like these are ubiquitous in biological systems and seem to have easily arisen many times in evolution, in association with many different processes.

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