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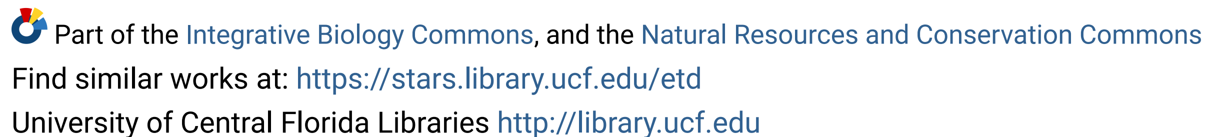

THE ROLE OF THE Y-CHROMOSOME IN THE EVOLUTION OF AUTOSOMALLY CODED TRAITS

by

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B.S. Florida State University, 2008

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
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ABSTRACT

Recent work indicates that the Y-chromosome of the fruit fly *Drosophila melanogaster* can influence gene regulation on the autosomes and X chromosome. This newly discovered function of the Y has the potential to dramatically shape the regulatory evolution of numerous genes that reside throughout the genome; even for genes that code for both male and female traits. Given that the mechanism underlying the Y-linked influence on gene expression in *D. melanogaster* appears to exist in other independently evolved heterogametic sex chromosomes, the evolutionary implications of Y-linked regulatory variation (YRV) deserves to be explored. These implications include the potential for Y-chromosomes to facilitate the adaptive evolution of sexually dimorphic gene expression, and the potential for the Y to constrain evolutionary rates in both males and females (depending on the nature of the YRV effect). Unfortunately, the evolutionary implications of this potentially widespread and significant phenomenon have yet to be explored. My dissertation addresses this knowledge gap by determining the influence YRV has on the evolution of autosomally coded traits in *D. melanogaster*. First, we address the potential for selection to shape YRV by determining if YRV (i) exists within natural populations (i.e. where natural selection operates), and (ii) has any influence on male fitness-related autosomal traits. Second, we address if YRV can facilitate the adaptive evolution of sexually dimorphic gene expression by testing for the presence of Y-linked additive genetic variation. To this end, we investigate the physiological properties of select Y-chromosomes across multiple genetic backgrounds. Third, we address if YRV can constrain adaptive evolution for autosomally coded traits by employing artificial selection on replicate populations that contain either multiple Y-chromosomes (i.e. contain YRV) or only a single Y-chromosome (no YRV).

The following studies present evidence that YRV does exist within populations where natural selection operates. We show significant levels of YRV on X-linked and autosomal immune gene expression in wild caught *D. melanogaster* from a single natural population. Furthermore, YRV effects on immune related genes show a significantly positive correlation to a male fly's ability to fight an immune challenge (an important aspect of organismal fitness). Estimated physiological properties of YRV support previous interpopulation studies showing strong non-additive effect dependent on the autosomal genetic background with which Y-chromosome's are paired with. Physiological epistasis can manifest as additive genetic variation on a population level, but our experimental evolution study suggest that YRV constrains rather than facilitates the evolution of the autosomal coded geotaxis behavior. Ultimately, this dissertation provides evidence that YRV has the potential to influence how autosomal traits evolve and that population level studies of YRV indicate a potential constraint to the adaptive evolution of autosomal traits. If these trends are common and YRV is a wide spread phenomenon, Y-chromosomes have the potential to influence how autosomal traits evolve.

For my family.

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CHAPTER 1: INTRODUCTION

Y-chromosome Evolution

The origin and contemporary maintenance of Y-chromosomes has been a major focus of evolutionary research for the past several decades. The current paradigm states that Y-chromosomes evolve from an ordinary autosome after the foundation of a sex determining locus (SDL) (Bull, 1983, Charlesworth, 1996). Sexually antagonistic genes, specifically genes that have alleles that are beneficial in males but detrimental in females, achieve a fitness boost when they become physically linked to the dominant male determining allele at the SDL. Male beneficial variants will accumulate due to their linkage with the male determining allele even when they are highly deleterious in females. Recruitment of more sexually antagonistic alleles to this region of the genome creates a genetic load for females as these variants will likely cross over to the X-chromosome at increasing rates. This genetic load is hypothesized to be the force that drives the development of repressed recombination of regions surrounding the SDL (Rice, 1996). The lack of recombination of some or most of the Y-chromosome and its unisexual inheritance are two characteristics that make the Y-chromosome unique in the genome (Rice, 1996). These two forces combine to result in an increased mutation rate resulting in loss of gene function (Muller, 1918, Muller, 1932). As a consequences of the Y-chromosomes unique evolutionary history, even independently evolved Y-chromosomes share common characteristics. Most old Y-chromosomes contain few protein coding genes and are largely heterochromatic comprised of densely packed tandemly repeated elements (Bachtrog, 2013). Additionally, they typically have low levels of nucleotide variation in their few remaining protein coding regions (Ashburner et al., 2005b, Zurovcova & Eanes, 1999).

Y-linked Regulatory Variation

Though Y-chromosomes are predicted to have minor influence on trait variation due to their few protein coding regions with reduced nucleotide variation, multiple *Drosophila melanogaster* studies in the late 90’s and early 2000’s observed detectable Y-chromosome effects on many traits not known to be

coded for on the Y. The *D. melanogaster* Y-chromosome has been shown to influence male fitness (Chippindale & Rice, 2001a), temperature sensitivity (Rohmer et al., 2004), behavior (Stoltenberg & Hirsch, 1997b), and longevity (Griffin et al., 2015). These findings were unexpected as theory suggested that the maintenance of any sequence polymorphisms on the Y might be limited (Clark, 1987), and empirical evidence showed a lack of protein-coding sequence variation in *Drosophila* populations (Zurovcova & Eanes, 1999). Bernardo Lemos and colleagues found evidence to reconcile these apparently contradicting pieces of evidence when they discovered that variation in the non-coding regions of Y-chromosomes influence the expression of hundreds of autosomal and X-linked genes (Lemos et al., 2010a). The proposed mechanism for this phenomenon is attributed to the great deal of structural polymorphism in the Y's non-coding regions (Lyckegaard & Clark, 1989a) and their downstream influence on the global chromatin structure of the X-chromosome and autosomes. Given that most heterogametic sex chromosomes are largely heterochromatic and share similar evolutionary histories, this effect may be wide spread across taxa with heterogametic sex chromosomes. Evidence for YRV in independently evolved Y-chromosomes has only been investigated in laboratory mice where Y-chromosomes have been shown to influence the transcriptomes of immune cells (Case et al., 2013).

Consequences of Y-linked Regulatory Variation

Ultimately, YRV provides a source of sex specific variation in X-linked and autosomal gene expression that could have some interesting implications for how autosomally coded traits evolve. Given that greater than 50% of the genome is differentially expressed between males and females (Meiklejohn et al., 2003a, Ranz et al., 2003), sexual conflict seems to be abundant throughout the genome. If selection could shape the Y-linked regulatory effect, YRV may provide a simple and untested mechanisms for the evolution of sexually dimorphic gene expression. However, if YRV cannot be shaped by natural selection, and is instead comprised of largely unpredictable non-additive effects, YRV may reduce the efficiency of selection while the X-chromosomes and autosomes are in the male background.

In order for YRV to have any influence on the adaptive evolution of autosomal traits, it must first exist within natural populations and influence fitness related traits. To date, these fundamental attributes of YRV have not been tested. In the first chapter of this dissertation, I test for YRV within a natural population of *Drosophila melanogaster* and its influence on immune function, a trait linked to organismal fitness. In order for selection to adaptively shape YRV, Y-chromosomes must provide additive genetic variation for autosomal traits. In the second chapter of this dissertation, I estimate the physiological effects of Y interactions with autosomal genes across two immune phenotypes to gain insight into the additive and non-additive effects attributed to the Y-chromosome. Finally, additive and non-additive genetic variance components are products of a population, not a set of interacting genetic elements. To determine how YRV influences genetic variation at the population level, in chapter three, I impose laboratory selection to artificial populations of fruit flies containing Y-chromosome variation or no Y-chromosome variation to determine the potential evolutionary consequences of YRV.

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CHAPTER 2: Y-LINKED VARIATION FOR AUTOSOMAL IMMUNE GENE REGULATION HAS THE POTENTIAL TO SHAPE SEXUALLY DIMORPHIC IMMUNITY[1]

Introduction

Males and females often exhibit different fitness optima for shared phenotypes, which drives the evolution of sexual dimorphism (Andersson, 1994). However, males and females share a genome, which ultimately constrains the evolution of dimorphism by not allowing sex-specific gene divergence (Griffin et al., 2013a). Despite this constraint, dimorphism does evolve and recent studies have shown that the majority of X and autosomal genes can be differentially regulated between the sexes (Meiklejohn et al., 2003b, Ranz et al., 2003, Jiang et al., 2001, Rinn & Snyder, 2005). Unfortunately, the underlying molecular mechanisms by which sexually dimorphic gene regulation manifests and evolves are unclear.

Recent work by Bernardo Lemos and colleagues has shown that the Y-chromosome in *Drosophila melanogaster* can influence the regulation of hundreds of genes throughout the genome (Lemos et al., 2010b, Jiang et al., 2010b), suggesting that Y-chromosomes may in part facilitate sexually dimorphic evolution. The mechanism underlying this effect appears to be associated with variation in the Y-chromosome's non-coding heterochromatin, which influences the formation of euchromatin boundaries throughout the genome (i.e. areas where tightly packed heterochromatin meets loosely packed euchromatin, which influences gene regulation (Lemos et al., 2010b, Paredes et al., 2011, Francisco & Lemos, 2014)). The effect found in *Drosophila* may be a common phenomenon in most heterogametic systems, as Y-chromosomes tend to have large tracks of heterochromatin (Bachtrog, 2013) and at least one other independently evolved Y-chromosome appears to exhibit a similar effect (e.g. mice; (Spach et al., 2009a, Teuschert et al., 2006, Case et al., 2013)). Unfortunately, it is currently unknown if Y-linked regulatory variation (YRV) is widespread among independently evolved Y-chromosomes.

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More importantly, it is still unclear if YRV can influence the evolution of autosome or X-chromosome coded traits, as most YRV studies have focused on the molecular mechanisms underlying the phenomenon and not its evolutionary potential (Lemos et al., 2010b, Jiang et al., 2010b, Paredes et al., 2011). In order for YRV to influence the continued evolution of male and female shared traits, it must (i) exist within populations where selection operates and (ii) directly influence male fitness. Regrettably, the original work reporting the YRV phenomenon precluded the detection of within-population variation by using Y-chromosomes captured from disparate geographic regions (i.e. non-coevolving populations; (Lemos et al., 2010b, Lemos et al., 2008)). Moreover, no subsequent study has examined if co-evolving Y-chromosomes newly derived from the same population can influence fitness-related traits that are simultaneously expressed by both sexes (e.g. body size, development time, etc.).

Here, we assess the potential for YRV to influence the evolution of immune function in *D. melanogaster*. Immunity is a central physiological trait that is shared between the sexes and often exhibits dimorphism within numerous animal systems (Rolff, 2002). Sexually dimorphic immunity has likely evolved in these systems due to differences in pathogen exposure or infection rates between the sexes, or due to different life-history strategies (Zuk et al., 1990, Zuk & McKean, 1996, Poulin, 1996). In *D. melanogaster*, the regulation of immune-related genes is sensitive to the Y-chromosome (Lemos et al., 2010b) and immune gene expression has been shown to be dimorphic (Rolff, 2002, Winterhalter & Fedorka, 2009). However, it is still unknown if contemporary selection can shape YRV. In this study, we use RT-qPCR to determine if immune-related YRV exists within a wild population of *D. melanogaster.* We then assess if Y-linked variation influences a male's immune-related fitness. These hitherto untested criteria are essential if the Y-chromosome is to influence the continued evolution of sexually dimorphic immunity.

Methods

Fly Stocks and maintenance

In the fall of 2010, isofemale lines were established by collecting 40 gravid *D. melanogaster* females from a single location in Orlando Florida. These lines were maintained via strict single-pair sibling matings to produce genetic homogeneity within each line. Just prior to each assay outlined below, corresponding Y-lines were created for each isofemale line (Figure 1). Y-lines consisted of a genetically identical genome across lines, but a unique Y-chromosome within lines. That is to say, all Y-lines had the same X-chromosome and autosomes derived from a common isogenic stock and unique Y's that were derived from the above isofemale lines.

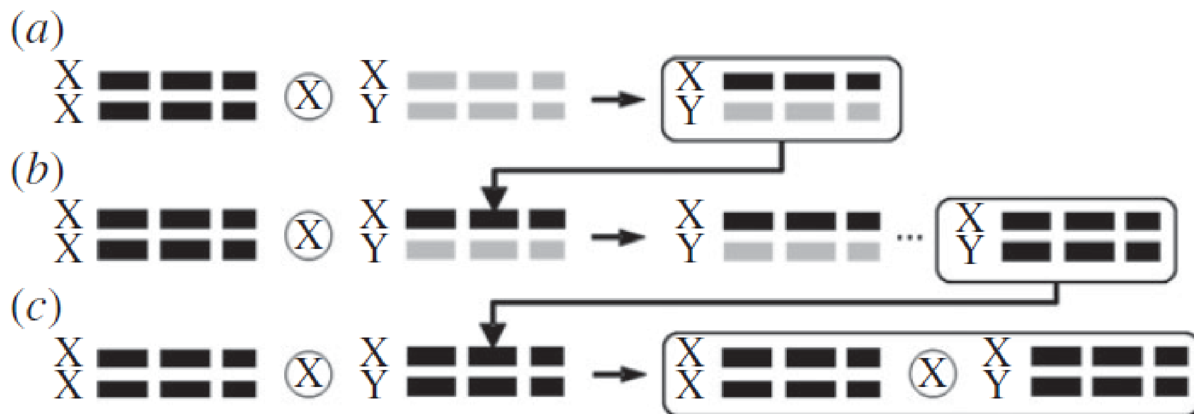

Figure 1. Establishment and maintenance of Y-lines. Here, the *Drosophila melanogaster* genome is depicted as 3 sets of autosomes (solid bars) and a pair of sex chromosomes (X and Y). Isogenic 4361 derived chromosomes with recessive markers on each chromosome are depicted in black, while the wild chromosomes from isofemale lines are depicted in grey. Y-lines were established by initially crossing an isogenic 4361 female to a male from each isofemale line (1a). The resulting heterozygote male F1 offspring were then backcrossed with 4361 females (1b), which produced a range of male genotypes represented by the ellipse. The F2 male offspring that exhibited all recessive markers (12.5% of the genotypes produced) were then used to establish the Y-lines, as they possessed a common isogenic background, but unique Y-chromosomes. These newly formed Y-line males were then maintained through continual backcrossing with 4361 females (1c).

To create the Y-lines, two crosses were conducted. The first cross paired a male from each isofemale line with a female from an isogenic stock (Bloomington Stock #4361; made isogenic with 10 generations of single-pair sibling matings prior to experimentation). The isogenic stock contained recessive markers on each chromosome (y[1]; bw[1]; e[4]; ci[1] ey[R]) allowing the detection of homozygous chromosome pairing. The resulting male offspring were all heterozygotes that contained a haploid set of isofemale autosomes and a haploid set of 4361 autosomes, as well as a 4361 X-chromosome (Figure 1a). These males were then backcrossed to 4361 to create a range of F2 genotypes. Males that possessed all of the recessive markers were collected to create the Y-lines, as they were genetically identical across lines, but contained a unique Y-chromosome derived from their original isofemale line (Figure 1b). Note that the F1 chromosomes are inherited intact, as male Drosophila do not undergo recombination during gametogenesis. The newly created Y-lines were then maintained by mating Y-line males with isogenic 4361 females (Figure 1c). All lines were maintained in vials on a cornmeal medium at 25°C 12:12 light:dark photoperiod using Percival incubators (Percival Scientific, Perry, IA).

YRV Assay for immune related genes

In the summer of 2011 (14 generations after the establishment of the isofemale lines), 30 isofemale lines were randomly chosen to create corresponding Y-lines and have their immune gene expression assayed. To remain consistent with the original work identifying the YRV effect (Lemos et al., 2010b), individuals were not immune-challenged prior to assessing immune gene regulation. From each Y-line, 20 male and 20 female offspring were collected upon adult eclosion and placed into sex-specific vials to ensure virginity. Four days after eclosion, males and females were placed in groups of 5 into a 1.5 µl microcentrifuge tube containing Trizol and disrupted using a motorized pestle. RNA was extracted according to the Trizol reagent protocol (Invitrogen, Carlsbad, CA), creating four independent RNA samples per sex per Y-line. RNA was then reverse transcribed using the iScript cDNA Synthesis kit (Invitrogen) and the resulting cDNA stored at -80°C. In total, 104 female samples and 111 male samples were used (25 samples were removed due to poor extraction results). If immune-related YRV exists

within a population, then Y-line males are predicted to differ in their immune gene expression while Y-line females should be the same.

To address our prediction, three immune effector genes associated with the IMD pathway were examined, including *attacin-A* (CG10146), *cecropin A1* (CG1365), and *diptericin* (CG12763). *Attacin-A* and *diptericin* are located on the second chromosome while *cecropin A1* is located on the third chromosome. These genes were chosen because they were shown to be influenced by across population YRV in a previous study (Lemos et al., 2010b). Gene expression was quantified using SYBR Green Supermix and the MyiQ Single Color Real Time qPCR Detection System (Bio-Rad, Hercules CA). All primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and gene sequences available from Flybase.org. The primer sequences are as follows: *attacin-A* (left - GGCCTGGATGGACGTGCTAA, right - GTTGGCAAACGGTCCACTCG), *cecropin A1* (left - CGTTGGTCAGCACACTCG, right - GACATTGGCGGCTTGTTG), and *diptericin* (left - AGAGTGCGTCGCCAGTTCCA, right - GGCTGTTGCCATAGGGTCCA). All primers exhibited PCR efficiencies above 95% and no off-target amplifications were detected. Expression estimates were normalized using the housekeeping gene *actin5C* (CG4027; left - TTGGGAATGGAGGCTTGCGG, right - AGCACGGTGTTGGCATACAGAT; PCR efficiency was 99.3%). For all female samples used in the analysis, *actin5c* CT-values ranged between 16.3 and 18.4, with a mean and standard error of 17.45 $\pm$ 0.12. Male *actin5c* CT-values ranged between 18.5-20.5, with a mean and standard error of 19.12 $\pm$ 0.11. Each 20μl qPCR reaction was run in triplicate. All technical replicates for a given gene were averaged prior to estimating gene expression. Immune gene expression estimates were generated by calculating the difference between the target gene's cycle threshold (CT) and *actin5c*'s CT within a given sample (i.e. we calculated each immune gene's ΔCT).

Functional Immune Response Assay

In the spring of 2013 (approximately 35 generations after the gene expression assay), 27 isofemale lines were randomly chosen to recreate the Y-lines (Y-lines from the YRV assay were destroyed after that assay). As above, virgin males and females from each newly established Y-line were collected after adult eclosion and maintained separately for four days. One male and female from each Y-line were then randomly chosen and injected with an LD_{90} of *S. marcescens* using a Nanoject II (Drummond Scientific, Bromall PA) under light CO_2 anesthesia (see below). After injection, each fly was placed in a Drosophila activity monitor (TriKinetics, Waltham MA) for 48 hours and their motion recorded every minute to establish an accurate time-to-death (TTD). The TTD assay was replicated twelve separate times (in series) and each Y-line replicate was derived from a new cohort of flies injected with a freshly reared bacterial solution. In total 212 females and 214 males were assayed across 27 lines. As with the YRV assay, if immune-related Y-linked variation translates into functional immune variation, then Y-line males are predicted to differ in their ability to defend against a live pathogen, while Y-line females should be the same.

Methods for infecting flies with *S. marcescens* were modified from Apidianakis & Rahme (Apidianakis & Rahme, 2009). In short, bacteria were incubated in sterilized LB broth for 18 hours at 37°C until log phase. This solution was diluted with sterile broth to an absorbance of 0.4 at 490 nm using a microplate reader (Bio-Rad Model 680, Hercules, CA, USA). 1 ml of the diluted solution was centrifuged at 11,000g for 2 minutes. The supernatant was discarded and 1ml of 10mM $MgSO_4$ wash was added to the remaining pellet and centrifuged at 11,000g for 2 minutes. Again, the supernatant was discarded and the pellet was re-suspended in 1ml of $MgSO_4$. Preliminary experiments showed that 90% of flies died within 48 hours of being infected with 60 nl of this concentration of $MgSO_4$ suspended bacteria. Negative controls injected with just the bacterial vehicle ($MgSO_4$) showed no mortality, indicating that *S. marcescens* was the source of death in these experiments. The *S. marcescens* used in this study was isolated from wild *Drosophila melanogaster* and graciously provided by B. Lazzaro.

Statistical Analysis

Relative immune gene expression values (ΔCT) were analyzed separately for each target gene via a random effects model ANOVA (REML method), with Y-line and replicate representing the independent random effects. The significance of each random effect was determined via a log likelihood ratio test. The sexes were analyzed separately, as female expression values serve as a methodological control (all females were isogenic) and we were uninterested in the effect of sex *per se* or its effect size. All Y-line by replicate interactions were not significant and were therefore not included in the final model. Considering that we examined three genes, we employed a sequential Bonferroni within each sex ($k = 3$) to minimize the potential for Type I error. Repeatability of our qPCR samples was high (0.91, 0.99, 0.96 and 0.85 for *actin5C, attacin-A*, c*ecropinA1* and *diptericin*, respectively). Repeatability was based on the intraclass correlation among our technical well replicates for three randomly chosen PCR plates (Sokal & Rohlf, 2012). To determine if expression levels of the different genes are correlated across Y-lines, we employed Pearson product moment correlation analysis based on family means, which provides an estimate of the genetic correlation between traits (Via, 1984).

Time-to-death values were analyzed separately for each sex using a proportional hazards model. Flies that survived the 48 hour period were censored and included in the analysis. Again, line by replicate interactions were not significant and were therefore not included in the final model. To examine the relationship between immune gene expression (2011 Y-line data set) and defense against *S. marcescens,* (2013 Y-line data set) we again employed Pearson correlation analysis based on Y-line means for those Y-lines used in both assays ($n = 19$ lines). Prior to correlation, gene expression data were transformed from their log based ΔCT values into the relative proportion of *actin5C* expression ($1/2^{(\Delta CT)}$).

Last, we estimated the intraclass correlation (ρ) for time-to-death and gene expression using a clonal analysis method (Lynch & Walsh, 1998). Prior to analysis, the influence of replicate was removed by generating the residuals between replicate and trait (i.e. gene expression or time-to-death). The resulting residuals were then used to estimate Y-linked genetic variation among Y-lines via a one-way

ANOVA. In this analysis, the intraclass correlation estimates the proportion of trait variation attributed to the Y-chromosome. Note that the small subsample of wild Y-chromosomes used in this study, coupled with the use of a single isogenic background, may downwardly bias intraclass correlation estimates. In contrast, pooling 5 isogenic individuals (i.e. clones) into each Y-line replicate sample may decrease our estimate of phenotypic variance, which can upwardly bias the intraclass correlation for the gene expression data (Krebs & Loeschcke, 1997, Krebs et al., 1998). Thus, the intraclass correlation estimates may not accurately reflect evolutionary potential in the original wild population. All analyses were conducted using JMPver10.

Results

If within population YRV exists for immune related genes, we predicted that Y-line males would exhibit differences in gene expression while Y-line females would not. We found that Y-line males did indeed exhibit differences in their gene expression for two of the three immune genes, *attacin-A* and *cecropin-A1* (Table 1). Furthermore, the proportion of gene expression variation attributed to the Y-chromosome was $\rho = 0.18$ for *attacin-A* ($p = 0.0150$) and $\rho = 0.20$ ($p = 0.0072$) for *cecropin-A1*. Y-line females showed no among-line variation for any of the immune genes. Male expression levels for all three genes were positively correlated (all $r > 0.79$ and all $p < 0.0001$) and Y-lines exhibited consistent expression across all three genes (e.g. consistently high or low; Figure 2). This is not surprising considering that these genes share the same regulatory pathway. These data indicate that immune-related YRV exists within the examined population.

Table 1. Evidence for within population Y-chromosome regulatory variation (YRV) for immune-related genes. As expected, Y-line males exhibited significant variation in immune gene expression for attacin A and cecropin-A1, while female Y-lines were not associated with expression variation. Significance of the random effects models was determined via log likelihood ratio tests (degrees of freedom = 1). Bolded values remain significant after a sequential Bonferroni test (k = 3).

Source	Var Comp	$\chi 2$	p <
Females			
Attacin			
Line	0.08	0.17	0.6766
Replicate	0.07	0.68	0.4102
Residual	3.04		
Cecropin			
Line	0.16	0.62	0.4296
Replicate	-0.05	0.55	0.4575
Residual	3.29		
Diptericin			
Line	-0.10	0.04	0.8415
Replicate	0.64	4.24	0.0395
Residual	8.41		
Males			
Attacin			
Line	0.51	6.25	**0.0124**
Replicate	0.27	9.44	**0.0021**
Residual	2.43		
Cecropin			
Line	0.43	7.10	**0.0077**
Replicate	0.32	20.7	**0.0001**
Residual	1.58		
Diptericin			
Line	0.43	0.90	0.3428
Replicate	0.48	5.39	**0.0202**
Residual	6.55		

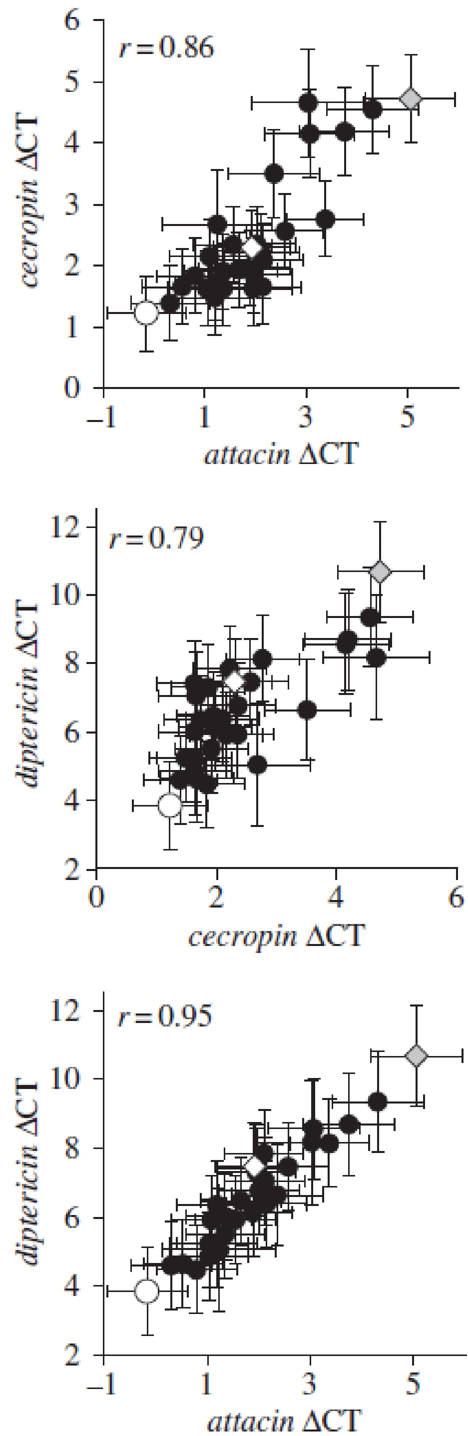

Figure 2. Correlation between immune gene expression levels across male Y-line means. All three immune genes were positively correlated (all $p < 0.0001$) and Y-lines exhibited consistency in their expression across the genes. For clarity, the Y-lines with the highest, lowest and middle levels of attacin-A expression have been identified on all three graphs (open circle, gray diamond and open diamond, respectively). Error bars represent standard errors of Y-line means.

As with the immune gene assay, if Y-line males but not females exhibit variation in their ability to defend against a pathogen, then it would suggest that YRV influences immune-related fitness. Accordingly, we found that only Y-line males differed in their ability to defend against the gram-negative bacteria *S. marcescens* (Table 2). The proportion of variation in time-to-death explained by the Y-chromosome was $\rho = 0.05$ ($p = 0.0583$). Moreover, we found a significant positive correlation between immune gene expression and defense against *S. marcescens* across male Y-lines for *attacin-A*, *cecropin-A1* and *diptericin* ($r = 0.64$, $p = 0.0029$; $r = 0.60$, $p = 0.0071$; $r = 0.57$, $p = 0.0109$, respectively). We found no significant relationship across female Y-lines (all $p > 0.2703$; Figure 3). These data suggest that Y-chromosomes that induce high baseline levels of immune gene expression in males also induce a greater defense against *S. marcescens* infection. Importantly, the lack of a female correlation does not imply that variation in female immune gene expression is unassociated with immune defense. Rather, it indicates that female Y-lines did not differ significantly in immune gene expression, which resulted in no significant relationship between expression and survival.

Table 2. Evidence for within-population Y-chromosome variation for defense against a bacterial pathogen. As expected, Y-line males exhibit variation in their time-to-death after infection with *Serratia marcescens*, while Y-line females exhibited no variation.

Source	DF	$\chi 2$	P <
Female			
Model	37	59.46	0.0110
Line	26	31.00	0.2281
Rep	11	31.24	0.0010
Male			
Model	37	74.00	0.0003
Line	26	41.95	0.0248
Rep	11	45.59	0.0001

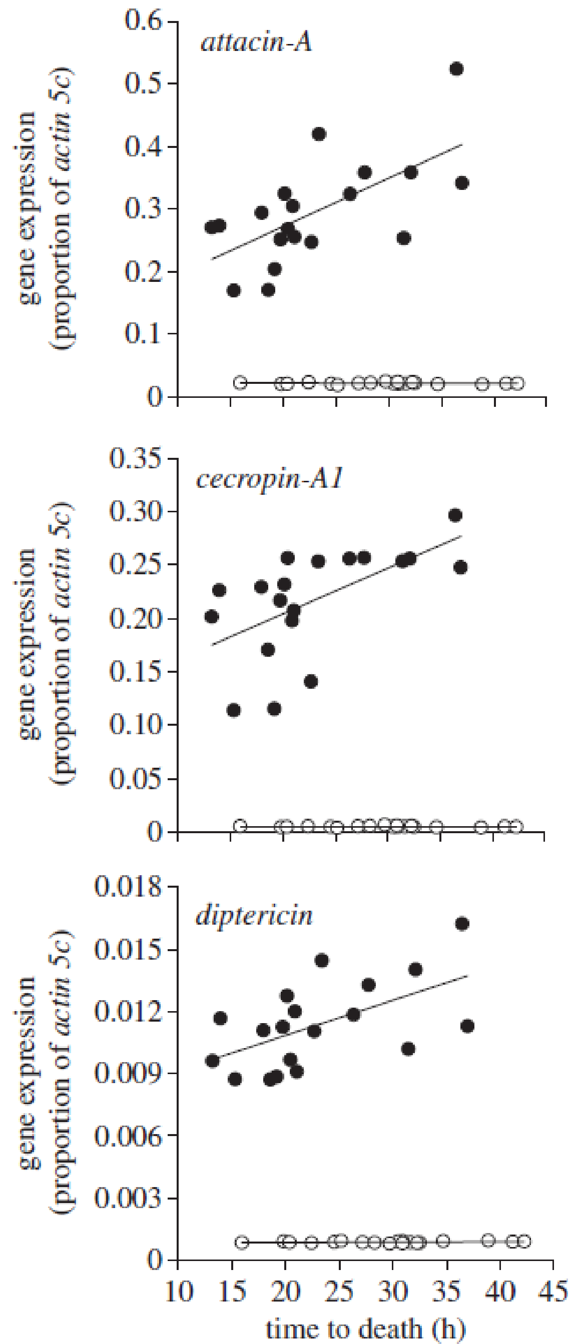

Figure 3. Male and female immune gene expression as a function of pathogen resistance for all three immune-related genes. As baseline expression increased for each immune gene, resistance against S. marcescens increased for males (closed circles), but not for females (open circles). Immune gene expression is relative to actin5C and expression for all three genes is male biased. Although female expression levels are low relative to the housekeeping gene (on average 2% of actin5C, making them appear close to zero), expression levels remain robust. Each data point represents the least squared Y-line means for the models presented in Tables 1 and 2.

Discussion

For the YRV phenomenon to have an impact on the continued evolution of immune gene regulation, immune-related YRV must exist within populations and have a functional influence on a male's ability to defend against pathogens. Here we show that Y-chromosomes sampled from a single wild population affect immune-related fitness variation when introgressed into an isogenic laboratory background. Specifically, we show that Y-line males differed significantly in their immune gene expression (Table 1) and functional immune response to a bacterial pathogen (Table 2). In contrast, Y-line females did not exhibit variation in either assay. Furthermore, immune gene expression level and ability to defend against a pathogen were genetically correlated in all three genes in males but not females (Figure 3). This correlation shows that baseline immune gene expression is sexually dimorphic, with males exhibiting higher values than females for all three genes; a trend that has previously been documented by our lab for IMD-related genes (Winterhalter & Fedorka, 2009). It also shows that Y-lines with the highest levels of expression were associated with the strongest pathogen defense (Figure 3). Although the proportion of variation in time-to-death explained by the Y-chromosome appears small (ρ = 0.05), small amounts of genetic variation can have profound implications on evolutionary time scales. Furthermore, this value is likely underestimated due to our use of only a small subset of wild population Y-chromosomes and a single isogenic background. These results suggest that the YRV phenomenon has the potential to influence the adaptive evolution of immune function in *D. melanogaster*. Future work should examine more genes and other types of fitness assays in order to obtain a robust understanding of how YRV influences male immunity.

Although these data suggest that YRV *can* influence immune system evolution, the question of *how* it influences immune system evolution remains to be answered. Specifically, the type of genetic variation on the Y-chromosome can influence immune evolution in different ways. If the Y-linked genetic variation detected in this study is entirely additive, then selection can efficiently shape adaptive sexual dimorphism. If the variation is a mix of additive and epistatic variance, then adaptive evolution could be

greatly hindered, depending on the proportion of the Y-linked variation that is epistatic. Epistasis can hinder adaptation by (i) making fitness landscapes rugged and (ii) reducing overall trait heritability; both of which reduce the efficiency of selection. In general, ruggedness is driven by local sign epistasis within the landscape, which can also cause a population to become marooned on a sub-optimal fitness peak and impede further evolution (Kvitek & Sherlock, 2011a, Poelwijk et al., 2011). The reduction in heritability stems from the fact that all types of epistasis increase phenotypic variance (the denominator of the heritability estimate; Roff, 1997) and can also decrease additive genetic variance (the numerator of the heritability estimate; Cheverud & Routman, 1995a).

Importantly, if Y-linked variation is entirely epistatic, then dimorphic evolution via the Y-chromosome cannot proceed. Moreover, it could hinder monomorphic selection (i.e. when males and females share the same fitness optimum), considering that alleles must spend 50% of their time in a male background (assuming a 1:1 sex ratio) where epistasis alters their phenotypic effects and fitness values in a non-additive manner. The extent to which monomorphic selection is hindered would depend on the magnitude of the YRV effect relative to the rest of the genome. Interestingly, one of the few studies to have estimated Y-linked epistatic contributions on a trait did so for male mating success (a male-specific trait) and found that at least 40% of the variation in male fitness was linked to the Y-chromosome and this variation was entirely epistatic (Chippindale & Rice, 2001b). If Y-linked epistasis similarly influences the regulation of a male-female shared trait (i.e. not sex-specific) experiencing monomorphic selection, then it may be a common mechanism constraining adaptive evolution throughout the genome.

It is important to note that the maintenance of Y-chromosome additive variation within populations may be quite difficult, requiring either frequency-dependent selection or strong interactions with other genetic elements (Clark, 1987). Unfortunately, the original work reporting the YRV phenomenon precluded the detection of within-population variation by using Y-chromosomes captured from disparate geographic regions (i.e. non-coevolving populations; (Lemos et al., 2008, Lemos et al., 2010b)). Recent work in *D. simulans* did find evidence for within-population YRV associated with sex

ratio distortion (Branco et al., 2013). However, the *D. simulans* population used in the previous study had been maintained in the laboratory as isofemale lines for approximately 200 generations prior to investigation. Thus, the within-population YRV reported may have evolved under laboratory isolation and not under wild conditions (due to the likely occurrence of independent evolution among *D. simulans* isofemale line genetic elements), making the determination of whether YRV persists within populations difficult.

In our study, the assessment of immune-related YRV was conducted in the absence of infection, and therefore represents a "baseline" investment in immune gene products. Some Y-chromosomes exhibited a higher baseline investment than others (Figure 2), which may induce a greater energetic cost on the bearer when no pathogen is present (i.e. a higher immune function maintenance cost). If the probability of infection is temporally or spatially variable, then a chromosome by environment interaction could help to maintain Y-chromosome variation within a population. This is an interesting result that deserves further investigation.

The original work describing the YRV phenomenon (Lemos et al., 2010b) noted several immune genes under the influence of the Y-chromosome, including *attacin-A, cecropin A1, and diptericin* (the three genes studied here). In contrast to the previous work, we found no effect of the Y-chromosome on *diptericin* (Table 1). The reason underlying this discrepancy may be due to the use of different Y-chromosomes, which influenced the formation of the euchromatin-heterochromatin boundaries differently (Lemos et al., 2010b). For instance, the Orlando population may lack a Y-chromosome that has a large influence on *diptericin*, or such a Y-chromosome was not captured during the initial phase of the study. These possibilities raise interesting questions. For instance, which immune genes are most sensitive to the YRV effect, and do sensitive genes share similarities in function (i.e. recognition proteins versus signal transduction proteins)? An assay of YRV sensitive genes may provide insight into which immune traits and/or pathways are readily being shaped by YRV.

There are hundreds of heterochromatin-euchromatin boundaries in the *Drosophila* genome (Kharchenko et al., 2011), each of which may be susceptible to the Y-effect. Furthermore, YRV is not limited to immune-related genes, but may affect any area of organismal physiology. The more fitness-related traits found to be influenced by YRV the broader its evolutionary implications. Additionally this phenomenon has only been studied in detail in a few *Drosophila* species and these species all share the same ancestral Y-chromosome. We still do not know if the within-population fitness consequences of YRV are simply a unique characteristic of this ancestral *Drosophila* Y, a characteristic of all Y-chromosomes, or a common characteristic of all heterogametic systems, including ZW systems. Work in mice does suggest that murid Y-chromosomes also influence the expression of autosomal genes, hinting that the YRV phenomenon may be widespread; however, these studies examined Y-chromosomes drawn from independent populations thereby limiting any conclusions about the ubiquity of within-population YRV (Spach et al., 2009a, Teuschert et al., 2006, Case et al., 2013). Thus, a widespread examination of Y-chromosomes drawn from natural populations across numerous evolutionary unique Y-chromosome systems could greatly increase our basic understanding of genome evolution.

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CHAPTER 3: THE SEARCH FOR ADDITIVE GENETIC EFFECTS OF Y-LINKED REGULATORY VARIATION IN *DROSOPHILA* IMMUNE FUNCTION[2]

Introduction

Sexual dimorphism is pervasive throughout the animal kingdom and manifests itself at numerous levels of biological organization including morphological, physiological and behavioral (Fairbairn et al., 2007). Ultimately, such dimorphism results from sex-specific gene regulation. But how such regulation evolves under the constraint of a male-female shared genome is not fully understood (Lande, 1980, Griffin et al., 2013b). The current paradigm suggests that an evolutionarily conserved gene from the *doublesex/mab-3* Related Transcription factor (DMRT) family is fundamental. In all metazoans, DMRT genes function as the molecular switch that initiates male or female gonad development (Figure 1), which itself is activated through a diverse array of genetic or environmental signals (Moniot et al., 2000, Shoemaker et al., 2007, Smith et al., 2009, Kato et al., 2011, Price et al., 2015). In vertebrates, the gonads then secrete sex hormones (estrogen and testosterone) that bind to thousands of genome-wide hormone receptors to alter regulation in a sex-specific manner (Carroll et al., 2006). However, invertebrates do not appear to possess sex hormones (but see De Loof & Huybrechts, 1998, Hentze et al., 2013). Instead, they likely utilize male and female splice variants of the DMRT gene *doublesex* (*dsx*) to achieve the same goal. In support of this hypothesis, *dsx* products in *Drosophila melanogaster* were recently shown to bind to numerous targets throughout the genome, which could allow for sex-specific regulation (Clough et al., 2014). Thus, sexually dimorphic selection appears to focus primarily on the creation/modification of thousands of genome-wide binding sites that respond to the concentration of two sex hormones or two sex-specific gene products (male and female *dsx* isoforms).

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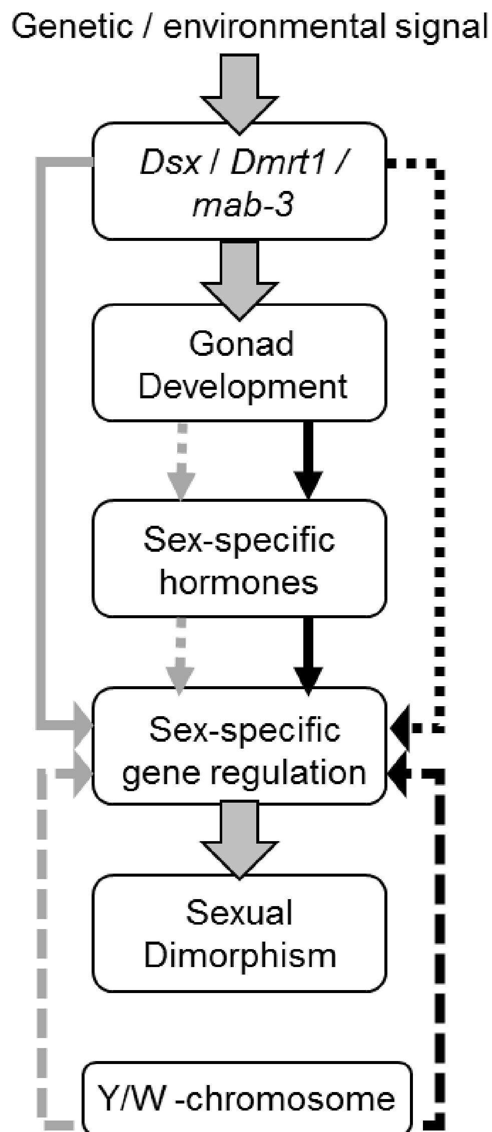

Figure 4. Sexual dimorphism and Y/W-chromosomes. In all metazoans, it appears that genes from the evolutionarily conserved Doublesex/Mab-3 Related Transcription factor (DMRT) family function as a molecular switch to initiate male or female gonad development, regardless if sex is initiated by genetic (Sry) or environmental (temperature) signals. In vertebrates, gonad-secreted sex hormones alter regulation at thousands of genes (solid black arrows). Invertebrates likely rely on Dsx sex-specific alternative splicing to induce dimorphic regulation (solid grey arrow). Invertebrate hormones and vertebrate DMRT genes are not sex-specific but might influence dimorphism via sex-specific titers; although these mechanisms are expected to be relatively minor (dotted arrows). Thus, dimorphic evolution likely centers on DMRT protein and

hormone binding sites. Y/W-chromosomes offer an independent and powerful mechanism by which adaptive dimorphic evolution may occur (dashed arrows).

An evolutionarily simple but unproven way that dimorphic phenotypes might also evolve is for selection to promote numerous trans-acting regulatory elements on hemizygous sex chromosomes, as they represent genetic material unique to one sex (i.e. Y or W; Stewart et al., 2010). However, most Y/W-chromosomes are heterochromatic and possess few protein-coding genes (Koerich et al., 2008, Carvalho et al., 2009). Nevertheless, *Drosophila* Y's have been shown to influence the regulation of hundreds of genes on the X-chromosome and autosomes (Lemos et al., 2008, Jiang et al., 2010a, Lemos et al., 2010b) suggesting that these chromosomes may help sexual dimorphism evolve. The Y-chromosome's regulatory influence stems from its heterochromatin, which may act as a sink for genome-limited transcription factors or chromatin regulators, altering chromatin structure and gene regulation on other chromosomes (Francisco & Lemos, 2014). Moreover, this phenomenon may be a hallmark of most heterogametic systems, given that most Y/W-chromosomes tend to be comprised of large swaths of heterochromatin (Bachtrog, 2013), Y-linked heterochromatin has been shown to be highly variable in both flies and humans (Lyckegaard & Clark, 1989b, Karafet et al., 1998, Repping et al., 2003), and a similar effect has been detected in an independently evolved murid Y-chromosome (Teuscher et al., 2006, Spach et al., 2009b, Case et al., 2013).

Still, in order for Y-chromosomes to play a role in the adaptive evolution of sexual dimorphism (i.e. differentially expressed male and female phenotypes; not sex-limited phenotypes), Y-linked regulatory variation (YRV) must (i) influence male fitness, (ii), exist within populations where selection operates, and (iii) be comprised in part of additive genetic variation. Our recent work on the *D. melanogaster* immune system (Kutch & Fedorka, 2015) has provided evidence for the first two criteria. Specifically, Y-chromosomes derived from a single wild population were shown to differentially influence the regulation of immune-related genes, as well as defense against a live replicating pathogen. Immune-related YRV was also positively correlated with pathogen defense; with Y-chromosomes that

induced the highest expression levels exhibiting the greatest ability to survive infection (Kutch & Fedorka, 2015). Although these data suggest that YRV has the potential to influence dimorphic evolution, we still do not know if the Y-linked effects are additive in nature. This is critical considering that additive genetic variation is essential for adaptive evolution (Roff, 1997, Lynch & Walsh, 1998).

It is important to note that YRV may not be entirely additive, as previous studies have shown that the *D. melanogaster* Y-chromosome can act epistatically with the rest of the genome (Stoltenberg & Hirsch, 1997a, Chippindale & Rice, 2001a, Jiang et al., 2010a). Epistasis occurs when the contribution of an allele to a phenotype is dependent on the alleles present at other loci (Cheverud & Routman, 1995a). This non-additive interaction among loci can have important consequences for adaptive evolution, including the impediment of natural selection by reducing trait heritability and increasing the ruggedness of the trait's fitness landscape (Cheverud & Routman, 1995a, Kvitek & Sherlock, 2011b, Poelwijk et al., 2011). If the phenotypic contribution of the Y-chromosome is purely the result of epistasis between the Y and autosomes, then the Y will not influence the evolution of sexual dimorphism. Moreover, it could significantly reduce evolutionary rates when a YRV-sensitive trait is under monomorphic selection (i.e. the sexes are selected for the same trait value), considering that the autosomal alleles under selection spend half of their time in a male background where their relative fitness values are altered. It is therefore best to assess both Y-linked additive and epistatic effects to gain insight into how YRV influences trait evolution.

To date, few studies have examined Y-linked influences on presumed sexually dimorphic traits. Griffin et al. (2015) found Y-linked effects associated with longevity and Jiang et al. (2010) found the presence of both Y-linked additive and Y-by-background epistatic effects on global gene expression. Dean et al. (2015) found a joint influence of Y-chromosome and mitochondrial variants on locomotive activity. However, any extrapolation of the reported Y-effects to adaptive evolutionary potential may be misleading because these studies either (1) did not explicitly examine Y-linked effects on fitness-related phenotypes (criterion 1 above), (2) estimated Y-effects using Y-chromosomes from geographically

disparate populations that were not co-evolving with each other or their genetic backgrounds (criterion 2 above), or (3) restricted assessment of Y-linked effects to a single genetic background precluding the detection of non-additive Y-linked effects. In short, the possibility for Y-chromosomes to contribute to sexually dimorphic evolution remains unclear.

In this study, we assessed the potential for the Y-chromosome to influence the evolution of two immune traits in *D. melanogaster*, where immunity is sexually dimorphic (Winterhalter & Fedorka, 2009). These traits included resistance against *Serratia marcescens* (gram-negative bacteria) and *Lactococcus lactis* (gram-positive bacteria). To address our objective, we tested for both Y-chromosome main effects (i.e. additive effects) and Y-chromosome by genetic background interactions (i.e. epistatic effects) on immune phenotypes by introgressing multiple Y-chromosomes from a single wild population into multiple, co-evolving genetic backgrounds (i.e. genomic haplotypes) in a fully factorial design. These data provide preliminary insights into how Y-chromosomes might influence the evolutionary dynamics of male and female shared traits.

If large additive Y-effects exist, then selection could easily shape sexual dimorphism via the Y-chromosome. However, if Y-effects are epistatic and not additive, then Y's would not actively contribute to dimorphic evolution. If a mixture of additive and epistatic effects are found, it would indicate that dimorphism could evolve via additive effects, but epistasis would reduce the evolutionary rate by reducing trait heritability. It is important to note that YRV can also influence sexually monomorphic selection (i.e. the sexes are selected for the same trait value). If selection was sexually monomorphic and YRV was additive, males would reach a new phenotypic optimum faster than females due to additional, male-specific additive genetic variance. This would create transitory sexual dimorphism until both sexes have reached the new optimum. However, if selection were sexually monomorphic and YRV was purely epistatic, then Ys could constrain the response to selection by reducing trait heritability in males and increasing the ruggedness of a male's fitness landscape (Cheverud & Routman, 1995a, Kvitek & Sherlock, 2011b, Poelwijk et al., 2011); especially if the epistatic effect is of large magnitude.

Methods

Fly Stocks and Maintenance

Isofemale lines were established from 40 gravid *D. melanogaster* females collected in the fall of 2010 from a single locality of in Orlando Florida. Each isofemale line was initially maintained via single-paired sibling matings for 10 generations after capture, ensuring that each line contained a single Y-chromosome and were at least 99.9% isogenic. After this period, isofemale lines were maintained at a population size of 8 breeding pairs per generation to prevent the accumulation of excess genetic variation.

To test for the presence of Y-linked additive and epistatic genetic effects in immune defense, four isofemale lines were selected. These lines were chosen because their Y-chromosomes had been previously shown to differ in baseline immune gene expression profiles and bacterial defensive capabilities when placed in an isogenic background (Bloomington stock 4361; Kutch & Fedorka, 2015). Prior to experimentation, the four isofemale lines were again maintained via single pair sibling matings for six generations to purge any accumulated genetic variation.

A corresponding "Y-line" was created from each isofemale line by introgressing each Y-chromosome into an isogenic background (Bloomington stock 4361; made isogenic via 10 generations of single sibling matings). Stock 4361 contains recessive markers on each chromosome (y[1]; bw[1]; e[4]; ci[1] ey[R]), allowing the introgression of Y-chromosomes into the isogenic background in just 2 generations. In short, a male from each isofemale line was crossed with a 4361 female. The resulting heterozygote F1 males were then backcrossed to 4361 females. Considering that male *D. melanogaster* do not exhibit recombination during gametogenesis, chromosomes are transmitted intact to the next generation (Ashburner et al., 2005a). F2 male offspring from this cross that exhibited all of the recessive markers were used to establish the new Y-lines, as these flies were genetically identical across lines, but contained a unique Y-chromosome derived from their original isofemale line. These Y-lines were thereafter maintained by mating Y-line males with isogenic 4361 females. All flies were maintained in

vials on a cornmeal medium at 25°C and a 12h:12h light:dark photoperiod using Percival incubators (Percival Scientific, Perry, IA).

Experimental Genotypes

Prior to experimentation, males from each Y-line were crossed with females from each isofemale line to create 16 unique genotypes (hereafter referred to as male Y-XA lines, considering they represent the pairing of a Y-line Y-chromosome with the X and autosomes of the isofemale line; Figure 2). Y-XA lines contained all possible Y-chromosomes paired with all possible isofemale genomic haplotypes. The remaining haploid set of autosomes was derived from the isogenic 4361 genetic background and thus, identical across all Y-XA background lines. Male Y-XA lines were then immune assayed. Note that the females produced from this line cross were also immune assayed and served as a negative control.

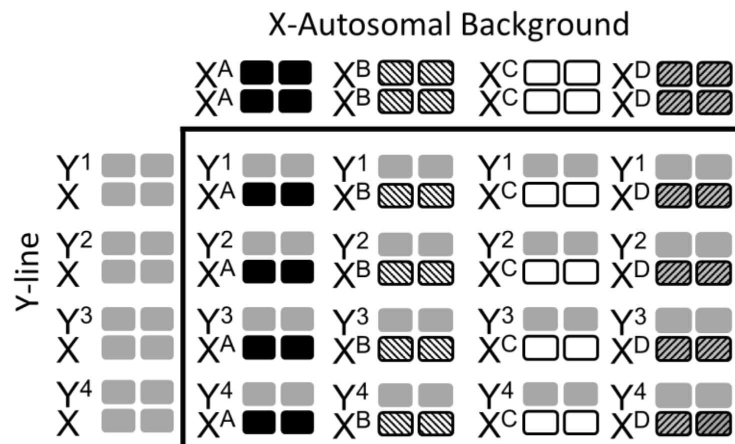

Figure 5. The creation of Y-XA lines. The Drosophila melanogaster genome is depicted as three sets of chromosomes (sex chromosomes depicted as X's or Y's and two sets of autosomes depicted as colored bars; the fourth dot chromosome is not depicted). Four different Y-line males were crossed with four different iso-female line females to pair different Y-4361 haplotypes with each iso-female X-autosomal haplotype.

The use of only four Y-chromosomes and four genetic backgrounds limits our ability to estimate natural levels of additive or epistatic variation, as these variances are a product of population allele frequencies. However, the goal of this study is to gain coarse insight into how the genetic effects are relatively partitioned, which allows us to draw broad conclusions about how YRV may influence trait evolution. To increase the power of detecting additive and epistatic effects in our 4x4 matrix, we selected Y-chromosomes shown to have significantly different effect on immune gene expression (Kutch & Fedorka, 2015).

Immune assay

To assess the immune function of each Y-XA genotype, four day old male virgins from each line (and their corresponding female controls) were infected with an LD_{90} concentration of *Serratia marcescens* (gram-negative) and *Lactococcus lactis* (gram-positive bacteria) using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA). Although we are interested in Y-chromosome effects in males, females were also tested as a negative control (i.e. if females who do not carry a Y-chromosome differed across Y-lines in their immune function, it would suggest that any Y-line effect in males was spurious). Because male and female flies vary dramatically in their body size, a unique concentration was used for each sex and pathogen combination that would elicit the mortality of about 90% of the flies infected. Time to death (TTD) in response to infection was measured using a Drosophila Activity Monitor (TriKinetics, Waltham MA) for a 48 hour window after infection. Flies were deemed dead once their last movement was recorded, which was confirmed through visual inspection at the end of the 48 hour window. Flies that died in the first 3 hours after infection were removed from the study as their mortality was more likely due to trauma than succumbing to the bacterial infection (a total of 6 flies were removed for this reason). Flies who survived throughout the entire 48 hour observation window were censored and included in the analysis. The infection experiment was replicated 13 times, where the 16 genotypes were created *de novo* for each replicate by crossing different Y-line males and isofemale line females from a new generation. Within each replicate, a male and a female from each of

the 16 Y by background genotypes were infected with a new bacterial culture. One female replicate from each infection type was lost due to a loss of power to the Drosophila Activity Monitors. In total 639 flies were tested among 16 genotypes; 157 males and 157 females were tested in response to *L. lactis,* and 159 females and 166 males were tested in response to *S. marcescens*.

Methods for infections of *S. marcescens* and *L. lactis* were modified from Apidianakis & Rahme (2009). In short, bacteria were incubated in sterilized LB broth at 37°C until log phase. This solution was diluted with sterile broth to an absorbance of 0.4 at 490 nm using a microplate reader (Bio-Rad Model 680, Hercules, CA, USA), aliquoted into 1ml micro centrifuge tubes, and stored in at 4°C to prevent bacterial growth. The 1ml aliquots of the diluted solution were centrifuged at 11,000g for 2 minutes just prior to each assay. The supernatant was discarded and 1ml of 10mM $MgSO_4$ wash was added to the remaining pellet and centrifuged at 11,000g for another 2 minutes. Again, the supernatant was discarded and the pellet was resuspended in 10mM $MgSO_4$. LD_{90} concentrations for each pathogen were determined by resuspending the pellet in different quantities of 10mM $MgSO_4$ and infecting ~50 flies to assess mortality after 48 hours. For *S. marcescens* ~90% mortality was observed when resuspending the pellet in 666µl 10mM $MgSO_4$ for males (1.5x concentration) and in 370µl 10mM $MgSO_4$ for females (2.7x concentration). For *L. lactis* ~90% mortality was seen when resuspending the pellet in 333µl 10mM $MgSO_4$ in males (3x concentration) and 222µl 10mM MgSO4 in females (4.5x concentration). Negative controls injected with just the bacterial vehicle (10mM $MgSO_4$) showed no mortality, indicating that *S. marcescens* and *L. lactis* were the source of mortality in these experiments.

Analysis

Prior to testing for additive and epistatic effects across backgrounds, we first tested for Y-chromosome effects on male "time-to-death" within backgrounds to determine if the four chromosomes used in this study were genetically distinct. To this end, we generated risk ratios for all Y-chromosome combinations within backgrounds using a Proportional Hazards model with Y-chromosome and replicate

as factors. Flies that lived longer than 48 hours were censored. We then assessed the overall probability of one Y differing from another Y via Fisher's Combined Probability Test across backgrounds.

To test for additive and epistatic effects on "time-to-death", we again used a censored Proportional Hazards model with genetic background, Y-chromosome, Y x background interaction and replicate as model factors. Here, replicate variation is due primarily to variation in bacterial culture, which induces differences in the mean time-to-death for each replicate. The analysis was conducted separately for both sexes and pathogens because each pathogen elicited very different responses in mortality, pathogen doses were different for each sex, and females served as a negative control (i.e. we did not expect females to differ across Y-lines). A significant Y-chromosome effect indicated the presence of Y-linked additive genetic effects. A significant background by Y-chromosome interaction indicated the presence of epistatic effects.

Least squared means for each Y-XA line were estimated via ANOVA using the previous model. The ANOVA also provided estimates of the model variance components, which reports the relative magnitude the background effect, Y effect, Y by background effect, and the replicate effect had on the immune phenotype. Note that we used bounded variance components in our analysis, which constrains variance components to be non-negative. As a result, negative variance components are reported by the statistical software as zero. All analyses were conducted in JMP v.12.1.0.

Results

In general, flies died more quickly when infected with an LD_{90} of *S. marcescens* compared with *L. lactis* and males died more quickly than females (Figure 3). In females, the X-Autosome genetic background had a significant influence on "time-to-death" for both pathogens (Table 3). These data indicate that genetic variation for *S. marcescens* and *L. lactis* defense existed in these backgrounds. Importantly, Y-chromosome effects were not significant in females (Table 3). The lack of a female effect serves as an important negative control indicating that any Y-chromosome effects in males were actually due to the Y-chromosomes and not some uncontrolled artifact among Y-XA lines.

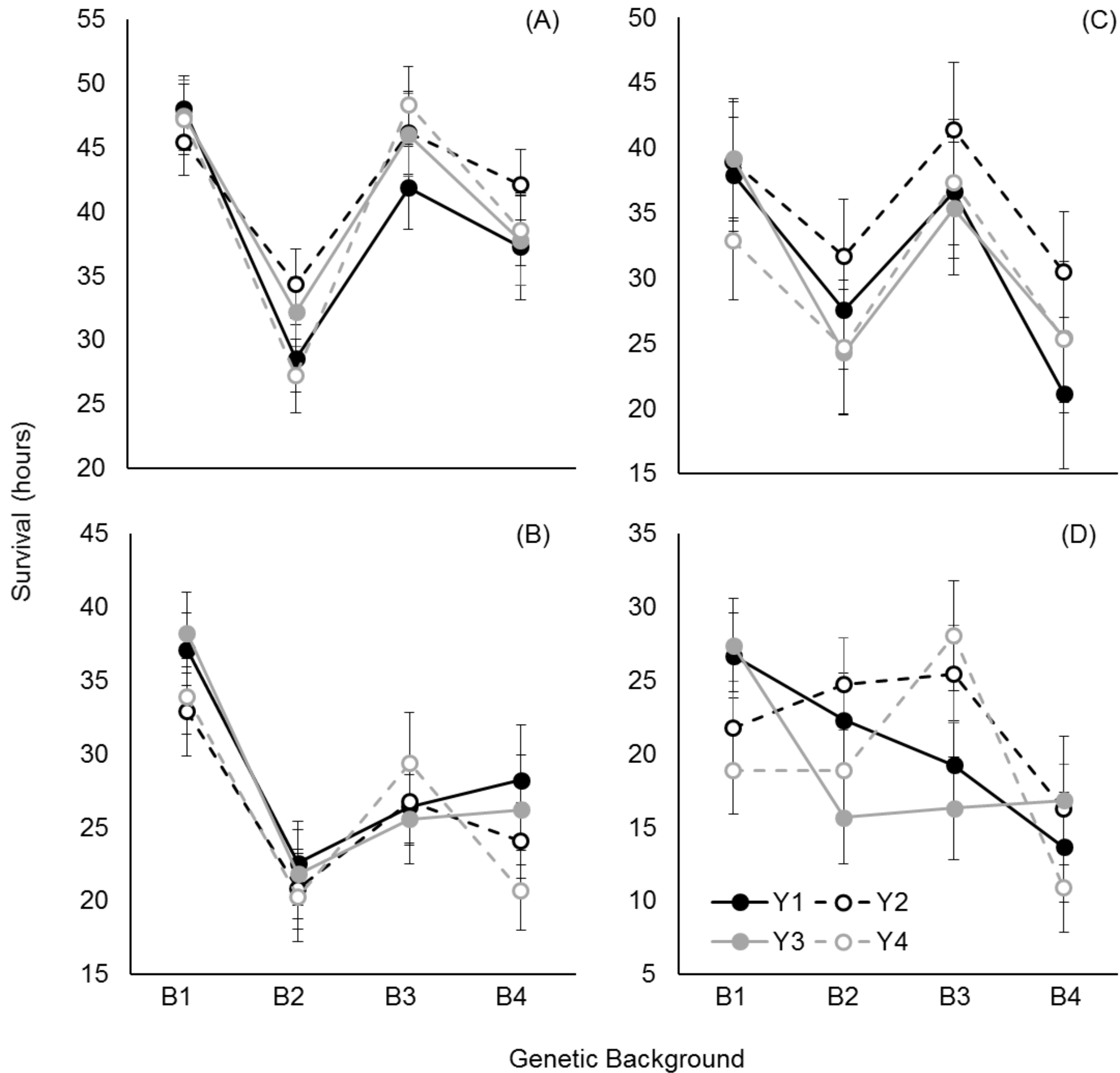

Figure 6. The effect of genetic background, Y-chromosome, and their interaction on bacterial survival. The genetic background significantly influenced both females (A) and males (B) response to *L. lactis*, however the Y-chromosome had no effect (note that females do not possess a Y-chromosome and are presented as a negative control). This pattern is the same for the female response to S. marcescens (C). Furthermore, the pattern of genetic background effects appear similar in (A-C). However, males showed a significant Y-chromosome by genetic background interaction when infected with S. marcescens (D), indicating significant epistatic effects.

Table 3. The effect of X-autosomal background (Background), Y-chromosome (Y-chrom) and Y by Background interaction (Y*B) on female (Top) and male (Bottom) time-to-death when infected with an LD_{90} of (A) *L. lactis* and (B) *S. marcescens*. Both sexes exhibited a significant X-Autosome effect for pathogen defense. Y-chromosome effects were not significant in females (this was expected due to the isogenic nature of females from the Y-XA design). Y-chromosome effects were also not significant for males, indicating the lack of Y-linked additive genetic effects. However, a significant background by Y-chromosome interaction for defense against *S. marcescens* was detected in males, indicating the presence of epistatic genetic effects.

A. Response to *Lactococcus lactis*

Females	df	x2	P-value
Model	26	99	< 0.0001
Background	3	84	< 0.0001
Y-chrom	3	0.9	0.8205
Y*B	9	13.6	0.1362
Replicate	11	20.3	0.041

Males	df	x2	P-value
Model	27	92.5	< 0.0001
Background	3	66.2	< 0.0001
Y-chrom	3	1.8	0.6068
Y*B	9	3.1	0.9590
Replicate	12	34.6	0.0005

B. Response to *Serratia marcescens*

Females	df	x2	P-value
Model	26	43.6	0.0166
Background	3	21.2	< 0.0001
Y-chrom	3	3.3	0.3420
Y*B	9	3.3	0.9516
Replicate	11	20.8	0.0361

Males	df	x2	P-value
Model	27	120.6	< 0.0001
Background	3	33.7	< 0.0001
Y-chrom	3	5.23	0.1555
Y*B	9	24.41	0.0037
Replicate	12	84.45	< 0.0001

In males, the X-Autosome genetic background also had a significant influence on “time-to-death” for *L. lactis* (Table 3). Furthermore, the genetic background influence on time-to-death exhibited the same rank pattern as in females, with B1 > B3 > B4 > B2 time-to-death (Figure 3), indicating that genetic backgrounds performed consistently among males and females when infected with *L. Lactis*. Background B1 and B3 also exhibited the longest time-to-death in females infected with *S. marcescens*, suggesting some consistency in genetic background performance across pathogens (Figure 3). A Y by background interaction in male *S. marcescens* defense precluded assessment of the genetic background main effects for this pathogen.

Prior to testing for Y-linked additive effects across genetic backgrounds, we first tested for the existence of Y-chromosome genetic variation by assessing Y-chromosome main effects within genetic backgrounds. Consistent with previous work (Kutch & Fedorka, 2015), the Y-chromosomes appeared to be genetically distinct and behaved consistently within genetic backgrounds (Table 3). When all backgrounds were analyzed simultaneously, no Y-chromosome effect was detected in males for either *L. lactis* or *S. marcescens* defense. However, a Y by background effect was detected in male *S. marcescens* defense (Table 3). These data indicate that Y-linked additive effects did not exist in our population of Y-chromosomes, but that Y by background epistatic effects were abundant (Figure 3). Not surprisingly, replicate effects were significant in each model. This effect is likely driven by the use of a newly created bacterial solution for each replicate, creating variation across replicates in the amount of pathogens transferred.

Table 4. Assessment of Y-chromosome genetic distinctiveness. Assessment based on Fisher's combined probability test of the risk ratio P-values generated from four independent Proportional Hazards models (one for each genetic background; see methods). These data indicate that at least three of the four Y-chromosomes (Y2, Y3 and Y4) are genetically distinct

A. Serratia marcescens

Y Comparison	X2	DF	P - Value
Y1 ≠ Y2	12.7	8	0.1226
Y1 ≠ Y3	15.6	8	0.0485
Y1 ≠ Y4	12.9	8	0.1189
Y2 ≠ Y3	21.8	8	0.0052
Y2 ≠ Y4	15.6	8	0.0485
Y3 ≠ Y4	27.9	8	0.0005

B. Lactococcus lactis

Y Comparison	X2	DF	P - Value
Y1 ≠ Y2	12.7	8	0.1226
Y1 ≠ Y3	20.5	8	0.9212
Y1 ≠ Y4	10.6	8	0.0087
Y2 ≠ Y3	3.2	8	0.2273
Y2 ≠ Y4	8.32	8	0.4047
Y3 ≠ Y4	17.3	8	0.0271

To better understand the magnitude of the additive and epistatic effects on immune phenotypes, we partitioned the model effects in Table 3 into their respective variance components. In females, the genetic background accounted for 39.4% of the total variance in *L. lactis* defense, but only 12.7% in *S. marcescens* defense (Table 5A). Thus, genetic background had a larger proportional influence on *L. lactis*. This trend was similar in males, although the total variance explained by genetic background was smaller compared with females (Table 5B). Most importantly, the Y by background epistatic component for *S. marcescens* defense exhibited a large magnitude effect relative to the X-autosome genetic background (3.7% versus 7.4%, respectively). This indicates that the epistatic effect on *S. marcescens* immune defense was a substantial part (~33%) of the total genetic effect.

Table 5. Magnitude of the additive and epistatic effects on immune phenotypes. Variance components were derived from the models in Table 2. Asterisks denote statistically significant model components as reported in Table 3.

A. Females

L. lactis	Variance Component	% variance
Background	52.1	39.4*
Y-chrom	0	0
B x Y	0	0
Replicate	0.3	0.2*
Error	79.8	60.4
Total	132.2	100
S. marcescens		
Background	33.4	12.7*
Y-chrom	4.3	1.6
B x Y	0	0
Replicate	7.5	2.9*
Error	217.3	82.8
Total	262.5	100

B. Males

L. lactis	Variance Component	% variance
Background	38.3	31.6*
Y-chrom	0	0
B x Y	0	0
Replicate	5.2	4.3*
Error	77.5	64.1
Total	100.21	100
S. marcescens		
Background	13.4	7.5*
Y-chrom	0	0
B x Y	6.7	3.7*
Replicate	50.7	28.3*
Error	108.5	60.5
Total	106.63	100

Discussion

Y and W chromosomes offer a theoretically powerful way in which sexual dimorphism can adaptively evolve. Consistent with this possibility, *D. melanogaster* Y-chromosomes have been noted to influence the regulation of hundreds of genes throughout the genome; particularly immune-related genes (Lemos et al., 2010b, Kutch & Fedorka, 2015). But for Y-linked regulatory variation to contribute to adaptive evolution it must be in part comprised of additive effects. That is to say, Y-chromosomes must exhibit some level of consistency in their capacity to affect phenotypes. In this study, we assessed the potential for Y-chromosomes to consistently influence immune function phenotypes by introgressing them across multiple genetic backgrounds that were drawn from the same wild population. Our data, however, indicated that no Y-linked additive effects existed for the immune traits examined here (Table 3). Therefore, we find no evidence that Y-chromosomes can adaptively shape sexually dimorphic immunity in this system via large additive YRV effects.

It is possible that the lack of Y-chromosome main effects presented here (Table 3) was limited by the use of only four Y-chromosomes and four genetic backgrounds. However, Y-chromosome main effects were detected when the analysis was limited to within autosomal backgrounds only (Table 4), which is consistent with previous work (Kutch & Fedorka, 2015). It is only when Y-chromosome by background interactions were considered that Y-chromosome main effects dissipated while autosomal main effects persisted. Thus, Y-chromosome effects appear to be significant and consistent within genetic backgrounds, but highly variable and non-significant among backgrounds. Our interpretation of this pattern is that, while small Y-linked additive effects may be present in the population, large Y-linked additive effects are likely to be absent. These data also suggest that the lack of Y-linked main effects were not due to anomalous genetic elements (i.e. Ys that do not impart additive effects, and/or backgrounds that interact oddly with the Ys), as Y main effects were detected within single autosomal backgrounds and the autosomes were randomly derived from the population. Although the assessment of more Y's and

more genetic backgrounds may uncover the presence of small Y-linked additive effects within the greater population, large additive Y-effects are likely absent.

As noted above, we detected a significant Y-chromosome by genetic background epistatic interaction when males were infected with *S. marcescens*. If this physiological epistatic effect translates into statistical epistasis at the population level, then phenotypic evolution outside of sexual dimorphism can be profoundly affected. Statistical epistasis (i) can increase the ruggedness of trait adaptive landscapes, which can cause a population to become marooned on a sub-optimal fitness peak (Kvitek & Sherlock, 2011b, Poelwijk et al., 2011), (ii) will increase trait phenotypic variances, which decreases heritabilities and evolutionary rates, and (iii) can reduce additive genetic variances, which limits evolutionary potential (Cheverud & Routman, 1995a). Thus, statistical epistasis can dramatically disrupt monomorphic selection in both sexes (i.e. males and females are selected for the same trait fitness optimum), considering that the autosomal alleles under selection must spend 50% of their time in a male genetic background where their relative fitness values are changed. This effect may be potent if epistasis causes the fitness values to be reversed (i.e. "sign" epistasis) and if it is of a relatively large magnitude (Cheverud & Routman, 1995a, Mackay, 2014). With complete sign epistasis in males, male heritability for a trait shared with females (e.g. immune function or body size) could drop to zero. This would reduce the population's response rate to monomorphic selection by half. In plotting the rank order changes of the Y-chromosome effects across our four genetic backgrounds, we see complete rank order reversal for some Y and background combinations; a signature of sign epistasis (Figure 3). Furthermore, the proportion of the phenotypic variance explained by the epistatic effect was relatively large when compared to the genetic background effect (Table 5).

Sign epistasis is most powerful when the interacting genetic elements are in equal frequency (Mackay 2014). Whether Y-chromosomes exist in equal frequency in the Orlando population is unknown. However, our previous work randomly sampled Y-chromosomes from the wild and found a continuous distribution of Y-effects on immune function. This suggests that there may be many Y-chromosomes present in a population at relatively equal frequencies (Kutch & Fedorka, 2015). If true, then immune-

related YRV in *Drosophila melanogaster* may play a significant role in constraining adaptive evolution in YRV sensitive traits. Such a hypothesis is intriguing, as YRV may be widespread and evolutionarily conserved among XY and ZW heterogametic systems, potentially providing a universal constraint to autosomal trait evolution.

The Y by background effect detected here is consistent with previous work that showed Y-chromosomes harbor genetic variation associated with *S. marcescens* defense (Kutch & Fedorka, 2015). In contrast, no Y-chromosome effect was observed in response to *L. lactis.* The difference between these results may stem from these pathogens eliciting different immune pathways. *L. lactis* is a gram-positive bacteria that elicits the Toll pathway while *S. marcescens* is a gram-negative bacteria that elicits the IMD pathway. Thus, YRV may simply not affect Toll genes; at least for the chromosomes and background used in this study.

Overall, our data indicate that the Y-chromosome cannot facilitate the continued adaptive evolution of sexually dimorphic immunity; at least for the Y-chromosomes and immune traits examined here. In contrast, the large Y by genetic background epistatic effect suggests that Y-chromosomes may actually constrain sexually monomorphic selection. This could be easily tested by creating genetically similar populations that differ only in their number of Y-chromosomes (e.g. 1 versus 50) and enacting monomorphic selection over successive generations. A slower evolutionary response by the population with more Y-chromosomes would be indicative of Ys acting as an evolutionary constraint (the reverse pattern would suggest that they could facilitate sexual dimorphism). That said, if some of the epistatically interacting Y-chromosomes in a population are lost to genetic drift, then additive genetic variation could be released (Goodnight, 1988, Goodnight, 2015), which could allow selection to adaptively shape dimorphic phenotypes via the Y. If the data presented here are consistent with other sexually dimorphic traits influenced by the Y, and of heterogametic chromosomes in other systems, then the Y-chromosome regulatory phenomenon may represent a large cost of sex, similar to the cost of males and the cost of meiosis. This possibility warrants further investigation.

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CHAPTER 4: ASSESSING THE CONSEQUENCES OF Y-LINKED REGULATORY VARIATION ON THE EVOLUTION OF AUTOSOMAL TRAITS IN EXPERIMENTAL POPULATIONS OF *DROSOPHILA MELANOGASTER*[3]

Introduction

Y-linked regulatory variation (YRV) is a recently discovered phenomenon where the non-coding regions of Y-chromosomes in several species have been shown to influence the expression of hundreds of autosomal and X-linked genes (Lemos et al., 2010b, Jiang et al., 2010a, Case et al., 2013). This phenomenon has been most extensively studied in *Drosophila melanogaster,* and serves to reconcile two seemingly contradictory observations: (i) Y-chromosomes possess few protein coding regions, all of which appear tied to male fertility (CITE), and (ii) Y-chromosomes influence as wide variety of traits including male fitness (Chippindale & Rice, 2001a), heat tolerance (Rohmer C, 2004), geotaxis (Stolenberg & Hirsch, 1997), and immune function (Kutch & Fedorka, 2015). The regulatory influence of the Y-chromosome found in *D. melanogaster* may be widespread throughout the animal kingdom, given that it is driven by non-coding, repetitive elements on the Y, and that such elements are common among most XY systems (Bachtrog, 2013).

Currently, little is known about the evolutionary implications of YRV, but the phenomenon could play an important role in how traits evolve in both sexes. For traits experiencing sexual conflict (i.e. where sex-specific optimal trait values exist), YRV may provide a mechanism for reducing conflict and achieving sexual dimorphism. This requires the presence of Y-linked additive genetic variance within a local gene pool. Conversely, strong non-additive interactions between the Y and other chromosomes can weaken selection on autosomal and X-linked genes when they resided in males, potentially constraining the adaptive evolution of autosomal traits. Such non-additive epistatic effects can constrain adaptive evolution in 3 ways: (i) by creating a rugged fitness landscape that maroons traits on a sub-optimal fitness

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peak (Kvitek & Sherlock, 2011b, Poelwijk et al., 2011), (ii) by increasing phenotypic variance that reduces heritabilities and by extension, evolutionary rates, and (iii) by directly reducing additive genetic variance, limiting evolutionary potential (Cheverud & Routman, 1995b). It is important to point out that non-additive effects can also increase additive genetic variation (Cheverud & Routman, 1995b). Whether additive variance is increased or decreased depends largely on the direction and magnitude of the non-additive effects (e.g. positive versus negative epistasis), and the frequencies of the interacting alleles.

Multiple studies have in some way addressed the genetic architecture of the Y-linked phenomenon, but all have failed to detect the additive effects that would be required for the continued evolution of sexual dimorphism. In 1997, Stoltenberg and Hirsh found that the effects of Y-chromosomes from lines selected for extreme phenotypes in climbing ability (geotaxis) were entirely dependent on the background in which they were placed; a signature of non-additive genetic effects (Stolenberg & Hirsch, 1997). In 2001, Chippendale and Rice examined 20 different Y-chromosomes in 3 autosomal genetic backgrounds and found a large, non-additive effect on male fitness (Chippindale & Rice, 2001a). In 2011, Jiang and colleagues crossed non-coevolving Y-chromosomes and autosomal backgrounds from disparate populations and found a strong Y by background interaction in gene expression, but reported no Y-linked additive effects (Jiang et al., 2010a). Recently, Kutch and Fedorka (manuscript under review) introgressed Y-chromosomes into multiple genetic backgrounds derived from the same population where the genetic elements were co-evolving and found only non-additive effects on male immune function.

It may be that a small amount of Y-linked additive genetic variance persist in populations, but has remained undetectable in previous studies. Importantly, additive genetic variation is not an attribute of specific Y-chromosomes or Y by background interactions, but is a statistical property of a given population. The frequencies at which the genetic elements exist in the population influence how genetic variance manifests (Mackay, 2014). Genetic variance components change as populations change (Goodnight, 1988), as selection and drift can both erode and release additive variance. Therefore, to test if YRV can facilitate the adaptive evolution of autosomally coded traits, we must directly test its effect in

populations. To date, no study has examined the impact of YRV on trait evolution within a free-breeding population.

In this study, we assess the impact of YRV on the evolution of geotaxis (a trait shared by both males and females) using co-evolving Y-chromosomes and genetic backgrounds from a single population of wild caught *Drosophila melanogaster*. This ensures that we are looking at naturally occurring variation in simulated populations. To this end, we created replicate populations that contained either a single Y-chromosome (no YRV) or multiple Y-chromosomes (YRV). Extensive backcrossing was used to homogenize the autosomal genetic variation in our experimental treatments. To determine the effect YRV has on the evolution of an autosomal trait, we imposed similar selective pressures to increase negative geotaxis; a trait coded for on the autosomes that has been shown to respond to selection (Watanabe, 1976). If YRV can facilitate the adaptive evolution of autosomal traits, the YRV populations must provide additive genetic variation (i.e. a statistical property of the population) and therefore should exhibit an accelerated response to selection relative to the No-YRV populations. If YRV constrains the adaptive evolution of autosomal traits due to its non-additive nature, we expect to see a reduced response to selection relative to the No-YRV populations.

Methods

Geotaxis apparatus

To quantify and measure a fly's geotaxis behavior, vertical mazes were construed out of 1/4' PVC cross fittings to create of series of up/down choices as flies traversed the maze to reach food at the other end (Figure 7). Each up/down junction was fitted with small funnels to reduce the probability of flies going back on their choice. In all, 7 mazes were constructed, each consisting of 6 consecutive up/down junctions before flies reached a collection vial filled with cornmeal, sugar, yeast, and agar *Drosophila* food. Flies that made 6 consecutive up choices exit the maze at the top collection vial and are scored with a geotaxis value of 7 where flies that made 6 consecutive down decisions would exit into the

collection vial at the bottom of the maze and receive a geotaxis score of 1. Any other combination of up/down choices resulted in flies exiting at intermediate exit vails. Flies were then scored individually and used to determine the mean population geotaxis behavior.

Population creation and fly maintenance

To test if YRV facilitates or constrains adaptive evolution of the autosomally coded geotaxis behavior, we created two treatment populations. To create our YRV treatment (populations that contain Y-chromosome variation) 1 male from each of 25 different isofemale lines caught from the wild in Orlando FL in the Fall of 2010 were backcrossed to 10 virgin females from an outbred population caught from the same location. These 25 isofemale lines each contained a potentially different Y-chromosome and data from immune response and immune gene expression indicate that there is significant Y-chromosome variation across these lines (Kutch & Fedorka, 2015). The resulting male offspring from this backcross contained 50% of their isofemale genetic background and 50% genetic variation from the outbred population. 10 male offspring from this backcross were again crossed with 10 virgin females from the outbred cross reducing the isofemale genetic background to 25% in the resulting offspring. This process was done 10 times to create flies where ~99.9% of their X chromosome and autosomal genetic background was derived from the outbred population but each line contained its original Y-chromosome that was collected from the wild. After 10 generations of backcrossing to the outbred population, 10 males from each line were pooled and crossed with 250 virgin females from the outbred population to create one replicate YRV treatment population. This was done three times to create three YRV treatment populations each containing similar X chromosome and autosomal genetic variation paired with a variety of Y-chromosomes. The same process was carried out to create the "No YRV" treatment but the originating 25 isofemale lines used to backcross to the outbred population were all copies of a single isofemale line (i.e. all contained the same Y-chromosome). Again, three replicate populations were created for the No YRV treatment resulting in populations where flies had similar X and autosomal genetic variation paired with a single Y-chromosome. The 3 replicate populations of the No YRV and the 3 replicate populations of the

YRV treatment were used to test how Y-chromosome variation influences the adaptive evolution of geotaxis.

Flies were maintained at 27°C in temperature controlled incubators with a 12:12hr light-dark cycle on a cornmeal, sugar, yeast, and agar media. Backcrosses were performed in 25 x 95mm polystyrene vails. After backcrosses were complete and lines were combined, each replicate population was continued by collecting 300 virgin males and 300 virgin females, aging flies 4±1 days, and allowing these flies to mate and lay eggs for 48 hours before being discarded. These 600 flies were randomly spread across 16 six ounce polypropylene bottles for each generation.

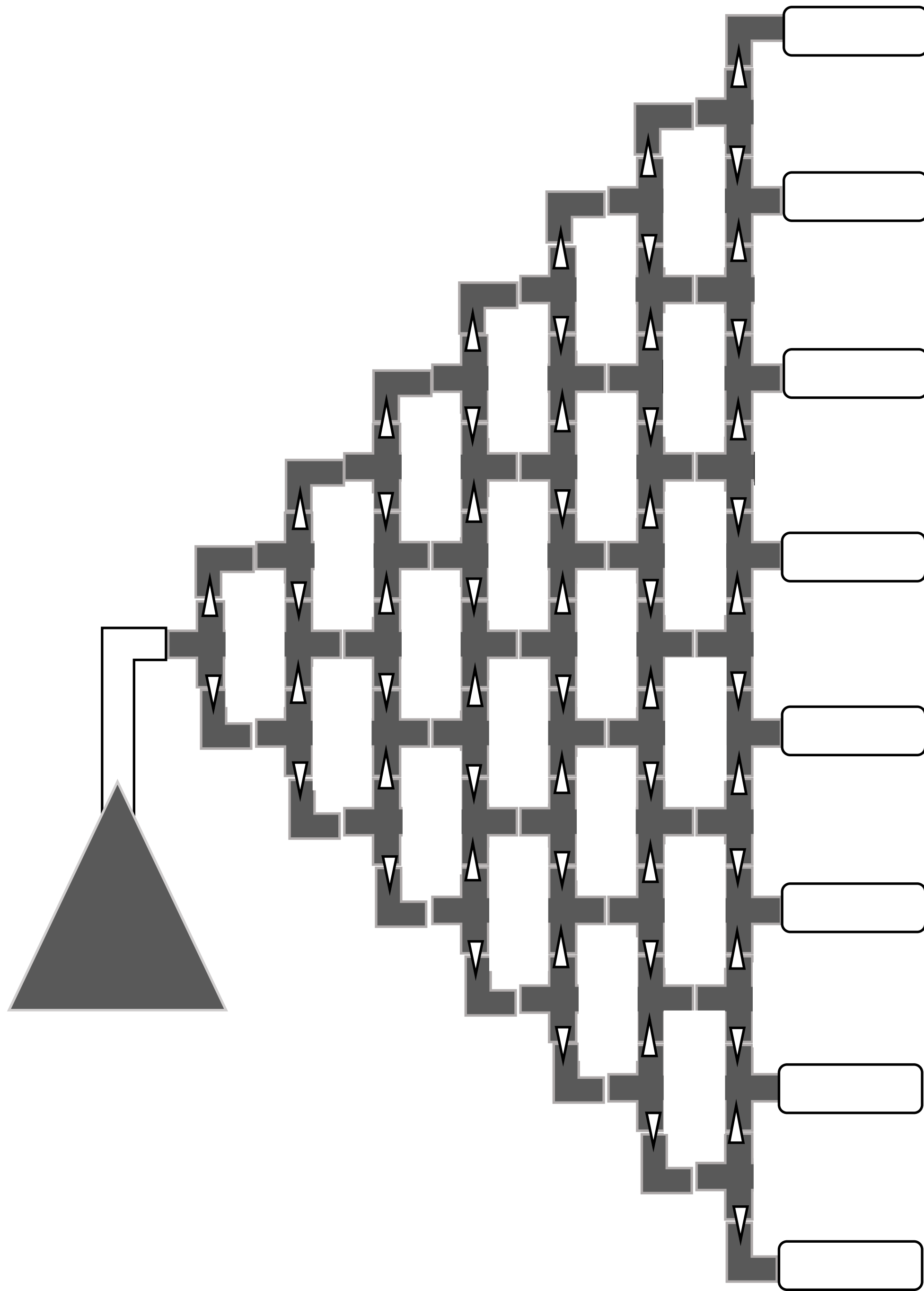

Figure 7. Schematic of the geotaxis apparatus used to phenotype flies and impose selection. Flies enter the maze on the left and are confronted with a series of up/down decisions. Flies that make 6 consecutive up choices exit the maze in the top vial and receive a geotaxis score of 7. Flies that make 6 consecutive down choices exit the maze in the bottom vial and receive a geotaxis score of 1. White triangles represent small funnels that were placed at up/down junctions to reduce the probability of flies going back on their decision.

Selection

300 males from each replicate population were collected as virgins, aged 4±1 days, and run through a single vertical maze. Their exit values were used to determine the population mean exit value. A selection gradient of .65 was then calculated in these males by coding males that would survive selection as having a fitness of 1 and the males that would be culled to have a fitness of 0 and creating a selection gradient (β) that was $\approx$ .65. This resulted in a selection gradient that was similar across all populations and all treatments. After culling those calculated to not pass the selection gradient, the selected males were then paired with 300 females to create the next generation. Because we were interested to how adaptive evolution would occur when genes experienced YRV, we only implemented selection in male flies, where YRV is experienced. Selection was run 15 generations.

Population Assessments

Population geotaxis scores for males were estimated continuously throughout the implementation of selection. These estimates however were the result of only one run of each population in a single given maze. In assessing these time series data, it was evident that there were significant effects of day and maze. Given that day was completely confounded with generation and within each generation the maze effect was confounded with the population effect, these weak estimates yield only minimal insight into the effects of our selection protocol (S1). To better understand what happened across the two treatments, robust estimates were taken for both males and females before the first generation of selection (G1) and after the 15th generation of selection (G15). These assessments measured each population, across multiple mazes, across multiple days. Each population was measured in 3 random mazes across 3 days for G1 and across 7 random mazes and 7 random days for G15. These robust measures allowed for a better understanding of the geotaxis behavior of the different treatments before and after 15 generations of selection. As a post-hoc test to see if our treatment populations responded to selection, the outbred population where the Y-chromosomes and autosomal genetic variation present in the experimental

treatments originated was estimated at the G15 time point. If adaptive evolution occurred in our treatment population, we would predict that the unselected outbred population would have a lower mean than the two selected treatments in males and females.

Statistical Analysis

Robust estimates at both G1 and G15 were analyzed using a Generalized Linear Model with the response variable (Exit Value) modeled under a Poisson distribution. The predictor variables of interest were the treatment effect (YRV vs No YRV), the population effects nested within their respective treatments, the effect of maze, and the effect of day. For the purpose of visualization of the data, a standard ANOVA was also run using the same model to obtain least squared mean estimates of the treatments given the effects of the other measured variables. Pairwise contrasts of the G15 treatments and the outbred population were also cared out via Generalized Linear Models. All analyses were carried out in JMP Pro v.12.

Results

G1 estimates for each treatment showed significant differences between the YRV and No YRV treatments in males but no significant differences in females (Table 7). As expected, the effect of both maze and day were significant in both sexes (Table 7). The No YRV treatment males showed a significantly lower Mean Exit Value in G1 than the YRV treatment males (Figure 8). In females, the G1 estimates for the YRV and No-YRV treatment are not significantly different (Figure 9)

Table 6. The effects of treatment, population nested within treatment, maze, and day on fly geotaxis behavior before selection. Significant effects of population, maze, and day are observed in both males and females. Males show significant differences in treatment while females do not.

G1 Estimates

Source	DF	χ2	P-value
Males			
Treatment	1	17.97859	<.0001
Pop[Treat]	4	21.73917	0.0002
Maze	5	153.6312	<.0001
Day	2	25.03026	<.0001
Females			
Treatment	1	0.504397	0.4776
Pop[Treat]	4	47.98667	<.0001
Maze	5	16.02188	0.0068
Day	2	63.01827	<.0001

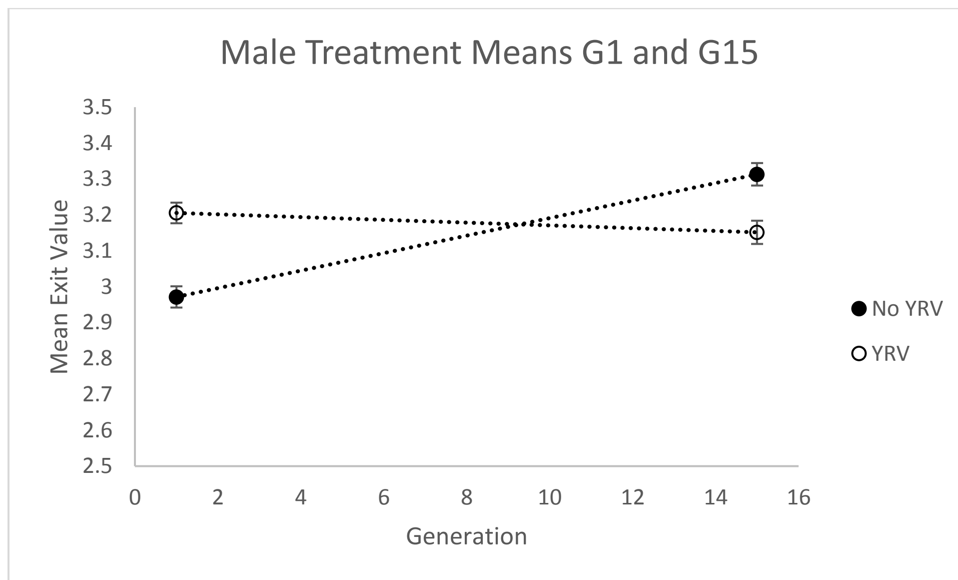

Figure 8. Least squared means of mean exit value for YRV (open circles) and No-YRV (closed circles) treatments for males at generation 1. Error bars represent standard error for the treatment effect. No-YRV treatment males had a lower mean exit value than YRV treatment males in G1. In G15, the No-YRV treatment was higher than the YRV treatment.

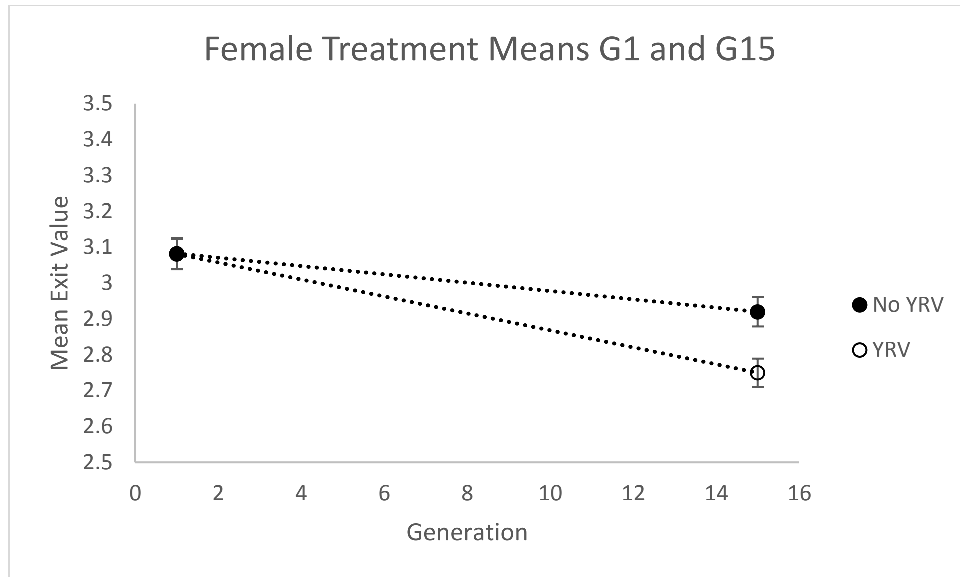

Figure 9. Least squared means of mean exit value for YRV, No-YRV, and Outbred treatments for males and females after 15 generations of selection. Error bars represent standard error for the treatment effect. YRV and No-YRV treatments show higher mean exit values than the Outbred treatment that experienced no selection in both males and females. Additionally, in both males and females the No-YRV treatment shows a higher mean exit value than the YRV treatment.

G15 estimates for each treatment showed significant effect of treatment in both males and females (Table 8). Again, as expected, the effect of both maze and day had significant effects in both sexes (Table 8). In both the males and the females, The No YRV treatment showed a significantly higher geotaxis score than the YRV treatment (Figure 8 & 9). Pairwise contrasts reviled significant differences between all treatments (Outbred, YRV, and No YRV) in both sexes (Table 9). All selected treatments (YRV and No YRV) showed a significantly higher Mean Exit Value when compared to the Outbred population which experienced no selection (Figure 10).

Table 7. The effects of treatment, population nested within treatment, maze, and day on fly geotaxis behavior after 15 generations of selection. Significant effects of treatment, population, maze, and day are observed in both males and females.

G15 Estimates

Source	DF	χ2	P-value
Males			
Treatment	2	4.41880	0.0355
Pop[Treat]	4	13.10050	0.0108
Maze	6	203.9989	<.0001
Day	6	180.8064	<.0001
Females			
Treatment	1	8.75162	0.0031
Pop[Treat]	4	61.16238	<.0001
Maze	6	87.39717	<.0001
Day	6	29.64879	<.0001

Table 8. Independent contrast comparing treatment populations to the outbred population after 15 generations of selection. Significant differences are detected between each treatment comparison for males and females.

Males

Population Comparison	Difference	Standard Error	X2	DF	P - Value
Outbred ≠ YRV	0.1132	0.0278	16.9	1	<.0001
Outbred ≠ No YRV	0.1563	0.0273	33.6	1	<.0001
YRV ≠ No YRV	0.0431	0.0184	5.5	1	0.0191

Females

Population Comparison	Difference	Standard Error	X2	DF	P - Value
Outbred ≠ YRV	0.3582	0.0330	126.0	1	<.0001
Outbred ≠ No YRV	0.4239	0.0330	179.0	1	<.0001
YRV ≠ No YRV	0.0657	0.0209	9.9	1	0.0017

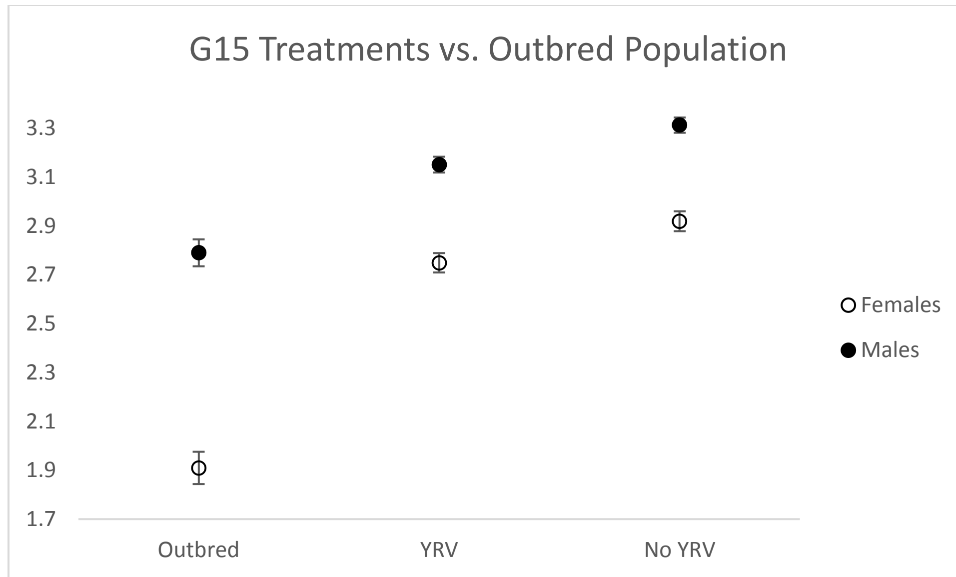

Figure 10. Post-hoc comparison of selected treatments (YRV and No-YRV) and the unselected outbred population. Both males (closed circles) and females (open circles) show the outbred population with significantly lower exit values than the two selected treatments.

Discussion

The G1 estimates of the YRV and No YRV treatments show a significant difference in males but no significant difference in females (Table 7). No difference in the females suggests that the autosomal and X-linked variation across the treatments is similar with regards to geotaxis behavior. In males, the No YRV treatment showed a significantly lower geotaxis score than the YRV treatment. This may be due to the fact that the No YRV treatment was seeded with a single, randomly selected, Y-chromosome. Previous studies have shown that Y-chromosomes influence geotaxis behavior (Stolenberg & Hirsch, 1997). Given their design and creation, the only difference across these treatments was their Y-chromosome make up. These data suggest that YRV exists within our chosen Y-chromosomes and that the Y-chromosome selected for the No YRV treatment displays a significantly lower geotaxis score than the 25 Y-chromosomes of the YRV treatment.

The G15 estimates show significant differences between all treatment contrasts (Table 9). Both selected treatments (YRV and No YRV) show a significantly higher geotaxis value than the outbred

population which experienced no selection suggesting that our selection regime elicited higher geotaxis scores in our Y-chromosome treatment populations. Furthermore, in both males and females, the No YRV population estimates are significantly higher than the YRV populations. In males, the No YRV treatment which started with a lower geotaxis score relative to the YRV treatment ended with a significantly higher geotaxis score. In females, where the starting geotaxis scores of the treatment populations were not significantly different show significant differences after 15 generations of selection, again with the No-YRV treatment showing a greater mean exit value (Figure 9). These data suggest that after 15 generations of selection, the No YRV treatments responded more efficiently to selection reaching a significantly higher geotaxis score than the YRV treatment and the outbred population.

The use of geotaxis as a trait to study the effects of YRV introduced some complications to our analysis. Population geotaxis scores varied greatly from day to day (S1). We hypothesize that changes in the weather outside contributes to phenotypic variation in this trait. There also seemed to be a strong effect of season on geotaxis behavior where flies seemed to climb higher in the summer (at the start of the experiment) and lower in the winter (at the end of the experiment) (S1). Furthermore, the mazes we created influenced fly behavior significantly (Table 7 & 8). However, given that all flies experienced similar environmental conditions on the days where selection occurred and experienced selection across all of the mazes throughout the course of the experiment, selection should still shape this behavior if genetic variation exists for geotaxis and no overwhelming GxE interactions are present. Ideally, differences in robust estimates for each population at each generation would provide the strongest evidence for YRV's influence on autosomal trait evolution. Unfortunately, it was not until we had collected significant amounts of data that we recognized these caveats and the number of flies and time required for each run hindered such data collection. Instead, robust estimates at the G0 and G15 time point give us the best glimpse into how YRV influenced the response to selection for geotaxis behavior.

Population parameters are expected to play a large role in how genetic variance components manifest in a given population. This study examined the effects of YRV on populations containing roughly ~550 flies experiencing a moderate to high strength of selection ($\beta \approx .65$ in males only). Smaller

population sizes would be more susceptible to drift which may change the frequencies of interacting genetic elements potentially releasing additive genetic variation where larger population sizes will be less susceptible to such stochastic changes. Additionally, stronger strengths of selection may influence the genetic architecture of the trait in question. We aimed to choose a strength that was strong enough to elicit a response in a reasonable number of generations but not too strong as to fix Y-chromosomes in a population and release additive genetic variation. Though this study provides evidence for YRV as a constraint to autosomal trait evolution, YRV may manifest in a variety of ways depending on the population parameters it experiences.

Despite the aforementioned caveats with this study, we still gain some insight into the effects of YRV on autosomal trait evolution though our robust starting and ending estimates. By estimating populations across multiple mazes and multiple days, we can better estimate the geotaxis behavior of our treatments. These data indicate that the No YRV treatment showed a greater response to selection after 15 generations than the YRV treatment. This suggests that the non-additive nature of Y-linked regulatory variation constrains the adaptive evolution of geotaxis behavior. The constraint observed at the 15 generation mark is evidence for a reduced rate of adaptive response to selection. It is still possible that the Y-chromosome variation contributed to the additive genetic variance of geotaxis in the long run due to allele frequency changes over time, but a much longer experiment would be required to determine YRV's role in the evolutionary potential of autosomal traits. Future studies should aim to directly detect the reduced heritability in populations containing variable Y-chromosomes in a much simpler and easy to estimate trait (i.e. morphology). Expanding these types of population level studies to other organisms may shed light on the extent of this effect across other independently evolved Y-chromosomes or other heterogametic sex chromosomes. Additionally, investigating how different population parameters like population size and strength of selection may yield insight into the variety of ways YRV can influence autosomal trait evolution.

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CHAPTER 5: CONCLUDING REMARKS

Due to their lack of recombination, heterogametic sex chromosomes like the Y, typically have reduced gene content with low levels of nucleotide variation (Reviewed in Tomaszkieqicz et al. 2017). Furthermore they are unisexually inherited making them a unique aspect of the genome. Previous research suggested that the Y should play little to no role in the evolution of autosomal traits as a result of these unique characteristics. Recently, a potentially wide reaching phenomenon detected in *Drosophila melanogaster* provided a mechanism by which the Y may have profound effects on autosomal trait evolution. The discovery of Y-linked regulatory variation reopened the door to evolutionary studies involving the Y-chromosome which has recently been neglected in most evolution studies.

Sex specific genetic variation in gene expression may provide a simple and untested mechanism for the evolution of sexually dimorphic gene expression. For this to occur, YRV must exist within natural populations and affect fitness related phenotypes. Prior to this study, all Y-linked regulatory variation studies looked at interpopulation Y-chromosome variation (Lemos et al. 2010, Jiang et al. 2010). If YRV only exits on the interpopulation level, selection will not be able to shape it to facilitate the adaptive evolution of sexually dimorphic gene expression. Our data suggests that YRV does exist within natural populations of *Drosophila melanogaster*. Furthermore, we provide the first evidence that YRV translates into phenotypic variation in immune response, a trait that influences organismal fitness. These data indicate that YRV is susceptible to selective forces.

However, selection cannot act on all types of phenotypic variation. In order to determine if selection will efficiently shape YRV, we aimed to determine if within population Y-chromosome variation contained any additive effects. Following similar designs as previous Y-chromosome studies, we paired Y-chromosomes with different genetic backgrounds to try and detect any additive properties of Y-chromosomes. Our intrapopulation study corroborated the previous interpopulation studies revealing no additive properties of Y-chromosomes, but large non-additive properties. These data do not provide evidence for a simple Y-linked mechanism for the evolution of sexually dimorphic gene expression. They

did however bring to light potential adverse evolutionary consequences of YRV. Large non-additive effects may manifest in populations as phenotypic variation that is not additive, introducing noise to male phenotypes, reducing trait heritabilities and thus evolutionary rates.

The genetic architecture of complex traits is a property of a population and is dictated by the frequencies of the genetic elements involved in phenotypic expression (Mackay 2014). In order to determine how YRV will influence the genetic architecture of autosomally coded traits, we needed to investigate populations, not specific genetic elements. The study of physiological properties of genetic elements can tell us a little about the genetic architecture of a trait, but selection acts on genetic variance at the population level. The data from our experimental populations experiencing similar selective pressures indicate that YRV can constrain evolutionary rates and reduce how autosomal traits respond to selection.

The data presented in this dissertation provide the foundation for further study of Y-linked regulatory variation. Though the data are only derived from a single natural population of a single species and limited to only a few traits, the techniques used and questions asked about natural YRV are fundamental to our understanding of this phenomenon. They provide evidence that YRV exists in a relevant context and may have interesting consequences for how traits evolve. Though the ubiquity of this effect is still not well understood, these data indicate that YRV is a phenomenon that deserves further investigation. Future studies should investigate how wide spread this effect is. Do other independently evolved Y-chromosomes show the same effect? Do young Y-chromosomes show similar effects across the genome or is YRV just a characteristic of old degraded Y's? What traits are susceptible to YRV and are these similar in independently evolved Y-chromosomes? What are the consequences of one genetic element influencing so many traits? Additionally, the effects of YRV on the genetic architecture of traits will change under different population parameters. Drift and selection may play a significant role in changing allele frequencies making it possible for non-additive YRV to manifest as additive genetic variance (Goodnight 1988). This would have significantly different evolutionary consequences for how

traits evolve. Future studies should also investigate how different population parameters influence how YRV manifests in natural populations.

Until now, Y-chromosomes have been excluded from most sequencing studies because of the difficulty of assembling chromosomes with large blocks of repeated sequences (Tomaszkieqicz et al. 2017). New technologies are available now that allow for the better assembly of repetitive elements (PacBio Sequencing). With the advancement of sequencing technologies, a better understanding of the mechanism behind YRV may be unearthed. These technologies may also provide a way to investigate Y-chromosome population genetics, a field of study that has been neglected for a long time.

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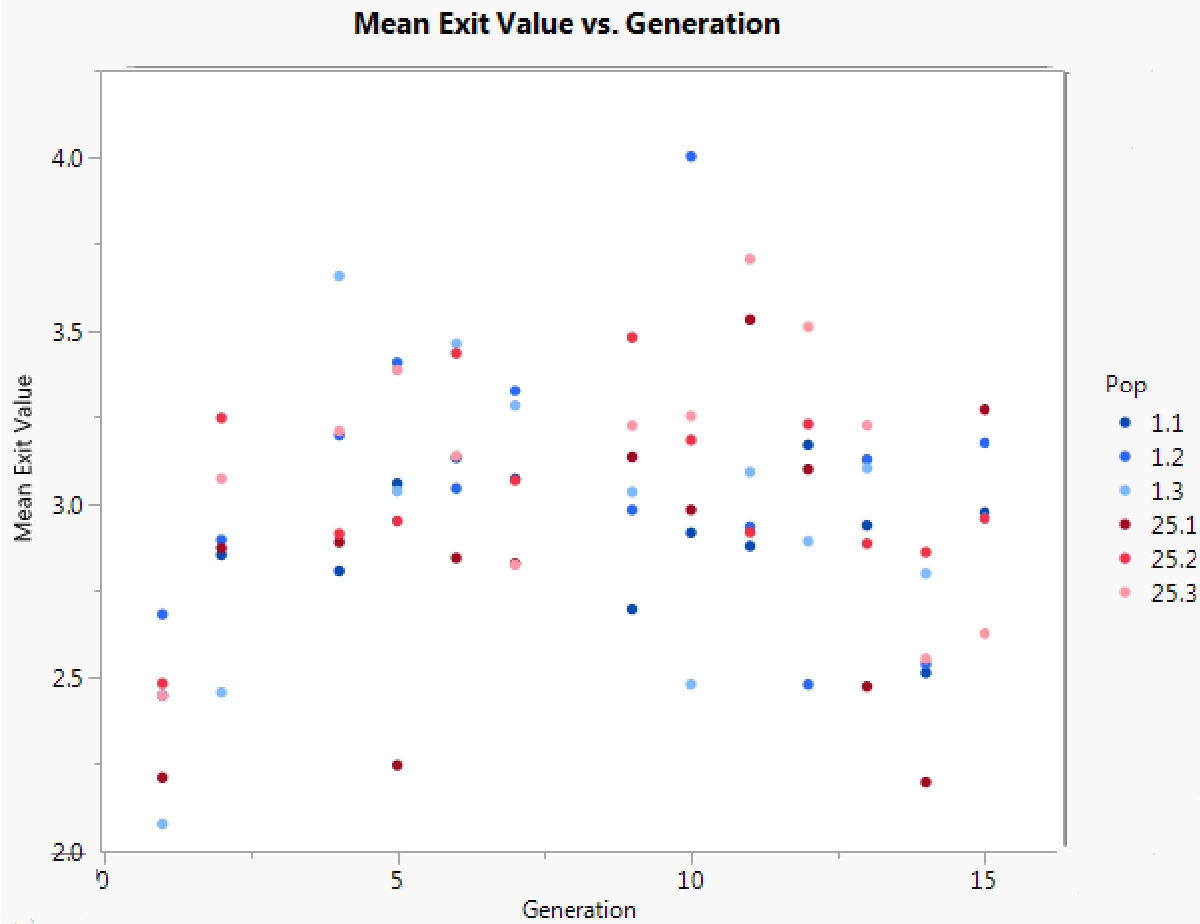
APPENDIX: SUPPLIMENTARY MATERIAL



S1: Weak estimates of each population across the 15 generations of selection. Population names are color coded where No-YRV populations are blue and YRV populations are red. As stated in Chapter 4; each estimate is the result of 300 males traversing the mazes. Each estimate is confounded with day and maze as each population was only measured in one maze on one given day. These caveats prevent any robust interpretation of the data given the significances of the day and maze effect. However, a general increasing in population means is seen for the first 8 generations followed by a general decreasing for the remainder of the study.