



Is the number of possible QTL for asymmetry phenotypes dependent on thermal stress?

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ABSTRACT

In bilateral organisms, fluctuating asymmetry (FA) was often used as an index of developmental instability but FA was not always found to be higher in stressful environments. An intercontinental set of recombinant inbred lines (RIL) was used to search for genetic variation in fluctuating asymmetry (FA) of both wing length (WL) and wing width (WW) in *Drosophila melanogaster* when reared at both benign (25 °C) and stressful (30 °C) temperatures. FA levels did not differ between benign and stressful temperatures. At benign temperature, no QTL was significant for FA. However, at stressful temperature, composite interval mapping revealed some QTL for FA in both WL and WW. QTL-based scans under stressful thermal environments may be an informative approach for either FA or developmental instability analyses, even when FA levels are similar between stressful and benign environments.

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1. Introduction

Fluctuating asymmetry (FA) can be defined as the non-directional deviation from perfect bilateral symmetry (Van Valen, 1962; Palmer and Strobeck, 1986). FA is sometimes used as an index of developmental instability. FA was also used for monitoring stress levels in wild populations, as an increase in FA may be directly associated with stochastic disturbances in homeostasis during development (Palmer and Strobeck, 1986). In addition, disruption of development homeostasis may take place at the molecular, chromosomal or epigenetic levels, plausibly increasing FA in adult morphology (Parsons, 1990).

One subject of intense debate is whether or not the phenotypic variation in FA has a genetic basis (Leamy and Klingenberg, 2005). FA is often not genetically related to fitness in *Drosophila* (Santos, 2001). In addition, the use of FA as an indicator of stress assumes that FA is entirely attributable to the environmental conditions (Palmer, 1994; Dongen, 2006). However, though FA is often considered to be environmental in origin, Quantitative Trait Loci (QTL) are often found for FA, suggesting that the variation in FA is also under some genetic control (Leamy et al., 1997; Burgio et al., 2009). The generation of predictable phenotypes requires the protection of developmental processes from the damaging impact of environmental and genetic perturbations (Trotta et al., 2005; Takahashi et al., 2010). Waddington (1942) suggested a conceptual

mechanism he called canalization to account for phenotypic robustness despite variation in the environment. Canalization acts as a buffer during development, guiding it toward the generation of constant phenotypes. Also, canalization allows for the accumulation of cryptic genetic variation, which is present in the genome but is not expressed phenotypically. Stressful environmental conditions during the development of an organism, for example high temperature in insects (Vishalakshi and Singh, 2008), may be strong enough to partially disrupt canalization, releasing some fraction of accumulated cryptic genetic and phenotypic variation (Rutherford and Lindquist, 1998). Molecular chaperones such as heat-shock proteins (Hsps) are implicated in the maintenance of protein homeostasis, providing a certain amount of protection against several forms of environmental stress (Morimoto, 1998).

Some heat-shock proteins, including Hsp90s, are of singular importance with regard to canalization. Several studies in *Drosophila*, *Arabidopsis* and the zebra fish *Danio rerio* have found that the inhibition of Hsp90 leads to increased phenotypic diversity (Rutherford and Lindquist, 1998; Queitsch et al., 2002; Yeyati et al., 2007). Hsp90 is involved in both signal transduction and the cellular stress response, suggesting a mechanistic link between developmental programs and environmental contingencies (Rutherford and Lindquist, 1998). In addition, other heat-shock proteins such as small Hsps have also been recently implicated in the determination of FA levels in *Drosophila* (Takahashi et al., 2010). More recently, Takahashi et al. (2011) used a deficiency mapping to identify some genomic regions where gene deficiencies affected FA for wing shape in an isogenic background in *D. melanogaster*, but no quantitative complementation study was

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yet performed for FA in order to test for differences between wild-type alleles as used by Pasyukova et al. (2000) for other traits. QTL are functionally variable regions of the genome influencing the phenotypic variation, and QTL for FA remain to be tested in *Drosophila*.

Here one we used a quantitative trait loci (QTL) approach to explore the genetic basis of wing FA using a subset of *Drosophila melanogaster* recombinant inbred lines (RIL), which were obtained by Norry et al. (2008) from parental stocks that differed for heat-stress resistance. Many genes might affect FA levels, and we performed a QTL-based scan to test the hypothesis that the number of significant QTL for FA is higher at stressful developmental temperature than at benign temperature, as developmental canalization is likely to be weaker in stressful environments. We used QTL mapping in an exploratory way to test asymmetry phenotypes (e.g., FA) in two wing traits, wing length (WL) and wing width (WW), using both benign (25 °C) and stressful (30 °C) developmental temperatures.

2. Materials and methods

2.1. Experimental RIL stocks

The construction of the lines used in this study is described in Norry et al. (2008). Briefly, two nearly -homozygous and heat-stress resistance divergent stocks, D48 and SH2 (heat-sensitive and heat-resistant lines, respectively), were used as parental lines. F1-females, progeny of D48 × SH2, were backcrossed to either D48 or SH2 males. The backcross progeny was randomly mated for two more generations, followed by the set-up of individual pairs subsequently inbred by full-sib mating to form RIL stocks. Here, we used the D48-backcross derived lines. Experimental individuals from each RIL-D48 line were reared in standard culture vials at either of two different developmental temperatures, benign (25 °C) or stressful (30 °C), with 30 larvae per vial. Standard culture vials were 95 × 20-mm² shell vials containing 6 mL of Carolina culture medium (Biological Supply, Burlington, NC, USA). All larvae were 1–2 h old when transferred and all vials were kept in a 12 h light–12 h dark cycle. In total, 28–30 RIL were scored for WL and WW of right and left wings, per development temperature and sex.

2.2. FA phenotypes

Wings were removed and placed in a lactic acid droplet on a microscope slide and covered with a cover slip. All wings were photographed using a microscope-mounted camera. Wing length (WL) and wing width (WW) were measured with the image processing software ImageJ (Rasband, 2001). Specific wing landmarks were used to obtain measurements, which were later converted to mm. WL was estimated as the distance between the humeral cross-vein and the distal tip of vein III. WW was estimated as the distance between the distal tips of veins II and V.

Exploratory analyses showed no directional asymmetry (Palmer and Strobeck, 1986), as a three-way ANOVA on data pooled across RIL, using side (L vs. R), sex (S) and temperature treatment (T) as fixed factors indicated no significant effects of side itself ($F_{1;606}=0.18$ for WL; $F_{1;606}=0.013$ for WW) and, in addition, all interaction terms involving side (L vs. R) were non-significant (results for WL were $F_{1;606}=0.17$ for side × T and $F_{1;606}=0.08$ for side × S, $F_{1;606}=0.01$ for side × S × T; results for WW were $F_{1;606}=0.15$ for side × T, $F_{1;606}=0.01$ for side × S and $F_{1;606}=0.23$ for side × S × T). FA was indexed as the unsigned values of the left-minus-right difference (L–R) between wings (Palmer and Strobeck, 1986). Unsigned FA was averaged for each

trait (WL, WW) across up to 6 individuals measured per RIL, sex and temperature treatment. In total, the number of flies measured was 612. To reduce measurement error, all measurements were repeated 3 times and averaged for each wing, as FA measurements are often replicated to reduce measurement error in the wings (Trotta et al., 2005). To remove any possible size-dependent asymmetry or antisymmetry variation (Palmer and Strobeck, 1986), we estimated relative FA by dividing unsigned FA by the mean value of the trait within each RIL, sex and developmental temperature treatment (Markow and Ricker, 1991). Thus, any possible associations between trait size (e.g., females are larger than males) and asymmetry were removed between sexes as well as among RIL (Markow and Ricker, 1991). Relative FA for each trait (WL-FA, WW-FA; Fig. 1) was subjected to two-way ANOVA with developmental temperature (DT) and sex as fixed factors. ANOVA yielded results similar to Mann–Whitney tests performed on FA using DT as independent variable (results not shown).

2.3. QTL analysis

Composite interval mapping (CIM) was performed on each FA trait. Marker genotypes were the number of SH2-alleles (0 or 2), as in previous studies (Norry et al., 2008; Gomez et al., 2009; Defays et al., 2011). All QTL-based CIM scans were carried out with QTL-Cartographer Windows Version 2.5 (Wang et al., 2010), using Ri2 design initially with 5 control markers and 10 cM window size. Reasonable modifications of these initial settings yielded no significant alterations and QTL profiles were consistent across a range of parameter combinations. Significance thresholds were determined for each scan by 1000 random permutations. The marker map associated to the RIL is based on 35 micro-satellite loci on chromosomes X, 2 and 3 (Fig. 2), and it is described in Norry et al. (2008). QTL profiles were assessed for both unsigned FA and unsigned relative FA, and results were quite similar in both cases. Deletion of any possible outliers (Fig. 1) did not affect the number and positions of significant QTL. Confidence intervals were estimated for each QTL using 1.5 LOD, as suggested by Dupuis and Siegmund (1999) for a confidence level higher than 95%.

Additionally, we performed an exploratory analysis for the differences in FA between haplotypes of QTL for each trait (FA for wing length, WL-FA; FA for wing width, WW-FA). If more than one QTL is found for a given trait, the haplotype term is here used to mean a combination of different alleles sharing similar effects on FA (i.e., either increasing or decreasing FA) across the different QTL for a given trait. RIL were chosen according their haplotypes for QTL previously identified by composite interval mapping. All lines from our RIL population sharing one QTL haplotype were used in the analysis. QTL alleles (from either D48 or SH2 parental

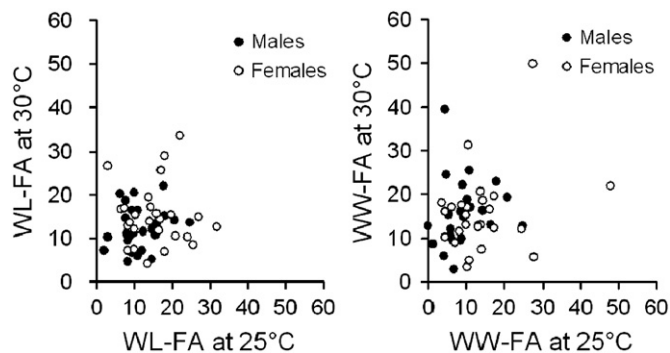


Fig. 1. Relative FA is shown for wing length (WL-FA) and wing width (WW-FA) in RIL lines at two developmental temperatures (25 °C and 30 °C).

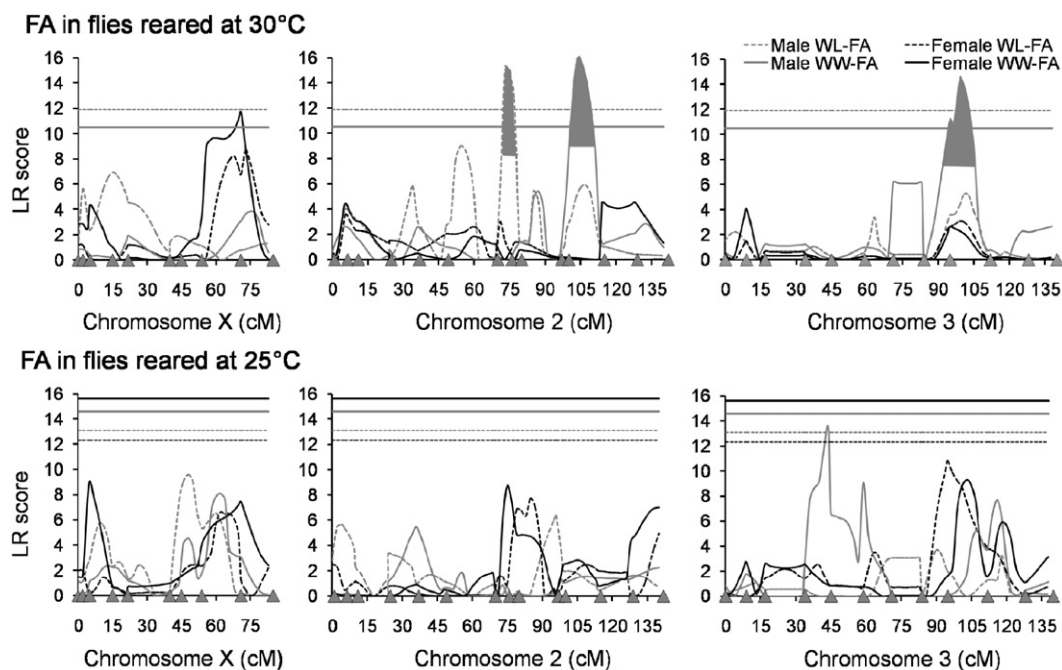


Fig. 2. Composite interval mapping LR scores are plotted against map position (cM). Significance thresholds (horizontal lines) were determined by 1000 random permutations and their style matches that of their corresponding LR profile. Significance thresholds at 30 °C are shown only for cases where at least one QTL was significant. All significance thresholds are shown for FA at 25 °C. The maximum width of hatched regions (shaded areas) corresponds to higher than 95% confidence intervals for significant QTL, using 1.5 LOD (i.e., 6.9 LR) according Dupuis and Siegmund (1999). Triangles on the X-axis indicate the location of the microsatellite markers in all chromosomes (see Norry et al., 2008, for genetic map of markers).

lines) were identified according to the allele of the microsatellite locus corresponding to the marker closest to the QTL peak in the genetic map (coinciding too with the marker within the QTL region explaining most of the FA variation in a linear regression analysis of FA on maker genotype). FA differences between QTL-haplotypes were evaluated using *t*-test (Fig. 3).

No QTL was significant for wing size traits (WL and WW) in the RIL-D48 panel, and these traits were otherwise not included in this study. Candidate genes were identified searching each QTL region for genes with known or inferred effects on wing phenotype or otherwise implicated in the stress response, as represented in the Flybase ontology database and previous studies (FlyBase Consortium, 2003).

3. Results and discussion

Temperature during development had no significant impact on FA levels when comparing mean FA between temperatures (Tables 1 and 2). However, FA differed between lines at 30 °C. For example, absolute FA differed between parental lines at 30 °C only, as mean values (\pm SD) for FA in WL were as follows: 0.018819 ± 0.0071 mm for D48 averaged males and 0.0271719 ± 0.0149 mm for SH2 averaged males at 30 °C (two-tailed *t*-test, $t_{41}=2.49$, $P=0.017$), whereas no significant differences were detected at 25 °C in both sexes (two-tailed *t*-test, $t_{41} < 1.5$). Thus, FA levels were lower in D48 than in SH2 flies when reared at high temperature. This variation is not surprising if, as observed in previous studies (Norry et al., 2004), heat-induced expression of Hsps is higher in D48 than in SH2 lines. In insects, effects of Hsps were found for asymmetrical phenotypes (Breuker and Brakefield, 2003; Takahashi et al., 2010).

Relative FA values are shown for each RIL in Fig. 1. There was no significant correlation for relative FA between developmental temperatures (Spearman's rank correlations for data shown in Fig. 1 were as follows: -0.09 and 0.18 for WL-FA in females and

males, respectively; 0.13 and 0.29 for WW-FA in females and males, respectively). This lack of association for FA across temperatures was also found for absolute FA (-0.08 and 0.15 for WL-FA in females and males, respectively; 0.13 and 0.30 for WW-FA in females and males, respectively).

Composite interval mapping revealed three QTL for wing FA in males, all of them at stress temperature (30 °C), and no QTL was significant for FA at 25 °C (Fig. 2). One large-effect QTL was found for FA in the pericentromeric region of chromosome 2 at 30 °C, and two other QTL were detected on chromosomes 2 and 3 at 30 °C (Fig. 2). As all three FA-QTL were found in males only, the results might suggest sex-specificity. Although most of the genes underlying a phenotypic trait are the same in both sexes, sex-specific QTL were often found for diverse traits (Mackay et al., 2009). In addition, a deficiency mapping recently carried out by Takahashi et al. (2011) revealed small regions of chromosome 3 with sex-specific effects on wing-shape FA, which is also consistent with a sex-specific genetic basis of FA.

Instability during development is a result of inability of the genome and developmental pathways to reduce or suppress the random noise affecting the process (Palmer and Strobeck, 1986; Zakharov, 1992; Trotta et al., 2005). Since both sides of bilateral organisms are produced by the same genome, FA was often considered as an index of developmental instability. The present results suggest that stress induces genetic variation in FA, as QTL for FA were significant at stressful temperature only. This is an interesting result, as the number and the magnitude of significant QTL are expected to be higher under stressful environments rather than in benign conditions. In fact, heat stress is a well-known factor of environmental perturbation on developmental systems in *Drosophila* (Rutherford and Lindquist, 1998). Using high temperature as a stress factor for egg-to-adult development, we found 3 significant QTL for FA of wings developing at 30 °C but no QTL at the benign test temperature (25 °C). A review on studies informing FA heritabilities reported mean FA heritability to be quite low (0.026 ± 0.015 ; Fuller and Houle, 2002). The present

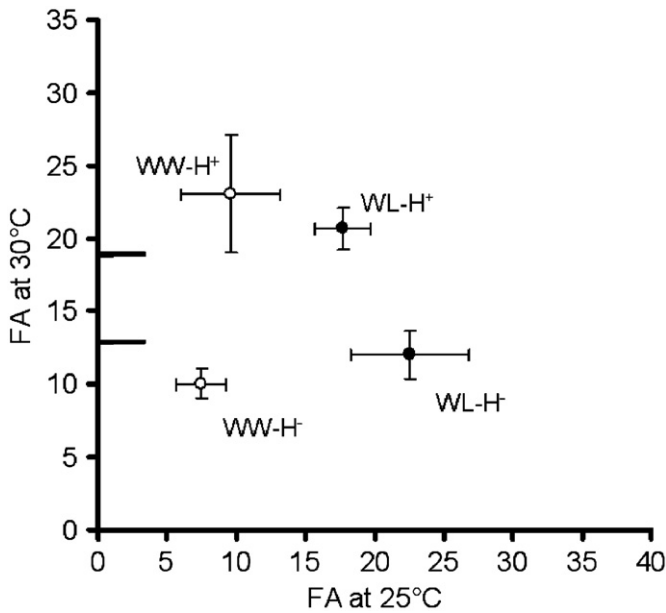


Fig. 3. Unsigned FA of wing width (WW) and wing length (WL; in $\text{mm} \times 10^3$) is shown for QTL-haplotypes in males at two developmental temperatures (25 °C and 30 °C). Haplotypes were defined as explained in Section 2.3. All QTL were identified by composite interval mapping at 30 °C (Fig. 2). All values correspond to non-relative FA to avoid overlapping of traits. Haplotype WL-H⁺ corresponds to all RIL sharing the allele that increases FA at Q1 ($n=22$ RIL). Haplotype WL-H⁻ corresponds to all RIL sharing the allele that decreases FA at Q1 ($n=6$ RIL). Haplotype WW-H⁺ corresponds to all RIL sharing the alleles that increase FA at Q2 plus Q3 ($n=3$ RIL). Haplotype WW-H⁻ corresponds to all RIL sharing the alleles that decrease FA at Q2 plus Q3 ($n=13$ RIL). Solid line marks on the vertical axis indicate the position for mean FA as averaged over the total of RIL measured at 30 °C (Table 1), for both wing length (upper case, $\text{FA}=18.82 \pm 1.35$) and wing width (lower case, $\text{FA}=12.81 \pm 1.15$). Error bars correspond to standard error of the mean. Significant differences were found between genotypic FA values at 30 °C only (t -tests for FA differences between haplotypes were: $t_{26}=2.99^*$ for WL-H at 30 °C; $t_{14}=4.77^{***}$ for WW-H at 30 °C and $t_{26}=1.13$ for WL-H at 25 °C; $t_{14}=0.52$ for WL-H at 25 °C. $^*P < 0.05$; $^{***}P < 0.001$).

Table 1

Unsigned absolute and relative FA (\pm SE) for wing length (WL-FA) and wing width (WW-FA) are shown for flies reared at 25 °C and 30 °C. Relative FA is the ratio of absolute FA to trait size in mm (i.e., wing length in mm; wing width in mm). All values were averaged across all RIL used in this study.

Trait	Sex	25 °C	N	30 °C	N
Absolute FA					
WL-FA	Females	26.97 ± 2.43	30	24.22 ± 2.22	28
WL-FA	Males	18.76 ± 1.79	29	18.82 ± 1.35	28
WW-FA	Females	14.66 ± 1.66	30	14.30 ± 1.68	28
WW-FA	Males	10.87 ± 2.41	29	12.81 ± 1.15	28
Relative FA					
WL-FA	Females	14.52 ± 1.29	30	14.38 ± 1.35	28
WL-FA	Males	11.73 ± 1.12	29	12.76 ± 0.93	28
WW-FA	Females	14.03 ± 1.61	30	14.98 ± 1.75	28
WW-FA	Males	11.65 ± 2.57	29	15.00 ± 1.42	28

results suggest that genetic variation in FA can be induced under stressful conditions of egg-to-adult development in insects, as some FA-QTL may appear with stress and disappear without stress (Figs. 2 and 3; Table 3), even if stress did not change FA phenotypic levels of population as in the present study (Table 2).

The QTL localizations did not generally correspond to those of deficiency mapping of FA in wing shape (Takahashi et al., 2011), possibly reflecting that we used natural alleles rather than loss-of-function mutants and both effects may be evenly relevant for FA itself (e.g., see above for sex-specificity). Another possible explanation for this discrepancy is the rather limited allele

Table 2

Two-way ANOVAs performed on relative unsigned FA (mean values for each RIL) to test for fixed effects of (1) developmental temperature (DT, 25 °C vs. 30 °C) and (2) sex. WL-FA is FA for wing length; WW-FA is FA for wing width.

Trait	Source of variation	d.f.	F	P
WL-FA	(1) DT	1	0.138	0.710
	(2) Sex	1	3.451	0.066
	(1) × (2)	1	0.242	0.624
	Error	111		
WW-FA	(1) DT	1	1.291	0.258
	(2) Sex	1	0.387	0.535
	(1) × (2)	1	0.397	0.529
	Error	111		

variation between parental lines that is inherent in QTL mapping experiments for FA. Interestingly, not only were none of the QTL detected in the absence of thermal stress, but also no significant differences, either for WL-FA or for WW-FA, were found when comparing the benign and stressful developmental temperatures (Tables 1 and 2). Some QTL controlling the severity of morphological developmental perturbation may appear under stressful developmental conditions and disappear under more benign conditions, as suggested in this study (Fig. 2; Table 2). Such FA-QTL segregating in populations could stabilize FA levels under stress (Fig. 3), as the overall FA level (i.e., mean FA pooling all RIL) did not differ between benign and stressful temperatures (Tables 1 and 2). Temperature is a relevant stress factor for FA levels in bilateral traits of insects, as temperature is one of the most important environmental factors regulating the growth and differentiation of imaginal disks. The insulin pathway is implicated in the coordination of imaginal disk proliferation in *Drosophila* (De Jong and Bochdanovits, 2003; Emlen and Allen, 2003). *InR* and *CG10702* (encoding insulin-like growth factor receptors) were included within FA-QTL regions (Table 3). Insulin and endocrine signaling may interact to coordinate the relative growth of insect body parts (Tu et al., 2002; Emlen and Allen, 2003; Tu and Tatar, 2003), and the ecdysone receptor gene (*Ecr*) was also included within FA-QTL on chromosome 2 (Table 3).

Hsp90 was not included within our FA-QTL. However, other stress response genes as well as some other loci implicated in growth, imaginal disk development and hormone biosynthesis were included within FA-QTL (Table 3). A previous study in the mouse model revealed FA-QTL for skeletal traits, many of which included growth factor, hormone and hormone receptor genes (Leamy et al., 1997). Our WL-QTL in the pericentromeric region of chromosome 2 (Fig. 2, cytological range 34C4-42A) not only co-localized with QTL for thermal-stress resistance (Norry et al., 2008, 2009; Loeschcke et al., 2011) but also included *ldgf1*, *ldgf2* and *ldgf3* as candidates involved in imaginal disk development. This QTL also included *Ddc*, a gene implicated in growth and hormone biosynthesis, as well as *trap1* and *Catsup*, all of them also implicated in the stress response (Wright, 1987; Baden et al., 1996; Sabban and Kvetnansky, 2001; Carbone et al., 2006). *Hsr-omega* is another heat-stress gene within one FA-QTL in this study (McKechnie et al., 1998). Presumably, QTL may often be the result of closely linked and co-expressed genes, as in Coffman et al. (2005) and Norry et al. (2009). We are aware that QTL positions could be approximated rather than narrow in the current study because of measurement complexity often associated with FA. In any event, this study suggests that QTL-based scans under stressful thermal environments may be an informative approach for FA analysis (Figs. 2 and 3), even if FA levels are often similar between stressful and non-stressful environments (Andalo et al., 2000). When compared to the benign temperature,

Table 3

QTL for FA of wings in *Drosophila melanogaster*. %Var is the proportion of total phenotypic variance explained by each QTL, and “a” corresponds to additive values (in relative units $\times 10^3$). Positional candidate genes within the significant QTL regions for WL-FA and WW-FA are shown. WL-FA is FA for wing length; WW-FA is FA for wing width; DT is developmental temperature.

QTL	Trait	Sex	DT	QTL range	a	% Var	Some candidates within QTL
Q1	WL-FA	Male	30 (°C)	34C4–42A	–13.3	26	<i>EcR</i> , ^a <i>catsup</i> , ^b <i>Ddc</i> , ^b <i>trap1</i> , ^b <i>ldgf1</i> , ^a <i>ldgf2</i> , ^a <i>ldgf3</i> , ^a <i>CG10702</i> . ^a
Q2	WW-FA	Male	30 (°C)	50C–54B2	–9.10	14	<i>Jhe</i> , ^a <i>Jhedup</i> , ^a <i>Jhl26</i> . ^a
Q3	WW-FA	Male	30 (°C)	90E–95C6	12.50	30	<i>InR</i> , ^a <i>hh</i> , ^a <i>hsr-omega</i> , ^b <i>fru</i> . ^a

^a Genes with known or otherwise inferred effects on wing development.

^b Some genes implicated in the stress response.

at which no QTL was found (25 °C), the number of possible QTL for asymmetry phenotypes increased with stressful temperature during egg-to-adult development.

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