EFFECTS OF THE HDAC INHIBITOR, CURCUMIN, ON POSITION EFFECT VARIEGATION IN DROSOPHILA

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Abstract

Naturally occurring genetic variation among members of a population is essential for the operation of natural selection and serves as the source of all adaptive evolution. Genomic data from a population sample in which allele frequencies vary naturally gives evidence for the basis of these observed phenotypic differences. In addition to genomic differences contributing to variance in phenotype, interaction with the environment in which members of a population find themselves may also contribute to change in gene expression, mediated through the epigenome. The epigenome is comprised of all the chemical alterations made to the DNA as well as changes made to the core histone proteins that organize chromatin into nucleosomes. The ability of the genome to respond to an individual's environment through modifications of chromatin state makes the epigenome particularly important. Moreover, it is unknown to what degree individuals harbor functionally important variation in the epigenome. In order to better characterize this variation in gene expression mediated by genetic interactions with the environment, we used five populations of *Drosophila melanogaster* and administered a chromatin-modifying drug to individual lines of each population. By crossing lines to an allele sensitive to change in chromatin state, we were able to assess and quantify the differences between and within populations in response to modified conditions. Our findings, that populations and individual lines differ in their genetic by environment interactions, shed light on the degree to which genetic variation contributes to variation in chromatin state at a single locus.

Introduction

Epigenetics is the study of changes in cellular function or gene expression that occur without alterations to the DNA sequence (Goldberg, *et al*. 2007). Epigenetic modification to histones or DNA is known to result in change in the gene expression (Handel *et al*. 2010), and while there have been many studies characterizing specific epigenetic mechanisms, there has been little research to quantify the magnitude of epigenetic variation in a population or to identify the underlying basis for the variation.

Previous research has made use of an X-ray induced, mutagenic allele that places the euchromatic *white* gene of *Drosophila melanogaster* adjacent to a region of pericentric heterochromatin (Muller 1930). This particular allele, designated *white-mottled-4* (w^{m4}), results in a "mottled" eye color phenotype where the normally solid red eye will instead exhibit patches of red and white. These patches appear to be stochastic in position and with no uniform size, shape, or location among the eyes. The eye color phenotype resulting from this inversion is described as showing position effect variegation (PEV). PEV is not unique to w^{m4} and may be observed in situations where one or more euchromatic genes is placed in close proximity to heterochromatic regions (Spradling and Karpen 1990).

Heterochromatin is highly compacted chromatin that generally undergoes lower rates of transcription; while genes present in euchromatin, less condensed regions, are transcribed more readily (Girton and Johansen 2008). In *D. melanogaster,* this difference in chromatin condition is mediated by chromatin interacting factors and through alteration to chromatin components such as

histones (Tartof *et al*. 1989). It has been observed that when euchromatin is juxtaposed with heterochromatin, there appears to be a "spreading" phenomenon, whereby the formerly euchromatic material undergoes compaction and gene inactivation characteristic of heterochromatin (Karpen 1994). It is thought that binding factors that produce heterochromatin domains are physically spreading from the breakpoint of the inversion into the newly adjacent euchromatin region, leading to this observed change and the variable inactivation of the *white* gene. This partial inactivation in some ommatidia is considered to be the visible manifestation of variation in spreading of heterochromatic epigenetic silencing factors into the newly adjacent euchromatic gene(s) (Talbert and Henikoff 2000).

Until recently, this silencing process was hypothesized to work by the 'oozing model;' this model postulated that heterochromatin-binding proteins were spreading continuously into the euchromatic region and silencing gene expression by inducing the condensation of the chromatin. According to this model, whether or not a gene was subject to heterochromatic inactivation should depend on the proximity of the gene to the breakpoint of an inversion or translocation, and variegation should appear to decrease as distance from the breakpoint increases (Tartof *et al*. 1989). Talbert and Henikoff found that the supposed continuous and directional propagation of the heterochromatin domain into euchromatic areas was actually occurring in a discontinuous manner as some genes proximal to the breakpoint were being expressed while some more distal were silenced (Talbert and Henikoff 2000). This suggested that a simple 'oozing model' was not sufficient in explaining variation in propagation giving rise to variegation among ommatidia.

More recent models have taken these findings into account, proposing that it is chromatin looping (a process in which the chromatin segment proximal to the inversion breakpoint form a loop outward bringing more distal genes nearer to the breakpoint) that allows for genes distal to the inversion point to be influenced by heterochromatin spreading while proximal genes are unaffected (Talbert and Henikoff 2000). Furthermore, some models have proposed that there exists a limited supply of heterochromatin-binding proteins and that more distal genes may simply express a higher sensitivity to silencing factors, leaving little for the less sensitive proximal genes (Girton and Johansen 2008).

In an attempt to shed more light on the mechanism of heterochromatin spreading leading to variable silencing in PEV, Tartof *et al*. created revertants of w^{m4}. Using X-ray induced mutagenesis (which acted to undo the original inversion), revertants of the *white* locus were created and it was discovered that at least 3 kb of heterochromatin was also being relocated. This finding indicated that it is not the heterochromatic sequence immediately adjacent to the relocated *white* gene that is inducing variegation but rather a site farther into the heterochromatic domain. While the exact mechanism surrounding the propagation of the heterochromatin domain into euchromatin and which genes are affected is unclear, it is known that the differences in this degree of propagation is causing the PEV phenotype of the w^{m4} allele (Talbert and Henikoff 2000).

In addition to the contributions of a relocated gene's immediate microenvironment on the manifestation of the PEV phenotype, *trans*-acting modifiers too can influence phenotypic expression. A *trans*-acting modifier is one

that can exert in influence on gene expression without being present in the region of the chromosome that contains the gene; while, a *cis*-acting modifier alters gene expression by altering a region immediate to the gene (Yandeau-Nelson *et al*. 2006). Modifiers of PEV can be grouped into two categories, large-scale effects such as temperature and drug dosage and single mutations both *cis* and *trans* (Girton and Johansen 2008).

Single mutations that enhance variegation are termed *E(var)*s while those found to suppress variegation are termed *Su(var)s*. The first single-gene, dominant mutation that was isolated and thoroughly characterized was of a *Su(var)* present on the *Drosophila* third chromosome (Spofford 1967). The w^{m4} flies with a wild type version of the gene had variegating eyes, while sibling flies with the *Su(var)* allele had nearly wild-type red eyes. Since this initial finding, that genes on other chromosomes had the potential to either suppress or enhance variegation, further experiments have shown that there exist between 50 and 150 other loci capable of influencing PEV (Girton and Johansen 2008).

In addition to single alleles being capable of exerting an effect on PEV, findings indicate that the overall amount of heterochromatin present in a genome, not just on the chromosome with the gene being affected, can also influence the PEV phenotype. Gowen and Gay initially discovered that adding an additional Y chromosome to male and female *Drosophila* suppressed variegation (Gowen and Gay 1934). This suppression was observed for several mottled stocks, suggesting that a fundamental feature of the Y chromosome was at work. The *Drosophila* Y chromosome's paucity of genes and highly heterochromatic state have greatly

limited its genomic investigation, thus it would have been easy to assume that its affect on PEV may be due to the presence of undetected *Su(var)*s.

To test whether the Y chromosome's affect on PEV was due to its high heterochromatin content (by some proposed models of heterochromatin spreading, it is the abundance of heterochromatic factors that enables PEV to manifest) or simply the presence of *Su(var)* genes, researchers added heterochromatic regions to the genome. The duplication and insertion of heterochromatic domains into centric regions of chromosomes were found to have the same effect on PEV as adding a Y chromosome (Hinton 1949). As expected, the deletion of heterochromatic regions had the opposite effect, enhancing PEV. These findings indicated that genome-wide heterochromatin levels, whether located on the Y chromosome or in the centromeric region of another chromosome, influence PEV.

In addition to other parts of the genome inducing epigenetic changes, environmental influences are also able to directly affect gene expression through changes made to an individual's chromatin structure. Kang *et al*. found that feeding a drug to a population of *Drosophila*, altered their cellular environment, resulting in longer lifespans compared to controls (Kang *et al*. 2002). The drug administered was 4-phenylbutyrate (PBA), a histone deacetylase (HDAC) inhibitor. HDAC inhibitors prevent the action of HDACs, which work to remove the acetyl groups from the tails of the histones that form the core of nucleosomes. By preventing the removal of acetyl groups, HDAC inhibitors prevent the negatively charged DNA from associating with the still negatively charged histone complex, thus histones are effectively released from their binding to DNA and genes are readily transcribed

(Handel *et al*. 2010). HDAC inhibitors can function to increase histone acetylation, and subsequently increase gene expression, across various cell types. Kang *et al*. postulated that the extension of lifespan may have been due to the increase in gene transcription inhibiting the normal accumulation of cellular damage over time, or perhaps by assisting in cellular repair mechanisms (Kang *et al*. 2002).

Curcumin, another drug that can also function as an HDAC inhibitor, was found to have the same effect on increasing longevity. Much like when fed PBA, flies given curcumin experienced a global increase in histone acetylation. Being dosed with curcumin resulted in altered patterns of gene expression, specifically in age-related genes and genes that enhance an individual's defensive responses to environmental stressors (Lee *et al*. 2010). Curcumin is the most active component of the spice *Curcuma longa*, also known as turmeric (Pari *et al* 2008). Curcumin makes up 2 to 5% of the spice turmeric, a spice that is widely consumed throughout Asia. Traditional medicine practices use turmeric in the treatment of a variety of diseases and ailments due to its purported anti-oxidant, anti-tumor, anti-inflammatory, anti-viral, and anti-bacterial effects (Pari *et al*. 2008). In fact, it's been demonstrated that people who consume high doses of curcumin regularly, generally have lower cancer rates compared to those who do not (Reuter *et al*. 2011). Curcumin was chosen for use in our study because it is an inexpensive drug that it is widely consumed by humans.

Along with its purported health benefits, curcumin is also capable of inducing epigenetic changes. It's been found to have an active role in mediating these changes through its direct interactions with: HDACs, HATs, miRNAs, and DNA

methyltransferase I (Reuter *et al*. 2011). Some studies have reported that curcumin and some of its derivatives are more effective HDAC inhibitors than either valproic acid or sodium butyrate, two well known and widely used HDAC inhibitors (Liu *et al*. 2005). They found that curcumin's mechanism of action included specifically inhibiting the amounts of HDAC proteins 1, 3, and 8, which normally acetylated histone H4.

In addition to its effects on acetylation by influencing the action of HDACs, curcumin can also act as a potent histone acetyltransferase (HAT) inhibitor (Marcu *et al*. 2006). A HAT is an enzyme that transfers acetyl groups to the tails of histones. The inhibition of its action would lead fewer acetyl groups to be present causing DNA to adhere more closely to the histone complex, thereby decreasing gene transcription (Handel *et al*. 2010). Marcu et al. demonstrated that curcumin acts as a selective HAT inhibitor on the family of p300/CBP HAT proteins, in tumor cells, while exerting no effect on other HATs (Marcu *et al*. 2006).

Based on the particular characteristics of the cell, curcumin has been found to act as either an HDAC inhibitor or HAT inhibitor, by targeting these enzymes directly, despite the fact that these mechanisms have opposite results. Thus, in theory, curcumin may act either to increase transcription of the *white* gene, thereby appearing to suppress PEV or, to decrease transcription of the gene, thereby appearing to enhance PEV. In preliminary and unpublished trials (conducted by Keegan Kelsey, a graduate student in the lab) of the drug, it was noted that curcumin acts to enhance PEV in *Drosophila*. These results suggested that, at the site of the *white* gene, curcumin acts to inhibit the action of HATs. In our study, we use

curcumin as means to modify PEV in Drosophila and assess variation in modified state across multiple populations of individuals.

We used the w^{m4} allele to quantify differences among lines in Position Effect Variegation and to quantify genetic differences in the response to the chromatin-modifying drug curcumin. Additionally, we sought to quantify interactions between the genetic and environmental factors (a common dose of curcumin) in mediating chromatin state (again, as ascertained by PEV). We used several lines from each of five naturally derived, inbred fly populations. In this case, a line can be thought of as an individual genotype within a population, because they are a result of multiple generational sibling-sibling inbreeding, and are originally derived from one female, the flies from each line are considered clones of one another. A population, then, is composed of several of these varying lines (or individuals).

We set up crosses between males from twenty-four lines to females of a different inbred line of genetically identical females carrying the w^{m4} allele. The stock of females carrying the w^{m4} allele on their X chromosomes also has an inversion spanning a portion of one second chromosome while the other features a deletion. Male progeny from this cross were raised on media with or without curcumin; observed differences in PEV subsequently reflect natural variation in chromatin state. We quantified responses to the drug by imaging the degree of variegation among the eyes of the males. We note that there were population-wide differences in the effect of curcumin on PEV consistent with there being differences among populations in allele frequencies for genes that influence chromatin modifications. In addition, we found that the response to curcumin was variable

among lines, also consistent with variation between the individuals (lines) in a population in genes such as histone deacetylases that are targeted by curcumin.

Materials and Methods

Drosophila melanogaster stock and populations

Experiments were performed using 85 isofemale derived, inbred *Drosophila melanogaster* lines previously collected from five geographic locations throughout the world (Beijing [17 lines], Netherlands [18 lines], Ithaca [14 lines], Tasmania [19 lines], and Zimbabwe [17 lines]) (Greenberg *et al*. 2011). These globally collected lines represent a sampling of the genetic diversity present within *D*. melanogaster. Additionally, stock 1712 [In(1)w^{m4}; Df(2L)2802/CyO], was obtained from the Bloomington Stock Center. Individuals from the 1712 stock harbor the w^{m4} allele on their X chromosomes. 1712 flies also feature a second chromosome deletion Df(2L)2802 (referred to as Df), with a deletion spanning 25F2-25F5; and the balancer Curly of Oster (*CyO*) on the other second chromosome. All flies were raised on standard fly medium and kept in an incubator at 25 °C on a twelve hour light-dark cycle.[1]

Experimental food and curcumin supplementation

Standard food was heated to about 38°C and separated into two batches. One batch was left unmodified (control) while 75 mM of curcumin, supplied by Sigma-Aldrich, was blended into the second (experimental) batch. Five mL portions of media from both batches were then pipetted into 30mL vials. Food was allowed to cool and solidify for one day.

Experimental populations

Five 1712 virgin females, aged five days, were mated to ten males from each globally collected line. Pairs were mated for 24 hours on standard food; mated females were then transferred to vials containing modified (75 mM of curcumin) or unmodified food. Females were allowed to lay eggs for three days and then removed from the vial. Resultant male progeny, aged 5 days, were then sorted according to a visible wing phenotype of curly (*CyO* chromosome) or non-curly (Df chromosome). Sorted flies were then frozen on dry ice. Males were stored at -80°C until imaging. All conditions were performed in duplicate (vial A and vial B).

Imaging and phenotype collection

Twenty-four experimental lines (Beijing [5 lines], Netherlands [5 lines], Ithaca [4 lines], Tasmania [5 lines], and Zimbabwe [5 lines]) were defrosted at room temperature and both left and right eyes were imaged. Individual eyes were imaged using a Canon EOS Digital Rebel XS mounted to a Nikon SMZ-10 microscope. Eyes were digitally isolated using a custom pipeline developed with the imaging software CellProfiler and Adobe Photoshop. The statistics package R was used to extract descriptive statistics (mean, median, and standard deviation) from the red, green and blue color channel distributions of each individual image, and Principal Component Analysis was used to reduce the dimensionality of the differences in eye pigmentation. Multiple individuals were imaged per experimental condition (mean = 15).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate groups based on eye pigmentation with the following model: PEV ~ vial + eye + deficiency + curcumin + population + line + d*p + d*l + d*c + d*p*l + d*p*c + d*l*c + c*p + c*l + c*p*l + d*p*l*c: where vial represents replicates (A vs. B), eye represents side of eye (left vs. right); deficiency represents second chromosome haplotype (deficiency vs. no deficiency); curcumin represents food conditions (curcumin vs. no curcumin); population represents geographic origin (B,I,N,T, or Z); and line represents individual lines from each of the geographic locations. "*" denotes interaction terms. Implementation and analysis of the model was performed using the statistics package R.

Results

The eye color phenotype of each individual male fly derived from the crosses performed of the 24 lines was analyzed by passing the collection of red, green and blue color intensities of all 15,000 pixels for each eye through a computer pipeline that generated summary statistics for each image. The high dimensionality of the data was reduced by applying Principal Component Analysis—a multivariate statistical technique that identifies a vector through the data points that explains the most variance (known as Principal Component 1, or PC1). Figure 1 displays the first two Principal components of all data points; PC1 and PC2 are built based on loadings with the loadings for PC1 being blue mean and green mean and the loading for PC2 being the red mean. Because every possible manifestation of eye pigmentation corresponds to a specific value in its red, green, and blue distributions, eye pigmentation of each individual can be considered a quantitative trait. Principal Component analysis, by assigning a numerical value to every pigmentation state possible, can then act to quantify the continuous trait of eye pigmentation. Thus, the biological phenomenon of natural variation along a spectrum of white to red eyes can be reflected in a plot of Principal Components.

Figure 1. Relationship between Principal Component values and eye pigmentation

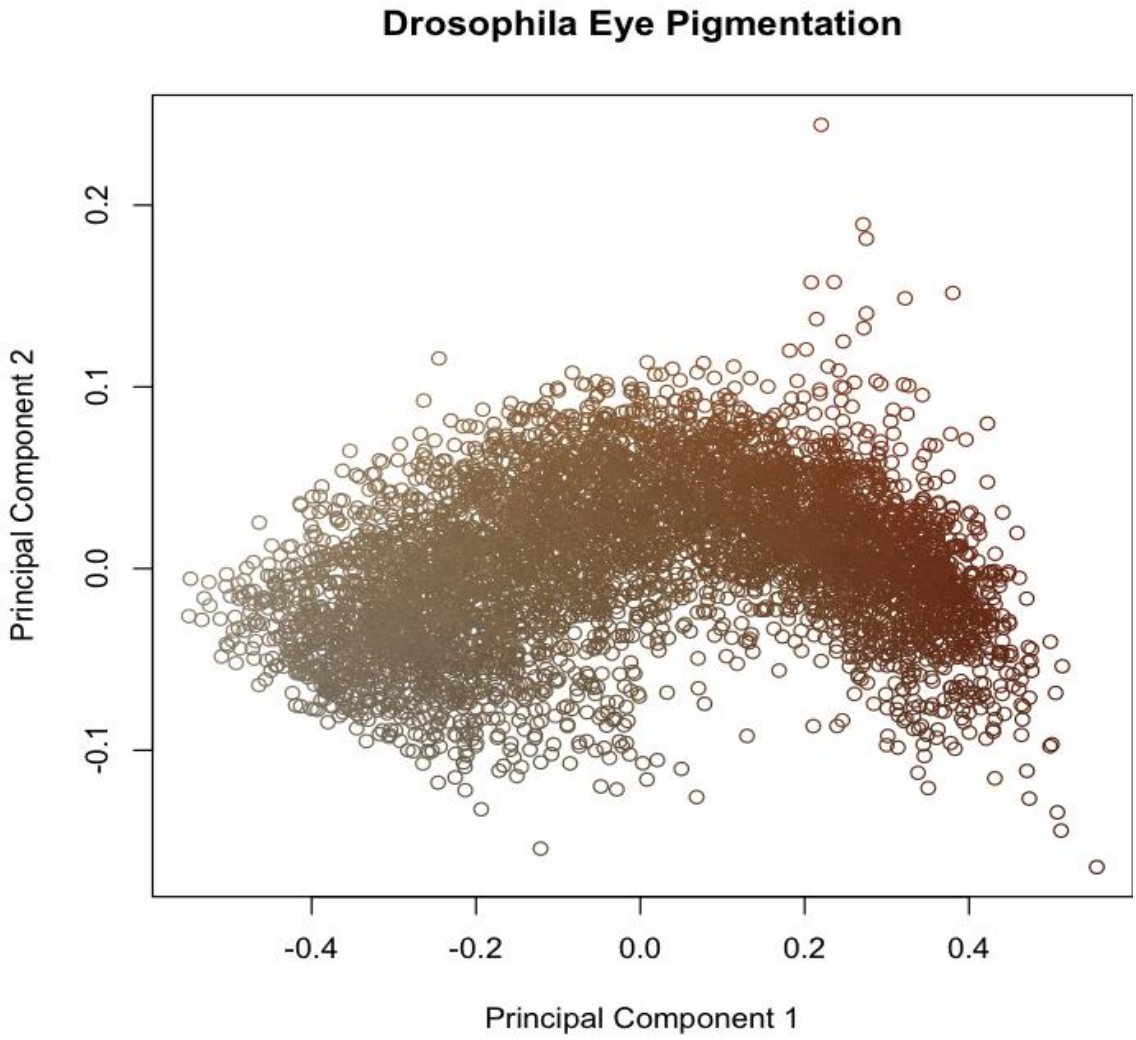


Figure is a scatter plot of Principal Component 1 values vs. Principal Component 2 values. Each point represents a single eye image (N = 5548) and the color of the point corresponds to the average of each red, green and blue color channel for the respective individual image. Flies with more variegation have a whiter eye phenotype, which corresponds to a lower PC1 value, while flies with less variegation and a redder eye phenotype have a higher PC1 value.

Analysis of variance (ANOVA) was used to evaluate groups based on eye pigmentation. The primary factors in this model include: "vial", which is the effect of the replicate vial growth conditions; "eye" which is the effect of the left vs. right eye on PEV; "deficiency" which refers to the Df(2L)2802 presence/absence among the segregating progeny of our test cross; and "curcumin" which is presence/absence of 75 mM of curcumin in the diet. The final ANOVA model is:

PEV ~ vial + eye + deficiency + curcumin + population + line + d*p + d*l + d*c + d*p*l + d*p*c + d*l*c + c*p + c*l + c*p*l + d*p*l*c,

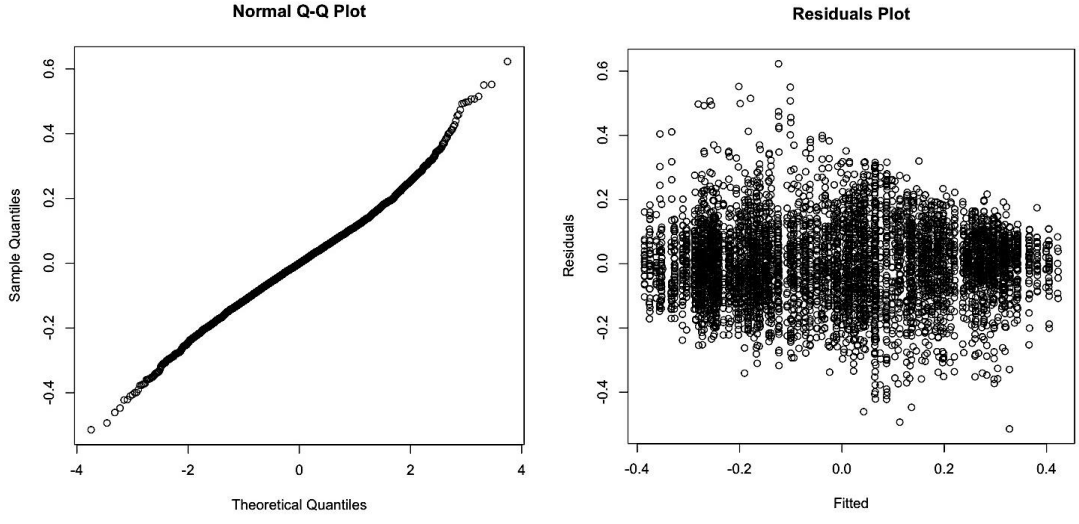
where (d) represents second chromosome haplotype; (c) represents food conditions; (p) represents geographic origin; and (l) represents individual lines. "*" denotes interaction terms. Implementation and analysis of the model was performed using the statistics package R.

Table 1. ANOVA Results

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
PEV ~ vial	1	0.018	0.018	1.2183	0.2697501
PEV ~ side	1	0.808	0.808	53.3585	3.179e-13
PEV ~ deficiency	1	137.122	137.122	9054.2616	< 2e-16
PEV ~ curcumin	1	19.608	19.608	1294.7008	< 2e-16
PEV ~ population	4	26.984	6.746	445.4420	< 2e-16
PEV ~ line	19	41.488	2.184	144.1838	< 2e-16
PEV ~ deficiency*population	4	3.160	0.790	52.1582	< 2e-16
PEV ~ deficiency*line	19	5.138	0.270	17.8565	< 2e-16
PEV ~ deficiency*curcumin	1	0.013	0.013	0.8461	0.3577053
PEV ~ curcumin*population	4	0.303	0.076	4.9975	0.0005093
PEV ~ curcumin*line	19	3.345	0.176	11.6264	< 2e-16
PEV ~ deficiency*curcumin*population	4	2.643	0.661	43.6373	< 2e-16
PEV ~ deficiency*curcumin*line	19	3.014	0.159	10.4744	< 2e-16
Residuals	5450	82.537	0.015		

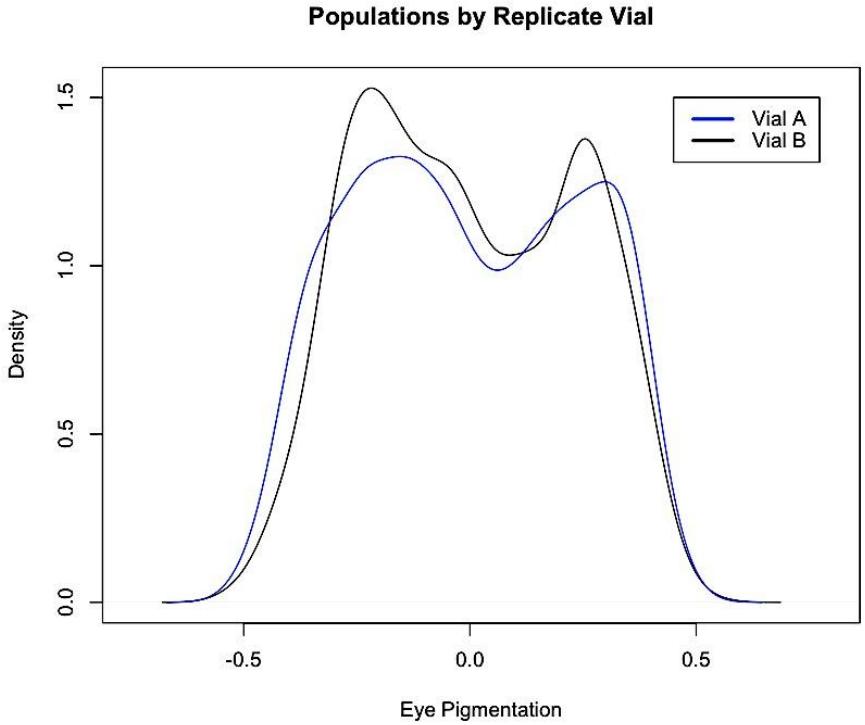
Before trying to interpret the P-values that the statistical tests produce, it is essential to assess whether the statistical model adequately fits the data. We did this by first examining the quantile-quantile plot, or "qq-plot," and secondly, by examining the distribution of residuals to the model fits. If the data (in this case, PC1 values) are normally distributed, then the qq-plot should have an array of points that follow the diagonal. As **Fig. 2** shows, the fit to normality is excellent. The residual plot also looks like the model is very well behaved, in that there is no strong trend to the residuals as a function of the fitted values. There were 38 values with unusually large residuals, implying they were outliers from the model. We attempted to assess the importance of these outliers by simply removing them and rerunning the analysis. This had little to no effect on the inferred P-values.

Figure 2. Q-Q Plot and plot of residuals



When we compare microenvironment replicates (vial A vs. vial B), we do not notice any significant differences between distributions **(Table 1 and Fig. 3)**. This proves there exists no vial effect as the observed reaction to modified environments between flies in vials A and B are very similar. Thus, observed PEV changes are due to actual biological determinants and are not simply stochastic.

Figure 3. Distributions of intra-individual and environmental replicates

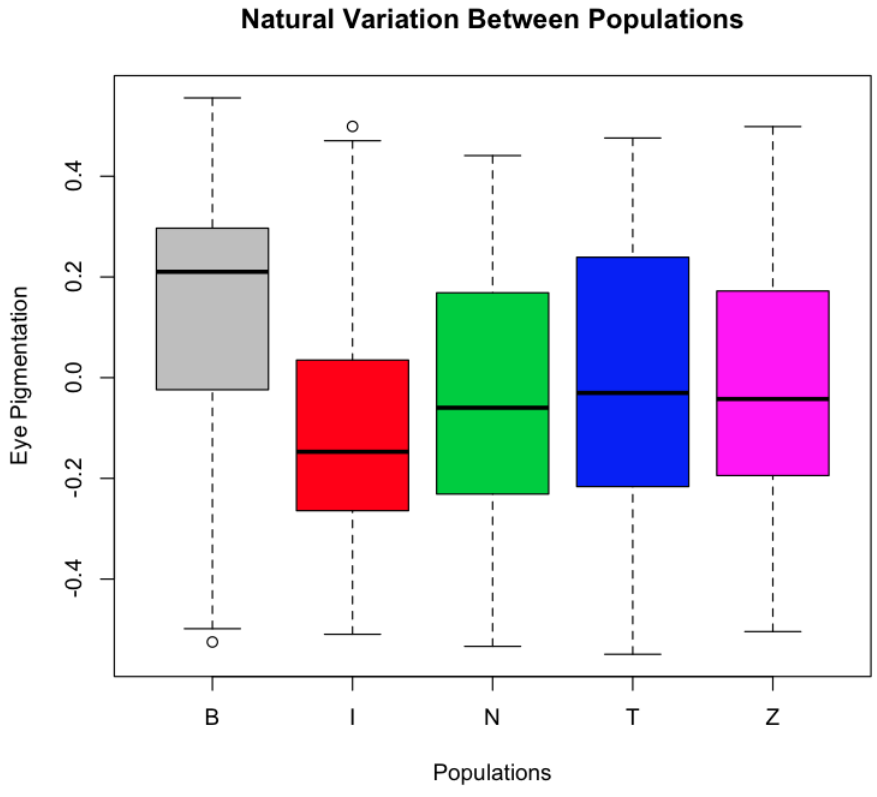


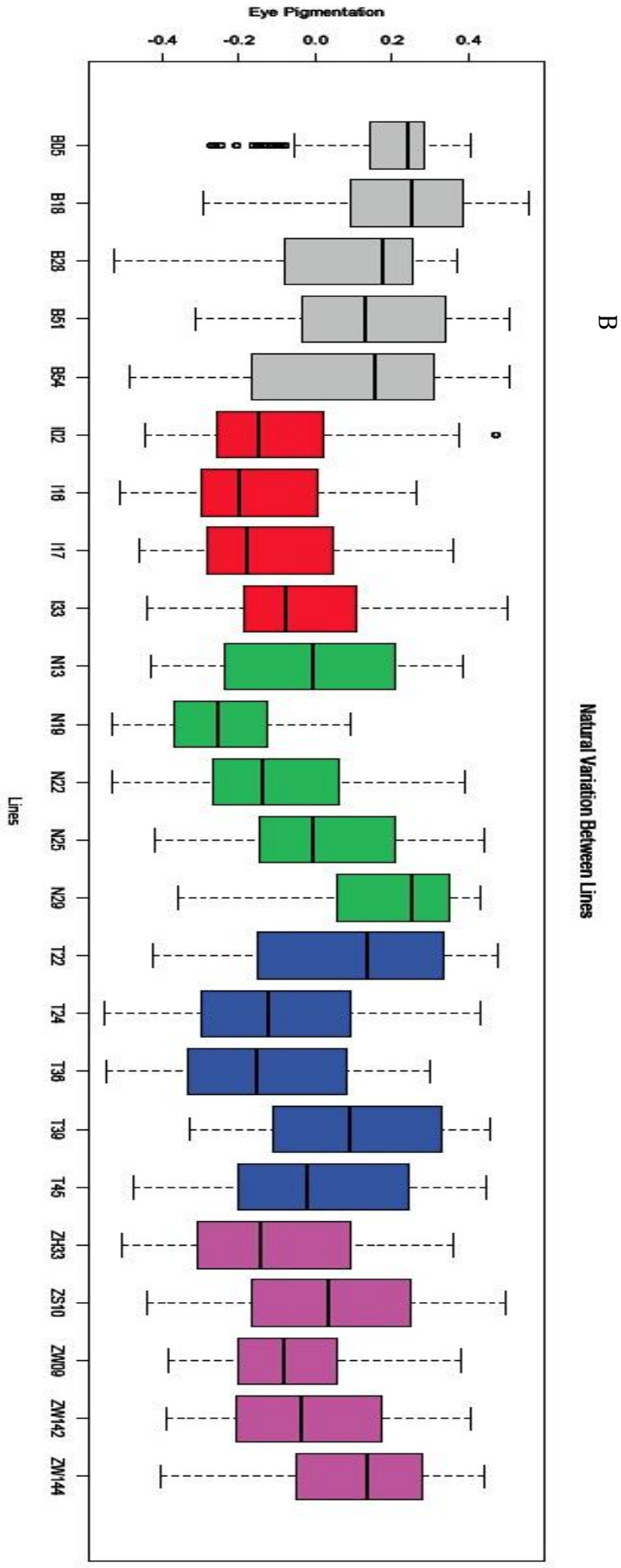
Figures show the distribution (density) of pigmentation values across replications. Lower pigmentation values represent greater variegation of the w^{m4} locus and higher pigmentation values represent less variegation. Replicates of microenvironment (vial A vs. vial B) were made for each genetic grouping. ANOVA indicates microenvironment has little effect on eye pigmentation ($F = 1.2183$, $p = 0.2697501$).

Twenty-four lines spanning five populations were imaged with the goal of quantifying natural genetic and subsequent epigenetic variation in PEV. What was unknown was the degree to which lines within the same population would vary from one another when compared to lines from other populations. ANOVA indicates a significant variance between lines spanning the five populations **(Fig. 4B)** as well as between the populations themselves **(Fig. 4A)**. "Further, this phenotypic variance was observed to be considerably greater than variance in eye color of the original 1712 stock (data not shown)." This indicates that variation between populations exceeds variation within them **(Table 1 and Fig. 4B)**.

Figure 4. Mean eye pigmentation values among lines and populations

A




Eye Pigmentation
-0.4
-0.2
0.0
0.2
0.4
B05
B18
B28
B61
B64
I02
I16
I17
I33
N13
N19
N22
N25
N29
T22
T24
T36
T39
T45
ZH33
ZS10
ZW09
ZW142
ZW144
Lines
Natural Variation Between Lines

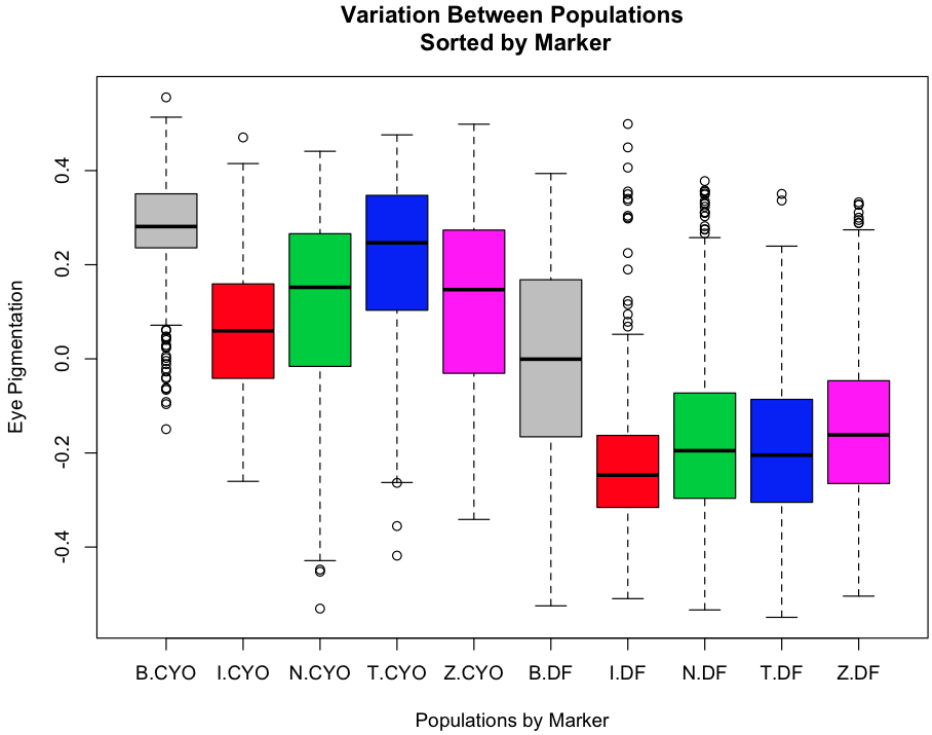
B

Figures show the distribution of pigmentation values across lines and the populations into which lines are grouped. **A)** Comparison of pigmentation values between populations. ANOVA indicates significant variance between populations in pigmentation ($F = 445.4420$, $p < 2 \times 10^{-16}$). **B)** Comparison of pigmentation values between lines. The same is reflected when the individual lines of populations are examined ($F = 144.1838$, $p < 2 \times 10^{-16}$).

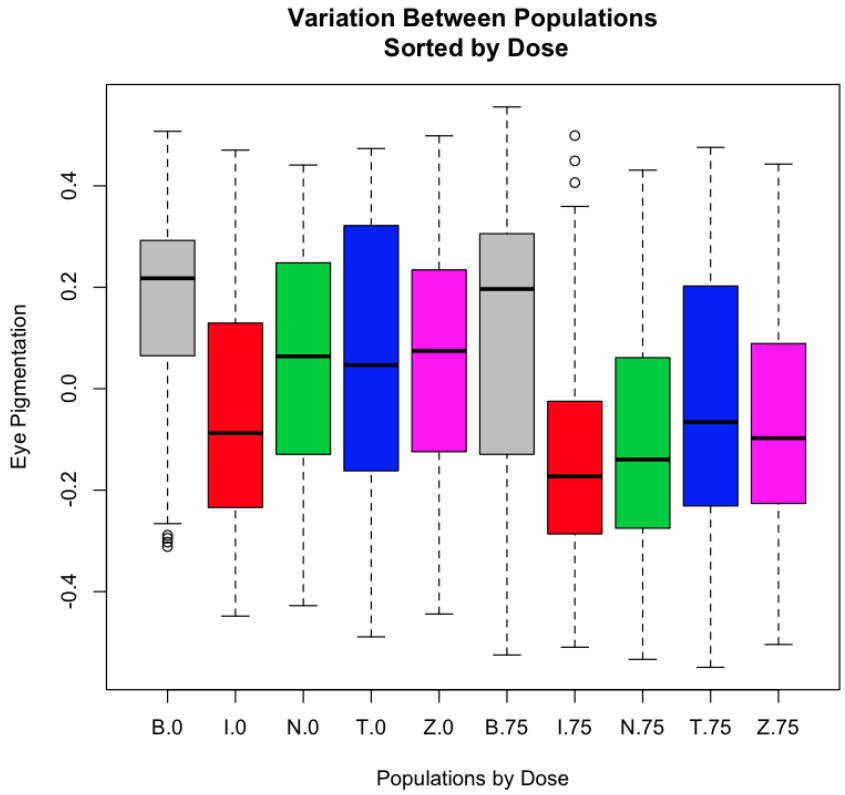
With the knowledge that the populations exhibit natural variation in PEV, the next question is how the second chromosome deficiency may affect PEV; how the curcumin dosage affects PEV; and whether the populations vary in their PEV response to these two conditions in consistent ways. The second chromosome deficiency (Df(2L)2802) spans a few Su(var) and E(var) loci, but the point of our interest in the deficiency is that it simply represents a different degree of sensitization to PEV. Possessing deficiency versus the balancer chromosome does appear to exert an influence over PEV **(Fig. 5A)** across populations. The same appears to be true of the dosage of curcumin administered **(Fig. 5B)**. ANOVA indicates a slight difference in distributions among populations when grouped by deficiency but less so when grouped by curcumin dosage **(Table 1)**. Plots of the means of populations grouped according to deficiency show that the flies with the Df(2L)2802 chromosome show enhanced variegation compared to those with the *CyO* balancer chromosome **(Fig. 5A)**. Similarly, plots of the means of populations grouped according to curcumin dosage indicate that the flies given 75mM of curcumin show enhanced variegation compared to those given no curcumin **(Fig. 5B)**.

Figure 5. Mean eye pigmentation values among populations grouped by deficiency and curcumin dosage

A



B



Figures show the distribution of pigmentation values across populations based on curcumin dosage (0 or 75mM) and second chromosome marker (*CyO* or Df). **A)** Comparison of pigmentation values between population with either *CyO* or Df haplotypes. ANOVA indicates slight variance between populations in pigmentation as influenced by second chromosome genotype ($F = 52.1582$, $p < 2 \times 10^{-16}$). **B)** Comparison of pigmentation values between populations with either unmodified food or food dosed with 75mM curcumin. ANOVA indicates that, there exists a Population × Curcumin effect ($F = 4.9975$, $p = 0.0005093$), implying that the effect of curcumin on PEV is significantly variable across populations.

It is apparent that both the second chromosome deficiency and the curcumin dosage can affect PEV; however, whether each population was reacting in a similar way to the same second chromosome deficiency and same dosage of curcumin was unknown. It appears that all but one population (Tasmania) has predicted interactions (the interactions that would be predicted by the results from the other four populations) between the second chromosome deficiency and mean eye pigmentation **(Fig. 6)**. Likewise, all but one population (Netherlands) has predicted effects of curcumin dosage on mean eye pigmentation **(Fig. 7)**.

Figure 6. Interaction plot of mean eye pigmentation among populations grouped by deficiency

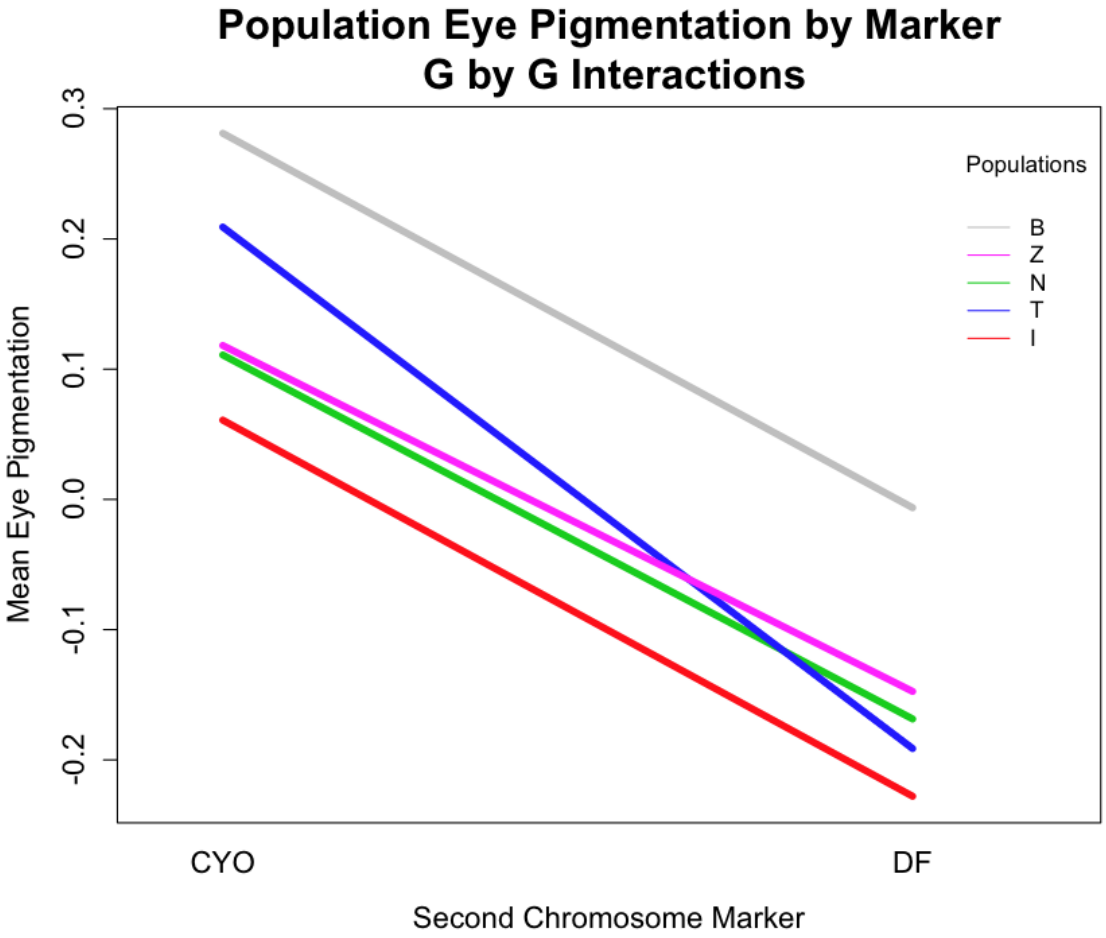


Figure shows the distribution of pigmentation values across populations based on second chromosome marker (*CyO* or Df). The interaction of the population used with the second chromosome marker and the subsequent effect on mean pigmentation values can be observed. Analysis of variance indicated that there was a significant Population × Deficiency interaction ($P < 2 \times 10^{-16}$).

Figure 7. Interaction plot of mean eye pigmentation among populations grouped by curcumin dosage

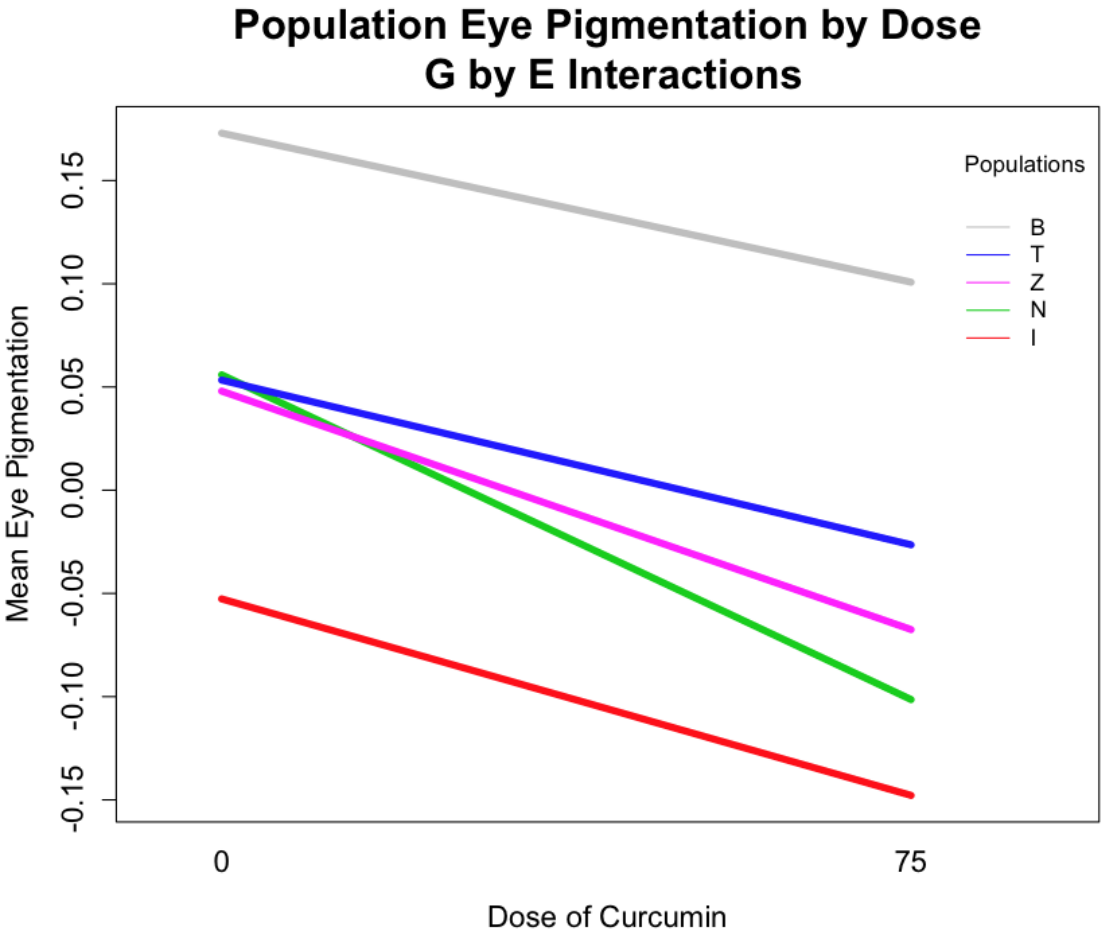


Figure shows the mean pigmentation values across populations based on curcumin dosage (0mM or 75mM). The interaction of population with dosage and the subsequent effect on mean pigmentation values can be observed. ANOVA indicated that the Population × Curcumin interaction was somewhat significant ($P < 0.0005$); this indicates that all populations responded to curcumin in somewhat different ways as indicated by the changes in slope of the lines in the figure.

Interaction plots indicate that most individual lines are reacting in a similar way to both curcumin **(Fig. 9)** and the chromosomal deficiency **(Fig. 8)**, with one notable exception. All but one line, Beijing 18, has predicted effects of curcumin dosage on mean eye pigmentation **(Fig. 9)**.

Figure 8. Interaction plot of mean eye pigmentation among lines grouped by deficiency

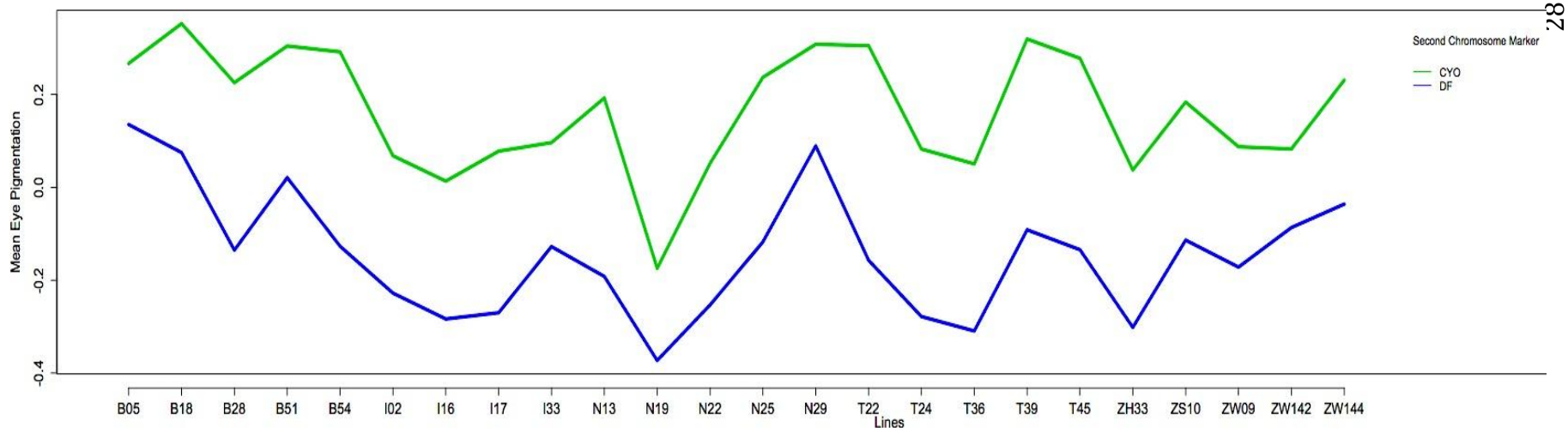


Figure 9. Interaction plot of mean eye pigmentation among lines grouped by curcumin dosage

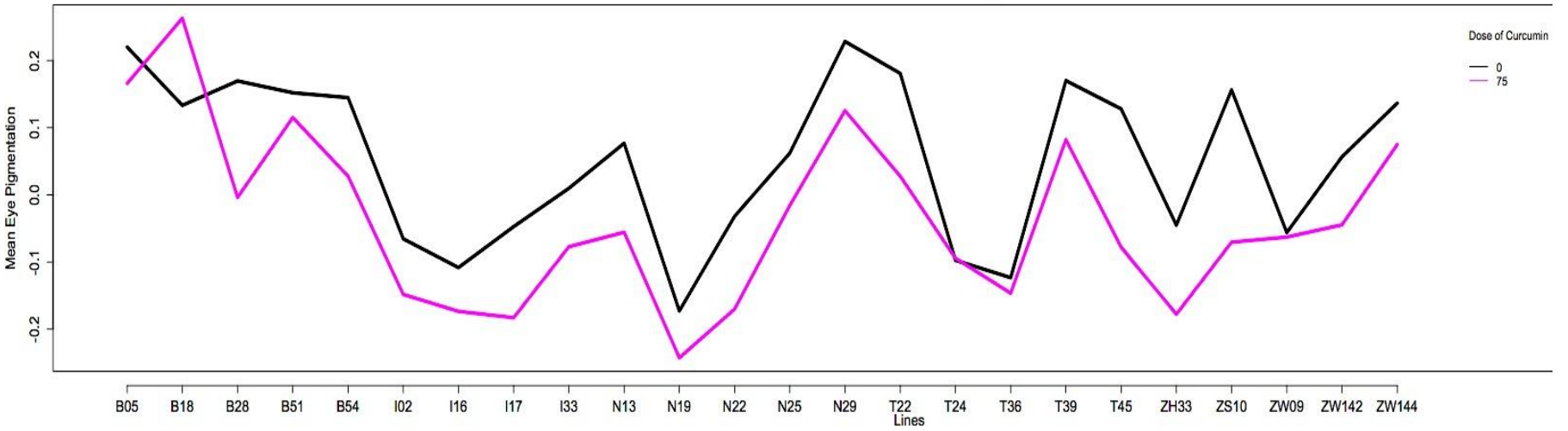


Figure 8 shows the distribution of pigmentation values across lines based on second chromosome marker (*CyO* or Df). The interaction of the line used with the second chromosome marker and the subsequent effect on mean pigmentation values can be observed. Analysis of variance indicated that there was a slight Line × Deficiency interaction ($P < 2 \times 10^{-16}$).

Figure 9 shows the distribution of pigmentation values across lines based on curcumin dosage. The interaction of the line used with the dosage of curcumin and the subsequent effect on mean pigmentation values can be observed. Analysis of variance indicated that there was a very slight Line × Curcumin interaction ($P < 2 \times 10^{-16}$).

Discussion

Observed effect of curcumin on PEV

It was observed that the individuals reared on the medium with curcumin present, on average, had lighter eyes than those reared on unmodified medium **(Fig. 5B)**. Curcumin appears to silence the expression of the w^{m4} allele, enhancing PEV. This suggests that curcumin acts to inhibit the action of HATs at the site of the gene, leading to enhanced PEV. This result is consistent with Marcu *et al.*'s finding that the mechanism of action of curcumin is through the specific inhibition of the p300/CBT HAT proteins (Marcu *et al.* 2006).

Genotype × environment interactions among populations

An interaction between the genotype and the environment, or dosage of a drug administered, arises from the differential influence environment has on gene expression among populations. ANOVA indicated that both the Population × Curcumin and Line × Curcumin interactions were only somewhat significant. This suggests that most populations and lines respond to curcumin similarly. Interaction plots and statistical testing have allowed us to note the occurrence of interaction effects between individual genotypes and the environment. Interaction plots allow for the visualization of mean pigmentation grouped by population and curcumin dosage. Assuming that all populations, despite natural genetic variation, will respond to the same environmental condition in the same manner, the lines of this plot should not cross and the slope of each line should be equal and negative. This assumption holds true across all populations with the exception of the population derived from the Netherlands **(Fig. 7)**. Males of this population seem to respond to

the same dose of curcumin as males in the other four populations with a greater enhancement of PEV. This indicates that, to some extent, the genetic variation of individuals in this population leaves them more susceptible to the effect of curcumin in preventing gene transcription at the w^{m4} locus.

Genotype × environment interactions among lines

Similarly, when an interaction plot of Line × Curcumin is observed, curcumin appears to be enhancing PEV in a predictable manner in most but not all lines. In fact, in one line in particular, Beijing 18, the data suggests that curcumin may be suppressing PEV, as the mean eye pigmentation when treated is redder than when not treated **(Fig. 9)**. In this case, an individual line's natural genetic variation has given rise to a characteristic chromatin state that causes it to react to the same environment as other individuals in an opposite manner.

Observed effect of deficiency on PEV

In addition to the genotype interacting with the environment, the genome can interact with itself to modify gene expression. *Drosophila* with no other genetic differences than the *CyO* inversion or Df(2L)2802 deletion present on the second chromosome seem to be epigenetically distinct at the site of the w^{m4} allele on the X chromosome. *Drosophila* with the Df(2L)2802 marker, across all populations, on average have a lighter eye when compared to those with the *CyO* chromosome **(Fig. 5A)**. It is unclear whether the Df chromosome's association with enhanced PEV is due to its genetic *E(var)* properties, or the intrinsic *Su(var)*properties of the *CyO*

chromosome, or some other factor—both of which could be a possibility based on our observations.

Genotype × genotype interactions among populations and lines

Since the genes present on or absent from) the second chromosome seem to be affecting PEV, we can then ask whether all populations are experiencing a similar effect. Analysis of variance indicated that there was a significant Population × Deficiency interaction ($P < 2 \times 10^{-16}$). It is apparent that, in the population derived from Tasmania, when compared to individuals from the other five populations, the deletion on the second chromosome is leading to a more pronounced enhancement of PEV **(Fig. 5A)**. Our observation that natural variation among members of a population can lead to dramatic, characteristic effects on how chromatin state can be altered by the genome or by the environment can then lead us to question to what degree, if at all, these three determinants of PEV can interact. Analysis of variance indicates that there exists a significant Population × Deficiency × Curcumin interaction ($P < 2 \times 10^{-16}$). This suggests that natural variation gives rise not just to differences in chromatin state that can mediate reactivity: to genomic enhancers/suppressors of PEV, or to environmental enhancers/suppressors of PEV, but also to how the two combine to produce phenotypes.

Significance of our findings: Natural genetic variation gives rise to functionally important epigenetic variation

Studying variation at the epigenetic level allows for a greater understanding of how natural genetic variation can give rise to epigenetic variation and how that

epigenetic variation is reflected in reactions to environmental stimuli. The finding that genome-wide heterochromatin levels and the presence of specific loci that affect variegation can influence chromatin state stresses the importance of the genome itself in affecting gene expression and resulting phenotypes. This implicates genomic variation in the variation individuals display in their interactions with their environment. This response to environmental conditions, that can be differentially mediated by the epigenome, can be beneficial or detrimental depending on the nature of the reaction. Thus, its basis in genetic material allows the degree of reactivity one shows to environmental conditions to be capable of undergoing selection, with individuals able to respond appropriately receiving a selective advantage.

PEV has mostly been studied at the molecular level, leaving the characterization of variation in PEV in a population relatively unexplored. Using the Global Diversity lines of *D. melanogaster* allowed us to observe natural variation involved in epigenetic modifications. We found that lines derived from different populations, possessing natural genetic variation, subsequently exhibit natural epigenetic variation as evidence by variation in PEV **(Fig. 3A)**. It appears that natural genetic variation is capable of giving rise to functionally significant natural epigenetic variation as evidenced by interaction plots and that this natural epigenetic variation is susceptible to differentials degrees of alterations by the chromatin-modifying drug curcumin (Fig. 6, 7 & 9).

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[1]http://flybase.org/reports/FBst0001712.html

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