Genome-Wide Deficiency Mapping of the Regions Responsible for Temporal Canalization of the Developmental Processes of Drosophila melanogaster

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Abstract

Developmental processes of organisms are programed to proceed in a finely regulated manner and finish within a certain period of time depending on the ambient environmental conditions. Therefore, variation in the developmental period under controlled genetic and environmental conditions indicates innate instability of the developmental process. In this study, we aimed to determine whether a molecular machinery exists that regulates the canalization of the developmental period and, if so, to test whether the same mechanism also stabilizes a morphological trait. To search for regions that influence the instability of the developmental period, we conducted genome-wide deficiency mapping with 441 isogenic deficiency strains covering 65.5% of the Drosophila melanogaster genome. We found that 11 independent deficiencies significantly increased the instability of the developmental period and 5 of these also significantly increased the fluctuating asymmetry of wing shape although there was no significant correlation between the instabilities of developmental period and wing shape in general. These results suggest that canalization processes of the developmental period and morphological traits are at least partially independent. Our findings emphasize the potential importance of temporal variation in development as an indicator of developmental stability and canalization and provide a novel perspective for understanding the regulation of phenotypic variability.

Key words: developmental period, developmental stability, Drosophila melanogaster, wing shape

Mechanisms that reduce phenotypic variability under environmental fluctuations may evolve through the natural selection of genotypes that produce a healthy phenotype with high reproducibility. Waddington (1942) suggested a mechanism called canalization that buffers developmental processes against internal and external perturbations to produce a constant phenotype. When the expression of a trait is environmentally well canalized, most environmental variation has little phenotypic effect and a population remains phenotypically uniform (Eshel and Matessi 1998). In fact, environmental canalization has been shown to be tighter for traits that have larger contribution to fitness (Stearns and Kawecki 1994). Resistance to nonspecific environmental variation such as among-individual environmental variation in a population has been considered to be the measure of environmental canalization (Debat and David 2001). The molecular machineries that control environmental canalization as well as buffer and stabilize developmental processes have been studied intensively (Stearns et al. 1995; Whitlock 1996; Palmer and Strobeck 1997; Klingenberg and Nijhout 1999; Leung et al. 2000; Van Dongen 2000; Milton et al. 2003; Dworkin et al. 2005; Leamy and Klingenberg 2005; Milton et al. 2006; Pelabon et al. 2006; Hall et al. 2007; Debat et al. 2009; Takahashi et al. 2010). In these studies, the stability of the developmental process was evaluated based on the variation of morphological traits such as flies’ wings, the mouse skull (Debat and David 2001), the hypocotyl region in plants (Queitsch et al. 2002; Sangster et al. 2008), and the zebrafish eyes (Yeyati et al. 2007).
Environmental canalization and the stability of developmental processes can also be evaluated by the stability of the developmental period. The developmental processes of organisms are programmed to proceed in a finely regulated manner and to be completed within a certain period of time depending on the ambient environmental conditions. Variation in the developmental period under controlled genetic and environmental conditions indicates innate instability of the developmental process. Because the instability of the developmental period summarizes a variety of perturbations in developmental processes, it can act as a general indicator of developmental stability and canalization. Stearns et al. (1995) measured the instability of the developmental period by defining it as the age at eclosion of Drosophila melanogaster using P element-inserted lines. They observed a general increase in the instability of the developmental period associated with both environmental and genetic perturbations, indicating that the instability of the developmental period reflects the degrees of environmental and genetic canalization. The developmental period is a heritable trait (Houle 1992) and is highly influenced by ecdysone because it determines the timing of metamorphosis (Lam et al. 1997). Although there has been extensive research on the coordination of ecdysone-regulated transcription during the onset of metamorphosis in D. melanogaster, the molecular mechanism of the canalization of the developmental period remains to be elucidated. Additionally, there has been no investigation of whether single molecular machinery stabilizes both morphological traits and a life-history trait such as the developmental period.

In this study, we examined whether a molecular machinery exists that can regulate the stability of the developmental period and, if so, to test whether the same mechanism also stabilizes a morphological trait. Because the developmental period is a polygenic trait and is influenced by competition and various environmental stresses such as thermal and nutritional stresses (Miller 1964; Burnet et al. 1977; Sgro and Blows 2003), genetic and environmental conditions need to be controlled carefully. A collection of isogenic deficiency strains provided by the DrosDel project (Ryder et al. 2004, 2007) is an ideal tool for genome-wide deficiency mapping of such a polygenic trait. In this study, we screened 441 deficiency strains covering 65.5% of the D. melanogaster genome to search for regions that influence the variation in the developmental period. We also examined whether regions that had an effect on variation in the developmental period also influenced variation in a morphological trait. We found that 11 independent deficiencies significantly increased the instability of the developmental period and 5 of these also significantly increased the fluctuating asymmetry (FA) of wing shape although there was no significant correlation between the instabilities of developmental period and wing shape in general. These results suggest that canalization processes of the developmental period and morphological traits are at least partially independent. Our findings emphasize the potential importance of temporal variation in development as an indicator of developmental stability and canalization and provide a novel perspective for understanding the regulation of phenotypic variability.

Materials and Methods

Flies

DrosDel isogenic deficiency strains obtained from the Drosophila Genetic Resource Center in Kyoto, Japan, were tested for instability of the developmental period. The RS element-Flippase recombination enzyme system used to construct these deficiency strains allows the breakpoints of the deletions to be determined with single base-pair resolution (Ryder et al. 2004). Ryder et al. (2004) established a control strain (DSK001) that was isogenic for the X, second, and third chromosomes and used it to create RS element-inserted strains. The control strain and all the deficiency strains had an isogenic background except for deletions and, therefore, provided an ideal tool to screen genome regions that are involved in quantitative polygenic traits, such as the duration of the preadult period. In this study, we used 441 DrosDel deficiency strains that cover about 65% of the whole genome region and 67% of the total number of genes in the Drosophila genome (Figure 1, Supplementary Appendix 1). Additional details of the deletion strains are available at the DrosDel web page (http://www.drosdel.org.uk/).

Experimental Conditions and Instability of the Developmental Period

We screened the genome for regions that affected the stability of the developmental period. Because most of the deficiencies used in this study are homozygous lethal, deficiency control heterozygotes (Df/+ ) were tested for the instability of the preadult period. Hundred eggs from each of the crosses between the control strain and the deletion strains were introduced into a glass vial along with fly medium. For deficiency strains that have a deletion on the second and third chromosomes, we crossed females of the control strain to males of each deficiency strain to control the maternal effect. For deficiency strains that have a deletion on the X chromosome, we crossed males of the control strain to females of each deficiency strain because of the lethality of these deletions in males. One liter of the fly medium comprised of water (1000 ml), dried yeast (35 g), soy flour (20 g), cornmeal (73 g), agar (50 ml), malt extract (46.25 g), and dextrose (75 g), and subsequently, the mixture was boiled thoroughly. Subsequently, we added 13.75 ml of acid mix (412 ml propionic acid plus 42 ml orthophosphoric acid made up to 1 l in water) and 16.5 ml of nipagin (100 g methyl-β-hydroxybenzoate made up to 1 l in 90% ethanol). The eggs were reared at 23 °C under constant light in incubators. Emerging adults were genotyped (target genotype, Df/+; nontarget genotype, balancer/+), recorded for eclosion every 24 h, and preserved in 70% ethanol for further morphological measurements. Five replicate vials were set up for each deletion strain. To obtain control
individuals (+/+), we collected 100 eggs from strain DSK001 and reared them as described above. The instability of the developmental period was characterized by the coefficient of variation (CV) of the preadult period in each vial. For deletion heterozygotes that showed a significant difference from the control, we calculated the mean preadult period and survival rate from egg to adult.

Morphology Measurement and Shape Analysis

Wing shape was used to assess developmental buffering in several previous studies (Milton et al. 2003; Breuker et al. 2006; Debat et al. 2006; Kellermann et al. 2007). In this study, we examined whether deletions that show a significant effect on the stability of the preadult period also have a significant effect on the stability of wing shape. We sampled 3 males and/or females of each deficiency strain per replication (in total 15 individuals) depending on the sex specificity of the effect of the deficiency on the instability of the developmental period, removed the right and left wings, captured their images using a microscope (SZX16; Olympus, Tokyo, Japan) with a CCD camera attached (DP25; Olympus), and obtained the x and y coordinates of 8 landmarks (Figure 2) with the TPSdig2 program (http://life.bio.sunysb.edu/morph/). The Procrustes generalized least squares procedure (Rohlf and Slice 1990; Bookstein 1991; Rohlf and Marcus 1993) with the “shapes” package of the statistical software R was used to obtain Procrustes coordinates. We performed principal components analysis (PCA) to visualize the effect of deletions on mean wing shape and then conducted multivariate analysis of variance.

**Figure 1.** Distribution of deletions on the second, third, and X chromosomes. Gray bars indicate chromosome arms, and the gray circles at their tips indicate centromeres. Genome regions that have deletions are filled with black, and black bars below each chromosome represent the location of each deletion.

**Figure 2.** Positions of the 8-wing landmarks used in this study.
on the principal component axes to examine the effects of deletion. We measured the right wings of 6 or 7 additional individuals for each replication to evaluate the variation among individuals using the CV. We performed PCA, used the first axis as a shape score, and used this to calculate the CV. To evaluate the FA of wing shape, we used a univariate measure of FA devised by Klingenberg and Monteiro (2005) based on the idea of a 1-sample standard distance (Flury and Riedwyl 1986; Flury 1997), which is equivalent to the 1-sample version of the Mahalanobis distance (Mardia et al. 1979). This measure of FA automatically provides a correction for directional asymmetry (DA) (Klingenberg and Monteiro 2005). The relative amounts of DA, FA, and measurement error in wing shape variation were assessed by Procrustes analysis of variance (ANOVA) (Klingenberg and McIntyre 1998) with degrees of freedom under the isotropic model (Klingenberg et al. 2002). In this analysis, we included individual, side, and their interaction terms, as well as added sums of squares across landmarks and coordinates, assuming equal and isotropic variation at each landmark.

In addition to those measurements, we randomly selected 100 of 441 deletions of which developmental period we measured and evaluated wing shape FA to test whether there is a general correlation between CV of developmental period and wing shape FA. We also checked the frequency distribution of wing shape FA scores of those randomly selected deletions and examined whether deletions that show a significant effect on the CV of the preadult period have high wing shape FA compared with the general tendency.

**Statistical Analysis**

To evaluate the effect of deletion on the instability of the developmental period, we performed the Dunnett test with

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**Figure 3.** CV of preadult period for control homozygotes (+/+ ) and deficiency heterozygotes (Df/+ ). Error bars represent standard errors. * P < 0.05, ** P < 0.001, *** P < 0.0001 (Dunnett test).
the CV of the preadult period as a dependent variable and strain as an independent variable. For the deficiency strains that showed a significant effect on the CV of the preadult period, we performed the Dunnett test with wing shape FA as a dependent variable and strain as an independent variable. We used mean FA score at vial level for this analysis. The normality of distributions was checked with the Kolmogorov–Smirnov test, and no significant deviation from the normal distribution was detected for any measure. These analyses were separately performed for females and males.

Results

Instability of Developmental Period

Because the number of genes deleted in each deficiency varies greatly (1–196), we tested whether the deletion of a number of genes in general influenced the instability of the developmental period. The correlation between the number of genes deleted and the CV of the preadult period was very weak and not significant either in females (correlation coefficient 0.041, \( P > 0.3 \)) or in males (correlation coefficient 0.001, \( P > 0.9 \)). Results of the Dunnett test on the CV of the preadult period indicated a significant increase in instability in 2 deletion heterozygotes in females and 10 deletion heterozygotes in males (Figure 3) out of 441 deficiency strains. Among these deletion heterozygotes, only \( Df(3R)ED5021/+ \) had a significant effect in both sexes; other deletion heterozygotes had a sex-specific effect.

Fitness Effect of the Deletions

In females, \( Df(3L)ED207/+ \) heterozygotes showed a significantly prolonged preadult period (13.5 ± 0.13 days for +/- and 14.91 ± 0.37 for \( Df(3L)ED207/+ \), \( P < 0.01 \)). In males, 3 deletion heterozygotes, \( Df(2L)ED234/+ \), \( Df(3R)ED5660/+ \), and \( Df(3R)ED5705/+ \), showed a significantly prolonged preadult period (13.45 ± 0.25 for +/-; 15.29 ± 0.82 for \( Df(2L)ED234/+ \), \( P < 0.01 \); 16.48 ± 0.47 for \( Df(3R)ED5660/+ \), \( P < 0.001 \); and 15.22 ± 0.83 for \( Df(3R)ED5705/+ \), \( P < 0.05 \)). For survival rate from the egg to the adult stage, only \( Df(3R)ED5705/+ \) males showed a significant reduction compared with the control (0.51 ± 0.18 for +/- and 0.104 ± 0.06 for \( Df(3R)ED5705/+ \), \( P < 0.001 \)).

Measurement Error

All main effects included in the Procrustes ANOVA were statistically significant except for “side” in females (Table 1).

### Table 1 Procrustes ANOVA for the wing landmarks

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>MS</th>
<th>( F )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>504</td>
<td>183.840</td>
<td>2.877</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Side</td>
<td>12</td>
<td>95.591</td>
<td>1.496</td>
<td>0.121</td>
</tr>
<tr>
<td>Individual × side</td>
<td>504</td>
<td>63.893</td>
<td>11.864</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Measurement error</td>
<td>2064</td>
<td>5.386</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>1920</td>
<td>257.360</td>
<td>3.846</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Side</td>
<td>12</td>
<td>152.783</td>
<td>2.283</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Individual × side</td>
<td>1920</td>
<td>66.919</td>
<td>11.394</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Measurement error</td>
<td>3864</td>
<td>5.873</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df, degrees of freedom; MS, mean square.

### Table 2 Results for the MANOVA of female and male wing shape

<table>
<thead>
<tr>
<th>Genotype</th>
<th>df1</th>
<th>df2</th>
<th>Wilk’s ( L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( Df(3L)ED207/+ )</td>
<td>2</td>
<td>7</td>
<td>0.148**</td>
</tr>
<tr>
<td>( Df(3R)ED5021/+ )</td>
<td>2</td>
<td>7</td>
<td>0.183**</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( Df(2L)ED234/+ )</td>
<td>2</td>
<td>7</td>
<td>0.454</td>
</tr>
<tr>
<td>( Df(2L)ED578/+ )</td>
<td>2</td>
<td>7</td>
<td>0.666</td>
</tr>
<tr>
<td>( Df(2L)ED1245/+ )</td>
<td>2</td>
<td>7</td>
<td>0.130**</td>
</tr>
<tr>
<td>( Df(2L)ED1454/+ )</td>
<td>2</td>
<td>7</td>
<td>0.308</td>
</tr>
<tr>
<td>( Df(2L)ED7733/+ )</td>
<td>2</td>
<td>7</td>
<td>0.293</td>
</tr>
<tr>
<td>( Df(2L)ED12487/+ )</td>
<td>2</td>
<td>7</td>
<td>0.383</td>
</tr>
<tr>
<td>( Df(3L)ED210/+ )</td>
<td>2</td>
<td>7</td>
<td>0.219*</td>
</tr>
<tr>
<td>( Df(3R)ED5021/+ )</td>
<td>2</td>
<td>7</td>
<td>0.232</td>
</tr>
<tr>
<td>( Df(3R)ED5660/+ )</td>
<td>2</td>
<td>7</td>
<td>0.078**</td>
</tr>
<tr>
<td>( Df(3R)ED5705/+ )</td>
<td>2</td>
<td>7</td>
<td>0.697</td>
</tr>
</tbody>
</table>

df, degrees of freedom.

\*\( P < 0.05 \), **\( P < 0.001 \) after Bonferroni correction.
Although significant DA was found in males, the FA measure used in the further analysis automatically provided a correction for DA (Klingenberg and Monteiro 2005). Therefore, interpretation of the results of FA analysis was not affected.

Mean Wing Shape and Developmental Instability of the Wings

Of the deletions shown to have an effect on the stability of developmental time, the mean wing shapes of $Df(3L)E:207/+$ and $Df/+$ were compared. No significant difference was found in the mean wing shape between these two groups. However, the CV of wing shape showed a significant difference between the two groups (Figure 5). The CV for $+/$ was lower than that for $Df/+$, indicating that the deficiency heterozygotes ($Df/+$) had a higher developmental instability than the control homozygotes ($+/$).
+ and \text{Df}(3R)\text{E}D5021/+ flies were significantly different from that of +/+ females (Table 2, Figure 4). These deficiencies tended to increase the CV of wing shape, but the effect was not significant (Figure 5), although they significantly increased the FA of wing shape in males (Figure 6). The mean wing shapes of 3 deficiency heterozygotes, \text{Df}(2L)\text{E}D1245/+, \text{Df}(3L)\text{E}D210/+, and \text{Df}(3R)\text{E}D5660/+, were significantly different from that of +/+ males (Table 2, Figure 4). The FA of wing shape in \text{Df}(3L)\text{E}D210/+, \text{Df}(3R)\text{E}D5660/+, and \text{Df}(3R)\text{E}D5705 males was significantly higher than that of +/+ (Figure 6). Although some deletions tended to increase the CV of wing shape, none showed a significant effect in males (Figure 5).

\textbf{Discussion}

Eleven out of 441 deficiencies significantly increased the instability of the developmental period. Furthermore, 5 out of these 11 also increased the bilateral asymmetry of wing shape. The lack of correlation between the number of genes

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{FA of wing shape for control homozygotes (+/+), and deficiency heterozygotes (\text{Df}/+). Error bars represent standard errors. *\textit{P} > 0.05, **\textit{P} < 0.001, ***\textit{P} < 0.0001 (Dunnet test).}
\end{figure}
deleted and the degree of instability of the developmental period indicates that lacking one copy of a number of genes by deletion in general does not affect the stability of the developmental period. Because the genetic background of these deletions is identical except for the size and location of the deletion, it is highly likely that genes encompassed by these deletions influenced the instability of developmental period and/or the FA of wing shape.

Four deficiency heterozygotes, Df(3L)ED207/+ in females and Df(2L)ED234/+ , Df(3R)ED5660/+ , and Df(3R)ED5705/+ in males, showed both increased instability of the developmental period and a longer mean developmental period, suggesting severe perturbations in the developmental processes. In fact, 3 of those deficiency heterozygotes, Df(3L)ED207/+ in females and Df(3R)ED5660/+ and Df(3R)ED5705/+ in males, also showed an increased FA of wing shape. On the other hand, Df(3R)ED5021/+ heterozygosity in females and Df(3L)ED210/+ heterozygosity in males did not affect the mean developmental period but significantly increased the FA of wing shape. The lack of general correlation between the CV of developmental period and FA of wing shape in the randomly selected 100 deletions suggests that such pleiotropic effect is limited to those deletions and not a general trend. In addition, only one deletion with significant effect on CV of developmental period in male Df(3R)ED5660/+ showed a very high FA of wing shape given the distribution of FA of wing shape for the randomly selected 100 deletions, suggesting again that CV of developmental period is not a general predictor of FA of wing shape. These results suggest that the stability of the developmental period is genetically regulated in a sex-specific manner and that stabilities of developmental period and wing shape are buffered by at least partially independent mechanisms.

Ecdysteroids are known to control molting and development in insects (Koolman 1989). In Drosophila,
prothoracic glands synthesize and secrete ecdysone, which, after undergoing 20-hydroxylation, yields the more active hormone 20-hydroxyecdysone (20E) (Rees 1995). Several successive pulses of 20E regulate the onset of metamorphosis (Riddiford 1993) and control the developmental period. A number of 20E-regulated genes have been isolated (Andres et al. 1993; Horner et al. 1995; Beckstead et al. 2005). In total, 104 genes that have been suggested to be regulated by 20E are listed in Andres et al. (1993) and Beckstead et al. (2005). In the present study, among the deficiencies that had a significant effect on the instability of the developmental period, 3 encompass 4 20E-regulated genes: brain tumor in Df(2L)ED1245, vrille in Df(2L)ED12487, and lamina ancestor and CG9192 in Df(3L)ED207. These genes are upregulated by 20E and may function in the 20E signaling pathway (Beckstead et al. 2005), suggesting their involvement in the instability of the developmental period. Among these genes, lamina ancestor is suggested to influence wing size (Carreira et al. 2009) and CG9192 is expressed specifically in the wing disc (Butler et al. 2003), suggesting their effect on wing morphogenesis. In fact, Df(3L)ED207, which encompasses both lamina ancestor and CG9192, also significantly increased the FA of wing shape. These results suggest that some of the present results might be explained by the deficiency of 20E-regulated genes, but they also suggest that non–20E-regulated genes are responsible for the instability of the developmental period in deletions with significant effect but that did not encompass 20E-regulated genes in this study.

In insects, the insulin/insulin growth factor (IGF) signaling pathway also plays a key role in regulating growth and metabolism (Colombani et al. 2005). Growth rate is mainly controlled by the insulin/IGF signaling pathway, whereas the developmental period is limited by the onset of the larval–pupal transition, which is timed by peaks of 20E secretion (Nijhout 2003; Colombani et al. 2005). Among the 29 genes, including ligands and secreted factors, insulin-like receptor and its substrates, signal transduction pathway, and targets (Kleijn and Proud 2000; Oldham et al. 2000; Schmelze and Hall 2000; Broggiolo et al. 2001; Raught et al. 2001; Britton et al. 2002), only Thor was encompassed by the deficiency Df(2L)ED234 that had a significant effect on the instability of the developmental period and on the mean developmental period. Other deficiencies did not encompass genes involved in the insulin/IGF signaling pathway, suggesting the existence of unknown mechanisms that are independent of the insulin/IGF signaling pathway and are involved in regulating the instability of the developmental period.

Genome-wide deficiency mapping of regions responsible for the instability of the developmental period was performed for the first time in the present study. We identified multiple genome regions with significant effect, and most of them did not affect wing shape asymmetry, suggesting at least partially independent buffering mechanisms for the instability of the temporal regulation of development and the instability of morphogenesis. The deficiencies that had a significant effect on the instability of the developmental period encompassed 562 genes. Although we have not identified the exact number of genes responsible for this effect, this novel finding may lead to a better understanding of the mechanism by which the temporal instability of development is canalized and how it affects the development of organisms.

### Supplementary Material


### Funding

Special Coordination Funds for Promoting Sciences and Technology of The Ministry of Education, Sport, Culture, Science, and Technology of Japan; Sumitomo Foundation to K.H.T.

### Acknowledgments

We want to thank Mses Yui Takahashi, Honami Saeki, and Megumi Nagano for the assistance for morphological measurements.

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Received November 18, 2010; Revised March 15, 2011; Accepted March 15, 2011

Corresponding Editor: James Thompson