EVOLUTION & DEVELOPMENT 7:2, 89–100 (2005)

Evidence for canalization of *Distal-less* function in the leg of *Drosophila melanogaster*

Ian Dworkin[1]

Department of Zoology, University of Toronto, Toronto, ON, Canada M5S 3G5

Correspondence (email: I_Dworkin@ncsu.edu)

[1]Present address: Department of Genetics, North Carolina State University, 3632 Gardner Hall, Raleigh, NC 27695-7614, USA

SUMMARY A considerable body of theory pertaining to the evolution of canalization has emerged recently, yet there have been few empirical investigations of their predictions. To address this, patterns of canalization and trait correlation were investigated under the individual and joint effects of the introgression of a loss-of-function allele of the *Distal-less* gene and high-temperature stress on a panel of iso-female lines. Variation was examined for number of sex comb teeth and the length of the basi-tarsus on the pro-thoracic leg of male *Drosophila melanogaster*. I demonstrate that whereas there is evidence for trait canalization, there is no evidence to support the hypothesis of the evolution of genetic canalization as a response to microenvironmental canalization. Furthermore, I demonstrate that although there are genetic correlations between these traits, there is no association between their measures of canalization. I discuss the prospects of the evolutionary lability of the *Distal-less* gene within the context of changes in genetic variation and covariation.

INTRODUCTION

Given the pleiotropic function of many genes, there may be a potential antagonism between conservation and divergence of function (Wistow 1993; Wilkins 2002). For example, *Ubx* is implicated in the differences in leg trichome patterning between *Drosophila simulans* and *D. melanogaster* (Stern 1998), suggesting that the molecular divergence in this gene is in part responsible for the morphological change. However, this rapid evolutionary divergence of *Ubx* function can be contrasted with the conservation of its function for anterior–posterior patterning within the arthropods (see Hughes and Kaufman 2002, for an extensive review). The conundrum of how gene function is conserved and yet still retains sufficient natural genetic variation for adaptive evolution has yet to be resolved. One potential explanation is that modular aspects of gene function have been canalized. For instance, evidence suggests that there is cryptic genetic variation for *Ubx* phenotypes (Waddington 1956; Gibson and Hogness 1996; Gibson et al. 1999), consistent with the canalization of *Ubx* function. This and additional work suggests that there is considerable natural genetic variation both for *Ubx* function and possibly for its canalization (Gibson and van Helden 1997; Gibson et al. 1999).

Canalization has received considerable attention, and some important theory has been developed examining under what conditions it might evolve (Wagner et al. 1997; Rice 1998; Siegal and Bergman 2002). One hypothesis that has garnered theoretical support is the congruence scenario (Wagner et al. 1997; de Visser et al. 2003) or the evolution of genetic canalization as a correlated response to environmental canalization (Ancel and Fontana 2000; Meiklejohn and Hartl 2002). Given our lack of understanding of the genetic architecture of most traits, let alone the mechanisms that may govern the buffering of genetic and environmental variation upon them, a considerable amount of empirical work is required to test such theory. In addition, it still remains unclear whether canalization is specific to a given trait, the genetic network underlying trait expression, or possibly a more "universal" mechanism (Rutherford and Lindquist 1998).

In this current study, I utilize the homeo-domain containing transcription factor *Distal-less* (*Dll*) as a model system for the study of canalization. *Dll* function is implicated in a number of processes during ventral appendage development within *D. melanogaster* including: patterning the proximal–distal axis of the ventral thoracic appendages (Sunkel and Whittle 1987; Cohen et al. 1989), specifying ventral from dorsal fates (Gorfinkiel et al. 1997), and decisions between antennal and leg cell fates (Dong et al. 2000). Loss of *Dll* function in *Drosophila* leads to truncation and fusion of distal leg segments, whereas in the antenna it leads to homeotic transformation of distal antenna to leg, with null mutations leading to complete loss of distal structures (Sunkel and Whittle 1987; Cohen et al. 1989). Evidence suggests that *Dll* function is conserved in appendage development within protostome and deuterostome lineages (Panganiban et al. 1997).

Within the arthropods, *Dll* function has evolved with appendage development (Panganiban et al. 1995, 1997), and has been co-opted for a novel function within the Lepidoptera as part of a patterning mechanism for eye-spot pigmentation on the wing (Brunetti et al. 2001). Variation in eye-spot size is associated with molecular polymorphisms in the *Dll* gene (Beldade et al. 2002) and phenotypic plasticity of eye-spot size is mediated through *Dll* function (Brakefield et al. 1996). Thus, *Dll* function seems to have both conserved genetic function across taxa, as well as being involved in the expression of intraspecific phenotypic variation.

I set out to explore the role of *Dll* with respect to the canalization and integration of trait variation. For this study, the Dll^{11} allele was introgressed into a panel of iso-female lines of *D. melanogaster* derived from several natural populations. Mutant and wild-type ($Dll^{+/11}$ versus $Dll^{+/+}$) con-genics from each line were studied under two developmental temperature regimes: benign (25°C) and stressful (30°C). High temperature is a known ecological stressor for *Drosophila* (Feder 1997), which has previously been shown to alter bristle trait expression and variation (Beardmore 1960; Bubliy et al. 2000), and sex comb teeth (SCT) number in particular (Combs 1937). I examined phenotypic variation for two morphological traits on the pro-thoracic leg of male *D. melanogaster*: the number of SCT and the length of the basi-tarsus, on which the sex comb is located.

This system was utilized to address specific questions pertaining to canalization. First, I demonstrate evidence for canalization of *Dll* function, as revealed by a number of metrics including a release in cryptic genetic variation, and changes in the macro- and microenvironmental variation associated with traits. Second, I provide evidence inconsistent with the coevolution of genetic and microenvironmental canalization as hypothesized by Wagner et al. (1997). Finally, I demonstrate that whereas there is a genetic correlation between basi-tarsus length and number of SCT, there is no evidence for a correlation between measures of canalization for these traits. These results are discussed within the context of the evolution of canalization, and the evolutionary lability of *Dll* function.

MATERIALS AND METHODS

Selection of *Dll* alleles

The $Dll^{11/B}$ allele (provided courtesy of J. P. Couso) is part of an inversion that occurs in the *Dll* gene (breakpoints include 48D–60E). Evidence suggests that $Dll^{11/B}$ may function as an antimorph, given that two wild-type copies of *Dll* cannot entirely rescue the phenotype of this allele (Cohen et al. 1989). It is likely that the entire inversion will be introgressed, not just the *Dll* allele.

Lines

A series of iso-female lines were established from single wild caught females collected in Toronto, Canada (10), and Algonquin Park, Canada (10). The remaining 10 lines were from the worldwide wild-type collection (Gibson and van Helden 1997; Polaczyk et al. 1998), and from inbred lab stocks w^{118}, Oregon-R, Canton-S, and Samarkand (provided by Dr. Trudy Mackay).

Introgression protocol

Females with the Dll^{11} allele were backcrossed repeatedly to males from each of the lines. Random selection of individual *Dll* females was based upon the dominant antenna-to-leg transformation, as opposed to traits scored in the experiment. This mode of selection reduces the bias of unconscious selection on the traits being studied. Flies were raised in a common incubator at 25°C, during the 11 generations of backcrosses.

Experimental details

Five pairs of flies (five females with the Dll^{11} allele and 5 "wild-type" [wt] males) from each line were placed in each of two replicate bottles, with standard medium and seeded with live yeast. The flies were allowed to lay eggs for 3 days, and were then transferred to new bottles. The first set was raised at 25°C, and a second set of bottles was incubated at 30°C. After 3 days of egg laying, the adults were removed, and sterilized cotton was placed within each of the bottles to provide additional space for pupation. After eclosion, flies were stored in 70% ethanol.

Measurements

The left pro-thoracic (first) leg of males from each line was removed and mounted on slides in glycerol. Twenty males from each line and genotype (wild type:$Dll^{+/+}$ vs. *Dll*:$Dll^{+/11}$) were used. For several lines, 20 males were not available (minimum 11). Digital images were captured using a Hitachi KP-250 camera (Hitachi, Tokyo, Japan) mounted on an Olympus BX-60 microscope (Olympus, Tokyo, Japan) at $\times$ 40 magnification using Image-Pro software (Media Cybernetics Inc., Silver Spring, MD, USA). The length of the basi-tarsus was measured using the digital caliper in Image-Pro. Number of SCT was counted on the Olympus BX-60 microscope at a magnification of $\times$ 200.

Dll individuals who showed any type of ectopic SCT phenotype were recorded as such. In comparison with the bristles on the first tarsal segment, SCT show three distinguishing characteristics (Hannah-Alava 1958): dark pigmentation, thicker diameter, and a relatively blunt tip morphology. Bristles that demonstrated at least two of these three characteristics were scored as SCT, and rare bristles that were ambiguous as to their identity were not included.

Scanning electron micrographs of flies

Flies stored in 70% ethanol were washed repeatedly in 70% ethanol, transferred to absolute ethanol, and then air dried. Flies were then mounted on stubs, gold coated, and observed using Hitachi S2500 scanning electron microscope.

Measuring canalization

Given that canalization remains an ill-defined concept (Debat and David 2001; Dworkin 2005a), it is important that the methods used for this study follow from particular definitions. For a given trait, the phenotypic variation can be partitioned into genetic and

environmental components (Falconer and Mackay 1996):

$$V_P = V_G + V_E$$

where V_P, V_G, and V_E are the phenotypic, genetic, and environmental variances, respectively. Although there are a number of distinct definitions of canalization (Debat and David 2001) it is evident that canalization leads to the suppression of phenotypic variation, V_P (Wagner et al. 1997). As is clear from the above equation this reduction can be due to a decrease in V_G or V_E. Furthermore, both V_G and V_E can be further partitioned, for instance:

$$V_G = V_A + V_{NA}$$

where V_A is the additive genetic variance and V_{NA} represents nonadditive components (dominance, epistasis). In particular, Wagner et al. (1997) defined genetic canalization as a reduction of V_A.

The environmental variance V_E can be broken down into components as follows:

$$V_E = V_{Em} + V_{Eg} + V_{Es}$$

where V_{Em} is the macroenvironmental variance, V_{Eg} is the general within-line, between-individual (residual) microenvironmental variance, and V_{Es} is the specific, within-individual variation usually measured as fluctuating asymmetry (Falconer and Mackay 1996). With respect to environmental canalization, there is currently disagreement as to which particular form of environmental variance is reduced (Debat and David 2001). Some have argued that canalization is the opposite of phenotypic plasticity (Waddington 1957; Nijhout and Davidowitz 2003), and canalization is expected to reduce V_{Em}. Under the definition of canalization reducing V_{Em}, it is worth distinguishing predictable macroenvironmental cues that lead to adaptive plasticity and polyphenisms from unpredictable stressful environments, as their genetic mechanisms may differ.

This form of environmental canalization can be contrasted with those that reduce V_{Eg} or V_{Es} (Gavrilets and Hastings 1994; Wagner et al. 1997). Although reduction in V_{Eg} or V_{Es} is likely to reflect a common mechanism to buffer against developmental noise (Clarke 1998), V_{Em} and V_{Eg} appear to be independent (Scheiner et al. 1991; DeWitt 1998).

By the definitions outlined above, most empirical studies have actually focused on examining genetic canalization, given that even when external environmental perturbations are used (Waddington 1952, 1956; Gibson and Hogness 1996) a release of cryptic genetic variation (increase in V_G) is used to infer de-canalization of the trait (Dworkin 2005a). Within the framework of an ANOVA, once a significant line-by-perturbation interaction is observed, its effect can be partitioned into a component due to crossing of line means and a component due to scaling effects (Lynch and Walsh 1998). If the genetic correlation across environments, $r_G > 0$, or if the between-line variance significantly increases, the results are consistent with an increase in genetic variation across treatments (Gibson and van Helden 1997; Dworkin 2005a). Related to the above measure, the difference in line means across perturbations was used to investigate patterns of macroenvironmental canalization (i.e., reduction in V_{Em}). For the measure of environmental canalization based upon the within-line, residual variance (V_{Eg}), the coefficient of variation (CV) is often used. However, I use the median form of Levene's statistic which is more robust and independent of the trait mean (Schultz 1985). Although there is no clearly defined measure of canalization based on this measure, the absolute difference in within-line variances across perturbations is used in this study. SAS code to calculate Levene's statistic for complex experimental designs is available from the author.

Analysis

All analyses were performed using SAS V8.2e (SAS Institute, Cary, NC, USA). For mixed models, the Proc Mixed command in SAS was used, using Type III sum of squares for tests of significance and restricted maximum likelihood estimates for variance components (Lynch and Walsh 1998). For each trait, data were fit to the following ANOVA model:

$$Y = \mu + L + G + T + T \times L + G \times L + G \times T + T \times L \times G + \varepsilon$$

where G is the genotypic effect of $Dll^{+/+}$ versus $Dll^{+/11}$ (fixed), T is the rearing temperature effect (25°C vs. 30°C, fixed), all terms including line (L) are random, and ε is residual error variance. Reduced models based on the main model were also used. Permutations of the data for ANOVA analysis using a modified SAS macro (Cassell 2002) were used to control for violations of model assumptions such as normality or homoscedasticity (Noreen 1989). For measures of canalization, estimate of line effects were obtained using best linear unbiased predictors for both means and Levene's statistic (within-line variances).

Standard correlation analysis was performed according to Sokal and Rohlf (1995). Genetic correlations were performed according to Robertson (1959), where

$$r_G = \mathrm{COV}_{1,2}/(\sigma_1\sigma_2)$$

where $\mathrm{COV}_{1,2}$ is the covariance between-line means in environments 1 and 2, and σ is the square root of the between-line variance components computed from models in each environment. The correlation coefficient was tested against the null hypothesis of $r_G = 0$, using a t-statistic (Zar 1999). Measures of canalization were compared using the Spearman rank correlation.

RESULTS

Loss of *Dll* reveals cryptic variation for the number, position, and arrangement of SCT

While the primary object of this study was to examine the effects of *Dll* on quantitative trait variation, there were also qualitative phenotypic effects of *Dll* (Fig. 1). Loss of *Dll* function alters both the number and positioning of the SCT (Figs. 1 and 2), and ectopic SCT appear to replace normal bristles in a line-dependent manner (Appendix).

Figure 2 demonstrates that number of SCT increases in a Dll^{11} background. Indeed, the individual and joint effects of Dll^{11} and high-temperature stress on SCT number explain a significant proportion of the variation for this trait (Tables 1 and 2), with the individual effects of Dll^{11} increasing mean number of SCT by 1.3 bristles (Table 1). Is there evidence for cryptic genetic variation for the number of SCT? As discussed in Dworkin (2005a,b), it must first be determined whether there is line-by-genotype ($L \times G$), line-by-temperature

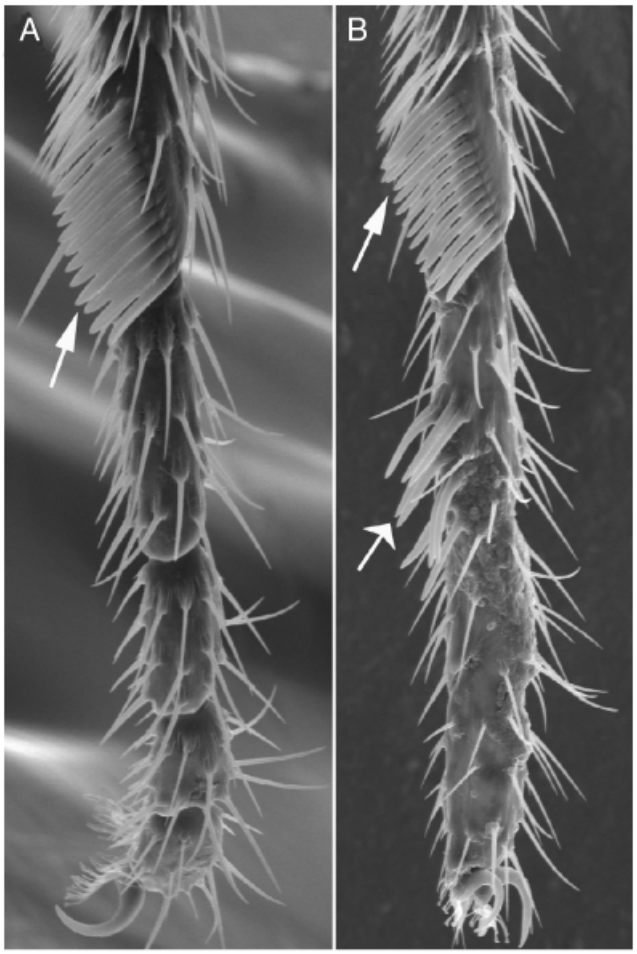

Fig. 1. The effects of *Dll* on sex comb teeth (SCT) position and arrangement. (A) Wild-type pro-thoracic leg from a male showing normal orientation and placement of SCT (marrow arrowhead). (B) In $Dll^{11/+}$ ectopic sex comb teeth are present, distal of the normal row of SCT. These SCT are replacing normal bristles located on the tarsal segment and elsewhere (broad arrowhead). Furthermore, there is a lack of external segmentation of the tarsi. This was a rare phenotype observed in the $Dll^{11/+}$ flies, and in virtually all cases, the remnants of segmentation could be made out under bright-field microscopic conditions.

($L \times T$), or a three-way $L \times G \times T$ interaction, where line refers to genetic background, genotype refers to $Dll^{+/+}$ versus $Dll^{+/11}$, and temperature refers to the rearing temperature of either 25°C or 30°C. All of the factors in this model interact strongly with one another, as shown in the significant $L \times G \times T$ term (Table 2; Fig. 3A). Furthermore, the treatment effects of rearing temperature and genotype demonstrate significant interaction.

Unfortunately, interpreting the third-order interaction is challenging; therefore, I focus my analysis on reduced models (Table 3). For the models performed on the 25°C and 30°C treatments separately, there are strong $G \times L$ interactions

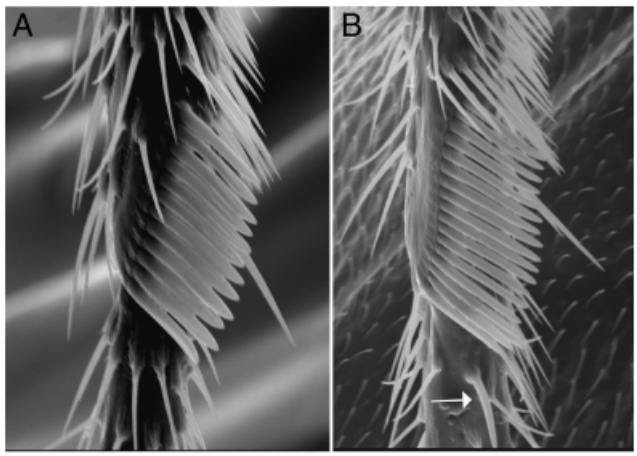

Fig. 2. SEM of sex combs of *D. melanogaster*. (A) Wild-type sex comb with 12 bristles. (B) The row of sex comb teeth for the $Dll^{11/+}$ individual contains 17 bristles, and the SCT are thinner than those observed in the wild-type leg. In addition, a leg bristle is transformed toward the fate of a sex comb tooth (arrow).

(Table 3B) suggesting that there is significant variation for *Dll* activity between lines, or that there is evidence for non-additive (epistatic) effects between genotype (*Dll* and wt) and line. Significant interactions are observed for the $L \times T$ interaction when considered across both genotypes separately (Table 3A; Fig. 3A). For the wt genotype this is consistent with genetic variation for phenotypic plasticity for the trait. This is also observed for the *Dll* treatment (Table 3B; Fig. 3A), and the interaction could be due to polygenic (background) effects, or genetic variation for allelic (temperature) sensitivity at the *Dll* locus. Thus, there is evidence for genotype-by-genotype (epistasis) and genotype-by-environment interactions for number of SCT.

In addition to changing mean trait expression, the individual and joint effects of temperature, and the genotypic effect of *Dll* increase the phenotypic variation for SCT number (Table 1A). Interestingly, the effects of *Dll* appear to have a more profound effect on overall levels of phenotypic variation. However, to test for evidence of canalization, it must be demonstrated that the interaction effects are due, in part, to a release of cryptic genetic variation, and not entirely due to crossing of line means. As discussed in the methods, this can be accomplished by examining relative amounts of genetic variation in the control and perturbing environments.

To specifically address whether the perturbations increased levels of genetic variation, the between-line variances were estimated for each treatment effect, which could then be compared with the control ($Dll^{+/+}$, 25°C). The results of the *F*-test on the coefficients of variation computed from line means demonstrate a marginally significant effect of the *Dll* mutation on variation for flies raised at 25°C, although a highly significant effect when the *Dll* flies reared at 30°C are examined

Table 1. Evidence for genetic canalization

(A) Summary of variance across treatment levels

G	*T*	*N*	Mean	SD	CV	*F*	*P*
Dll	25	418	12.27	1.79	0.14	1.795	2.59E − 11
Dll	30	440	10.81	2.40	0.22	4.152	9.40E − 58
wt	25	603	10.98	1.19	0.11		
wt	30	488	10.80	1.27	0.12	1.172	0.032

(B) Summary composed from line means

G	*T*	CV	*F*	Levene	r_G	σ^2_L	σ^2_E	H^2	CV_G
Dll	25	0.077	1.81 (0.08)	0.036	0.40	1.02	2.13	0.25	7.0
Dll	30	0.16	8.52 (1.3E − 6)	0.037	0.36	2.55	3.44	0.35	12.2
wt	25	0.057				0.29	1.16	0.15	4.18
wt	30	0.061	1.15 (NS)	NS	0.74	0.37	1.18	0.19	4.80

Summary of measures of variation, *F*-tests, and Levene's test for differences in variation for SCT number. *F*-tests are always performed versus the wt 25°C treatment. Levene's was simply a *t*-test based upon the median form of Levene's statistic. Differences in significance are largely due to degrees of freedom, given that the *F* statistic is similar whether measured across line means or treatment levels (*P* values). *G*, genotype; *T*, temperature; *N*, number of individuals (or lines in B); SD, standard deviation; CV, coefficient of variation; NS, not significant; r_G, genetic correlation; H^2, broad-sense heritability; SCT, sex comb teeth.

(Table 1B). Similarly, the results from the *t*-test for Levene's statistic derived from line means (Dworkin 2005a) are significant for genotypic effects at either temperature, although only marginally so. Interestingly, the genetic correlation between the 25°C and 30°C environment is significantly different from zero (Table 1B), suggesting that a significant fraction of the $T \times L$ interaction effect is due to an increase in between-line variance. The results are strongest for the *Dll* flies raised at 30°C, consistent with a synergistic effect between temperature and genotype. All treatments showed an increase in both heritability and the coefficient of genetic variation relative to the $Dll^{+/+}$ 25°C treatment (Table 1B). These results suggest a relative increase in genetic variation in the *Dll*, and possibly the high-temperature treatments, as compared with the wild-type, consistent with a release of cryptic genetic variation.

Alternatively, de-canalization due to perturbation can be considered as an increase in the residual within-line variance (V_{Eg}). Both the genetic and temperature perturbations increase V_{Eg} (Table 4), and indeed genetic variation for residual variance is affected by the synergistic interaction between these stressors (Table 4). Thus, for both number of SCT and their residual variance, there is evidence that both genetic and environmental perturbations lead to trait de-canalization.

Table 2. ANOVA summary for the full model for SCT number

Source	DF	SS	MS	*F*	Prob *F*
G	1	255.68	255.68	20.39	1.0E − 4 (<0.001)
T	1	252.15	252.15	26.62	2.7E − 5 (<0.001)
$G \times T$	1	153.15	153.15	14.91	7.9E − 4 (0.003)
L	26	899.31	34.59	2.49	0.06
$G \times L$	26	382.68	14.72	1.33	0.25
$T \times L$	23	243.37	10.58	0.92	0.58
$G \times T \times L$	22	252.54	11.48	6.10	2.3E − 17
Residual	1847	3475.93	1.88		

G, genotype; *T*, temperature; *L*, line; SCT, sex comb teeth. Significance of tests for fixed effects by 1000 permutations of the data are shown in parentheses. While the assumption of homoscedasticity across treatments is violated, the results appear robust given the results of the permutations, and log-transformation has no significant impact on the results (not shown).

The effects of *Dll* and temperature on the length of the basi-tarsus

Both the effects of temperature and loss-of-function mutations are known to affect the length of segments in the *Drosophila* leg (Sunkel and Whittle 1987; Cohen et al. 1989; Gorfinkiel et al. 1997; French et al. 1998); however, it is unclear how these factors interact with each other and genetic background. Table 5 provides the summaries for the ANOVA on basi-tarsus length. As with SCT number, the third-order ($G \times T \times L$) interaction term is highly significant (Fig. 3B). For each of the reduced models the line-by-perturbation interaction term is highly significant (Table 5, B and C). These results suggest that there is genetic variation for response to both genotype at the *Dll* locus and rearing temperature across this sample. When the analyses are broken down across each treatment (i.e., for each of the $G \times T$ treatments),

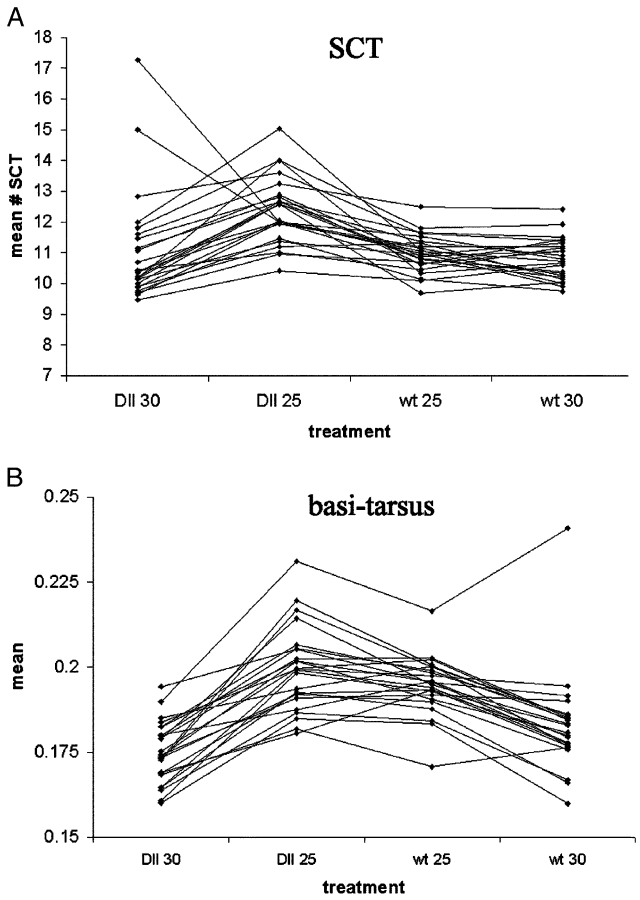

Fig. 3. Reaction norm plot of line means across all treatments. While there is considerable crossing of line means across treatment levels, the treatment-by-line interaction is due in part to an increase in between-line variance (scaling).

a significant line component is always observed. Overall these results suggest that not only is there substantial natural genetic variation for the length of the basi-tarsus, but also genetic variation for the interactions with *Dll* genotype and rearing temperature.

Is there evidence for canalization of the basi-tarsus?

Are these interactions in part due to changes in scaling effects between lines, consistent with an increase in the estimated genetic variation? Phenotypic variation generally increases due to either the effects of *Dll* or high temperature (Table 6). Overall, the results suggest that like SCT number, genetic perturbation of leg development via *Dll* leads to an increase in phenotypic variation for leg segment lengths. Similarly, rearing flies at 30°C also increases levels of phenotypic variation relative to the 25°C treatment. Interestingly, the levels of residual, within-line variance (V_{Eg}) are synergistically affected by temperature and *Dll*; however, genetic variance for this residual variance interacts solely with temperature (not shown).

Is any of this increase in expressed phenotypic variation due to a release in cryptic genetic variation? To address this, I examined the results of the *F*-tests with respect to the line means (Dworkin 2005a). Surprisingly, the pattern of the results differ from that observed for the levels of phenotypic variation. Whereas the genetic perturbation via *Dll* did not appear to significantly increase observed levels of genetic variation (Table 6B), the temperature stress (30°C) did. However, in each case the genetic correlation is significantly different from 0 (Table 6). Curiously, there was no observed synergism between the genetic and environmental perturbation with respect to increasing levels of observed genetic variation. Thus, it appears that even though there are common developmental mechanisms between basi-tarsal growth and SCT number, there are differences between the cryptic genetic variants they harbor.

Are measures of canalization correlated?

There have been numerous definitions of canalization proposed (Debat and David 2001; Nijhout and Davidowitz 2003; Dworkin 2005a), and from each an associated metric can be derived. However, it is unclear how these different measures are related to one another. To deal with this question I examined the correlation between two commonly represented measures of canalization; the first based on the absolute deviation of line means across perturbations, and the second the absolute deviations of within-line variation across treatments. Essentially, this addressed whether there is evidence for a common mechanism between how a perturbation affects changes in the mean and variance for a trait.

For both the basi-tarsus and SCT number, there are mixed results. For the basi-tarsus there is some evidence for a correlation for measures of canalization, but only for the treatment of *Dll* genotypes raised at 30°C, compared with wt raised at 25°C ($r = 0.62$, $P = 0.0015$). None of the other stress effects (either *Dll* or 30°C alone) showed any evidence for such a correlation. For SCT number, the same pattern is found, with only the *Dll*, 30°C treatment showing any significant effect ($r = 0.57$, $P = 0.0007$). It is striking that in both cases it requires the combination of both environmental and temperature stressors to allow the observation of these correlations. Thus, these results suggest that if such a correlation exists between the measures of canalization (implying a common mechanism), it may require severe perturbations to observe its effects.

Is there evidence for a common mechanism of canalization for basi-tarsus length and SCT number?

As discussed earlier, the SCT are arranged on the basi-tarsus, and the *Dll* mutation affects both of these traits. Table 7 summarizes the pattern of phenotypic and genetic correlations

Table 3. ANOVA summaries for reduced models

(A) Across genotypes

Genotype	Source	DF	SS	MS	*F*	Prob *F*
Dll	*T*	1	361.26	361.26	24.61	4.95E – 05
Dll	*L*	26	833.03	32.04	1.98	0.05
Dll	$L \times T$	22	369.36	16.79	5.99	3.8E – 16
Dll	Residual	807	2260.43	2.80		
wt	*T*	1	6.43	6.43	2.33	0.14
wt	*L*	26	358.64	13.79	4.66	1.74E – 04
wt	$L \times T$	23	69.20	3.01	2.57	7.01E – 05
wt	Residual	1040	1215.50	1.17		

(B) Across temperatures

Temperature	Source	DF	SS	MS	*F*	Prob *F*
25	*G*	1	370.90	370.90	38.19	2.11E – 06
25	*L*	24	446.09	18.59	1.65	0.12
25	$G \times L$	23	258.76	11.25	7.23	1.34E – 21
25	Residual	971	1510.14	1.56		
30	*G*	1	13.60	13.60	0.80	0.38
30	*L*	25	887.73	35.51	1.65	0.11
30	$G \times L$	25	538.82	21.55	9.60	4E – 32
30	Residual	876	1965.79	2.24		

between SCT number and basi-tarsus length for means, CVs, and measures of canalization across these traits. While there appears to be a strong association between basi-tarsus length and SCT number measured across individuals (Table 7A), there appears to be evidence for a genetic correlation in the *Dll* genetic background (Table 7B). This is consistent with a model of a release of genetic (co)variation in the *Dll* background, as would be expected for traits that have been de-canalized.

Table 4. ANOVA for within-line variation using the median form of Levene's statistic

Source	DF	SS	MS	*F*	Prob *F*
G	1	0.238	0.238	26.232	1.04E – 05
T	1	0.034	0.034	5.190	0.03
$G \times T$	1	0.042	0.042	3.570	0.07
L	26	0.506	0.019	5.339	0.31
$G \times L$	26	0.246	0.009	0.776	0.74
$T \times L$	23	0.149	0.006	0.521	0.94
$G \times T \times L$	22	0.274	0.012	1.895	0.007
Residual	1847	12.148	0.007		

The individual and joint effects of the environmental and genetic perturbations increase levels of within-line variation. The analysis suggests that the genetic variance for within-line variation is absorbed in the interaction terms, although the results of reduced models are consistent with overall line effects.

To test for a common mechanistic basis for the canalization of SCT number and basi-tarsus length, I examined the Spearman rank correlation between the measures of canalization for the two traits. It does not appear as if there is evidence for a common mechanism for the canalization of the two traits, either by the mean or variation measure of canalization (Table 7). The one exception is the mean measure of canalization for *Dll* raised at 25°C. Given that the strongest evidence for a genetic correlation between traits is also for the *Dll* 25°C treatment, it may be an artifact dependent upon the mean. When this relationship is examined across all treatments, there is no evidence for a statistically significant effect. I interpret these results to suggest that there is no support for a common mechanism for canalization across basi-tarsus length and SCT number. Thus, this argues against canalization of *Dll* function per se, and suggests that it is trait specific.

No evidence for a common mechanism of genetic and microenvironmental canalization

Wagner et al. (1997) hypothesized that genetic canalization should evolve as a correlated response to microenvironmental canalization. If this hypothesis is correct, then it is predicted that there should be a significant positive association between measures of canalization. To test this I computed measures of genetic and microenvironmental canalization for each line, and examined the Spearman rank correlation between them (Table 7C). I could find no evidence for such an association

Table 5. Summary of the ANOVA results for the basi-tarsus

(A) Analysis of the full model, demonstrating a highly significant three-way interaction

Source	DF	SS	MS	*F*	Prob *F*
G	1	0.0004	0.0004	1.02	0.32
T	1	0.119	0.119	148.37	7.42E – 12
$G \times T$	1	0.015	0.015	25.09	4.19E – 05
L	26	0.103	0.004	5.74	7.39E – 03
$G \times L$	26	0.011	0.0004	0.71	0.80
$T \times L$	23	0.021	0.0009	1.39	0.22
$G \times T \times L$	22	0.014	0.0006	4.06	8.32E – 10
Residual	1856	0.295	0.0002		

(B) Subdividing the data by genotype suggests that there are highly significant interactions between line and temperature

Genotype	Source	DF	SS	MS	*F*	Prob *F*
Dll	*T*	1	0.09	0.092	149.50	7.53E – 12
Dll	*L*	26	0.05	0.002	2.81	7.80E – 03
Dll	$T \times L$	22	0.02	0.001	4.11	1.23E – 09
Dll	Residual	814	0.14	1.71E – 04		
wt	*T*	1	0.03	0.029	49.12	2.64E – 07
wt	*L*	26	0.06	0.002	3.78	9.27E – 04
wt	$T \times L$	23	0.02	0.001	4.84	9.53E – 13
wt	Residual	1041	0.14	1.38E – 04		

(C) Similarly, reduced models examining the data at each temperature regime suggest that there are interactions between genotype and line as well

Temperature	Source	DF	SS	MS	*F*	Prob *F*
25	*G*	1	0.003	0.003	6.10	0.02
25	*L*	24	0.073	0.003	5.83	3.59E – 05
25	$G \times L$	23	0.012	0.001	3.31	2.84E – 07
25	Residual	976	0.155	1.58E – 04		
30	*G*	1	0.008	0.008	21.67	6.02E – 05
30	*L*	25	0.060	0.002	5.16	5.57E – 05
30	$G \times L$	25	0.012	4.68E – 04	3.22	2.34E – 07
30	Residual	879	0.128	1.45E – 04		

G, genotype; *T*, temperature; *L*, line.

between measures of microenvironmental and genetic canalization either for basi-tarsus length or SCT number; thus, the evidence is inconsistent with the hypothesis of a common mechanism. However, there was evidence for a significant correlation between measures of genetic and macroenvironmental canalization (Table 7), that is, genotypes that are well buffered against the effects of *Dll* show reduced phenotypic plasticity in response to temperature shifts.

DISCUSSION

Testing models of canalization

Although considerable theory has been developed examining mechanisms of trait buffering and the evolution of canalization, there is still a relative paucity of empirical data pertaining to these issues. I have investigated a number of questions with regard to hypothesized evolutionary and mechanistic relationships for developmental buffering in general and canalization in particular. From a conceptual standpoint, I have explicitly addressed the model of Wagner et al. (1997) with respect to the prediction of a common mechanism between genetic and microenvironmental canalization. I have examined the individual and joint effects of the introgression of the Dll^{11} allele and high-temperature perturbation on a panel of iso-female lines on four traits on the pro-thoracic leg of male *D. melanogaster*. I did not observe any evidence that supports the hypothesis of Wagner et al. (1997) with respect to either the number of SCT or basi-tarsus length. In another study, I examined the effects of the introgression of the mutation *Sternopleural* and high-temperature stress on number of sternopleural bristles, using the same set of lines used in the

Table 6. Summary of measures of total phenotypic and genotypic variation for the basi-tarsus

Phenotypic variation							
					Genetic correlations		
	Dll-25°C	*Dll*-30°C	wt-25°C	wt-30°C	*Dll*-25°C	*Dll*-30°C	wt-30°C
Trait	CV	CV	CV	CV	r_G	r_G	r_G
Tarsus	8.91 (1.5, 4E − 6)	8.64 (1.4, 6E − 5)	7.29	7.78 (1.1, 0.07)	0.88	0.66	0.96

Genetic variation								
	Dll-25°C	*Dll*-30°C	wt-25°C	wt-30°C	*Dll*-25°C	*Dll*-30°C	wt-25°C	wt-30°C
Trait	CV	CV	CV	CV	H^2	H^2	H^2	H^2
Tarsus	2.88 (1.2, NS)	3.67 (2.0, 0.04)	2.60	6.13 (5.8, 3E − 5)	0.31	0.19	0.22	0.46

Phenotypic variation was measured from the coefficients of variation across treatment levels. The between-line component allows an estimate of the genetic contribution (F statistic, P value). H^2 is the broad-sense heritability for the traits in each environment. r_G is the genetic correlation across environments (significant in each case).

current study (Dworkin 2005b). As with the results from this study, I did not observe any evidence for a common mechanism of genetic and environmental canalization, nor for a joint analysis of the data (Dworkin 2005b). To date, there is no empirical support for this hypothesis, although it must be tested in other systems, utilizing other methods. It is possible that the lack of correspondence between the theory and these data are a result of one or more of the model assumptions being violated in this system. If this is the case, the prediction may still hold, but for a more limited number of biological systems.

Alternatively, a new theoretical study has suggested that cryptic genetic variation may be an inherent property of genetic systems with epistasis or genotype-by-environment interaction (Hermisson and Wagner 2004). If the conclusions are valid, then inferring de-canalization via the release of cryptic genetic variation is suitable under limited circumstances. Although there are a number of assumptions in the model that may not be biologically realistic, such as the approach to modeling the genetic architecture and the incorporation of genetic and environmental perturbations on trait variation, it is worth considering whether canalization can be inferred from the experiments performed in the current study. One concern outlined by this model (Hermisson and Wagner 2004) is that lines derived from a single population may share a common history of environments thus stabilizing selection, leading to the accumulation of cryptic genetic variation in rare environments, without selection for canalization. As discussed by Hermisson and Wagner (2004), this issue is not of a concern if lines were sampled from a number of populations (Gibson and van Helden 1997; Polaczyk et al. 1998). In the current study, not only were fresh flies sampled from two different locales in Ontario, Canada, but a third of the lines used were collected broadly across the globe, and are unlikely to share a common selective history. However, whereas there is substantial evidence for selection due to geographic variation, there is also extensive gene flow in *Drosophila*, and it is unclear how this may effect the interpretation of the results. In addition, the result of the model by Hermisson and Wagner (2004) requires a novel or rare perturbation. The temperature stress used in this study is a common, ecologically relevant environmental factor for *Drosophila* (Feder et al. 1996; Feder 1997). Thus, it is likely that the results of the current study are robust, even if the conclusions of the model are generally correct.

In addition, I tested for patterns of canalization across traits, examining whether metrics of canalization were correlated between basi-tarsus length and SCT number. Given that the SCT are located on the basi-tarsus, and that many mutations, including *Dll*, affect both, it is plausible that these could be correlated. Although I observed phenotypic and genetic correlations between trait means, there was no evidence for an association between measures of canalization. Hypotheses that suggest a common mechanism for canalization for all traits (Rutherford and Lindquist 1998) have been criticized on both theoretical and empirical grounds (Wagner et al. 1999) given the general deleterious effects observed of such mutants. My evidence also shows no evidence for a common mechanism for canalization across traits. It is plausible that a global mechanism exists, which is then further modified by particular genetic effects in the context of specific phenotypes. Alternatively, the effects of HSP83 are in general highly pleiotropic, though not universal. Introgressing alleles of *Egfr* and *Sevenless* into a panel of inbred lines (Polaczyk et al. 1998) showed little correlation with respect to their effect on photoreceptor determination, even though they are involved with the same signaling cascade. Thus, it remains unclear how widely used particular mechanisms of canalization are, and if

Table 7. Correlations and tests of canalization between SCT number and basi-tarsus length

(A) Phenotypic correlations

	r	*P*	*N*
All	0.28	<0.0001	1933
wt-25°C	0.06	0.12	596
wt-30°C	0.18	<0.0001	485
Dll-25°C	0.27	<0.0001	417
Dll-30°C	0.27	<0.0001	435

(B) Genetic correlations

	r	*P*	*N*
All	0.39	<0.0001	102
wt-25°C	0.12	0.29	25
wt-30°C	0.20	0.16	26
Dll-25°C	0.56	0.002	25
Dll-30°C	0.44	0.01	26

(C) Genetic versus macroenvironmental canalization

Trait	Method	*r*	*P*
Tarsus	Mean	0.12	NS
Tarsus	LS	0.33	NS
SCT	Mean	0.52	0.01
SCT	LS	0.08	NS

While there are phenotypic (A) and genetic correlations (B) between traits, there was no evidence for a correlation for measures of canalization. (C) Summary of tests for genetic versus environmental canalization for basi-tarsus length or SCT number. In neither case is there a significant positive correlation between measures of environmental and genetic canalization. *R*, correlation coefficient; *P*, significance; *N*, sample size; LS, Levene's statistic; SCT, sex comb teeth.

it is trait specific. Considerable work still needs to be done on this issue in particular.

Finally, I addressed whether different measures of canalization, based on line means or within-line variances, are themselves correlated. There was some evidence for a correlation between these measures, under the most severe perturbation. It is unclear why the results differ from that of the previous study on sternopleural bristles (Dworkin 2005b), and why the effects could only be observed under severe perturbation. However, a joint analysis combining the data for all of these traits found no such association, utilizing a stratified permutation test (Dworkin 2005b). Therefore, it is likely that measures of canalization based on line means and within-line variances measure complementary aspects of developmental buffering, and should be examined jointly in addressing questions of canalization.

One common feature of the results of this and an additional study (Dworkin 2005b) is that it leads to a different perception of the de-canalization of a trait, especially with respect to genetic variation. Genetic de-canalization is not simply a shift of nonadditive to additive variance, but results in an overall increase in phenotypic variation, with a relative increase in the proportion of variance explained by the genetic component. This study is not able to distinguish between the additive and nonadditive components, but clearly demonstrates an overall increase in genetic variation.

Atavism, constraint, or other?

I demonstrate that loss of *Dll* function leads to changes in SCT number, and causes rearrangement or transformation of bristles into SCT (Figs. 1 and 2; Appendix). However, the position of the ectopic SCT (via transformed bristle identity) seems to occur along a "vector" of interspecific variation observed in different species of *Drosophila* (Kopp and True 2002). One potential explanation is that loss of function of *Dll* reveals some sort of underlying "pre-pattern" or atavism for SCT positioning, as has been previously suggested for the *Drosophila* wing vein pattern (Palsson and Gibson 2000) and for mammalian skeletal processes (Mark et al. 1995; Smith and Schneider 1998). Another possible (related to the pre-pattern concept) explanation for the apparent atavism is a developmental constraint, where genetic variation in developmental processes is limited as to the direction and magnitude of effects. Thus, genes that affect SCT could only modify the phenotypes in certain (developmentally possible) ways. Although this provides an entertaining hypothesis and a "just-so" story, it needs to be tested explicitly, possibly by the utilization of perturbation to reveal phenotypes not observed in other species (Alberch 1989; Dworkin et al. 2001; Wilkins 2002). There is some evidence against the developmental constraint hypothesis as ectopic expression of Homothorax (Hth) causes arrangements of SCT on the leg never observed in nature (Azpiazu and Morata 2002). Specifically, ectopic expression of Hth causes ectopic SCT to be situated on the fifth tarsal segment (most distal) without appearing on the intervening proximal ones. This arrangement is not observed in nature, suggesting that the developmental variation is present for such phenotypes, and that other factors (such as selection) explain its absence.

Dll function and the evolvability of phenotypes

Like *Ubx*, aspects of *Dll* function appear to be highly conserved in divergent lineages (Panganiban et al. 1997), whereas other aspects appear responsible for intraspecific patterns of trait variation (Beldade et al. 2002). Thus, there appears considerable evolutionary lability with respect to *Dll* function and trait evolution. In this study, I demonstrated that the introgression of the Dll^{11} allele was sufficient to increase genetic variation for traits (Tables 1 and 6), while significantly altering patterns of phenotypic covariances. This suggests that altering *Dll* function could simultaneously lead to a release of

available genetic variation while altering its pleiotropic functions. Whereas the arrangement and number of SCT appear to be a rapidly evolving trait within *Drosophila* (Kopp and True 2002; Schawaroch 2002), to date there is no evidence for polymorphisms in *Dll* being responsible for intra- or interspecific variation of SCT number (True et al. 1997; Nuzhdin and Reiwitch 2000; Kopp et al. 2003). Given the results demonstrating genetic variation for interactions with *Dll*, one possible explanation is that the variants in other genes are responsible. Alternatively, it is possible that *Dll* remains a source of cryptic genetic variation for this trait (Gibson and Dworkin 2004). Cryptic genetic variation for photoreceptor number under the perturbation of the $Egfr^{E1}$ allele was recently mapped to segregating polymorphisms in the *Egfr* locus itself (Dworkin et al. 2003). Surprisingly, the variants that affected photoreceptor determination had no effect on two other traits, and Gibson *Egfr* functions in wing shape (Palsson and Gibson 2004; Dworkin et al. 2005) and dorsal–ventral patterning of the eggshell (Goering and Gibson 2005). This suggests that in some instances canalization has effectively suppressed the allelic effects of these variants under wild-type conditions. The evolutionary potential of this cryptic genetic variation will hopefully be explored in future theoretical and empirical studies.

Acknowledgments

Special thanks to Dr. E. Larsen, in whose lab this work was performed, A. Greene and B. Ing for help with dissections, and Dr. G. Gibson, Dr. E. Larsen, and Dr. T. F. C. Mackay for various discussions on the subject and statistical advice. Thanks to Dr. L. Goering, Dr. G. Wray, and the anonymous reviewers for comments on the manuscript. This work was supported by an Ontario Graduate scholarship to I. D.

REFERENCES

Alberch, P. 1989. The logic of monsters: evidence for internal constraint in development and evolution. In B. David, J. L. Dommergues, J. Chaline, and B. Laurin (eds.). *Ontogenese et Evolution*, Geobios, memoires speciale 12: 21–57.

Ancel, L. W., and Fontana, W. 2000. Plasticity, evolvability, and modularity in RNA. *J. Exp. Zool.* 288: 242–283.

Azpiazu, N., and Morata, G. 2002. Distinct functions of *Homothorax* in leg development in *Drosophila*. *Mech. Dev.* 119: 55–67.

Beardmore, J. A. 1960. Developmental stability in constant and fluctuating environments. *Heredity* 14: 411–422.

Beldade, P., Brakefield, P. M., and Long, A. D. 2002. Contribution of *Distal-less* to quantitative variation in butterfly eyespots. *Nature* 415: 315–318.

Brakefield, P. M., et al. 1996. Development, plasticity and evolution of butterfly eyespot patterns. *Nature* 384: 236–242.

Brunetti, C. R., Selegue, J. E., Monteiro, A., French, V., Brakefield, P. M., and Carroll, S. B. 2001. The generation and diversification of butterfly eyespot color patterns. *Curr. Biol.* 11: 1578–1585.

Bubliy, O. A., Loeschcke, V., and Imasheva, A. G. 2000. Effect of stressful and nonstressful growth temperatures on variation of sternopleural bristle number in *Drosophila melanogaster*. *Evolution* 54: 1444–1449.

Cassell, D. L. 2002. A randomization-test wrapper for SAS PROCs. SAS Users' Group International Proceedings 27: 251. http://www2.sas.com/proceedings/sugi27/p251-27.pdf.

Clarke, G. M. 1998. The genetic basis of developmental stability. V. Inter- and intra-individual character variation. *Heredity* 80: 562–567.

Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G., and Jackle, H. 1989. *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* 338: 432–434.

Combs, J. 1937. Genetic and environmental factors affecting the development of the sex-combs of *Drosophila melanogaster*. *Genetics* 22: 427–433.

Debat, V., and David, P. 2001. Mapping phenotypes: canalization, plasticity and developmental stability. *Trends Ecol. Evol.* 16: 555–561.

de Visser, J. A. G. M., et al. 2003. Perspective: evolution and detection of genetic robustness. *Evolution* 57: 1959–1972.

DeWitt, T. J. 1998. Costs and limits of phenotypic plasticity: tests with predator-induced morphology and life history in a freshwater snail. *J. Evol. Biol.* 11: 465–480.

Dong, P. D., Chu, J., and Panganiban, G. 2000. Coexpression of the homeobox genes *Distal-less* and *Homothorax* determines *Drosophila* antennal identity. *Development* 127: 209–216.

Dworkin, I. 2005a. Canalization, cryptic variation and developmental buffering: a critical examination and analytical perspective. In B. Hallgrimsson and B. K. Hall (eds.). *Variation*. Academic Press, New York (in press).

Dworkin, I. 2005b. A study of canalization and developmental stability in the sternopleural bristle system of *Drosophila melanogaster*. *Evolution*. (in press).

Dworkin, I. M., Palsson, A., and Gibson, G. 2005. Replication of an *Egfr*-wing shape assocation in a wild-caught cohort of *Drosophila melanogaster*. *Genetics*, in press.

Dworkin, I., Palsson, A., Birdsall, K., and Gibson, G. 2003. Evidence that *Egfr* contributes to cryptic genetic variation for photoreceptor determination in natural populations of *Drosophila melanogaster*. *Curr. Biol.* 13: 1888–1893.

Dworkin, I. M., Tanda, S., and Larsen, E. 2001. Are entrenched characters developmentally constrained? Creating biramous limbs in an insect. *Evol. Dev.* 3: 424–431.

Falconer, D., and Mackay, T. 1996. *Introduction to Quantitative Genetics*. Longman, Essex.

Feder, M. 1997. Natural thermal stress and heat-shock protein expression in *Drosophila* larvae and pupae. *Funct. Ecol.* 11: 90–100.

Feder, M., Blair, N., and Figueras, H. 1996. Oviposition site selection and temperature: unresponsiveness of ovipositing *Drosophila* to cues of potential thermal stress on offspring. *Anim. Behav.* 53: 585–588.

French, V., Feast, M., and Partridge, L. 1998. Body size and cell size in *Drosophila*: the developmental response to temperature. *J. Insect Physiol.* 44: 1081–1089.

Gavrilets, S., and Hastings, A. 1994. A quantitative-genetic model for selection on developmental noise. *Evolution* 48: 1478–1486.

Gibson, G., and Dworkin, I. 2004. Uncovering cryptic genetic variation. *Nat. Rev. Genet.* 5: 681–690.

Gibson, G., and Hogness, D. S. 1996. Effect of polymorphism in the *Drosophila* regulatory gene ultrabithorax on homeotic stability. *Science* 271: 200–203.

Gibson, G., and van Helden, S. 1997. Is function of the *Drosophila* homeotic gene *Ultrabithorax* canalized? *Genetics* 147: 1155–1168.

Gibson, G., Wemple, M., and van Helden, S. 1999. Potential variance affecting homeotic *Ultrabithorax* and *Antennapedia* phenotypes in *Drosophila melanogaster*. *Genetics* 151: 1081–1091.

Goering, L. M., and Gibson, G. 2005. Genetic variation for dorsal–ventral patterning of the *Drosophila melanogaster* eggshell. *Evol. Dev.* 7: 81–88.

Gorfinkiel, N., Morata, G., and Guerrero, I. 1997. The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Genes Dev.* 11: 2259–2271.

Hannah-Alava, A. 1958. Morphology and chaeotaxy of the legs of *Drosophila melanogaster*. *J. Morphol.* 103: 281–310.

Hermisson, J., and Wagner, G. P. 2004. The population genetic theory of hidden variation and genetic robustness. *Genetics* 168: 2271–2284.

Hughes, C. L., and Kaufman, T. C. 2002. Hox genes and the evolution of the arthropod body plan. *Evol. Dev.* 4: 459–499.

Kopp, A., Graze, R. M., Xu, S. Z., Carroll, S. B., and Nuzhdin, S. V. 2003. Quantitative trait loci responsible for variation in sexually dimorphic traits in *Drosophila melanogaster*. *Genetics* 163: 771–787.

Kopp, A., and True, J. R. 2002. Evolution of male sexual characters in the oriental *Drosophila melanogaster* species group. *Evol. Dev.* 4: 278–291.
Lynch, M., and Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer, Sunderland, MA.
Mark, M., Rijli, F. M., and Chambon, P. 1995. Alteration of *Hox* gene expression in the branchial region of the head causes homeotic transformations, hindbrain segmentation defects and atavistic changes. *Semin. Dev. Biol.* 6: 275–284.
Meiklejohn, C. D., and Hartl, D. L. 2002. A single mode of canalization. *Trends Ecol. Evol.* 17: 468–473.
Nijhout, H. F., and Davidowitz, G. 2003. Developmental perspectives on phenotypic variation, canalization, and fluctuating asymmetry. In M. Polak (ed.). *Developmental Instability: Causes and Consequences*. Oxford University Press, Oxford, pp. 1–12.
Noreen, E. W. 1989. *Computer-Intensive Methods for Testing Hypotheses*. Wiley and Sons, New York.
Nuzhdin, S. V., and Reiwitch, S. G. 2000. Are the same genes responsible for intra- and interspecific variability for sex comb tooth number in *Drosophila*? *Heredity* 84: 97–102.
Palsson, A., and Gibson, G. 2000. Quantitative developmental genetic analysis reveals that the ancestral dipteran wing vein prepattern is conserved in *Drosophila melanogaster*. *Dev. Genes Evol.* 210: 617–622.
Palsson, A., and Gibson, G. 2004. Association between nucleotide variation in *Egfr* and wing shape in *Drosophila melanogaster*. *Genetics* 167: 1187–1198.
Panganiban, G., et al. 1997. The origin and evolution of animal appendages. *Proc. Natl. Acad. Sci. USA* 94: 5162–5166.
Panganiban, G., Sebring, A., Nagy, L., and Carroll, S. 1995. The development of crustacean limbs and the evolution of arthropods. *Science* 270: 1363–1366.
Polaczyk, P. J., Gasperini, R., and Gibson, G. 1998. Naturally occurring genetic variation affects *Drosophila* photoreceptor determination. *Dev. Genes Evol.* 207: 462–470.
Rice, S. H. 1998. The evolution of canalization and the breaking of von Baer's laws: modeling the evolution of development with epistasis. *Evolution* 52: 647–656.
Robertson, A. 1959. The sampling variance of the genetic correlation coefficient. *Biometrics* 15: 469–485.
Rutherford, S. L., and Lindquist, S. 1998. Hsp90 as a capacitor for morphological evolution. *Nature* 396: 336–342.
Schawaroch, V. 2002. Phylogeny of a paradigm lineage: the *Drosophila melanogaster* species group (Diptera:Drosophilidae). *Biol. J. Linn. Soc.* 76: 21–37.
Scheiner, S. M., Caplan, R. L., and Lyman, R. F. 1991. The genetics of phenotypic plasticity .3. Genetic correlations and fluctuating asymmetries. *J. Evol. Biol.* 4: 51–68.
Schultz, B. B. 1985. Levene's test for relative variation. *Syst. Zool.* 34: 449–456.
Siegal, M. L., and Bergman, A. 2002. Waddington's canalization revisited: developmental stability and evolution. *Proc. Natl. Acad. Sci. USA* 99: 10528–10532.
Smith, K. K., and Schneider, R. A. 1998. Have gene knockouts caused evolutionary reversals in the mammalian first arch? *Bioessays* 20: 245–255.
Sokal, R. R., and Rohlf, F. J. 1995. *Biometry*. Freeman, New York.
Stern, D. L. 1998. A role of *Ultrabithorax* in morphological differences between *Drosophila* species. *Nature* 396: 463–466.
Sunkel, C. E., and Whittle, J. R. S. 1987. *Brista*—a gene involved in the specification and differentiation of distal cephalic and thoracic structures in *Drosophila-melanogaster*. *Roux's Arch. Dev. Biol.* 196: 124–132.
True, J. R., Liu, J. J., Stam, L. F., Zeng, Z. B., and Laurie, C. C. 1997. Quantitative genetic analysis of divergence in male secondary sexual traits between *Drosophila simulans* and *Drosophila mauritiana*. *Evolution* 51: 816–832.
Waddington, C. H. 1952. Selection of the genetic basis for an acquired character. *Nature* 169: 625–626.
Waddington, C. H. 1956. Genetic assimilation of the *Bithorax* phenotype. *Evolution* 10: 1–13.
Waddington, C. H. 1957. *The Strategy of the Genes*. Allen & Unwin, London.
Wagner, G. P., Booth, G., and BagheriChaichian, H. 1997. A population genetic theory of canalization. *Evolution* 51: 329–347.
Wagner, G. P., Chiu, C. H., and Hansen, T. F. 1999. Is Hsp90 a regulator of evolvability? *J. Exp. Zool.* 285: 116–118.
Wilkins, A. S. 2002. *The Evolution of Developmental Pathways*. Sinauer, Sunderland, MA.
Wistow, G. 1993. Lens crystallins: gene recruitment and evolutionary dynamics. *Trends Biol. Sci.* 18: 301–306.
Zar, J. H. 1999. *Biostatistical Analysis*. Upper Saddle River, NJ, Prentice Hall.

APPENDIX

The ectopic SCT sometimes occurred in groups (Fig. 1B), although they also occur individually (Fig. 2B) or as replacements for the transverse row of bristles proximal to the normal row of SCT (not shown). The data (see Table A1) suggest line-specific phenotypic effects of *Dll*, and these are correlated with number of SCT.

Table A1. Summary of the frequencies of ectopic SCT (%) in $Dll^{11/+}$ individuals

Line	T_1 (%)	T_2 (%)	T_R (%)	Rows (%)
1	0	0	0	0
2	0	0	5	0
3	50	0	0	0
4	23.8	0	0	0
6	0	18.2	0	0
7	9.1	9.1	0	0
8	5.9	0	5.9	0
9	0	0	0	0
11	7.1	10.7	10.7	0
12	25	0	5	0
13	16.7	0	33.3	0
15	14.3	0	0	0
16	17.7	0	58.8	0
17	5	0	0	0
18	13.1	0	0	0
19	0	0	10	0
20	0	0	18.2	0
21	0	4.8	14.3	0
22	0	5	5	0
23	10	0	20	0
24	0	0	0	0
27	0	0	0	0
w	27.8	16.7	11.1	38.9
OreR	0	0	0	0
CanS	0	0	0	0
Sam	0	5.9	52.9	0

SCT, sex comb teeth; T1, ectopic SCT on the basi-tarsus; T2, ectopic SCT on the second tarsal segment; TR, transverse row of bristles proximal to sex comb transformed to SCT; Row, extra row of SCT adjacent to normal sex comb.