Ethanol sensitivity and rapid tolerance in *Drosophila*

by

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Abstract

Overall, the goal of this project is to understand how the response to ethanol in males and females has diverged between species, which is an essential first step in connecting phenotypic differences in this response to molecular evolution in underlying gene networks. *Drosophila* is an important model organism in the study of ethanol tolerance and sensitivity. This is due to the ease of genetics in this system, the wealth of genomic knowledge and widely conserved physiological and behavioral responses. In addition, *Drosophila* feed on fermenting fruit and frequently encounter ethanol in their environment. While lethal effects of ethanol have been surveyed in many *Drosophila* species, little is known about species diversity, and sex dimorphism, of ethanol sensitivity and tolerance. This thesis set out to better understand species and sex differences in the response to ethanol. To do this, behavioral assays of sensitivity to sedating effect of ethanol, as well as tolerance to multiple exposures, were used to examine the response in males and females of fifteen *Drosophila* species. I find that measure of ethanol sensitivity and tolerance are highly variable. Despite this, likely environmental, variance within species there are significant differences among species including presence and absence of ethanol tolerance. Some species show statistically significant sex dimorphism individually, but differences between adult males and females using our methodology was not common. When significant sex differences were detected, they were primarily in tolerance rather than in sensitivity. To lay the groundwork for future studies of the molecular basis of these differences we examined both data from the existing literature on regulation of sex differential expression, including our own, and newly collected data on the genes downstream of *fruitless* a major regulator of neurological differences between sexes, as well as an essential genes in both sexes. I find that ethanol tolerance and sensitivity vary substantially across species, and in some cases differences are sex specific or dimorphic. Additionally, I identified eighty six potential candidates for the regulation of sex differences in ethanol tolerance and sensitivity that are downstream of both major branches of the sex determination pathway.

Acknowledgments

I would like to thank, first and foremost, my advisor Dr. Rita M. Graze for her support and guidance throughout my studies at Auburn University. Without her commitment to this work and to me as a graduate student, this thesis would not have been possible. I would also like to thank the other members of my graduate committee, Drs. Leslie Goertzen and Aaron Rashotte for all their beneficial insight they provided to this project. In addition, I would like to thank all the members of the Graze lab past and present for their assistance and discussions on this work. Finally, I would like to thank my family and friends for their encouragement and support as I pursued this thesis.

Table of Contents

List of Tables

List of Illustrations

List of Abbreviations

ADH	alcohol dehydrogenase
ALDH	acetaldehyde dehydrogenase
ST50	time to knock out 50% of the flies in a sample
LD50	the amount and/or percent of ethanol that kills 50% of the flies in a sample
SD50	the amount and/or percent of ethanol that knocks out 50% of the flies in a sample

Introduction

The goal of this project is to expand current knowledge of species diversity in ethanol sensitivity and one form of tolerance, rapid tolerance, in the genus *Drosophila* by characterizing diversity at the phenotypic level in males and in females. These are essential first steps, which lay the groundwork for understanding evolution of the response to ethanol at the level of the underlying gene regulatory networks and placing this diversity in a phylogenetic context. These experiments develop interspecific behavioral assays (Chapter 1) with model system gene expression analyses (Chapter 2) to develop testable hypothesis as to the molecular evolution of ethanol response for future genetic and genomic studies. Collectively, the work presented will help expand our understanding of ethanol sensitivity and tolerance in *Drosophila*.

Drosophila is a key model system in the genetics of ethanol sensitivity and rapid tolerance. Behavioral studies have shown that exposure to ethanol affects activity level and eventually leads to sedation, and have also revealed differences between males and females in these behavioral responses (Pfeiler and Markow, 2001; Colon-Parrilla and Perez-Chiesa, 1999; Devineni and Heberlein, 2012; McKenzie and Parsons, 1972; Mercot et al., 1994; Urizar et al., 2007). Molecular studies in this model system have identified many of the genes involved in sensitivity and rapid tolerance, with the aim of understanding their role in alcoholism and alcohol related diseases (*e.g.*, Devineni and Heberlein, 2013; Edenberg et al., 2006). Ethanol response related phenotypic traits are complex, the role of hundreds of genes in ethanol metabolism and in the neurological and behavioral response to ethanol have been well-characterized at the molecular level (Mihalek et al., 2001; Pandey et al., 2003) and quantitative genetic studies suggest approximately a hundred genes have additional, potentially small and cumulative effects on these traits.

Ethanol is a ubiquitous abiotic factor in the natural environment of a fly. Thus, ethanol sensitivity and tolerance are ecologically and evolutionarily important. Natural variation in the response to ethanol in the environment is thought to be genetic, with evidence for a role of variation in alcohol dehydrogenase (ADH) activity. ADH is a short-chain dehydrogenase with a different structure and enzymatic mechanism than vertebrate ADH (Persson, et al., 1991; Persson, Hedlund, and Jornvall, 2008). When a fly is exposed to ethanol, ADH first converts

ethanol to acetaldehyde and then acetaldehyde is metabolized to acetate by acetaldehyde dehydrogenase (ALDH). Both ADH and ALDH promote resistance to ethanol toxicity in flies (Devineni and Heberlein, 2013). The ADH locus has historically been thought to explain much of the ethanol response and its evolution in flies. However, see Siddiq et al., 2017, which corroborates the importance of the fast/slow alleles in present-day populations. This paper does refute the ADH adaptive hypothesis. The diversity of ADH activity has been examined in several species in the genus Drosophila (Mercot et al., 1994; Urizar et al., 2007; Pfeiler and Markow, 2001; Colon-Parrilla and Perez-Chiesa, 1999). However, the majority of these studies have not looked at other genes, have used a single measure of ethanol response or have used only *Drosophila melanogaster* or *Drosophila simulans* as models.

Quantitative genetic and expression studies indicate greater complexity with potentially many genes of diverse function playing a role in the response to ethanol (Morozova, Anholt and Mackay, 2006). They found down-regulation of genes affecting olfaction, upregulation of biotransformation enzymes and with the development of tolerance, altered transcription of transcriptional regulators, proteases, and metabolic enzymes (Morozova, Anholt, and Mackay, 2006). A complex genetic architecture may explain why comparative studies have found that species can have a response to ethanol which conflicts with predictions made on the basis of their ADH activity level alone (Mercot et al., 1994). For instance, *Drosophila bipectinata* has low rapid tolerance even though it has high ADH activity (Mercot et al., 1994; Urizar et al., 2007). Therefore, the genetic basis of species diversity in the response to ethanol is likely to have a complex architecture, motivating the experiments described here and future regulatory genomic investigations.

In addition to overall differences in sensitivity and tolerance, species may differ in sexual dimorphism of the response to ethanol. An extreme example is *Drosophila pachae,* where mature adult males have no ADH activity in wild populations or laboratory stocks compared to the females (Pfeiler and Markow, 2001). Sex dimorphism in ethanol sensitivity or tolerance has also been observed in *D. melanogaster* and *D. simulans*, but it is modest in degree (McKenzie and Parsons, 1972). In Chapter 1, a series of behavioral studies investigate not only species differences, but also male and female differences in tolerance.

Differences between the sexes in *Drosophila* are likely to be genetic and downstream of the known sex determination hierarchy pathway, specifically the terminal transcription factors *doublesex* and *fruitless*. The sex determination gene *transformer* acts in the developing the nervous system to promote sex-specific splicing of *fruitless*. *Fruitless* has been suggested to mediate a subset of the sex differences in behavioral responses to ethanol (Devineni and Heberlein, 2012). Considering that rapid tolerance has a strong neurological component and that *fruitless* is primarily responsible for sex dimorphism in the Drosophila brain, as well as results from the behavioral analyses in Chapter 1, the potential ethanol response gene targets of *fruitless* are the primary focus of the analyses presented in Chapter 2.

Overall, my work has characterized species diversity and sex differences in sensitivity and rapid tolerance to ethanol sedation. In Chapter 1, a series of behavioral assays determined species diversity of ethanol sensitivity and tolerance by assaying males and females of fifteen different species with sequenced genomes. Additional assays were conducted among more closely related species only, in order to understand phenotypic differences prior to genomic studies of allele level regulatory responses to ethanol exposure. Sensitivity, rapid tolerance and sex dimorphism of these traits were examined using a mixed model and pairwise contrasts to account for block effects. In Chapter 2, the potential role of key sex determination genes in regulating ethanol response was explored using both publically available data and newly collected expression data which identifies genes regulated downstream of the male isoform of *fruitless*, separately from the role of isoforms common to both males and females. The goal of this analysis was to identify candidates for sex differences in ethanol response that can anchor future genomic studies and be tested in comparative molecular genetic studies.

Chapter 1

The species diversity of ethanol sensitivity and tolerance

1.1. Introduction

Drosophila is a key model system in the genetics of ethanol sensitivity and rapid tolerance. Molecular studies in this model system have identified many of the genes involved in sensitivity and rapid tolerance, with the aim of understanding their role in alcoholism and alcohol related diseases (Devineni and Heberlein, 2013; Edenberg et al., 2006). Sex dimorphism in behavioral response may be adaptive; female flies oviposit on rotting fruit and are more likely to be exposed to higher levels of ethanol than male flies (Becher et al., 2012). Some studies have looked at sex dimorphism as well in *Drosophila pachea*, *Drosophila acutilabella*, *Drosophila belladunni*, and *Drosophila melanogaster* using different ethanol exposure techniques (Pfeiler and Markow, 2001; Colon-Parrilla and Perez-Chiesa, 1999; Devineni and Heberlein, 2012).

Ethanol sensitivity is defined as the overall sensitivity to the effects of ethanol exposure, including both an initial period of hyperactivity and ultimately sedation. If an organism has decreased ethanol sensitivity then the organism will take longer to be effected by ethanol. On the other hand if an organism has increased ethanol sensitivity then the organism will be affected by ethanol sooner. Rapid tolerance occurs when sensitivity is attenuated in a second exposure, following a single initial ethanol exposure and recovery period (Urizar et al., 2007).

Ethanol sensitivity is affected by both metabolism of ethanol itself and response to downstream products of ethanol metabolism (Fry et al., 2014). Alcohol dehydrogenase is the key enzyme, which affects ethanol sensitivity. However, other major effect loci affect ethanol sensitivity. For example, the gene *amnesiac* (amn) is believed to encode a neuropeptide that stimulates cAMP production. Proper activation of the cAMP pathway plays an important role in regulating ethanol sensitivity in *Drosophila* (Moore et al., 1998). After the fly absorbs ethanol, it is degraded by a number of enzyme systems to acetaldehyde. Acetaldehyde is converted to

acetate and acetyl-CoA, which initiates the pathway that degrades more than 90% of the total ethanol in the fly (Geer et al., 1993).

Rapid tolerance, in contrast, is affected both by metabolism and by adaptive changes in the central nervous system (Scholz et al., 2000). *Drosophila* mutants with structural abnormalities in certain brain regions have a reduced ability to develop tolerance (Scholz et al., 2000). In *Drosophila*, the octopaminergic systems and the circadian clock in the brain are also involved in tolerance acquisition. The circadian clock genes work together to create the twenty-four hour cycle seen in flies and other organisms. However, the role of fly circadian genes in ethanol rapid tolerance is independent of their role in producing circadian rhythmicity. Mutations in *per*, *tim*, and *cyc* can completely block ethanol tolerance (Pohl et al., 2013). Some genes play roles in both ethanol sensitivity and rapid tolerance. For example, *homer* regulates ethanol sensitivity and tolerance through its function in the central nervous system especially the ellipsoid bodies (Urizar et al., 2007).

Is ethanol a significant selective pressure in natural populations? The natural breeding sites of wild *D. melanogaster* contain up to nine percent ethanol (Gibson and Wilks, 1988) and it is prevalent in both larval and adult environments. Environmental ethanol can exert strong selective pressure during larval development (McKechnie and Morgan, 1982; Hoffmann and Parsons, 1993; Fry, 2001). Variation in ethanol tolerance may also be related to presence of other environmental alcohols (David et al., 1986). A classic example of molecular evolution is the polymorphism involving electrophoretic variants of ADH. The two major variants are ADH^F and ADH^S. The fast-migrating allele (ADH^F) appears to have a selective advantage in high environmental concentrations of certain alcohols. The slow-migrating allele (ADH^S) has a selective advantage at high temperatures and in the presence of some alcohols (Thompson, Jr. and Kaiser, 1977). More recent studies have shown that enzymes involved in metabolism of ethanol or downstream metabolites of ethanol are also likely to be important in variation and evolution of ethanol sensitivity and tolerance (Fry, 2014; Chakir et al. 1996).

An additional complexity of understanding adaptation to ethanol is that some *Drosophila* species have a sexually dimorphic response. In some laboratory strains, *D. melanogaster* male flies have increased hyperactivity and greater resistance to ethanol sedation compared with

females in response to high ethanol vapor concentration (Devineni and Heberlein, 2012). However, earlier studies in natural population found that females were less sensitive to ethanol than males (McKenzie and Parsons, 1972). Therefore, it is unclear if there is a consistent effect of sex. There are, additionally, potentially sex by environment or by genotype interactions affecting ethanol sensitivity. Beyond subtle differences found in *D. melanogaster*, *Drosophila pachea*, *Drosophila acutilabella*, and *Drosophila belladunni* display extreme sex dimorphism in ADH activity, which may be linked to sex differences in ethanol sensitivity and/or tolerance (Pfeiler and Markow, 2001; Colon-Parrilla and Perez-Chiesa, 1999).

The most extensive surveys of ethanol response in Drosophila, to date, have focused on relating ADH expression, ADH activity level, and ethanol induced mortality (Figure 1). For instance, the ADH of *D. funebris* has a high specific activity, which may improve the ethanol tolerance due to a rapid metabolic flux. *D. melanogaster*, *D. labanonensis*, and *D. virilis* show the highest levels of ethanol tolerance in the genus *Drosophila,* under standard laboratory conditions. However, many species display responses to ethanol that are not strongly correlated with ADH activity level. *D. funebris*, *D. littoralis*, and *D. mercatorum* have low ADH activity, but they are tolerant to ethanol. On the other end of the spectrum are *D. bipectinata*, *D. parabipectinata*, and *D. ercepeae*. These species have low ethanol tolerance even though they have high ADH activity (Mercot et al., 1994). The decoupling of ethanol tolerance and ADH activity suggests the importance of gene regulation and potentially other genes in the ethanol tolerance pathway. Sex specific interactions imply as yet uncharacterized sex specific regulation in related gene regulatory networks. Unfortunately, sex dimorphism in ethanol sensitivity and tolerance has not been examined in many of the sequenced species, and the exemplar species are not among the sequenced species of *Drosophila*. This gap in knowledge is addressed in this chapter.

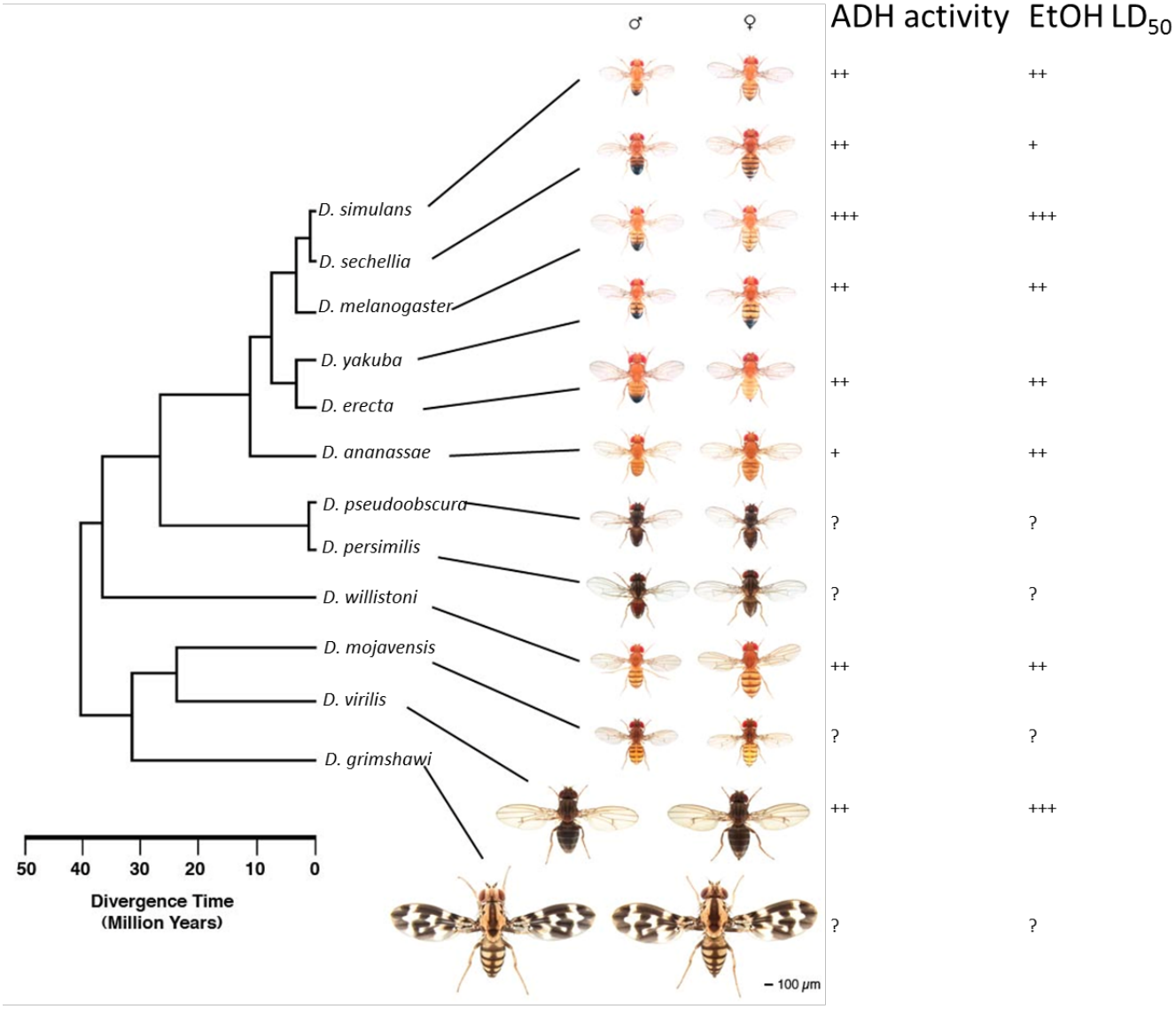


Figure 1. ADH activity, EtOH LD50, and sex dimorphism in *Drosophila*. ADH activity, EtOH LD_{50}, and mean ST50 (this study) are shown. Pluses indicate relative activity level or LD50, respectively. Sex dimorphism in tolerance has been observed in some, but not all species and in many cases has not been tested. Images by Nicolas Gompel. The relationship between ADH activity and LD50, a measure of toxicity, shows that ADH activity is not always predictive of survival rates upon exposure to ethanol vapor. Some species have sex dimorphism in ADH activity and LD50 (Raganayakulu and Reddy, 1994; Mercot et al., 1994).

D. simulans (*sim*) N = 4 Blocks 1

D. mauritiana (*mau*) N = 4 Blocks 1, 2

D. sechelia (*sec*) N = 6 Blocks 7, 8

D. yakuba (*yak*) N = 6 Blocks 1, 2

D. erecta (*ere*) N = 6 Blocks 6, 7

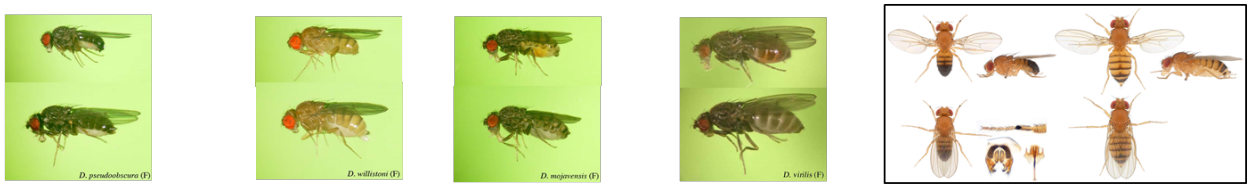
D. biarmipes (*bia*) N = 4 Blocks 1, 2

D. ficusphila (*fic*) N = 5 Blocks 3, 4

D. annanasae (*ana*) N = 7 Blocks 1, 2

D. bipectinata (*bip*) N = 5 Blocks 1, 4

D. kikkawai (*bip*) N = 6 Blocks 2, 4



D. Pseudoobscura (*pse*) N = 4 Blocks 8

D. willistoni (*wil*) N = 5 Blocks 1, 2

D. mojavensis (*moj*) N = 4 Blocks 2, 3

D. virilis (*vir*) N = 20 Blocks 2-6, 8

D. melanogaster (*mel*) N = 17 Blocks 1, 5-7

Figure 2. All species assayed. The minimum number of replicates per sex and exposure in experiment 1 is given (N). The blocks (1-8) in which each species was assayed is shown. Blocks 1, 5-7 had *D. melanogaster* as a common control and blocks 2-6 and 8 had *D. virilis* as a common control. Blocks 5, 6, and 8 included both of the control species. Images by Nicolas Gompel and Ehime Fly Stock Center.

The behavioral assays conducted here (Chapter 1) expand on the previously mentioned earlier studies, but focus on sensitivity and rapid tolerance, rather than survival. Further, the assays have been done in both males and females for all species- using a standardized protocol, allowing for statistical analysis of sex differences in multiple species.

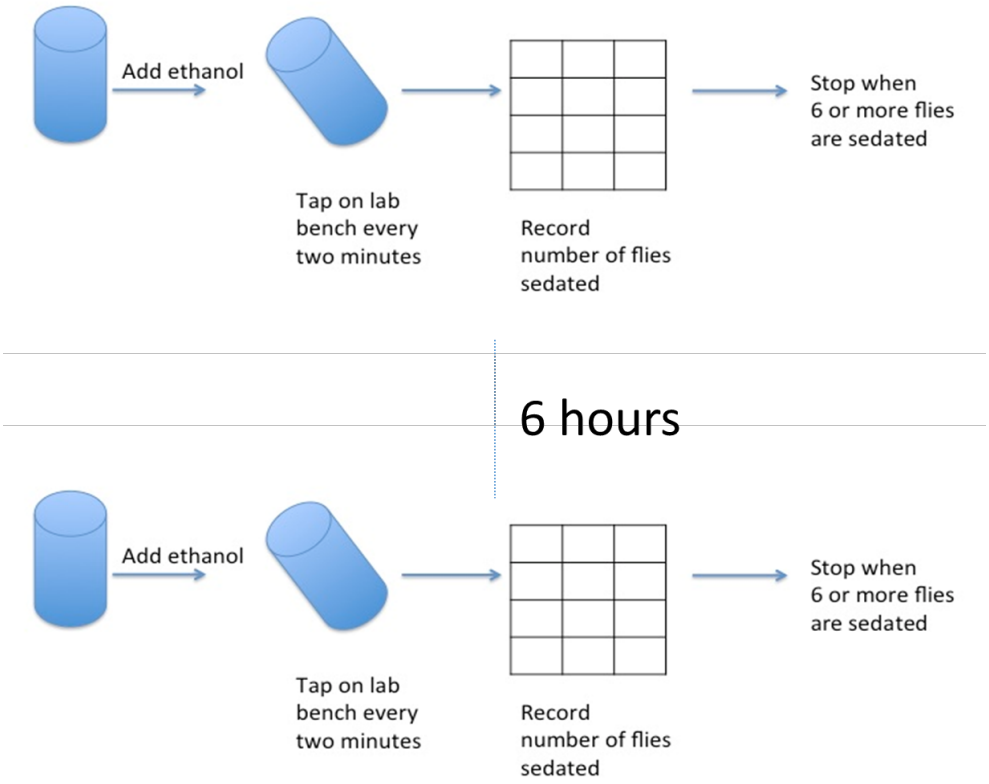


Figure 3. Ethanol sensitivity and rapid tolerance assay. Ten individuals per replicate were exposed to ethanol in plastic chambers. Chambers were randomized and assays were blind with respect to sample type. Each person measuring sedation received a random set of sample types. Some measurers participated in all blocks, but by necessity different blocks often involved different people participating in the assay.

1.2. Materials and Methods

(a) Study Design

To determine which species have high ethanol tolerance and whether there are differences between the sexes within a species, fifteen fully sequenced strains (DSSC collection) have been assayed for sensitivity and rapid tolerance to ethanol induced sedation. Data were collected in eight blocks of assays. Over all blocks four to twenty independent replicates were

assayed for males and females of each species, with most species having six replicates (Figure 2). Each block contained different groups of species and one control species common to multiple blocks, either *D. melanogaster* or *D. virilis*.

Three different behavioral assays were conducted, a broad species survey including all species shown in Figure 2 (Experiment 1), a focused survey of species in the *melanogaster* subgroup (Experiment 2) and a longer assay of the two controls included in each block *D. melanogaster* (two week life cycle species) and *D. virilis* (three week life cycle species) (experiment 3).

Experiment 1: Ethanol induced sedation was measured in ethanol exposure chambers following Maples and Rothenfluh, 2011 (Figure 3). Each chamber was constructed from a 28.5 x 95 mL plastic vial. To measure sensitivity, ten flies were transferred to an exposure chamber. A vial plug was coated with 1 mL dyed 85% ethanol and inserted into the exposure vial. Immediately after ethanol was added, the vial plug was pushed into the exposure chamber and a second plug was used to prevent escape of ethanol vapor during exposure. The number of flies sedated was assayed immediately (time zero). Every two minutes, the exposure chambers were tapped on the lab bench to startle the flies and the flies were observed for ten seconds. The number of sedated flies was recorded at each two minute interval. The time to ST50 was calculated for a thirty minute exposure. After thirty minutes has passed, the flies were transferred to regular food vials and allowed to recover for six hours. The flies were re-exposed and the ST50 of the second exposure was determined.

Experiment 2 and 3: Ethanol induced sedation was measured similarly to the approach described for experiment 1 with the following exceptions. To measure sensitivity, ten flies were transferred to an exposure chamber. Half of a Q-tip was soaked in dyed 85% ethanol and inserted into a hard white plug. Then the hard white plug was inserted into the exposure vial. This protocol was used to assay sensitivity and tolerance for *D. melanogaster*, *D. simulans LHR*, *D. yakuba*, *D. mauritiana*, and *D. sechellia* with a thirty minute exposure and for *D. melanogaster* and *D. virilis* for a one hour exposure.

(b) Analysis

Experiment 1: The ST50 (time to 50% sedation) was $\log_2$ normalized and a mixed model was fit with $Y_{ijklm} = \mu + B_i + P_j + S_k + E_l + BP_{ij} + BS_{ik} + BEil + PS_{jk} + PE_{jl} + SE_{kl} + BPS_{ijk} + BPE_{ijl} + BSE_{ikl} + PSE_{jkl} + BPSE_{ijkl} + \varepsilon_{ijklm}$. The dependent variable Y_{ijklm} is the normalized ST50 for each of *i* blocks (1-8), *j* species (see Figure 2), *k* sexes (male, female), and *l* exposures (1, 2), for *m* replicates (1, 2, 3 … *m*). To account for the incomplete blocking structure and heterogeneity of variance we fit a cell means model, $Y_{ij} = \mu + t_i + \varepsilon_{ij}$, where Y_{ij} is the is the normalized ST50 estimate for the I^{th} treatment type (*ana*, *bia*, *bip*, *ere*, *fic*, *kik*, *mau*, *mel*, *moj*, *pse*, *sec*, *sim*, *vir*, *wil* and *yak*; F-female or M-male; exposure 1 or exposure 2) and J^{th} replicate. Contrasts were constructed to test each differential expression comparison of interest for each species separately. For example, to test sex and exposure differences in *mel* the male and female sensitivity (exposure 1) for each combination of factors (denoted as μ1F and μ1M) will be compared using an *F* test (μ1F = μ1M). The differences between males and females at exposure 2 (rapid tolerance) and the differences between exposure times for males and females (μ2F = μ2M, μ1F = μ2F, and μ1M = μ2M) were tested using the same approach. Sex dimorphism can be present in either one or both of the exposure times, with either males or females showing significantly lower ST50 values if one sex is more sensitive than the other sex. Significant differences between exposure 1 and exposure 2 are evidence of rapid tolerance, when the two differ and exposure 2 has a longer ST50 than exposure 1. In a few cases that time to sedation was greater than the maximum assay length or less than the minimum time point assayed. In the first case a maximum time to 50% sedation was substituted as ST50 = 30 minutes and in the second case a minimum time to 50% sedation as ST50 = 1 minute.

Experiment 2 and 3: The two additional assays did not require blocking. The ST50 (time to 50% sedation) was $\log_2$ normalized and a mixed model was fit with $Y_{ijkl} = \mu + P_j + S_j + E_k + PS_{ij} + PE_{ik} + SE_{jk} + PSE_{ijk} + \varepsilon_{ijkl}$. The dependent variable Y_{ijkl} is the normalized ST50 for each of *i* species (Experiment 2: *mau*, *mel*, *sec*, *sim* LHR strain, and *yak;* Experiment 3: *mel*, *vir*), *j* sexes (male, female), and *k* exposures (1, 2), for *l* replicates (1, 2, 3 … *m*). *F* tests were constructed to

test the difference between the first and second exposure for each sex and species (e.g., $\mu 1M = \mu 2M$) and to test for an interaction of sex and exposure for each species ($\mu 1F - \mu 2F = \mu 1M - \mu 2M$).

1.3. Results

Experiment 1, fifteen Drosophila species: In this experiment a complex blocking structure was necessary due to the number of assays to complete and the differences in life cycle across species (Figure 2). To determine if there were significant effects of block an initial mixed model with all factors (block, species, sex and exposure) was fit. The mean normalized ST50 for each species sex and exposure is shown in Table 1 for all species. Note that the variance for the measure of sensitivity (ST50 at exposure 1) and rapid tolerance (ST50 at exposure 2) was very large. It is likely that only large effects were detectable in this data (see Figure 4). The results for the main effects and for interaction terms are reported in Table 1 A-B. There was a significant main effect of block, as well as a significant interaction between block and species or exposure. The four way interaction (Species*Sex*Exposure*Block) was also significant. The main effect of species (Figure 5A) was also significant and differences between species were generally larger than differences between sexes or exposures. However, the interaction between species and block was significant, which is shown in Figure 5B. For some species there was little or no difference in mean normalized ST50 across blocks, but for others large differences across blocks were observed. For example, for *bip* females exposure 2, the $\log_2$ST50 for block one was 3.60, which is similar to exposure 1, but in block four the mean $\log_2$ST50 1.79. To account for the effect of block, as well as heterogeneity of variances, we fit a cell means model to test pairwise contrasts (Table 3).

Table 1 A-B. Mean log_2ST50 for fifteen sequenced Drosophila species (experiment 1).

A. Melanogaster group

Species	Sex	Exposure	Log_2ST50	Species	Sex	Exposure	Log_2ST50
***melanogaster* subgroup**				Other subgroups			
D. melanogaster	M	exp1	3.98	*D. biamarpes*	M	exp1	3.76
	M	exp2	4.27		M	exp2	3.48
	F	exp1	4.04		F	exp1	3.56
	F	exp2	4.17		F	exp2	3.64
D. mauritiana	M	exp1	3.91	*D. ficusphila*	M	exp1	3.75
	M	exp2	3.95		M	exp2	3.65
	F	exp1	3.88		F	exp1	3.79
	F	exp2	3.86		F	exp2	3.99
D. simulans	M	exp1	4.12	*D. kikkawai*	M	exp1	3.30
	M	exp2	4.39		M	exp2	3.33
	F	exp1	4.60		F	exp1	3.57
	F	exp2	4.45		F	exp2	2.95
D. sechellia	M	exp1	4.16	*D. ananassae*	M	exp1	3.80
	M	exp2	4.17		M	exp2	3.05
	F	exp1	4.60		F	exp1	3.52
	F	exp2	4.45		F	exp2	3.41
D. erecta	M	exp1	3.57	*D. bipectinata*	M	exp1	3.30
	M	exp2	3.20		M	exp2	3.26
	F	exp1	3.98		F	exp1	3.69
	F	exp2	4.03		F	exp2	3.00
D. yakuba	M	exp1	3.56				
	M	exp2	3.56				
	F	exp1	3.48				
	F	exp2	3.38				

B. Other groups

Species	Sex	Exposure	Log_2ST50
		***obscura* group**	
D. pseudobscura	M	exp1	4.42
	M	exp2	4.21
	F	exp1	4.43
	F	exp2	4.23
		***willistoni* group**	
D. willistoni	M	exp1	2.53
	M	exp2	3.03
	F	exp1	3.19
	F	exp2	3.34
		***repleta* group**	
D. mojavensis	M	exp1	4.80
	M	exp2	3.85
	F	exp1	4.43
	F	exp2	4.23
		***virilis* group**	
D. virilis	M	exp1	4.86
	M	exp2	4.83
	F	exp1	4.90
	F	exp2	4.91

Table 2. Experiment 1 full model results.

Source	*DF*	*Type III SS*	*Mean Square*	F value	*Pr > F*
Block	7	4.844876	0.692125	2.22	0.0332
Species	14	97.49712	6.96408	22.29	<.0001
*Species*Block*	13	11.28351	0.867963	2.78	0.001
Sex	1	1.260952	1.260952	4.04	0.0455
*Sex*Block*	7	1.538623	0.219803	0.7	0.6692
*Species*Sex*	14	4.956375	0.354027	1.13	0.3283
*Species*Sex*Block*	13	6.779651	0.521512	1.67	0.067
Exposure	1	0.099816	0.099816	0.32	0.5724
*Exposure*Block*	7	3.655701	0.522243	1.67	0.1158
*Species*Exposure*	14	6.47605	0.462575	1.48	0.1173
*Species*Exposure*Block*	13	5.14209	0.395545	1.27	0.233
*Sex*Exposure*	1	0.003043	0.003043	0.01	0.9215
*Sex*Exposure*Block*	7	1.470735	0.210105	0.67	0.6954
*Species*Sex*Exposure*	14	4.549609	0.324972	1.04	0.4134
*Species*Sex*Exposure*Block*	13	10.07659	0.775122	2.48	0.0032

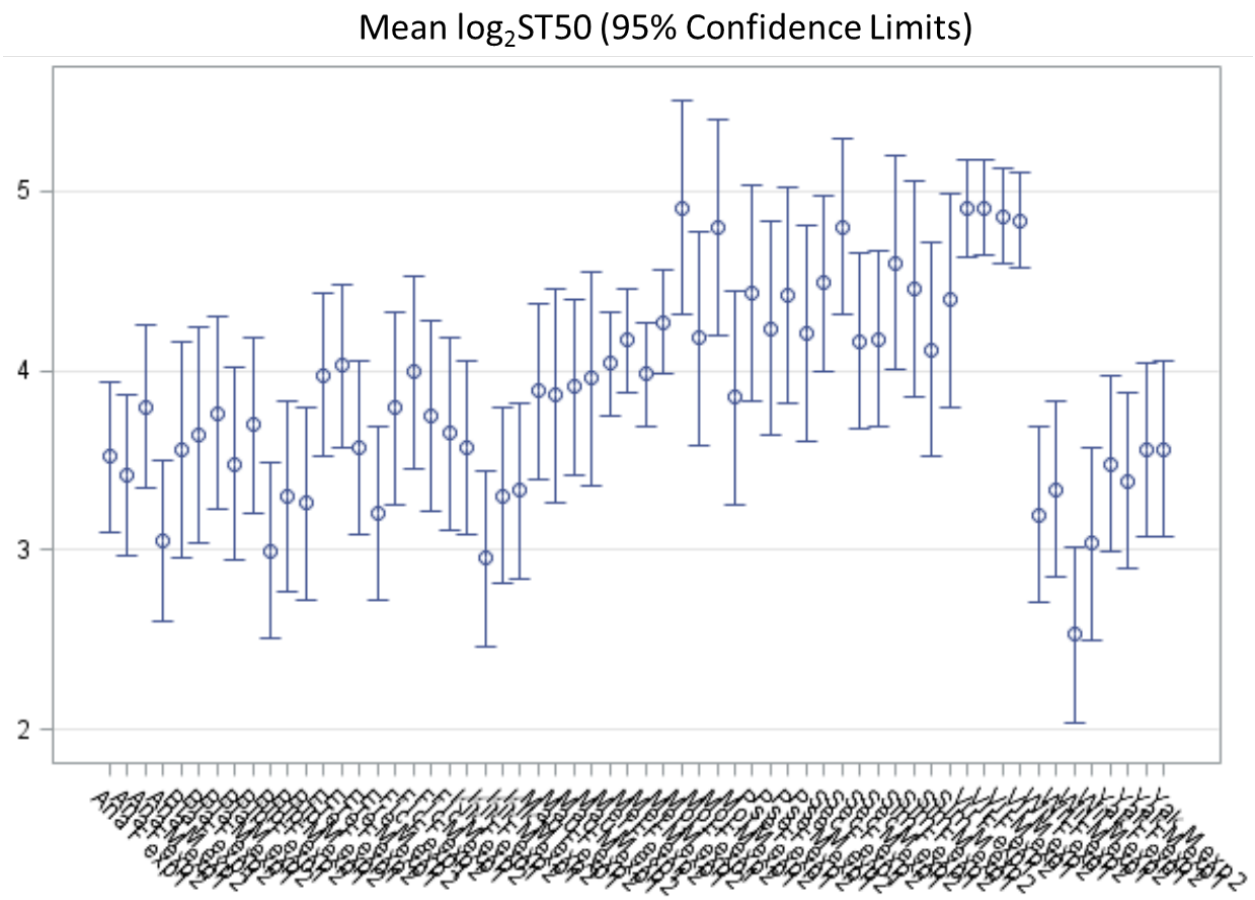
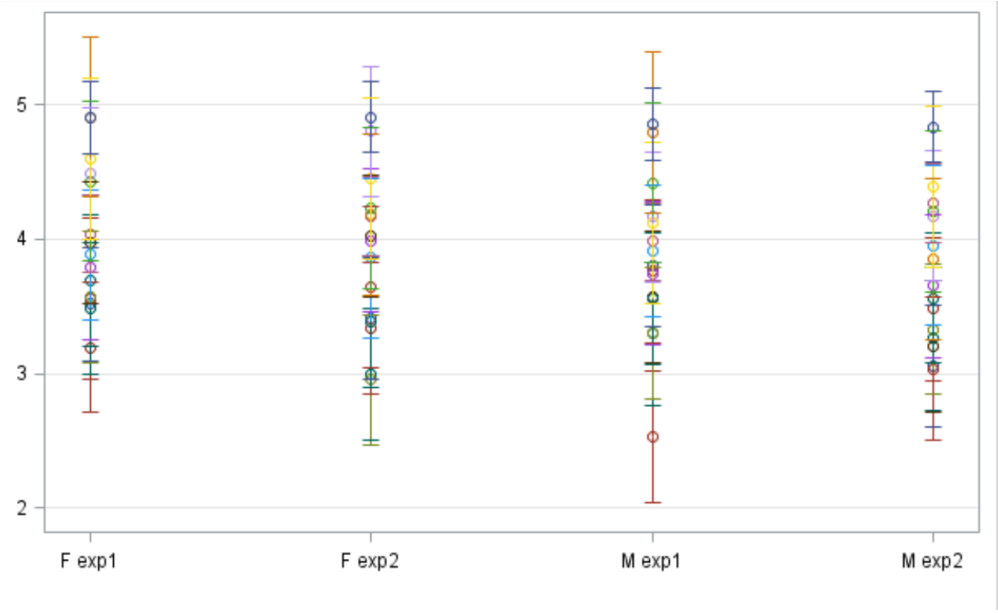


Figure 4. Mean normalized ST50 for each species, sex and exposure in experiment 1. Open circles denote the mean and whiskers the 95% CI.

A) Mean log_2ST50 (95% Confidence Limits) Grouped by Sex and Exposure



B) Mean log_2ST50 (95% Confidence Limits) Grouped by Sex and Exposure

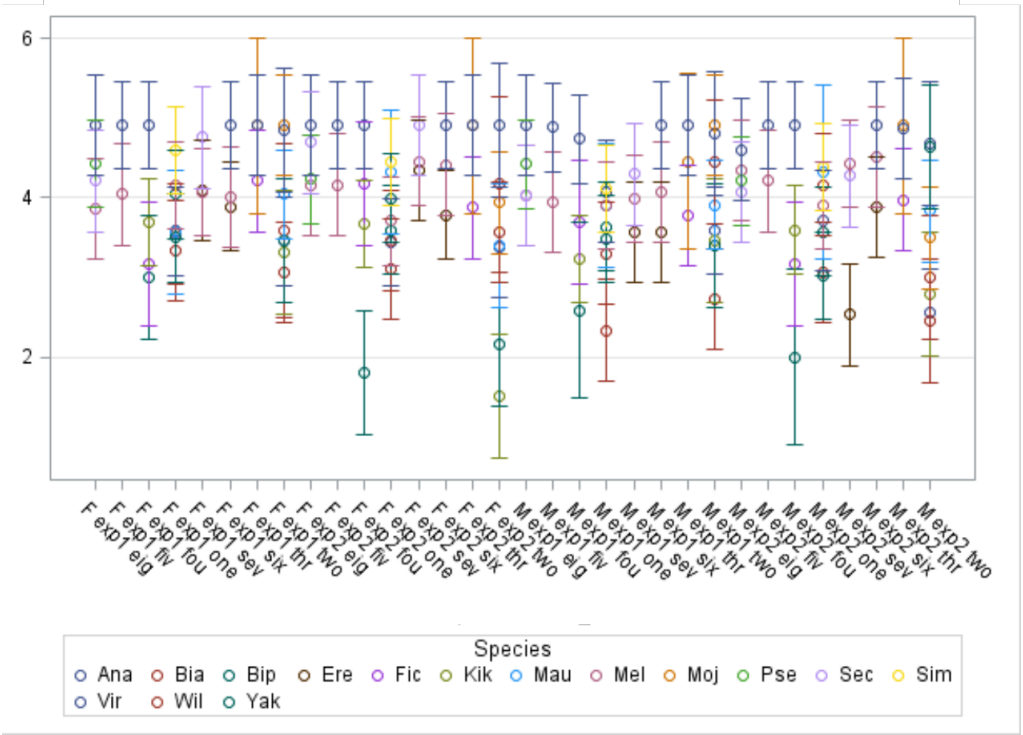


Figure 5 A-B. Mean normalized ST50. A) Grouped by sex and exposure and B) grouped by sex, exposure and block. Blocks are denoted by a three letter code ('one' for 1, 'two' for 2, 'thr' for 3 and so on). Not all species are included in each block, due to differences in life cycle.

Table 3. Pairwise contrasts for fifteen Drosophila species (experiment 1, P < 0.05).

Label	Num DF	Den DF	F Value	Pr > F
ere μ2F = μ2M	1	360.7	6	0.015
bip μ1F = μ2F	1	360.4	4.03	0.045
ana μ1M = μ2M	1	360.4	5.36	0.021
moj μ1M = μ2M	1	360.4	4.92	0.027

Experiment 2, melanogaster subgroup species: In order to decrease variance in measurements of ethanol and tolerance, and to collect phenotypic data in preparation for expression experiments using an interspecific hybrid F1 design, a second experiment was conducted using a modified protocol that reduced the amount of ethanol and kept the ethanol concentrated at a single point in the exposure chamber. A different, hybrid rescue, strain of *D. simulans* was used in this assay (*D. simulans LHR*; Stock #14021-0251.023 (Watanabe, 1979)). A linear model was fit to determine the effects of species, sex and exposure. The mean normalized ST50 is reported in Table 4 and plotted in Figure 6, with 95% confidence intervals. Model results are reported in Table 5. The model did not fit the data, which indicates that unaccounted for factors may have had large effects in this experiment. Only the main effect of sex was significant. No pairwise comparisons were significant. One hypothesis for the poor model fit was that the specific person counting sedated flies might have a significant effect on ST50, which was not accounted for in the initial model. We examined this in two ways; first we fit a second model (model 2), including the effect of person. This model fit the data significantly better than did the model, which not include person as a factor (Model 1: Model SS = 3.97, Error SS = 35.47, and P = 0.57; Model 2: Model SS = 26.29, Error SS = 13.15, and P < 0.0001). In model 2, the main effect of person was

significant, as was the interaction of person and exposure (Table 6). The mean and standard deviation for each person are reported in Table 7.

Table 4. Mean log_2ST50 for the *melanogaster* subgroup (experiment 2).

Species	Sex	Exposure	Log_2ST50
D. melanogaster	M	exp1	4.44
	M	exp2	4.31
	F	exp1	4.51
	F	exp2	4.30
D. mauritiana	M	exp1	4.40
	M	exp2	4.37
	F	exp1	4.35
	F	exp2	4.42
D. simulans LHR	M	exp1	4.56
	M	exp2	4.35
	F	exp1	4.84
	F	exp2	4.58
D. sechellia	M	exp1	4.28
	M	exp2	4.19
	F	exp1	4.48
	F	exp2	4.51
D. yakuba	M	exp1	4.09
	M	exp2	4.26
	F	exp1	4.47
	F	exp2	4.35

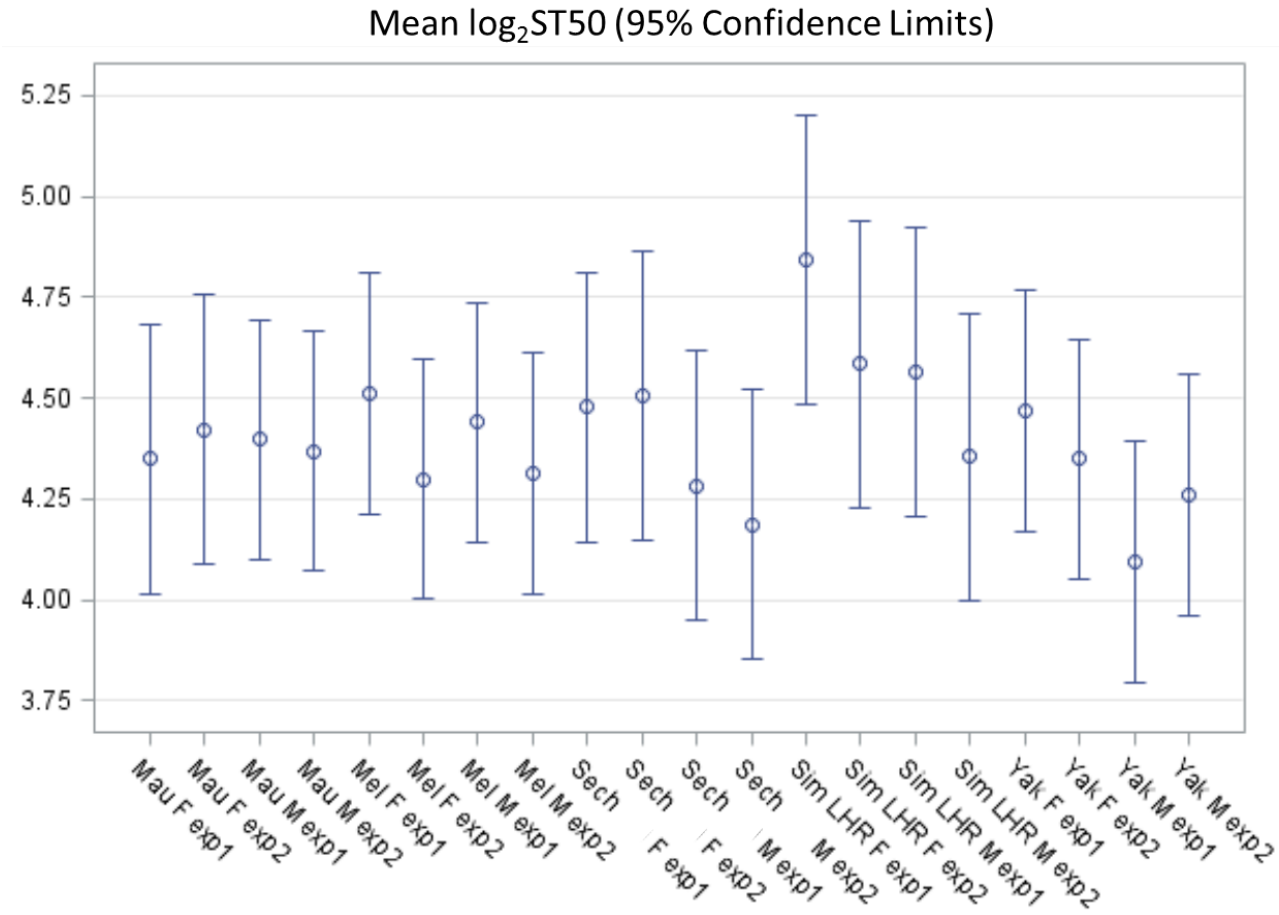


Figure 6. Mean normalized ST50 for each species, sex and exposure in experiment 2. Open circles denote the mean and whiskers the 95% CI.

Table 5. Experiment 2 full model results (model 1).

SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
SPECIES	4	1.476972	0.369243	1.61	0.17
SEX	1	1.032933	1.032933	4.51	0.04
SPECIES*SEX	4	0.569672	0.142418	0.62	0.65
EXPOSURE	1	0.265161	0.265161	1.16	0.28

SPECIES*EXPOSURE	4	0.461728	0.115432	0.5	0.73
SEX*EXPOSURE	1	0.016493	0.016493	0.07	0.78
SPECIES*SEX*EXPOSURE	4	0.250563	0.062641	0.27	0.89

Table 6. Experiment 2 full model results (model 2).

Source	***DF***	***Type I SS***	***Mean Square***	***F Value***	***Pr > F***
Name	3	16.0479	5.3493	45.16	<.0001
Species	4	1.64522	0.4113	3.47	0.0103
Species*Name	12	2.71135	0.22595	1.91	0.0408
Sex	1	0.66191	0.66191	5.59	0.0198
Sex*Name	3	0.38329	0.12776	1.08	0.3613
Species*Sex	4	0.52249	0.13062	1.1	0.359
Species*Sex*Name	11	1.78747	0.1625	1.37	0.1962
Exposure	1	0.3928	0.3928	3.32	0.0713
Exposure*Name	2	0.73165	0.36583	3.09	0.0495
Species*Exposure	4	0.25898	0.06475	0.55	0.7019
Species*Exposure*Name	6	0.32196	0.05366	0.45	0.8415
Sex*Exposure	1	0.0142	0.0142	0.12	0.7299
Sex*Exposure*Name	2	0.13914	0.06957	0.59	0.5576
Species*Sex*Exposure	4	0.2983	0.07458	0.63	0.6425
Species*Sex*Exposure*Name	5	0.36929	0.07386	0.62	0.6822

Table 7. The mean normalized ST50 by person.

Person	N	log_2ST50	
		Mean	Std Dev
I	48	4.71	0.25
II	17	4.63	0.30
III	22	3.71	0.51
IV	88	4.34	0.40

Experiment 3, control species: In order to eliminate the effect of person, a second experiment using the same protocol, but keeping the measurer constant, was conducted for the two controls and with double the assay time (experiment 3). The assay time was doubled because one of the controls had a large body size and often did not have half of a sample sedated in thirty minutes. The mean normalized ST50 is reported in Table 8 and plotted in Figure 7, with 95% confidence intervals. Model results are reported in Table 9. There was a large and significant effect of species, which is expected given that *D. melanogaster* species group and *D. virilis* species group last shared a common ancestor an estimated 40 million years ago (Petrov and Hartl, 1998). Interestingly, with the reduction of confounding factors such as person measuring and block the main effect of sex and exposure are significant, as well as the three way interaction. This is primarily explained by a species difference in the rapid tolerance of females only (Figure 8). It was also apparent that the new assay has generally higher ST50, and that future assays of this type should be conducted with the extended (1 hour) time period in order to avoid species in which ST50 is not reached within the assay window.

Table 8. Mean log_2ST50 for the control species (experiment 3).

Species	*Sex*	*Exposure*	*log_2ST50*
mel	F	exp1	4.793849
mel	F	exp2	4.408707
mel	M	exp1	4.956012
mel	M	exp2	4.810022
vir	F	exp1	5.258281
vir	F	exp2	5.615133
vir	M	exp1	5.580481
vir	M	exp2	5.43608

Table 9. Experiment 3 full model results.

Source	*DF*	*Type III SS*	*Mean Square*	*F value*	*Pr > F*
Species	1	7.642831	7.642831	89.94	<.0001
Sex	1	0.447152	0.447152	5.26	0.0259
*Species*Sex*	1	0.158219	0.158219	1.86	0.1783
Exposure	1	0.090947	0.090947	1.07	0.3057
*Species*Exposure*	1	0.495147	0.495147	5.83	0.0193
*Sex*Exposure*	1	0.061519	0.061519	0.72	0.3988
*Species*Sex*Exposure*	1	0.490925	0.490925	5.78	0.0198

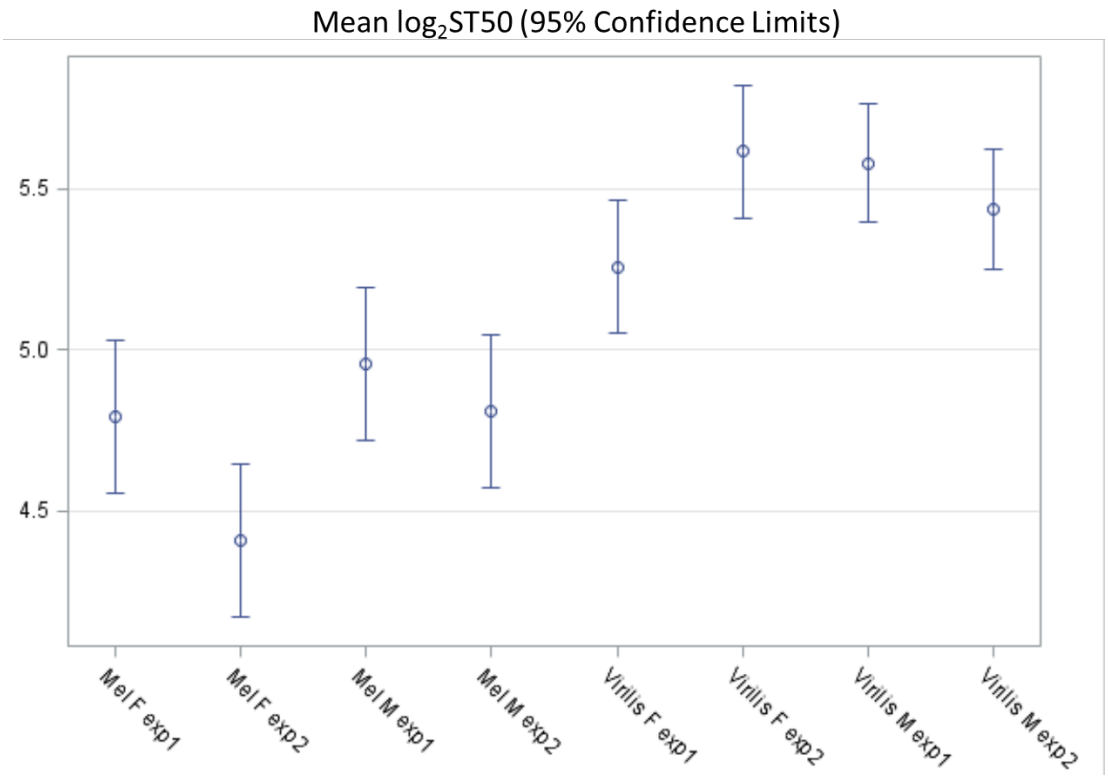


Figure 7. Mean normalized ST50 for each species, sex and exposure in experiment 3. Open circles denote the mean and whiskers the 95% CI.

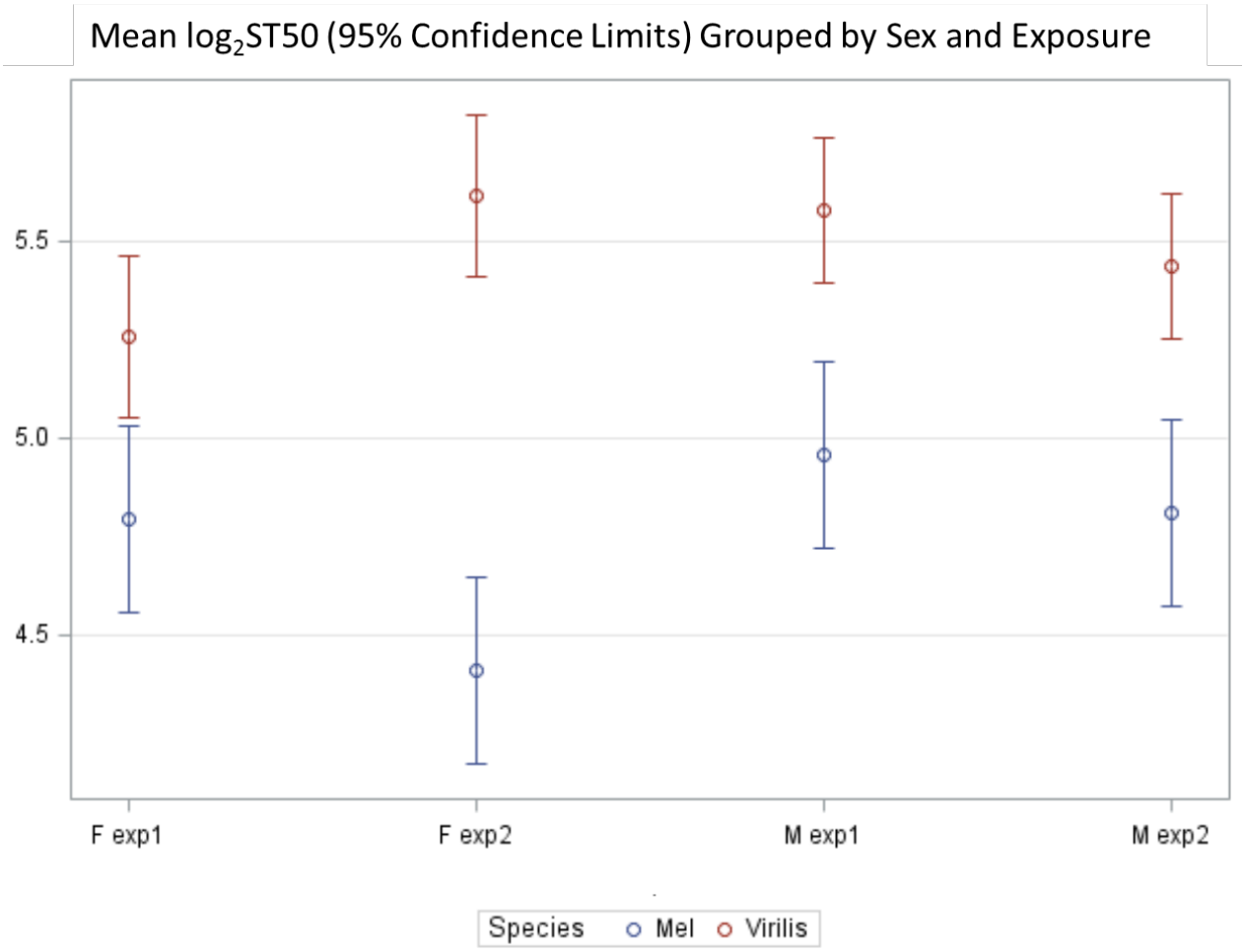


Figure 8. Mean normalized ST50 for grouped by sex and exposure for experiment 3. Opencircles denote the mean and whiskers the 95% CI.

1.4. Discussion

I found that sex dimorphism in ST50 is present for some, but not all species. Rapid tolerance also differs between species and sexes. Finally, not all species show rapid tolerance. This result is similar to work done by A.-Z. K. Muhammad-Ali and B. Burnet in 1995, Wilma V. Colon-Parrilla and Ivette Perez-Chiesa in 1999, and Mercot et al., in 1994, which are summarized below. Muhmmad-Ali and Burnet tested the prediction that *Drosophila mauritiana* might be ethanol-sensitive like *Drosophila simulans* based on taxonomic affinity. Their

comparison of larval behaviors shows that *Drosophila mauritiana* has a lower ethanol tolerance than *Drosophila melanogaster* or *Drosophila simulans* (Muhammad-Ali and Burnet, 1995). I found that adult Drosophila mauritiana has higher SD50 than *Drosophila melanogaster* or *Drosophila simulans*, which makes *Drosophila mauritiana* more tolerant to ethanol than *Drosophila melanogaster* or *Drosophila simulans*.

Colon-Parrilla and Perez-Chiesa examined the level of ethanol tolerance in the *cardini* species group of *Drosophila* to see if these traits have evolved independently in these species. They found that all of the species were ethanol sensitive (Colon-Parrilla and Perez-Chiesa, 1999). The main species group that was looked at was the melanogaster species group. In this species group, some species were ethanol sensitive like *Drosophila ananassae* and others were tolerant like *Drosophila yakuba*. An interesting species group to study in the future would be the *cardini* species group because they have not been extensively studied for ethanol tolerance. Mercot et al did a comprehensive study in 1994 that looked at ADH activity and LD50 in 61 species from the *Drosophila* and *Zaprionus* genus. They found that species breed in artificial, man-made, high alcoholic resources had high average ADH activity and high LD50s, which includes *Drosophila melanogaster* and *Drosophila virilis*. However, species breed using nonsweet substrates had low average ADH activity and low LD50s, which includes *Drosophila funebris* and *Drosophila repletea* (Mercot et al., 1994). I did not look at LD50. I only looked at ST50 and found that *Drosophila virilis* had the highest ST50 of the species studied. Determining the ST50 of *Drosophila funebris* and *Drosophila repleta* would be an interesting extension of this work.

Chapter 2

Links between the *doublesex* and *fruitless* sex determination and ethanol response genes

2.1. Introduction

Drosophila melanogaster is a model organism with many functionally characterized genes and pathways which greatly enables the generation, and testing, of genetic hypotheses. However, while core functions of many genes and networks are well characterized, the connections between pathways and genes are not as well understood. One of these uncharacterized connections is between sex determination and dimorphism of the ethanol response. Ethanol is a naturally occurring substance in *Drosophila's* environment and females encounter ethanol more than males do (McKenzie and Parsons, 1972). Females encounter ethanol more frequently because they prefer ovipositng in ethanol containing sites (Devineni and Heberlein, 2013). Since females encounter ethanol more frequently, the response to ethanol exposure in females may differ quantitatively or qualitatively from that of males. One hypothesis is that these differences could be regulated downstream of the somatic sex determination pathway. In this chapter, published and newly collected data are used to identify possible candidates, which might explain how sex dimorphism in the ethanol response arises, at the molecular level.

The balance between X chromosomes and autosomes is the primary signal triggering sex determination pathway in *Drosophila melanogaster*. Some of the specific genes whose dosage is responsible for the male/female decision in somatic cells are *sex-lethal* (*sxl*), *sisterless-a*, *runt*, *deadpan*, and *daughterless*. (Cline, 1993) Sex determination in *Drosophila melanogaster* is specified by an alternative splicing cascade called the sex determination hierarchy, starting with functional splicing of *sxl* in females and including the genes *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*), *intersex* (*ix*), and *fruitless* (*fru*). The two terminal branches of this pathway are downstream of the transcription factors *dsx* and *fru*.

Dsx regulates somatic sexual differentiation due to sex-specific alternative processing of *dsx* precursor messenger RNA (Inoue et al., 1992). If there is a mutation of *dsx* where the regulation of *dsx* RNA processing is disrupted then *dsx* pre-mRNA will be spliced in the male-specific pattern regardless of the chromosomal sex of the fly (Nagoshi and Baker, 1989). Proper splicing of *dsx* results in male-specific and female-specific forms of *doublesex*. *Dsx* polypeptides are transcription factors and bind through a zinc finger-like domain to specific sites in an enhancer. The consensus target sequence for the DNA binding domain is a palindromic sequence made of half-sites around a central base pair (Erdman, Chen, and Burtis, 1996). The dsx transcription factor is thought to regulate sex dimorphism in morphology and physiology. For example, the male- and female-specific *dsx* proteins bind to three DNA sequences located in an enhancer region that regulates female-specific expression of yolk protein genes 1 and 2 (Burtis, Coschigano, Baker, and Wensink, 1991). In females, this results in upregulation of these genes. In males, the *dsx* locus represses the genes responsible for female sexual differentiation and promotes male differentiation (Baker and Wolfner, 1988). Ultimately this results in sex-specific expression of yolk protein gene.

Alternative splicing of *fru* transcripts produces male-specific proteins belonging to the BTB-ZF family of transcriptional regulators (Ryner et al., 1996). These transcription factors regulate sexual orientation and courtship behavior in Drosophila and *fruitless* is the first gene in a branch of the sex-determination pathway found to function in the central nervous system (Ryner et al, 1996; Dahanukar and Ray, 2011). Alterations in splicing of this gene are sufficient to elicit changes in courtship behavior as well as aggression, for example by causing males to mate indiscriminately or by causing female fighting patterns in males or vice versa. Subgroups of *fru* neurons are involved in the control of these sexually dimorphic patterns and in some cases this has been worked out at a neuron specific level. For example, specific *fru*-positive octopaminergic neurons in the subesophageal ganglion have been implicated in the decision between aggressive or courtship behavior (Zwarts, Versteven, and Callaerts, 2012).

Currently, 1500 neurons have been shown to express the sex-specific transcripts of the *fru* gene (Jai et al., 2010). These neurons are believed to play a critical role in generating sexually dimorphic behavioral responses to other flies. The core of the *fru* circuit in the brain is

the structure termed the lateral protocerebal complex. This region is enriched in *fru* projections and is where multiple sensory inputs are integrated and discrete motor actions are selected and coordinated (Jai et al., 2010). fru^{M}, the male-specific *fru* isoform is expressed in ~2% of neurons in the male. Some olfactory and gustatory neurons are also *fru* positive (Dahanukar and Ray, 2011). The activation of *fruitless* expressing neurons results in increased rates of ethanol sedation, thus *fru* been postulated to mediate sexual dimorphism in ethanol sedation (Devineni and Heberlein, 2012).

In addition to *fruitless,* the gene *tank* has been shown to encode a protein, which regulates ethanol responses in a sexually dimorphic manner. The sex differences in acute ethanol responses in Drosophila, specifically increased male hyperactivity and sedation resistance, are partially mediated by *fru* and *tank*. These genes define distinct sets of neurons that interact to regulate ethanol sensitivity, forming potential synaptic connections and exhibiting a cooperative interaction in regulating ethanol sensitivity. Thus, *fru* and *tank* not only contribute to the generation of sexually dimorphic ethanol responses, but also define distinct sets of neurons that interact to regulate these behaviors and may form part of a large neural circuit (Devineni, 2012).

However, the molecular basis of the effects of *fru* and *tank* are unknown. In addition, *doublesex* can also regulate neural differences between males and females. However, a role for *dsx* in sex dimorphism of the response to ethanol has not been previously documented. In order to understand what genes acting downstream of *dsx* and/or *fru* might also be involved in sex dimorphism of the ethanol response we examined published data sets identifying *doublesex* targets and sex-biased expression in *fruitless* neurons. We also examined genes putatively regulated by male-specific isoforms of *fruitless* and by common isoforms of fruitless using newly collected data from over-expression lines. These three datasets were compared to a manually curated list of ethanol response genes.

2.2. Materials and Methods

(a) Study Design

For this chapter, I looked at three different datasets, which identified potential targets of the somatic sex determination pathway to try and define the role of sex determination in dimorphism of the ethanol response. The first dataset identified likely targets of *doublesex* regulation in two different wild type strains (Arbeitman et al., 2016). In this study, the authors identified the gene expression differences between wild type females, pseudo males, *doublesex* null females, and *doublesex* null males. The second dataset identified genes responding to overexpression of male-specific *fruitless* isoforms (Dalton et al., 2013). In this study, male specific isoforms of *fruitless* were containing one of three alternative DNA binding domains (denoted as FruMA, FruMB or FruMC). The third dataset also identified genes, which respond to overexpression of *fruitless* isoforms, but with a different approach to comparing the effects of these isoforms in males and females (Arbeitman and Graze, 2017, unpublished). They overexpressed two isoforms of the *fruitless* gene in males and females to find similarities and differences between the responses to one of the male specific isoforms (FruMA), as compared to the commonly occurring transcripts that are expressed in both males and females (termed COMA). Below are summaries of the methods from each study.

From Arbeitman et al., 2016, experiments on different genotypes were conducted at the same time and flash frozen flies (stored at -80) were separated into heads and bodies. Heads were then pooled (200 in each library) into independent replicates and mapped to the D. melanogaster Release 5 genome (Dalton et al., 2013; Fear et al., 2015). Sex differences in transcript isoform expression level was accounted for by measuring and analyzing the expression at the exonic level, which were classified as single or overlapping across isoforms. Expression was normalized as the natural log of the number of reads per kilobase per million mapped reads (RPKM) per exon. (Mortazavi et al, 2008). Then a linear model was fit and contrasts were performed to detect differential expression between each strain and each gender with the Fisher's exact test being used to determine which ontology terms were overrepresented. The dataset from this study that

was used by us to find response to ethanol in the sex determination pathway was Supplemental Table 3 Sheet D (*doublesex* dataset).

Next is a summary of the methods from Dalton et al., 2013. The experiments for different genotypes were conducted at the same time and snap frozen (stored at -80) after recovering for 8 hours from CO_2 exposure. Approximately 200 adult heads were mechanical separated and pooled into independent replicates and made into cDNA libraries. Total RNA was extracted using TRIzol Reagent (Invitrogen), and RNA was precipitated by addition of isopropanol and NaCitrate. Some of the total RNA was DNase treated to remove any trace amounts of DNA. PCR was to done to amplify the product. The samples were sequenced on the Illumina Genome Analyzer GAII platform and the reads were matched to their corresponding sample via an index. Next, a sequential mapping pipeline was used to map to the *Drosophila* genome. The reads had the barcode, primer, and adaptor trimmed and then aligned using Bowtie. The unaligned reads were aligned using Tophat and a linear model was fit for each exon separately. Contingency tables and conducting a Fisher's exact test tested enrichments for chromosomal locations. Finally, gene ontology enrichment analysis was performed using gene ontology enrichment analysis and visualization tool. The dataset from this study that was used was Supplemental Tables 1 and 12 (*fruitless* dataset-A). The only data from Supplemental Tables 1 and 12 were FruMA males and FruMA 2-fold males. This data was compared to the ethanol response gene list to find genes related to ethanol response.

(b) Analytical approach

This new data will be called *fruitless* dataset-B from now on. The genotypes used for this experiment were white Canton S, UAS-Gal4, Fru-Gal4, UAS-FruMA, and UAS-FruComA. The wild type controls did not have induced male specific isoform overexpression or induced common region overexpression, which the treated flies did. All flies were maintained on standard cornmeal medium. Flies were collected after eclosion using CO_2. After recovering from anesthetization, flies were snap-frozen in liquid nitrogen and stored at -80C. Heads were then separated from bodies mechanically on dry ice. 200 heads from 24h old unmated, virgin, flies

were collected for each replicate of each sex and genotype. Heads were homogenized in Trizol reagent and total RNA was extracted following the manufacturers recommended protocol. Total RNA was DNase digested, mRNA isolation was performed using the MicroPoly(A) Purist™ Kit (Ambion), and 100 ng of Ambion ArrayControl RNA spike-ins (Spikes 3-7) were added.

Indexed Illumina sequencing libraries were prepared for each sample; samples were multiplexed and sequenced on two lanes (Hiseq 2500 100 BP SE). The RNA sequences had quality determined using FASTQC. Then the RNA sequences were aligned to the Drosophila genome using BWA-MEM (Li, 2013). BWA-MEM is an aligner that is splice aware and able to find matches with split reads. Afterwards samtools was used to create .sam files and to create .bam files. Mpileup (samtools) was used to generate pileup format files with alignment counts per base per chromosome. Finally, a custom script was used to count reads aligning to each exonic region and to normalize counts as the $\log_2$APN (log base 2 of the average per nucleotide coverage).

To account for heterogeneity of variance across male and females samples we fit a cell means model, $Y_{ij}= \mu+t_i+\varepsilon_{ij}$, where Y_{ij} is the normalized expression estimate for the I^{th} treatment type (wild type or overexpression, FruMA or COMA, genotypes and male or female samples for each genotype) and J^{th} replicate. Contrasts were constructed to test each differential expression comparison of interest for each sex separately. For example, to test the differences between wild type expression and FruMA overexpression in males an F test was constructed with the contrast μM-WT = μM-FruMA. Overall, we tested four comparisons identifying DE between control and overexpression genotypes (FruMA or COMA) in each sex separately (M, male, or F, Female).
Ethanol response genes were identified using the Vocabularies tool in Flybase to batch download genes with keywords response to ethanol, behavioral response to ethanol, cellular response to ethanol, and response to alcohol in the biological process, molecular function or cellular process GO ontology terms. Then combined with genes identified from the literature to create a master ethanol response gene list. For the each dataset (*doublesex*, *fruitless* dataset-A, or *fruitless* dataset-B) gene lists were constructed and matched to the curated list using SAS.

2.3. Results

There were 901 total genes identified in Arbeitman *et al.* (2016) as being likely regulatory targets of *dsx*. These were further categorized as female (n=438) or male biased (n = 274). There were 102 ethanol response genes that were also regulated by *dsx* and female biased and 56 ethanol response genes that were regulated by *dsx* and male biased.

The *fruitless* dataset-A genes that were induced or repressed in males by FruMA (and comparable to *fruitless* dataset-B) were considered. In males there were 1,920 genes induced by FruMA and 1,314 repressed (752 and 204, respectively, differed from controls 2-fold). In *fruitless* dataset-B we considered genes that were downstream of the male specific isoform of *fruitless* in males (n = 46) and in females (n=325) and the common isoform (COMA) in males (n = 62) and in females (n = 320). We identified 86 ethanol response genes found in one or more of these datasets. We highlight selected ethanol response genes in Table 10.

Table 10. Candidate genes for dimorphism in the response to ethanol, in *D. melanogaster.* Selected, relevant, annotation reported

Symbol	*FlyBase ID*	*GO Annotation*
slo	FBgn0003429	circadian rhythm
slo	FBgn0003429	locomotion involved in locomotory behavior
slo	FBgn0003429	response to drug
homer	FBgn0025777	behavioral response to ethanol
homer	FBgn0025777	positive regulation of circadian sleep/wake cycle, sleep
homer	FBgn0025777	regulation of locomotion
homer	FBgn0025777	response to ethanol
Tig	FBgn0011722	axon guidance
Tig	FBgn0011722	phagocytosis
Ilp6	FBgn0044047	insulin receptor binding
Tbh	FBgn0010329	aggressive behavior
Tbh	FBgn0010329	behavioral response to ethanol

Tbh	FBgn0010329	locomotion
Tbh	FBgn0010329	memory
Tbh	FBgn0010329	octopamine signaling pathway
Drat	FBgn0033188	cellular response to ethanol
Drat	FBgn0033188	response to hypoxia
Drat	FBgn0033188	sleep
tim	FBgn0014396	circadian behavior
tim	FBgn0014396	locomotor rhythm
tim	FBgn0014396	sleep
hang	FBgn0026575	behavioral response to ethanol
hang	FBgn0026575	response to heat
hang	FBgn0026575	response to oxidative stress
pan	FBgn0085432	negative regulation of Wnt signaling pathway
Aldh	FBgn0012036	acetaldehyde metabolic process
Aldh	FBgn0012036	response to ethanol
rad	FBgn0265597	anesthesia-resistant memory
Cat	FBgn0000261	aging
Cat	FBgn0000261	response to ethanol
fus	FBgn0023441	epidermal growth factor receptor signaling pathway
pum	FBgn0003165	behavioral response to ethanol
pum	FBgn0003165	long-term memory

2.4. Discussion

The genes *Catalase* and *timeless* are regulated downstream of *doublesex*, are male biased and have a role in ethanol response. *Catalase* is a protein-coding gene whose tissue activity is suppressed by dietary ethanol (Greer et al., 1985). The gene *timeless* is a circadian clock gene when mutated can block ethanol tolerance (Pohl et al., 2013). This gene is also induced by FruMA overexpression in males 2-fold in *fruitless* dataset-A and was detected as responding to

fruitless overexpression, and not common isoform expression, in *fruitless* dataset-B. This could mean that timeless is male based, but needs to be studies further. The female biased genes from the *doublesex* dataset included ethanol response genes *pumilio, slowpoke,* and *pangolin*. *pum,* also known as *pumilio*, has a role in mitotic cell cycle and negative regulation of transcription (Berger et al., 2008). The gene *slowpoke* is required for rapid tolerance in Drosophila (Cowmeadow et al., 2005). Finally, the gene *pangolin* is part of the Armadillo pathway and functions downstream of Armadillo to transduce the Wingless signal (Brunner et al., 1997). Interestingly, all three of these genes are induced by overexpression of FruMA in males (in *fruitless* dataset-A or –B).

With *fruitless* dataset-A, genes induced by FruMA two-fold in males that play a role in the ethanol response included *hangover*, *radish*, *homer* and *Tiggrin.* The alcohol metabolism gene *Aldh* is repressed in males upon FruMA overexpression, but not 2-fold. *Homer* is a gene that positively regulates the circadian sleep/wake cycle. The ellipsoid body requires *homer* for normal expression of ethanol sensitivity and tolerance (Urizar et al., 2007). *Hang,* also known as the gene *hangover,* is required for normal development of ethanol tolerance. *Hangover* encodes a large nuclear zinc-finger protein and a cellular stress pathway that is needed for ethanol tolerance (Scholz et al., 2005). The gene *radish* is involved in odor avoidance memory (Khurana et al., 2009). Finally, *Tiggrin* is an extracellular matrix protein with an unknown role in ethanol sensitivity and tolerance (Fogerty et al., 1994). *Aldh* is the enzyme aldehyde dehydrogenase and is essential for ethanol resistance in both adults and larvae (Fry and Saweikis, 2006). Both *homer* and *radish* also respond to overexpression of the common regions of *fruitless*, *homer* is female biased and downstream of *dsx* as well. These genes may be important for ethanol responses in both sexes.

Genes identified in *fruitless* dataset-B as regulated downstream of FruMA in males included *Drat* and *pangolin*, downstream of FruCOMA in male included *Cry* and *Hsp27* and downstream of FruCOMA in females included *Drat*, *Ilp6* and *Tbh*. *Ilp6* is an insulin-like peptide. Insulin-like peptides modulate metabolism, growth, lifespan, and behavior including sexual receptivity in virgin females (Watanabe and Sakai, 2015). So these genes could have a role in ethanol sensitivity and tolerance. *Pangolin* is part of Wnt pathway, which has been shown to

affect *Onthophagus binodis* and *O. sagittarius* horn growth (Wasik and Moczek, 2011). So *pangolin* could have a role in sexual dimorphism in Drosophila. Whereas *Drat* responses to hypoxia and a cellular response to ethanol. *Drat* also confers protection from alcohol-induced apoptosis (Chen et al., 2012).

Future Directions

The future directions for this work would be determining the *cis* and *trans* regulatory regions for ethanol sensitivity and tolerance using hybrid crosses, CRISPR/CAS9 based gene editing, more species in the ethanol assays, aggression behavioral assays, and qPCR. The hybrid crosses work has been started as part of this thesis. Finally, all future would involve sequencing and ethanol tolerance assays.

With the hybrid crosses to determine *cis* and *trans* regulatory regions, some of that work has been done. So far hybrid crosses have been done between the cinnabar brown spec *D. melanogaster* strain and the sequenced *D. mauritiana* strain and the cinnabar brown spec *D. melanogaster* strain and *D. simulans* LHR. The first cross produced only females, which is not expected. However, the second cross was supposed to produce males and females, which did not happen. I am currently testing four new sequenced melanogaster strains to determine which ones will have both female and male offspring when crossed with *D. simulans LHR.* The crosses that were original done had offspring from hybrid crosses and parental crosses exposed to ethanol then frozen. Those frozen samples will have RNA extracted using the RNA direct micro kit from Life Sciences. Then the sequences will be analyzed to determine *cis* and *trans* regulatory regions.

Next CRISPR/CAS9 based gene editing could be used to verify regulatory differences involved in the adaptations to ethanol. Third, the behavioral assays could be expended to include more species including *Drosophila funebris*, *Drosophila replete*, and some species that are not sequenced, to better understand how species diversity in ethanol tolerance is related to adaptive evolution in ethanol response pathways. Fourth aggression behavioral assays followed by crossing aggressive flies then RNA sequencing to determine which genes in addition to *fruitless* determine how aggressive a fly will be. The aggression behavioral assays could be followed by ethanol exposure for aggressive flies then RNA sequencing to determine how aggression affects ethanol sensitivity and tolerance. Lastly, qPCR could be done to confirm all the results I found.

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