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Genetic variation for expression of the sex determination pathway genes in *Drosophila melanogaster*

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Summary

Sequence polymorphisms result in phenotypic variation through the pathways of interacting genes and their products. We focused on transcript-level variation in the splicing pathway for sex determination – a model network defining downstream morphological characters that are dimorphic between males and females. Expression of *Sex lethal*, *transformer*, *transformer2*, *doublesex*, *intersex* and *hermaphrodite* was assayed with quantitative RT-PCR in 0- to 1-day-old adult males and females of 36 *Drosophila melanogaster* inbred lines. Abundant genetic variation in the transcript levels was found for all genes. Sex-specific splices had high concentrations in the appropriate sex. In the other sex, low but detectable concentrations were also observed. Abundances of splices strongly co-varied between sexes among genotypes, with little genetic variation strictly limited to one sex. The level of sexually dimorphic *Yolk protein1* expression – an immediate downstream target of the pathway – was modelled as the target phenotype of the upstream sex determination pathway. Substantial genetic variation in this phenotype in males was explained by leaky splicing of female-specific transcripts. If higher transcript levels of the appropriate isoform of sex determination genes are beneficial in a sex, then stronger leakiness of the inappropriate transcript might be deleterious, perhaps contributing to the fitness trade-offs previously observed between the sexes.

1. Introduction

Understanding agriculturally, evolutionarily and medically important complex traits requires inferring the nature of their variation. Factors contributing to variation are usually resolved by recombination analysis, with the underlying genes pinpointed by quantitative complementation (Mackay, 2001) or expression comparisons (Wayne & McIntyre, 2002). Associations between DNA polymorphisms and the phenotype in a large panel of natural genotypes also hold promise for inferring the causal genes (for a review see Reich & Lander, 2001). When the genes for complex characters are known, one may further ask how their molecular variation results in variation of phenotypes. Clearly, the networks of genes and their products shall be considered to reach the answer (von Dassow *et al.*, 2000; True & Haag, 2001). Research reported here focuses on the sex determination splicing pathway of *Drosophila melanogaster* – the central hub in the network of genes involved in sexual differentiation.

Two ways in which variation at a locus may affect a quantitative trait are: (i) variation in expression regulation, as is the case for maize apical dominance, affected by the *tb1* gene, and tomato fruit size, affected by the *fw2.2* gene (Paran & Zamir, 2003), and (ii) polymorphisms for protein efficiency, as in the case of the fast and slow alleles of *Alcohol dehydrogenase* (*Adh*) in *Drosophila melanogaster* (Stam & Laurie, 1996). This gene also exhibits variation in expression levels (Parsch *et al.*, 1999, 2000): fast alleles of *Adh* – better processors of metabolic ethanol due to increased protein activity – also possess a deletion in a regulatory site, which causes higher expression levels than slower alleles. Across taxa and characters, the contribution to phenotypic variation from protein coding sequence and expression regulation appears comparable. Of 10 cloned plant quantitative trait loci (QTLs) three exhibit altered protein function, three exhibit a loss of function (two of these affect transcription factors), two have differences in expression

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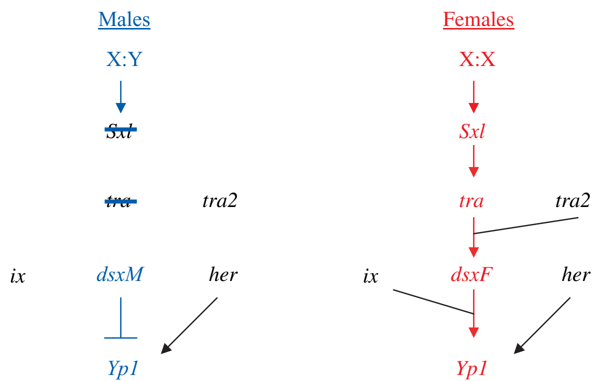

Fig. 1. The cascade of sex determination genes in males (blue) and females (red). Non-sex-specific transcripts are in black. Non-protein-coding transcripts are crossed through.

levels and two have unknown mechanisms that affect the phenotype (one, *Dwarf 8*, is a transcription factor) (Paran & Zamir, 2003). Our study focuses on the variation in the levels of transcripts in the pathway.

We chose to work on a well-understood genetic pathway for sex determination in *D. melanogaster* (see for review Cline & Meyer, 1996) for two reasons. First, every cell in the body constitutively maintains male or female identity (Fig. 1), thus genetic variation in transcript level can be assayed with whole-body RNA. Second, the pathway alters splices, amounts of which are easy to assay with quantitative PCR. More specifically, the X-chromosome to autosome ratio is 1 : 1 in females, and this causes the gene *Sex lethal* (*Sxl*) to be functionally spliced. *Sxl* protein then splices t*ransformer* (*tra*) into a functional transcript and *tra* protein interacts with *transformer 2* (*tra2*) protein to splice the gene *doublesex* (*dsx*) to its female-specific isoform (*dsxF*). *dsxF* codes for a transcription factor that affects the majority of structural and behavioural aspects of female differentiation. In males, the X to autosome ratio is 1 : 2, and *Sxl* is spliced into a non-functional transcript (*SxlM*), causing *tra* to be mis-spliced and non-functional. In the absence of t*ra* activity, *dsx* is spliced to *dsxM* that causes most male somatic cell differentiation. *dsx* transcripts share a common DNA binding domain, but have different protein interaction domains.

The sex determination pathway appears binary, with splices turned on or off depending on the sex of the cell (Cline & Meyers, 1996). More realistically, the levels of splicing may vary quantitatively (Kozak, 1999), affecting the extent of downstream sex-specific differentiation. To test this hypothesis, we focus on expression of *Yolk protein* (*Yp*) genes (Burtis *et al.*, 1991). Males express low levels of *Yp*s in the fat body and females express high levels of *Yp*s in the fat body and in the ovaries under the control of *dsx* and *her*. *her* protein causes *Yp* expression in both sexes and *dsxF* protein enhances expression of *Yp*s in females, while *dsxM* represses the effect of *her* on *Yp* expression in males. *intersex* (*ix*) is also known to be involved in sex determination in both sexes and its product physically interacts with *dsxF* protein in female sex determination (Garrett-Engele *et al.*, 2002). Transformants with strongly altered expression of *her* and *dsx* exhibit quantitative effects on the expression of *Yps* (Li & Baker, 1998). Here, we test whether there is natural genetic variation for sexually dimorphic *Yp* expression, and estimate the contribution of transcript level variation in the sex determination pathway to this variation.

2. Materials and methods

(i) *Analysed genotypes*

The 34 *D. melanogaster* lines were developed from flies caught in Wolfskill orchard (Winters, CA) by full-sib mating for 40 generations. To this panel, we added the lines 2b (Pasyukova & Nuzhdin, 1993) and Oregon R (Nuzhdin *et al.*, 1997). The stocks were kept at a moderate density in 150 ml bottles on cornmeal with yeast and paper. Bottles were maintained at room temperature in a temperature-controlled building and transferred every 3–4 weeks.

(ii) *Primers*

Primers for measuring concentrations of the sex determination transcripts for S*xl*, *tra*, *tra2*, *ix*, *her*, *dsx*, *Yp1*, *Glycerol 3 phosphate dehydrogenase* (*Gpdh*) and *bonsai* were chosen with the Taqman primer design program. They are listed in Table 1. *SxlM* primer is inside the male-specific abortive product. *Sxl* primer is homologous to both female and male processed transcripts. The *tra* primer is for the female-specific extension of the second exon. *tra2* has several alternative sex-specific splices. As its role in the sex determination pathway is identical in both sexes, we chose primers from the exon shared between sexes. *dsx* has male- and female-specific exons. We called primers homologous to those *dsxM* and *dsxF* correspondingly. *her*, *Yp1* and *ix* have no alternative splices. For every primer and sex, we tested whether the primers yield amplification of the expected size with no aberrations detected. Note, that since oligo-dT primer was used as a template in the reverse transcription cycle of PCR reaction, the amplifications should have been processed solely from mature RNA. We also used the dissociation curve to ensure that only reactions that had curves that matched the standard were used for analysis.

(iii) *Measurements of transcript level*

To sample flies for RNA extractions, the bottles were cleared and the freshly emerging flies collected in 24 hours. Growing and all subsequent stages were

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Table 1. *Primers used for amplifications of sex determination pathway and housekeeping genes*

Gene name	Forward primer	Location	Reverse primer	Location	Reference sequence
Gpdh	CAACAATAACAAAATATGGCGGATA	Exon 1	CTATGGCCGAACCCCAGTT	Spans exons 1&2	FBgn0001128
bonsai	GGCCAAATTGGCAAAAATGA	Spans exons 1 and 2	GCGGATGCTTGTCCATGTG	Exon 2	FBgn0026261
Yp1	TGAGCGTCTGGAGAACATGAA	Exon 1	GCGACAGGTGGTAGACTTGCT	Exon 1	FBgn0004045
her	TTGGTATTACGCAGTCCAAGCTAT	Exon 1	GACGCCTTGTCGGTGTTGTA	Exon 1	FBgn0001185
ix	AGCAGAACTCCTCCAATCACTATCTC	Exon 1	GTCCGGCATAAAGGTCTCCAT	Exon 1	FBgn0001276
dsxM	TCGAACAGGGTCGCTATGG	Male-specific exon	TCTGGAGTCGGTGGACAAATC	Male-specific exon	M25294
dsxF	TCAACACGTTCGCATCACAAA	Female-specific exon	TAGACTGTGATTAGCCCAATAACTGA	Female-specific exon	M25292
tra	GGAACCCAGCATCGAGATTC	Exon 1 and female-specific exon	ATCGCCCATGGTATTCTCTTTC	Female-specific exon	FBgn0003741
tra2	AGGTAAGCAAAAAGCCAATGGA	Exon 2	TCTGGCGCTGCAATGGA	Exon 2	FBgn0003742
SxlM	CAGAAAGAAGCAGCCACCATT	Male-specific exon	CCGTGCAAATAAAAACTACATACTAT-CTTAAG	Male-specific exon	FBgn0003659
Sxl	TTGTTGTTGCCGAAGAAGGAA	Exon 1	CAGGAGGAGCAAAGAAAAATTACC	Exon 1	FBgn0003659

replicated in two blocks. Total RNA was prepared using TRIzol. The preparations were cleaned in a Qiagen Rneasy column utilizing the optional DNAse treatment protocol with twice the DNAse and twice the incubation time indicated in the Qiagen protocol. Preparations of cDNA were made using the ABI Taqman reverse transcription reagents in 100 μl volumes. cDNAs were put in two replicates in 96-well plates, each containing: three negative controls with no DNA added and three treatments of pooled no-RT controls as a precaution against contamination with residual DNA. Each plate also had a 12-well 10-fold dilution series of a standard PCR product for the target gene scored on this plate. Standard PCR products were acquired by PCR reactions with cDNA samples, and cleaned in a Qiagen QIAquick PCR clean up kit. One microlitre of the clean product was analysed in a fluorimeter and 1 μl was diluted into a 10 ml solution.

Quantitative RT PCR reactions were done for 50 cycles and analysed on an ABI 7900 with Sybrgreen master mix and 3 μM primers. A 2% temperature gradient was run at the end of the reaction to determine a dissociation curve for products in the plate. This curve was used to determine whether correct or erroneous products are amplified. The few curves (resembling those for primer dimers) that did not match the curve of known PCR product were thrown out. We recorded the cycle threshold (CT) – the number of cycles it takes to reach linear stage of amplification.

(iv) *Statistical analysis*

We used standard procedures to equalize amounts of total RNA between samples. To account for residual variation in RNA amounts and possible differences in cDNA syntheses, we measured CT values for two control housekeeping genes, *Gpdh* and *bonsai*, in every cDNA sample. Correlation between their amounts among samples was 0·91 ($P<0{\cdot}01$, 17). Thus, most of the differential cDNA concentration effect can be removed by regressing transcript levels of measured genes on that of a control gene. We accomplished this with Proc REG (SAS Institute, 1988). We pursued downstream analysis on residual values. Transcript level of a control gene might genetically vary between lines. Residuals from regression might, then, co-vary between the standardized genes. As a precaution, we standardized transcript levels in four different ways, about: (i) *Gpdh*, (ii) *bonsai*, (iii) *Gpdh* and *bonsai* and (iv) the predicted value of the correlation between *Gpdh* and *bonsai*. The results of the analyses were little changed. We chose approach (iii) for the following analyses.

CT value is a negative logarithmic function of the transcript concentration; a higher concentration of

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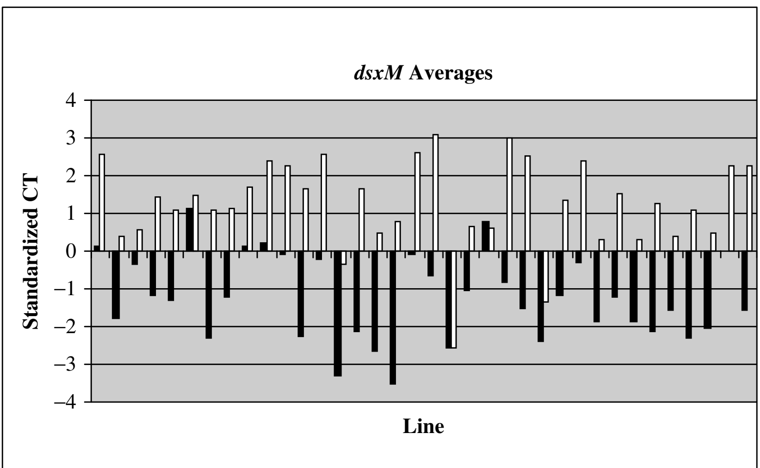

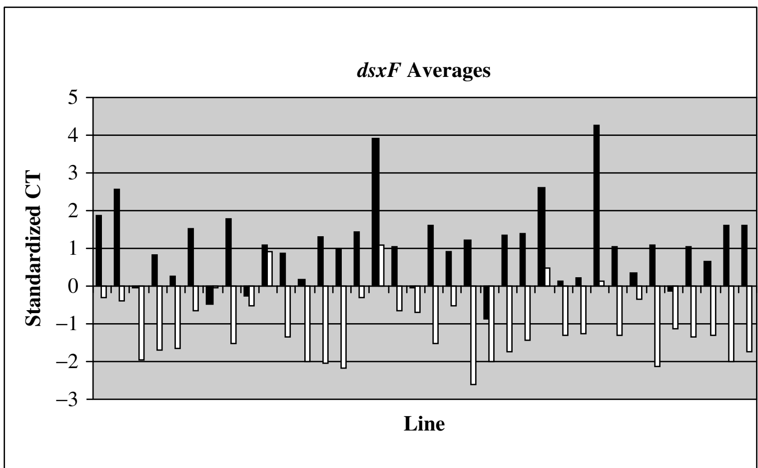

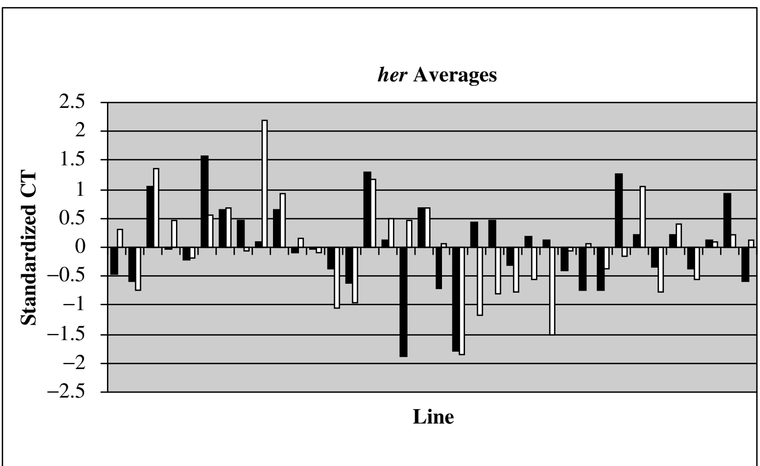

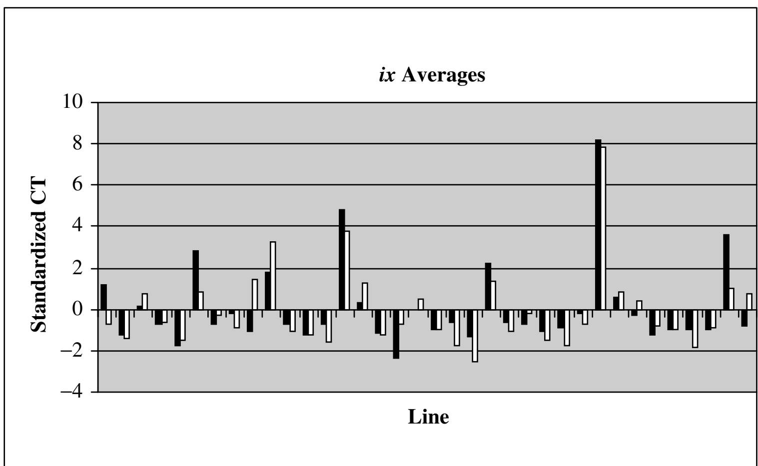

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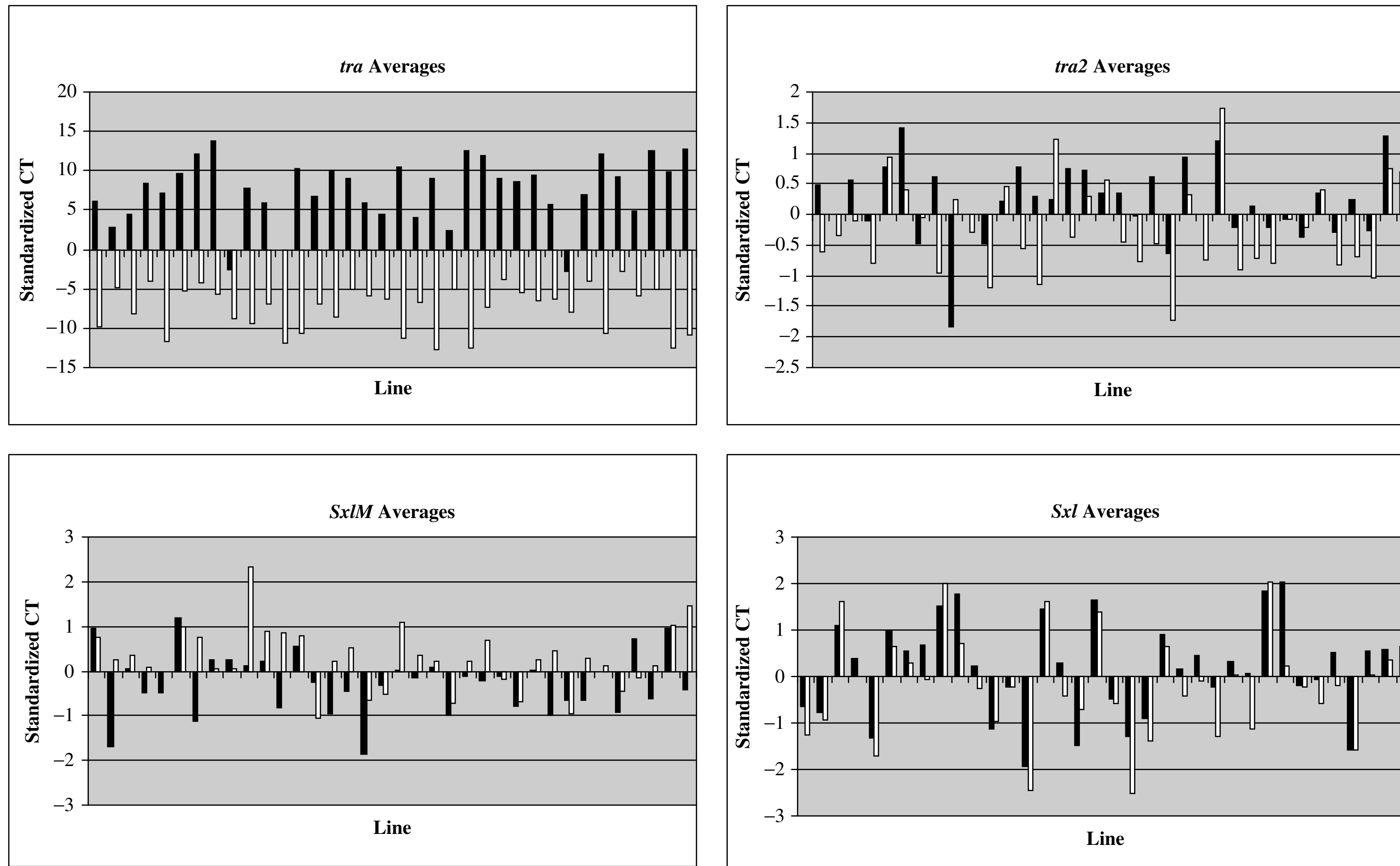

Fig. 2. Relative CT levels of sex determination splices in males and females among 36 inbred lines. Male CT averages are black. Female CT averages are white. Residuals after regression of the CT value for measured splice on that of control genes *Gapdh* and *bonsai* are plotted (see Section 1 for a fuller explanation). Note the difference in scales for *tra* and *dsx* between the sexes. For *Sxl* note the difference in scale between total *Sxl* and *SxlM* transcript within each sex.

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Table 2. *Genetic variation in the transcript levels of sex determination pathway genes in males and females*

Males			Females		
Product name	Genotype mean square/Error	r^2/Significance	Product name	Genotype mean square/Error	r^2/Significance
her	1·15/0·60	0·66/0·03	*her*	1·48/0·59	0·42/0·004
tra2[a]	0·78/0·44	0·64/0·05	*Sxl*	2·60/0·83	0·77/0·0007
dsxM	2·69/1·17	0·71/0·005	*tra*	15·55/4·49	0·79/0·0003
ix[a]	8·61/0·97	0·90/<0·0001	tra2	1·16/0·41	0·75/0·002
			dsxF	1·58/0·59	0·73/0·003
			ix[a]	7·55/1·12	0·87/<0·0001

[a] Line components of variation for these genes were also significant with a non-parametric Kruskal–Wallis test (*tra2*: $\chi^2_{35}=67{\cdot}7$, $P=0{\cdot}0008$; *ix* in males: $\chi^2_{35}=52{\cdot}9$, $P=0{\cdot}03$; *ix* in females $\chi^2_{35}=55{\cdot}4$, $P=0{\cdot}02$).

molecules causes a PCR reaction to reach the linear amplification stage with fewer cycles. The number of target molecules in a sample can be calculated from the CT value by incorporating information about the dilution curve, i.e. the efficiency of amplifications of a transcript with a given primer; the concentration of purified PCR product; and the target molecular weight (http://mcdb.colorado.edu/labs/singh/public_html/tools/). Standardized CT values were used to make Fig. 2 using Microsoft Excel. For ANOVAs, we chose standardized CT values rather than molecular concentrations because confidence intervals of the multiple parameters needed for calculations are undefined. The Kolmogorov–Smirnov test (Proc UNIVARIATE, SAS Institute, 1988) was used to check whether CT numbers are normally distributed. While in general normality was not violated, transcript level in males was not normally distributed for *dsxF* ($D=0{\cdot}17$, $P<0{\cdot}01$), *ix* ($D=0{\cdot}25$, $P<0{\cdot}01$), *tra2* ($D=0{\cdot}11$, $P=0{\cdot}04$) and *SxlM* ($D=0{\cdot}11$, $P=0{\cdot}02$); and in females for *Yp1* ($D=0{\cdot}11$, $P=0{\cdot}04$) and *ix* ($D=0{\cdot}21$, $P<0{\cdot}01$). No transformation was found that would fully normalize the complete data set. We used non-normalized data, and supplied our analysis with non-parametric tests when necessary.

Analysis of variance (ANOVA) was employed to test effects of genotypes (random), sex (fixed) and their interactions on standardized CT values (Proc GLM, SAS Institute, 1988). Correlations between line mean (averaged over replicates) expression levels of different genes were calculated separately within males and females (Proc CORR, SAS Institute, 1988). Multiple regressions (Proc REG, SAS Institute, 1988) were employed to partition the contributions of upstream genes known to jointly affect expression of downstream targets. As the pathway structure is well known, we form testable hypotheses about splicing variation of what gene might affect which downstream target. For instance, *tra* is expected to be affected by *Sxl* but not by *tra2* (Fig. 1). As the direction of influence has also been previously established, we use one-tailed tests, and limit our analyses to such *a priori* defined sets of interactions.

3. Results

(i) *Genetic variation in expression of the sex determination pathway genes*

We assayed the transcript levels of genes in the splicing pathway for sex determination in *D. melanogaster* – *Sxl*, *tra*, *tra2*, *dsx*, *ix* and *her* – in 1-day-old males and females from a panel of 36 genotypes (Fig. 2). ANOVAs established abundant genetic variation in the level of every transcript (Table 2). For example, the CT values for *dsxF* splice were different by 3·7 between lines with the extreme expression levels.

We analysed female-specific splices of *tra* and *dsx* in males, and male-specific splices of *dsx* and *Sxl* in females, as well as splices expected in both sexes, to assay the quantitative extent of the sex-specificity of the pathway. The primers were designed so that cross-amplification from immature RNA, or from alternative sex transcripts, was impossible (see Section 2). Sex-specific transcripts of an alternative sex were detected for *SxlM* (note that quantitative estimates for its proportion are unavailable due to the existence of a male-specific exon only). For *dsxM* and *dsxF*, levels of mis-spliced transcripts were roughly 4% compared with the level in the appropriate sex. Mis-splicing of *tra* was also observed but at a substantially lower level.

The results of two-way ANOVAs – for genotype and sex – are summarized in Table 3. The sex component of variance was highly significant for all transcripts known to be sex-specific (*Sxl*, *tra*, *dsxF* and *dsxM*). Of the transcripts expected to be present in both sexes (*tra2*, *ix* and *her*), only *tra2* was significantly differently expressed among sexes. The genotype component of expression variance was significant for

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Table 3. *Significance of components of variance in transcript level*

Transcript	Source	Mean square	*F* value	Significance
Sxl	Genotype	4·45	16·41	<0·0001
	Sex	3·32	12·47	0·0011
	Sex × Genotype	0·27	0·33	NS
SxlM[a]	Genotype	1·38	2·28	0·0085
	Sex	12·34	20·29	<0·0001
	Sex × Genotype	0·61	0·67	NS
tra	Genotype	24·91	1·00	NS
	Sex	8368·52	338·92	<0·0001
	Sex × Genotype	24·98	2·28	0·0018
tra2	Genotype	1·40	2·62	0·0027
	Sex	6·95	13·07	0·0009
	Sex × Genotype	0·53	1·25	NS
dsxF	Genotype	2·76	2·40	0·0056
	Sex	167·54	146·73	<0·0001
	Sex × Genotype	1·15	1·29	NS
dsxM	Genotype	4·35	4·32	<0·0001
	Sex	233·94	231·41	<0·0001
	Sex × Genotype	1·01	0·86	NS
ix	Genotype	14·99	15·63	<0·0001
	Sex	0·58	0·61	NS
	Sex × Genotype	0·96	0·91	NS
her	Genotype	1·82	2·65	0·0025
	Sex	0·028	0·04	NS
	Sex × Genotype	0·69	1·16	NS

[a] Non-protein coding transcript.

all genes except *tra*. An example of sex-specific CT values and their variation for the *dsx* gene is presented in Fig. 3. Note that while lines connecting transcript levels in males and females within lines sometimes cross, the line by sex component of variance is not significant for this gene. Also, while the levels of sex-specific transcripts are significantly different between sexes, estimates for individual lines appear to be overlapping between sexes. Both observations illustrate that quantitative RT-PCR yields measurements that are rather noisy, though they are sufficiently precise to establish genetic variation in transcript level, and partition it between genotype and sex.

(ii) *Does variance in the transcript level of an upstream gene affect its downstream target?*

Since the position of genes in the pathway are known (see the female cascade in Fig. 1), we tested what portion of expression variation in the downstream gene is associated with *trans*-acting upstream factors (Cline & Meyer, 1996). We used multiple regressions where the downstream target is a predicted value and upstream splicing factors are predictor variables. Both *tra* and *tra2* affected the amount of *dsxF* in females (*dsxF* ~0·18(*tra*)+0·43(*tra2*), $P=0·0007$ for the first gene, 0·02 for the second). *tra* transcript level was not significantly affected by the *Sxl* expression level (Table 4). As the causal role of pathway switches

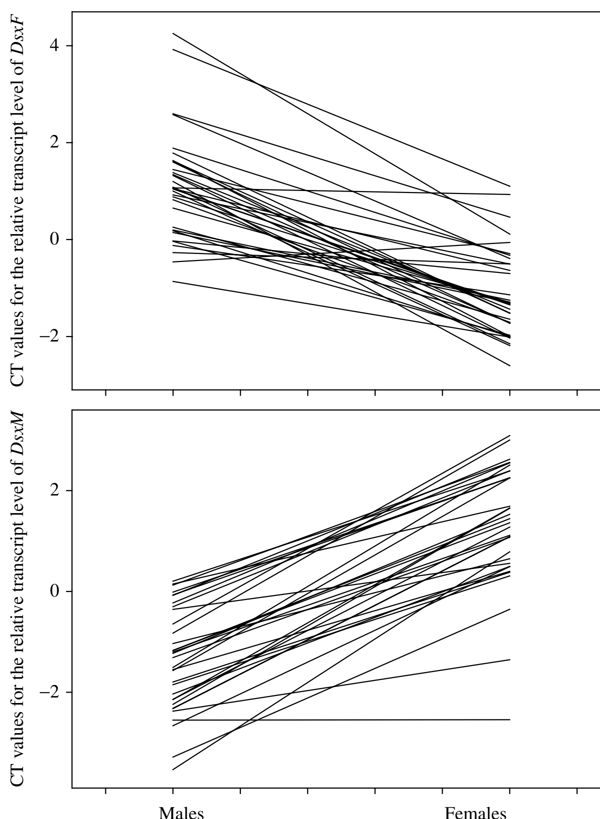

Fig. 3. Comparison of CT values for *dsxF* and *dsxM* splices in the two sexes among isogenic lines.

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Table 4. *The regression models tested*

Sex	Regression model	Estimates and significances			R^2
♀	*Yp1 = ix dsxF her*	−0·03 (NS)	0·37 (NS)	0·34 (NS)	0·11
♀	*dsxF = tra tra2*	***0·18*** (0·0007)	**0·43** (0·020)		0·33
♀	*tra = Sxl*	0·07 (NS)			0·00
♀	*tra = SxlM*	−**1·72** (0·008)			0·17
♀	*dsxM = tra tra2*	−***0·22*** (0·0003)	−0·34 (NS)		0·35
♂	*Yp1 = ix dsxF her*	−0·10 (NS)	**0·77** (0·030)	−0·32 (NS)	0·16
♂	*dsxF = tra tra2*	0·01 (NS)	**0·71** (0·017)		0·17
♂	*tra = Sxl*	−0·16 (NS)			0·00
♂	*tra = SxlM*	0·98 (NS)			0·03
♂	*dsxM = tra tra2*	−0·02 (NS)	0·00 (NS)		0·01

has been established in previous research, we interpret detected correlations as probable causations. We hypothesize that natural quantitative variation in the gene transcript level is partially explained by the transcript level variation of upstream splicing factors.

Does the transcript level of upstream splicing factors affect the amount of mis-spliced transcripts? We regressed the *dsxM* (*dsxF*) transcript level in females (males) on the transcript levels of *tra* and *tra2* genes jointly. In females, $dsxM \sim -0{\cdot}22$ (*tra*) ($P = 0{\cdot}0003$). Mis-splicing of *dsx* in females appears to be caused by low levels of *tra* expression. In males, $dsxF \sim 0{\cdot}73$ (*tra2*) ($P = 0{\cdot}007$). Similar associations have been detected at the other steps of the pathway. In females, $tra \sim -1{\cdot}72$ (*SxlM*) ($P = 0{\cdot}009$). Table 4 presents models we tested and lists significant correlations after correcting for multiple testing. Expression levels of splicing factors appear to have a strong influence on the natural variation in abundance of corresponding alternatively spliced transcripts. Genotypes that express lesser amounts of splicing factors possess more incorrectly spliced transcripts.

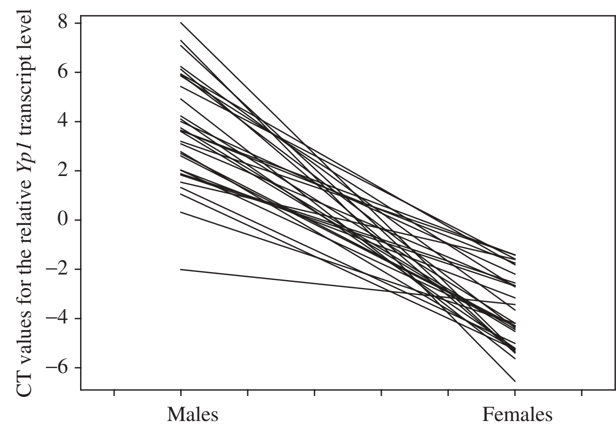

Fig. 4. Comparison of CT values for *Yp1* between the two sexes among isogenic lines.

(iii) *Genetic variation of the Yp1 gene transcript level is partially explained by variation in the expression of upstream genes in the sex determination pathway*

The relative expression of *Yp1* in females was roughly 250-fold higher than in males, as expected from earlier Northern blot hybridization data (Burtis & Baker, 1989). The extent of sexual dimorphism varied between genotypes (Fig. 4). In a two-way ANOVA (sex and genotype), sex effect (mean square 1912·36, $P < 0{\cdot}0001$) and sex by genotype interaction (mean square 10·62, $P = 0{\cdot}0004$) were highly significant, while genotype was not (mean square 7·78, NS).

We described *Yp1* expression in terms of the transcript level of the genes directly upstream of *Yp1*. In males, the level was affected by natural variation in expression of *dsxF* but not *her* ($Yp1 \sim 0{\cdot}82$ (*dsxF*), $P = 0{\cdot}011$), thus mis-splicing of *dsx* appears to contribute to *Yp1* expression in *D. melanogaster* males. Note that mis-splicing of *dsx* in males is in turn affected by the level of *tra2* transcript ($R^2 = 0{\cdot}17$, $P = 0{\cdot}007$). Deciphering which of those genes accounts for *Yp1* variation will require further experiments. *Yp1* transcript level in females was not significantly associated with the variation in expression levels of upstream sex determination genes.

4. Discussion

Recent technological (high-throughput genotyping, RNA and protein analysis) and conceptual (theoretical and simulation analyses of genetic network) advances are in shifting the focus of evolutionists from single genes to networks (von Dassow *et al.*, 2000). Here, we studied one of them – the sex determination pathway which switches the splicing of several genes in a sex-specific way (Cline & Meyer, 1996). It also defines male-specific expression of *fruitless* required for male brain differentiation (for review see Wolfner, 2003) and affects many less understood aspects of development (Burtis & Baker, 1989). We first studied whether or not the pathway is binary (i.e. possessing no variation in transcript level) or quantitative (i.e. with abundance and sex-specificity of transcripts varying among genotypes).

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Evolutionary considerations favour the latter. Mutations are purged from the population only when they affect the phenotype (Lynch & Walsh, 1998) – sexual differentiation in this case. Their transient segregation should maintain variation at the level of sexual dimorphism at the cellular and organismal levels. Consistent with this prediction, we observed abundant genetic variation for every gene in the pathway, and found erroneously spliced transcripts, in one case (*dsxF*) resulting, perhaps, from a limited amount of co-factor of splicing (*tra*).

Does genetic variation of switch pathways resolve in downstream phenotypic variation? We found that *Yp1* expression in males was partially accounted for by the splicing variation of an upstream gene (*dsx*). *Yp1* transcript level in females was not significantly associated with the variation in expression levels of upstream sex determination genes. This might be due to multiple reasons. First, variation in any of the genes in the pathway may not have an overwhelming influence on the *Yp1* expression in females. Instead multiple genes, including those from different pathways (for instance the ecdysone response pathway), simultaneously contribute. Second, the relationships between expression of the genes might not be linear as we assumed in the regression analysis. Indeed, the binding of the *dsx* protein to the *Yp1* enhancer is a cooperative process (Garrett-Engele *et al.*, 2002). Larger panel of genotypes will have to be assayed with more precision to incorporate such non-linear terms. Third, variation in *Yp1* expression may be primarily accounted for by the sequence variation of its *cis*-regulatory regions, with minute if any influence of *dsxF* and *her*. Future experiments should attempt to define variation in expression level in terms of upstream protein sequence, regulatory sequences and transcript variation levels. Finally, it is possible that females express upstream products in excess and protein function is more important for *Yp1* expression levels.

We found high concordance of levels of sex-specific transcripts among sexes. The genotypes with stronger *dsxM* transcript level in males mis-expressed *dsxM* in females at higher rates. Similarly, the genotypes with stronger *dsxF* transcript level in females mis-expressed *dsxF* in males. This observation is, perhaps, evolutionarily relevant. Theory predicts that when contributions of the two sexes in reproduction are unequal an antagonistic 'arms race' between the sexes evolves. Experiments show that when sexual selection improves the fitness of one sex, it decreases that of the other (Holland & Rice, 1998). Further, chromosomes defining high reproductive fitness of one sex underperform in the other sex (Chippindale *et al.*, 2001; Gibson *et al.*, 2002). The double role of the *dsx* gene, with a common DNA binding domain, might contribute to such antagonism. We hypothesize that in the genotypes exhibiting slight biases in the splicing of *dsx* in the direction of one sex or the other, if this bias affects one or more fitness-related pathways connected to the sex determination pathway, then it is likely that it could affect the fitness of these lines in a sex-specific manner that is advantageous to one sex and a disadvantage to the other. Whether or not such trade-offs exist, and how they contribute to the maintenance of genetic variation in the sex determination pathway, will be a subject of future tests.

Studying the sex determination pathway is useful in that it is the central pathway to the network establishing differences between the sexes. *dsx* interacts with the Anterior/Posterior patterning genes *decapentaplegic* (*dpp*) and *wingless* (*wg*) to cause the development of gonads, with *breathless* (*btl*) to give rise to male paragonia, with dachshund (*dac*) to cause the development of the spermathecal ducts in females and claspers in males, and with *bric-a-brac* (*bab*) to create the sexually dimorphic pigmentation of *D. melanogaster* (Christiansen *et al.*, 2002). There are many more sex-limited and sexually dimorphic traits that *dsx* affects, development of which remains a mystery. Variation in *dsx* could affect any of these phenotypes and contribute to the maintenance of variation in the extent of sexual dimorphism.

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