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Genetic and environmental factors influence Drosophila ethanol sedation

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Genetic and environmental factors influence *Drosophila* ethanol sedation

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Statement of Contributions

My main dissertation projects were to explore the contribution of diet (Chapter 3), the gene Myocyte enhancement factor 2 (Mef2, Chapter 4), and the gene Nitric Oxide Synthase (Nos, Chapter 5) in *Drosophila* ethanol sedation. Initially, my main project was to explore mechanisms underlying the effect of Nos on ethanol sedation. Nos had been implicated in ethanol sedation via a genetic screen performed by Ian Hines, Lara Lewellyn, Matt Hewitt, and Jena Butler in the Grotewiel laboratory. Starting with their preliminary data I conceived all follow-up studies, identified the major hypotheses, questions and approaches, performed hands on experimentation, analyzed and interpreted the data, and provided overall project direction. Despite the initial promise of the Nos project and considerable additional effort (summarized in Chapter 5), the role of Nos in ethanol sedation was challenging to pursue. I therefore turned my attention to the projects exploring diet and Mef2 in ethanol sedation.

In the diet project, my main role initially was designing experiments, analyzing/interpreting data with Dr. Grotewiel, and assisting Dr. Grotewiel in guiding others in the laboratory while they were completing their experiments. Monica Messick, Ian Hines, and Brandon Shell performed much of the initial experiments on diet. Ultimately, as I started to decrease my effort on the Nos project, I took over the day to day planning, execution, and interpretation of experiments to address the mechanism of action of diet in ethanol sedation. Additionally while working on the diet project, Dr.

Grotewiel and I were planning and conceptualizing the validation of a method to determine the amount of solid food flies consume, while Brandon Shell performed a large portion of the laboratory work for this piece.

In the Mef2 project, Dr. Alexis Edwards (VIPBG, VCU) performed a GWAS analysis that implicated several human genes in the level of response to alcohol. Dr. Grotewiel and I designed the follow-up experiments in flies where Brandon Shell performed the initial screen of candidate genes and backcrossing the Mef2 mutants fly lines. I, with Dr. Grotewiel, conceived the studies summarized in Mef2 (Chapter 4). I designed the experimental setup for: i) the candidate gene screen, ii) the Mef2 mutant backcross, iii) Mef2 mutant sequencing confirmation, and iv) Mef2 RNAi knockdown confirmation studies using immunohistochemistry. I was also involved with the statistical analysis and interpretation of the data.

Given the collaborative nature of my dissertation projects, experiments performed by others in the laboratory are noted in the legend of the individual figures. All experiments not noted in the figure legends as being performed by others, were performed by me.

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The last five years have been an overall amazing experience for me. It is funny to me how everything seems to be working out in the end. Being a scientist has helped me appreciate the world more than I ever thought possible.

My mentor, Dr. Mike Grotewiel, is the most important/influential/supportive person I have come across in my time here at VCU. He has been with me through many of my ups and downs and has been willing to go the extra mile (really the extra thousand miles) to help me get where I need to be. I cannot thank him enough for all of his help and mentorship over the last few years.

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5-HIAA	5-hydroxyindole-3-acetic acid
5-HIAL	5-hydrosyindole-3-acetaldyhyde
5-HT	5-hydroxytryptamine (aka serotonin)
5-HTP	5-Hydroxytryptophan
5-HT2A	5-hydroxytryptamine (aka serotonin) receptor 2A
5-HTOL	5-hydroxytryptophol
5-HTT	Serotonin transporter protein
5-HTTLPR	Serotonin transport-linked polymorphic region
ACE	Adverse Childhood Experiences
AD	Alcohol dependence
ADH	Alcohol Dehydrogenase
ADH1A, 1B, 1C	Alcohol Dehydrogenase, class I, genes A, B, and C
ADH4	Alcohol Dehydrogenase, class IV
ALDH	Aldehyde Dehydrogenase
ALDH1A1, 1B1	Aldehyde Dehydrogenase, class I, genes A and B
ALDH2	Aldehyde Dehydrogenase, class II
ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of Variance
APP	Amyloid beta precursor protein

Appl ..... Beta Amyloid Protein

Arf6 …… ADP ribosylation factor at 51F

ATC ……... Antibiotics

ATG5 ..... Autophagy Related 5

AUD ..... Alcohol use disorder

AUTS2 ..... Activator of Transcription and Development Regulator 2

BL6 ..... C57BL/6J

BMC ..... Bonferroni's Multiple Comparisons

BMI ..... Body Mass Index

BORCS8 ..... BLOC-1 Related Complex Subunit 8

BX ……... Backcross

C2B2 ..... Continuous 2-Bottle Choice

Cad99c ..... Cadherin 99C

CAFÉ ……. Capillary Feeder

cGMP ……..cyclic GMP

CHRM2 …….. Muscarinic acetylcholine receptor M2

Clic …….. Chloride Intracellular Channel

COGA ……. Collaborative Study on the Genetics of Alcoholism

CPNE5 ……Copine 5

d ….. day(s)

D2 ..... DBA/2J

da …….. daughterless

Ddc ……. Dopa decarboxylase

DID ..... Drinking in the Dark

DIOPT ..... DRSC Integrative Orthology Prediction Tool

DNA ..... Deoxyribonucleic Acid

DSM ..... Diagnostic and Statistical Manual of Mental Disorders

E1 ..... Ethanol Exposure #1

E2 ..... Ethanol Exposure #2

E75 ..... Ecdysone-induced protein 75B

elav ..... Embryonic lethal abnormal vision

Efa6 ..... Exchange factor for Arf6

EPA ..... Eicosapentaenoic acid

ExVial ..... excreted dye

fat-3 ..... Fatty acid desaturase

Fdh ..... Formaldehyde dehydrogenase

FGF21 ..... Fibroblast Growth Factor 21

FMR1 ..... Fragile X Mental Retardation 1

FTO ..... Alpha-Ketoglutarate Dependent Dioxygenase

FUMA ..... Functional Mapping and Annotation of GWASs

GABA ..... γ-Aminobutyric Acid

GABRB1 ..... β1 GABA Receptor

GFAP ..... Glial Fibrillary Acidic Protein

GPD2 ..... Glycerol-3-phosphate Dehydrogenase 2

Gpo-1 ..... Glycerophosphate oxidase-1

GS ..... GeneSwitch

GSNOR …….. S-nitrosoglutathione reductase

GWAS ..... Genome Wide Association Study

h^2 ..... heritability estimate

hr(s) ..... hour(s)

IASPSAD …….. Irish Affected Sib Pair Study of Alcohol Dependence

ics …… icarus

INT …….. Internal dye

ISL1 ..... ISL LIM homeobox 1

KLB ..... Klotho Beta

LCO3 ..... Long Chain ω-3 Fatty Acid

LORR ..... Loss of Righting Reflex

LS …….. Lausanne-S

MADS ..... MCM1, agamous, deficiens, SRF

MAOA ..... Monoamine Oxidase A

Mef2 ..... Myocyte Enhancer Factor 2

MEF2A, B, C, D ..... Myocyte Enhance Factor 2A, B, C, D

min …..... minute(s)

MP Bak …….. MP Bakers Yeast

MP Brew …… MP Brewers Yeast

NO ..... Nitric Oxide

Nos ..... Nitric Oxide Synthase

NPF …… Neuropeptide F

NPY ..... Neuropeptide Y

OR …… Oregon-R

PCDH15 ..... Protocadherin Related 15

PKC ……. Protein Kinase C

PSD1-3 …….. PH and SEC7 domain-contain protein 1-3

repo …… reversed polarity

RNA ..... Ribonucleic Acid

RNAi …….. RNA interference

RSU1 ..... Ras Suppressor Protein 1

RU486 …… Mifepristone

RYR3 ..... Ryanodine Receptor 3

S4S ..... Spit 4 Science

Sam ……..... Samarkand

Seq ……. Sequencing

sGC ……. Soluble guanaylyl cyclase

SFSWAP ..... Splicing Factor SWAP

slo ……. Slowpoke

SI Bak ……. Saf-instant Bakers Yeast

SNP(s) ..... Single Nucleotide Polymorphism(s)

SRE ..... Self-Rating of the Effects of Alcohol

ST50 ..... Sedation Time 50

su(w[A]) ..... Suppressor of white-apricot

SWI/SNF ..... Switch/Sucrose Non-fermentable

tay ……. tay bridge

TEG …….. Tegosept

TeTxLC …….. Tetanus toxin light chain

Th …… Tyrosine 3-monooxygenase

Trh ……..Tryptophan hydroxylase

tup ..... tailup

UAS …… Upstream activating sequence

UNF …… Hormone receptor 51

Abstract

GENETIC AND ENVIRONMENTAL FACTORS INFLUENCE *DROSOPHILA* ETHANOL SEDATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Alcohol use disorder is a global health issue that affects a significant portion of the population, with affects including both negative mental and physical consequences. Currently, there are few treatment options available to those who suffer from alcohol use disorder, alcohol abuse, or alcohol dependence. Identifying candidate genes or environmental influences would therefore improve the means for possible treatments or identification of those people at risk for alcohol use disorder. Previous studies in humans have demonstrated an inverse association between initial sensitivity and risk for alcohol abuse. This connection allows investigators, and our laboratory, to investigate genetic and environmental factors that may influence initial ethanol sedation. Our laboratory utilizes *Drosophila melanogaster* (flies) as a model organism to identify these such factors influencing acute behavioral responses to alcohol. Our lab has found

evidence for both environmental and genetic factors that influence initial alcohol sensitivity in flies. In one study, flies that are fed increased amounts of dietary yeast are resistant to ethanol. We have found that this ethanol resistance is related to the amount of nutrients that is consumed, which then affects alcohol uptake/metabolism, to influence initial alcohol sensitivity. Importantly, we found that serotonergic neuron function is essential for regulating the consumption of high dietary yeast media for the increased nutrient intake to occur. In two separate projects, we identified a role for myocyte enhancer factor 2 (Mef2) and nitric oxide synthase (Nos) in initial alcohol sensitivity. Mef2 was obtained via a GWAS study identifying genes with an association with initial sensitivity in humans. We found that decreasing or altering Mef2 expression, using mutants or Mef2 RNAi, resulted in flies having decreased sensitivity to alcohol. The gene Nos, came out of a previous genetic interaction screen in the laboratory. Multiple reagents to assess Nos's role in alcohol behavior were obtained and consistent evidence from three piggyBac transposon insertion flies and, importantly, a Nos null fly, demonstrate that decreased Nos expression results in increased ethanol sensitivity. Other preliminary results suggest that Nos expression during adulthood, as well as the mechanism of S-nitrosation, may be important for ethanol sedation in *Drosophila.*

Chapter 1 – Introduction

1. Overview of problematic alcohol consumption in humans

1.1. Alcohol use disorder, alcohol abuse, and alcohol dependence

Alcohol use disorder (AUD) can broadly be described as the harmful use of alcohol. AUD is a significant global health problem[1]. There was an estimated 283 million adults (5.1%, aged 15+ years) anticipated to suffer from AUD in 2016, while in the United States alone there was an estimated 15.1 million adults (6.2%, aged 18+) in 2015[1]. A substantial portion of global deaths (5.3%) in 2016, approximating 3 million, resulted from the contribution of alcohol[1]. Some of the categories for the cause of alcohol related deaths include: 21.3% digestive diseases, 20.9% unintentional injuries, 19% cardiovascular diseases and diabetes, 12.9% infectious diseases, and 12.6% malignant neoplasms[1]. Globally, cardiovascular disease is the top cause of mortality and 3.3% of these deaths are attributed to alcohol[1]. Irregular heavy drinking, or high volume consumption, of alcohol leads to an increased risk for cardiomyopathy, rapid/irregular heart rate, and brain bleeds[1]. Additionally, it has been found that a person can develop asymptomatic alcoholic cardiomyopathy when large amounts of alcohol are consumed over multiple years and there is an ~50% 4 year mortality rate[2]. Furthermore, it is thought that acetaldehyde (first metabolite in alcohol metabolism) may also contribute to the development of alcohol cardiomyopathy[3].

Research has linked alcohol consumption to the development of numerous cancer types in humans and rodent models. The risk to develop cancer caused by alcohol is typically higher for women than men, and can be caused by damage to DNA or by inhibition of the DNA repair process[1]. Alcohol exposure in rats led to increased cell death (as seen by expression of cleaved caspase 3), increased levels of double stranded DNA breaks (as indicated by increased γH2AX), decreased DNA repair pathway genes, and increased oxidative stress in the hippocampus[4]. These different measures, taken together, demonstrate the toxicity of alcohol and its negative impact on regions of the brain[4]. Of the 9 million deaths in 2016 caused by cancer, alcohol attributed to 4.2% of them[1]. In addition to cancer, high mortality has been commonly associated with alcoholic hepatitis and liver cirrhosis.

The widespread range of negative effects of alcohol also extends to negative mental health aspects[5]. This can include different types of mood or anxiety disorders, dementia[6], Alzheimer's[7], or even increased suicidalidy[8] [1]. Moreover, infectious disease account for almost 13% of all alcohol related deaths[1]. Alcohol consumption has been shown to lead to an increased risk for transmission of sexually transmitted diseases, by increasing the probability of risky sexual behavior[1, 9]. This includes having unprotected sex or having multiple sexual partners, which subsequently increases the likelihood of transmission and infection of sexually transmitted diseases. A second way alcohol can influence the risk for infections and transmission, is by the suppression of the immune system, which allows for easier infection by a disease[1, 10]. In fact, it has been shown there is a threefold increase in the risk for tuberculosis in the population of people who are diagnosed with AUD[1, 11]. As a disease that has far reaching effects, any treatment

or diagnostic tools to combat problem alcohol drinking would be beneficial to the world population.

1.2. Diagnostic criteria for alcohol use disorder

The diagnostic criteria for AUD is outlined by the 2013 version of the Diagnostic and Statistical Manual of Mental Disorders (DSM), fifth addition. To qualify as having AUD, someone must have experienced 2 of the 11 symptoms listed in the past twelve months[12]. Also, a person can be categorized as having mild (2-3 criteria), moderate (4-5 criteria), or severe (6+ criteria) AUD[12]. Previously, the fourth addition of the DSM broke the diagnosis into two categories, alcohol abuse and dependence. Abuse was diagnosed when one of four criteria were present in the last 12 months, whereas alcohol dependence would be diagnosed with three of seven criteria if present in the last 12 months[12]. The current criteria is considerably different from the DSM-IV criteria, released in 1994 and then the revision, DSM-IV-TR, in 2000[12]. The DSM-IV included legal issues as a potential criterion, which is not part of the DSM-V. Importantly, the DSM-V added alcohol cravings as a possible symptom, which was missing from the DSM-IV[12]. Some of the themes that have stayed the same between the two sets of diagnostic criteria include consuming more alcohol than wanted, alcohol affecting everyday life or causing issues with friends/family, gaining tolerance to the effects of alcohol, and not being able to stop use or having alcohol withdrawal symptoms[12].

1.3. Alcohol use disorder, alcohol abuse, and alcohol dependence as a complex disorder

The cause of alcohol use disorder, alcohol abuse, and alcohol dependence is of interest to help combat the negative mental/physical consequences it poses on society. Twin studies have been used to identify the main components involved in the risk for alcohol abuse. Kendler et. al. found via a female twin study that genetics and individual environment are the main factors leading to risk of alcohol abuse[13]. In this study 2,060 female twins were interviewed and, based on DSM-III-R criteria, 185 had alcohol dependence and 357 met the broad criteria for alcoholism[13]. It was found that of the ACE (additive genetics, common environment, and unique environment) model, AE fit best across all definitions of alcoholism used[13]. This suggests common environment does not play a significant role in the risk for alcoholism, whereas genetic and unique environmental factors do. The authors also found that, no matter the definition of alcoholism, the amount of variance genetic factors were estimated as contributing towards alcoholism was ~50-61% and unique environment contributing ~39-50%[13]. Other studies have found similar results for both, or either, males and females[14-16]. Therefore, it is widely accepted that the risk for alcohol abuse is ~50% based on genetics and the other ~50% is based on unique environmental factors.

One important finding is that initial sensitivity of alcohol is associated with the risk for alcohol abuse. If a person is more tolerant, or resistant, to the effects of alcohol, they have an increased associated risk for abusing alcohol; whereas those that have a larger response, or are sensitive to alcohol, have a decreased associated risk for alcohol abuse[17]. In addition, there is a numeric score that rates a person's initial sensitivity to

alcohol, called the Self-Rating of the Effects of Alcohol (SRE)[18]. This score is compiled from the answer to four questions about the first five times they consumed alcohol. Participants relayed the number of drinks it took to start feeling the following: i) different, ii) dizzy/slurred speech, iii) uncoordinated movements, and iv) sleep or pass out[18]. Furthermore, this SRE score is also associated with the outcome of alcoholism[18].

1.4. Current methodology used to identify genetic factors influencing alcohol use disorders, alcohol abuse, and alcohol dependence

Different methods have been used to identify genes that may be contributing to the risk for alcoholism. Some of the earliest studies began using linkage studies which principally used large families that have affected and unaffected individuals. It is determined whether any markers, or single nucleotide polymorphisms (SNPs), that have known locations, segregate with the affected individuals more than expected. Importantly, this method is helpful for when there is not prior knowledge about what the genetic contributions/mechanisms are because it is an unbiased approach and takes the whole genome into consideration[19]. As an example, from the COGA samples, strong linkage was found between alcohol dependence and a region on chromosome 4[20]. Chromosome 4q contains the cluster of alcohol dehydrogenase (ADH) genes and has been replicated in other studies[21]. A major limitation to this method is that the chromosomal locations identified are typically large (>10 megabases)[22] and can contain dozens to multiple dozens/hundreds of candidate genes due to the marker likely being in linkage disequilibrium with the potential influencing gene[23]. Therefore this method

requires follow up work that is necessary to investigate the region to find the gene that may be influencing the association with the phenotype of interest.

A second method that has been used to identify genes involved in a phenotype of interest is the candidate gene approach. A hypothesis can be made, and tested, about whether a gene/allele may be seen more commonly in an affected population compared to a control population. This process allows for the initial genome wide approach of investigation to be skipped because the investigator is able to test a specific question[24]. On the other hand, a significant limitation to this approach is that it is only useful when there is previous knowledge of the genes/mechanisms involved in a phenotype[24].

Association studies have made it to the forefront of investigation into genetic factors influencing a phenotype. This method looks across hundreds of thousands of SNPs/alleles to determine if any appear more often than expected in an affected sample compared to control[23]. One major advantage of using genome wide association studies (GWAS) is that that common variants that may influence a phenotype can be identified, so finding multiple common variants can be used to explain a portion of risk for a disease[25]. On the other hand, finding numerous common variants could lead to frustration due to the fact that very little of the variance for a phenotype can be accounted for (i.e., height[26]). A major downfall of this approach is that there is little statistical power to detect the variants that may be contributing to a phenotype of interest. Large sample sizes are typically necessary to increase the power of the analysis to find meaningful associations worth further investigation[23]. Additionally there is a lot of heterogeneity in alcohol phenotype. This can be seen in the criteria that used

to diagnose an individual with AUD, possibly making it harder to replicate findings across studies.

1.5. Known genetic contributions influencing risk for alcohol use disorder, alcohol abuse, and alcohol dependence

Genes that have been found to be involved in the risk for AUD, alcohol abuse, and alcohol dependence are very limited. There is, however, consistent evidence for the genes involved in alcohol metabolism – alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). For both ADH and ALDH, there are multiple family members or classes of genes. There are 7 ADH genes that are classed into 5 categories and class I ADHs, specifically ADH1A, ADH1B, and ADH1C, can act as heterodimers and typically are the most involved in the first oxidation step of alcohol to acetaldehyde[27]. ADH1B is more highly expressed than all other ADH genes in the liver and is thought to contribute the most out of the class I ADH genes to the oxidation of alcohol[27]. There are three isoforms of ADH1B and each have a different rate of alcohol metabolism as well as having altered allele frequencies depending on the population. ADH1B*1 has the lowest rate of alcohol metabolism and, when compared to a second allele, ADH1B*2, it is associated with an ~3 fold increase in risk for alcohol dependence[28]. ADH1B*2 is highly prevalent in American and Asian populations, with lower frequencies in European and south Asian groups, and is a protective allele against alcoholism and is associated with decreased alcohol consumption[27]. The third isoform, ADH1B*3, also has a higher rate of metabolism for alcohol over that of ADH1B*1[27]. The ADH1B*3, though, is almost exclusively found in populations of African descent and is protective against alcoholism.

ADH1C has two isoforms. ADH1C*1 is associated with protective effects against alcohol dependence, has high allele frequencies in most population groups, and is more active at metabolizing alcohol than ADH1C*2[27]. A smaller, potential significant role in ethanol metabolism is the class II ADH, ADH4[27].

ALDHs have 19 genes in a total of 9 major family groups, but only three genes from 2 classes, I and II (ALDH1A1, ALDH1B1, and ALDH2) are typically found to be important for oxidation of acetaldehyde to acetate. The reaction of acetaldehyde to acetate is the second step of alcohol metabolism and is largely carried out in the mitochondria[27]. ALDH2 has two main isoforms (ALDH2*1 and ALDH2*2) and depending on which version a person has, can substantially change the effects of alcohol on them. Specifically, ALDH2*2 has been found to be protective against alcohol dependence and is mainly found in East Asian populations, with a very low frequency in African and American populations, and essentially causes the enzyme to be inactive[27]. This subsequently results in buildup of acetaldehyde which can have toxic side effects such as nausea, flushing, and headaches[27]. These side effects are typically unpleasant and people who carry this isoform typically tend to consume less alcohol. Compared to ALDH2, ALDH1A1 and ALDH1B1 are thought to contribute minorly to acetaldehyde oxidation, but play a bigger role if ALDH2 is nonfunctioning[27].

Other genes that have been shown to be associated with some aspect of alcohol use disorder, alcohol abuse, or alcohol dependence include genes encoding γ-Aminobutyric Acid (GABA) receptors, dopamine receptors, the serotonin transport gene, and neuropeptide Y (NPY)[28]. Briefly, the $GABA_A$ receptor is made up of multiple subunits, one specific subunit of this receptor has fairly consistent evidence suggesting

a role in alcohol dependence or alcoholism susceptibility and is encoded by the β1 GABA receptor (GABRB1) gene[28]. Some examples include linkage studies that found there was a nominal association (p=0.0002) with chromosome 4q, near GABRB1, and alcohol dependence[29] and family based association study using the COGA sample[30]. DRD2, or the dopamine D2 receptor gene, is one of five dopamine receptors and is the most widely agreed upon dopamine receptor for a potential role in alcoholism. Even as it is the most agreed upon dopamine receptor of influence, there is a wide range of evidence for[31, 32] and against[33, 34] DRD2 association with alcohol dependence. Another gene with data suggesting association with aspects of alcohol dependence is the gene encoding the serotonin transporter protein (5-*HTT*). In the promoter of the 5-HTT gene, there can be an inclusion of a repeated sequence that classifies the gene as being the long or short allele[35]. The short and long allele have been shown to have different abilities in basal transcription activity with the long version being ~3 fold higher[35]. There are both positive and negative reports for its involvement in alcohol dependence[28]. Even with the contradictory findings with the studies on the serotonin reuptake transporter, subsequently serotonin signaling, it demonstrates the potential link between serotonin and alcohol responses in humans. This further supplies the necessity for further identification of factors (either genetic or environmental) that may be modifying the effects of the L and S alleles on alcohol responses in humans. Additionally, neuropeptide Y (NPY), has been associated with consumption of alcohol, but the direction of the specific variants influence is mixed. One study found that a specific variant, a leucine to proline substitution, caused a 34% increase in alcohol

consumption[36] while others have seen this specific variant appear more often in non-alcoholics[37].

The genes above, besides ADH and ALDH, have pretty controversial findings regarding associations with alcohol dependence or symptoms of AD. There are many factors that may be contributing to this ambiguous data such as the large heterogeneity of alcohol abuse, dependence, and AUD. This suggests there needs to be some refining of linkage and association studies to better improve the capability of confidently identifying genes with potential roles in any of these phenotypes.

1.6. Known environmental influences contributing to risk for alcohol use disorder, alcohol abuse, and alcohol dependence

There is a wide variety of factors outside genetics that can influence the risk for AUD, especially for young children and adolescents. Environmental factors can influence risk for alcohol dependence at ~50%. A large contributor to the risk for alcohol abuse as an adult is different adverse childhood experiences (ACE). ACEs include different categories such as sexual, mental, and physical abuse, and parental separation/divorce or mental illness. Multiple studies have been able to find associations between ACEs and alcohol misuse[38, 39]. In addition to ACEs, there are also other factors including type of friends an adolescent has, parental monitoring, and availability of alcohol. Dick, et.al found that the highest environmental risk factors for increasing alcohol consumption in children ages 12-14 and 15-17 are i) peer group deviance, ii) low parental monitoring, and iii) alcohol availability, while for young adults 18-21, the top factors are the same except low parental monitoring drops out[40]. Another

group found adolescent girls have an increased risk for alcohol abuse when there is low supervision by parents as well as having older aged friends[41].

Another environmental factor, which is largely unstudied, includes diet and how it affects alcohol behaviors in human. In humans, there is some evidence implicating direct, as well as indirect, connections between diet and alcohol behaviors. Evidence includes multiple studies that have found an association between body mass index (BMI) and alcohol abuse. Specifically in a study with participants from Finland, it was found males have a positive association of consuming larger amounts of alcohol (ten or more drinks a week) and obesity; whereas women had the opposite, where lower alcohol consumption associated with obesity[42]. A second study found similar results where males who were overweight/obese, or had a BMI score of greater than 25, had increased lifetime odds to suffer from alcohol abuse or dependence[43]. Consistently, obese women, those with a BMI score of greater than 30, had a decrease in odds for alcohol abuse in the last year[43]. Lichenstein and colleagues similarly found that teenage males who had high BMI values were at an increased risk for development of alcohol dependence[44]. This group was also able to find that *CHRM2*, a gene previously associated with alcohol dependence [45], is associated with increased BMI in the teenage male population[44], potentially making a connecting link between BMI and alcohol dependence.

Furthermore, a gene associated with BMI was also found to be associated with alcohol dependence, was further explored by Wang and colleagues[46, 47]. Initially, the A allele of the *FTO* gene was found to increase the likelihood of a person being overweight by 1.31 fold or for being obese at 1.18 fold[48]. Wang and colleagues found

that 16 SNPs in FTO were associated with alcohol dependence[46]. Still, another study determined that CPNE5 also had significant associations with obesity and alcohol dependence[47]. The group used the SAGE (the study of addiction – genetics and environment) to determine whether any number of the 77 SNPs located in CPNE5, in which 17, were associated with obesity and 10 with AD[47]. Together, these studies implicate that the same genetic factors can influence both obesity and alcohol dependence, suggesting the possibility of there being a further connection between them.

There is little evidence surrounding serotonin, its precursor tryptophan (which is largely acquired via diet), and its effects on alcohol behavior. Nesic et. al., found that non-binge drinkers given a tryptophan rich diet had an increase in desire for alcohol consumption after being through a stressful event, whereas there was an increase in desire for alcohol in binge drinkers when given a tryptophan low diet[49].

2. Drosophila melanogaster – a way to investigate and model human disease

2.1. Conservation between humans and flies

An important key factor of *Drosophila melanogaster* is that they contain orthologs for 62% of human genes that cause disease[50]. This has allowed flies to be used as a tool to understand molecular mechanisms or to identify therapeutic options of diseases when the task, if using other model organisms, would be much more challenging or time consuming. As an example, Fragile X can be modeled in flies to help identify the mechanisms underlying its development. In the human population, Fragile X has a prevalence of ~1/3500 boys and is characterized by developmental delay, intellectual

disability, and autistic-like behavior[51]. Loss of function mutations in the *FMR1* gene, which encodes the protein FMRP, results in the development of Fragile X. Drosophila have an orthologous gene, *dfxr*, that is expressed in neurons like the *FMR1* gene expression in humans and mutations in *dfxr* lead to failure of axon extension[51]. This phenotype in flies is important because it is anticipated that *FMR1* in humans regulates morphology of cell body projections[51], allowing for mechanistic inquires to be investigated into this process.

As a second example of the utility of *Drosophila* as a model of human disease, Alexander Disease is based on astrocyte dysfunction and leads to neurodegeneration and eventual loss of life[52]. The dominant mutations in GFAP, accounting for >95% of patients with this disease, result in protein aggregates formed in astrocytes called Rosenthal fibers, defects in the myelin sheath, seizures, and neurodegeneration[52]. Although the causative gene for Alexander Disease is known, there are no further mechanistic aspects known as to how the GFAP mutations lead to disease progression[52]. Expression of mutated human GFAP in flies causes recapitulation of symptoms seen in humans: seizures, cell death, and Rosenthal-like fibers[53]. The authors utilized this model in flies to identify that nitric oxide (NO) is released from glia and is important for mediating neuronal cell death[54]. These studies demonstrate the utility of the fly in identify mechanisms that may be influencing human disease.

Importantly, in relation to investigating alcohol-related behaviors in flies, there are conserved neurotransmitter systems, synaptic machinery, secondary messengers, and channels[55]. Conserved behavioral responses between flies and mammals, even humans, to alcohol start at the initial level of hyperactivity when exposed to low doses of

ethanol, and sedative effects when exposed to higher doses of alcohol. To identify this phenomenon, one group of authors used an acrylic chamber to determine locomotion of flies exposed to ethanol vapor. In this study, the wings of flies were clipped and placed in the acrylic chamber, where once exposed to ethanol vapor, the path and amount of lines travelled by the fly were recorded[56]. They found that flies had peak locomotor activity at ~10 minutes after the initial ethanol exposure start and that sedation occurred at ~25 minutes[56]. Other techniques have been developed to measure the sedative effects of alcohol such as the inebriometer. In the inebriometer flies are exposed to alcohol and the length of time required for them to elute from a tube with spaced slats is recorded[57]. In addition, other methods measure ethanol sedation via some measurement of, or loss of, a flies natural tendency to demonstrate negative geotaxis: such as counting the amount of flies that become sedated or lose postural control overtime when exposed to ethanol vapor[58, 59], alcohol sedation recovery[60], loss of righting reflex[61], the eRING assay[62], and calculating the speed at which the flies move when exposed to alcohol[63].

Rapid tolerance is another form of alcohol behavior that mammals, and flies[56], develop. Tolerance is developed when there is a first exposure alcohol, a recovery period, and then a second exposure to alcohol. In the second exposure, the amount of time it takes for a similar response as seen in the first exposure, takes longer[56]. Genetic manipulations have been able to influence the development of both ethanol sedation and rapid tolerance, such as a mutation in the gene *homer* which results in initial ethanol sensitivity and decreased development of rapid tolerance[64]. On the other hand, some genetic manipulations affect either ethanol sedation or rapid tolerance, but not

both. Such was found when RNAi against *Akap200* was expressed in glia had normal ethanol sedation but decreased development of rapid tolerance[65].

Flies, like humans and rodents, can also develop withdrawal symptoms. Flies going through withdrawal have increased susceptibility to seizures, seen as a lower stimulus voltage needed for induction of seizures[66]. This method is performed by applying a shock to the fly brain via electrodes and the induced seizure response is recorded[66]. When *slo*, a BK channel, is overexpressed in the fly, there is a decrease in the voltage stimulus that is needed to induce seizures, similar to the levels seen when flies are going through withdrawal[66] , suggesting a role for this gene in the development of ethanol withdrawal.

Additionally, flies will also prefer to consume liquid food that contains ethanol over a non-ethanol containing choice. One study found that the flies having access to liquid food with or without ethanol, measured via CAFÉ[67], had a significant preference for alcohol containing food and the preference increased over time[68]. Even in conditions where quinine, an aversive substance to flies, was added to the liquid food containing ethanol, flies were willing to intake the aversive substance as long as the ethanol was present[68].

2.2. Current approaches used to investigate genetic contributions in flies

One prominent genetic manipulation tool that is used commonly throughout *Drosophila* studies, is the UAS-Gal4 system[69]. This system allows for tissue specific expression of Gal4, a protein that binds to an upstream activating sequence (UAS), and activate transcription of a specific transgene of interest[69]. This system allows for tissue

specific genetic manipulations throughout the life span of *Drosophila.* A second method that is also commonly used is the GeneSwitch system[70]. GeneSwitch is very similar to the UAS-Gal4 system, except in order for transcriptional activation to occur, a steroid must be present to allow for binding of Gal4-like protein to the UAS sequence[70]. This system allows for temporal and tissue specific control of transgene expression. It can be used to determine what stage, development or adulthood, the gene is required for a phenotype or used to surpass developmental lethality issues.

2.3. Known genetic contributions influencing alcohol-related behaviors in Drosophila

Ethanol metabolism occurs in a similar manner as in vertebrates. Flies contain ADH and ALDH that are involved in alcohol metabolism[71]. Interestingly, similar to humans, natural variation in fly Adh can influencing alcohol metabolism[71]. Two alleles, Adh^F and Adh^S have differences in enzymatic activity, where Adh^F is higher and causes flies to be resistant to alcohol[71]. By 2015, ~95 genes were published and found to affect aspects of alcohol responses in flies[72]. More importantly, there are genes that have been found to play a role in different aspects or symptoms of alcohol use disorder, alcohol abuse, or alcohol dependence in humans (mentioned in section 1.5) that have altered behavioral responses to alcohol in flies[72]. Dzitoyeva, et. Al (2003) used two approaches, RNAi targeting of the $GABA_BR1$ subunit of the $GABA_B$ receptor in flies as well as treatment with an antagonist of the $GABA_B$ receptor, to demonstrate the role of GABA receptors in alcohol behavior in flies[73]. The authors found that there was effects on sedation recovery with both the RNAi and antagonist approach[73]. This relates to the fact that GABA has been implicated in human alcohol dependence[28-30].

When considering dopamine and its response in flies, a few studies have shown its importance in ethanol related behaviors, just as has been found in humans[31, 32]. Bainton et. al, found that treatment of flies with 3IY, a competitive inhibitor for the rate limiting enzyme in dopamine synthesis, significantly reduced the amount of time of ethanol induced hyperactivity[61]. Furthermore, serotonin has also been suggested as playing a role in fly alcohol sedation, whereas the serotonin reuptake transporter has been more studied for associations with alcohol dependence in humans[28]. Chen et. al, found that when flies have a subtype of protein kinase C (PKC) knocked down in Ddc positive neurons, but not Th positive neurons, flies are resistant to alcohol[74]. This would suggest that expression of this specific PKC isoform in serotonergic neurons is important for ethanol sedation. Additionally, treatment of these flies with two different serotonin reuptake inhibitors (citalopram and Prozac), the ethanol resistance phenotype of the PKC knockdown flies was rescued back to control levels[74].

Furthermore, just as the mammalian gene NPY has been shown to alter consumption and sedation in mice when knocked down, the fly ortholog NPF plays a role in fly alcohol behavior[75]. One study ablated NPF neurons in flies by expressing DTI (a diphtheria toxin that inhibits protein synthesis[76]) and resulted in flies being resistant to alcohol sedation compared to controls[75].

Some studies have even combined human GWAS studies and utilized *Drosophila* to further investigate their candidate genes. In one study, authors used the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD) and participants had to have been diagnosed with AD by the criteria in the DSM-IV to be included in the study[77]. They found a significant association with a SNP in the ryanodine receptor 3 (RYR3)

gene and AD in this sample, $p=1.47x10^{-7}$ [77]. The fly orthologous gene to human *Ryr3* is *RyR* and two loss of function mutations cause a decrease in the development of rapid tolerance[77]. A second study performed a GWAS for genes that are associated with alcohol consumption, and identified the autism susceptibility candidate 2 (*AUTS2*) gene[78]. The *Drosophila* ortholog of human *AUTS2* is called *tay* and the authors found that mutations in *tay*, or pan-neuronal expression of *tay* RNAi, resulted in flies being resistant to alcohol[78]. A third study found that Ras suppressor 1 (*RSU1)* was associated with AD in adults as well as for an association with lifetime frequency of drinking in a younger population sample[79]. The fly ortholog is *icarus (ics)* which encodes Rsu1, causes ethanol resistance when the gene interrupted by a P element and expression of UAS-Rsu1 was able to rescue the phenotype[79].

In a more recent article, *Arf6* and *Efa6* loss of function mutant flies demonstrated a higher preference for alcohol when having not been previously exposed to the drug compared to control fly preference[80]. Additionally, *Efa6* mutants are sensitive to the effects of alcohol compared to controls[80]. The human ortholog of *Efa6* includes genes *PSD1-4,* and specifically *PSD3,* had an association with the amount of alcohol consumed by a human within the last 30 days[80]. These examples show the importance of the conserved biology and behavioral responses to alcohol between flies and humans. This allows for identification, or follow-up studies from GWAS investigations, of genes that may be involved in the risk for alcohol dependence, abuse, or AUD in humans. Using flies allows for specific questions to be asked and mechanisms to be probed that would otherwise be harder to perform in humans or other vertebrate models.

2.4. Known environmental influences contributing to alcohol-related behaviors in Drosophila and other model organisms

Altering environmental conditions, such as rearing conditions, is an easier task to accomplish in flies compared to other types of model organisms. Studies have used hypoxia or hypercapnia to investigate genetic contributions in flies that can alter the development of seizure-like activity[81]. Other studies have used alterations in the fly diet to study how a high fat diet influences responses to stress[82].

Sekhon and colleagues set out to address the specific questions whether there is an overlap between genes influencing food intake and alcohol intake in flies. The authors used recombinant inbred fly lines and assessed food intake, ethanol intake, and preference of these different lines. Through correlations of the various measures, the authors identified that factors influencing consumption of food or ethanol, from capillary tubes specifically, are positively correlated[83]. This suggests that there may be some overlap specifically in genes responsible for consumption of food and alcohol, relating the two processes.

Looking further into the connection of diet and alcohol behaviors, minimal work has been previously done in other model organisms. The few studies, which demonstrate there is a relationship between diet and alcohol, are very important for supporting the idea that human risk for alcohol use disorder, alcohol abuse, and alcohol dependence may be in part affected by diet. One study investigated whether six different mouse “standard” chows could alter alcohol-related behaviors across two different laboratory tests, drinking in the dark (DID) and continuous 2-bottle choice

(C2B2), in C57BL/6J (BL6) mice[84]. The authors found that the DID mice had altered ethanol consumption and C2B2 mice had changes in ethanol preference as well as consumption when given access to these six different diets[84]. A second study in mice investigated how different genetic backgrounds, BL6 or DBA/2J (D2), responded differently in alcohol behavioral tests when subjected to food with altered concentrations of long-chain ω-3 fatty acid (LCO3)[85]. They found that a diet with low LCO3 levels resulted in altered alcohol induced locomotor activation in BL6 and D2 mice, and with higher levels of LCO3 in D2 mice[85]. Their study also demonstrated a difference in ethanol preference in BL6 mice when fed low or high levels of LCO3, but no changes in the D2 mice ethanol preference[85]. Furthermore, D2 mice had a significant increase in the duration of the loss of righting reflex (LORR) test when given high LCO3 diets compared to low LCO3 diets, but BL6 mice demonstrated no difference in effects in LORR[85]. These data, demonstrate that diet can play an important role on alcohol-related behaviors in mice, and that genetic background can alter diet induced responses to alcohol[85]. An additional rodent study probed whether calorie restriction influences responses to alcohol in rats. The investigators were able to identify that a 25% calorie restricted diet in rats lead to a decrease in the self-administrative setting in an ethanol reward challenge as well as having a decrease in relapse behavior compared to control animals[86]. Together, these studies suggest that altering dietary components influence alcohol responses in mammals.

A study using *C. elegans* also demonstrates the role diet can play on alcohol-related behaviors. *C. elegans* develop acute functional tolerance (AFT), which means they develop tolerance to ethanol while they are being exposed to the drug, allowing the

animal to recover from the effects of alcohol resulting in decreased speed[87]. A *fat-3* mutant strain, *fat-3* is necessary for the synthesis of long-chain polyunsaturated fatty acids, does not develop acute functional tolerance as seen by no increase in speed after 30 minutes of ethanol exposure compared to the 10 minute time point[87]. Using mutant strains of other enzymes needed for the metabolism of other long-chain polyunsaturated fatty acids, the investigators found that eicosapentaenoic acid (EPA) is necessary for *C. elegans* to develop acute functional tolerance and supplementation with EPA is sufficient to rescue *fat-3* mutant strains lack of development of acute functional tolerance[87]. These data, in combination with the rodent data above, highly suggest a role for diet in alcohol related behaviors spanning across both invertebrates and vertebrates.

3. Myocyte enhancer factor 2 (Mef2) in mammals and flies

The family of human myocyte enhancer factor 2 (MEF2) is an important family of transcription factors that is expressed in most tissues at both adult and embryonic time points[88]. In mammals there are four MEF2 genes, A-D, all with highly conserved MADS and Mef2 domains, which are important for MEF2's ability to bind target DNA[88]. *Drosophila* have one orthologous gene, Mef2, which is orthologous to human (highest>lowest) MEF2A>MEF2C> MEF2D>BORCS8-MEF2B>MEF2B[89] . Mef2 has been mainly studied for its role in muscle development in the fly[88]. Not only is Mef2 important in myogenesis in flies, but the MEF2 family in mammals is important for muscle differentiation [90, 91], human disease/cancer [92, 93], and neuronal differentiation [94-96]. As MEF2 is a versatile transcription factor, it is opens the gateway to investigation of

mechanisms that could be important in ethanol-related behaviors in flies, and in a translatable manner to mammals. Identification of these processes could open the avenue for development of new drug therapies for treatment of alcohol use disorder, or identification of people who may be at an increased risk for abusing the drug.

4. Nitric oxide synthase (Nos) in mammals and flies

Nitric oxide synthase (Nos) is an enzyme that converts L-Arginine to Citrulline and NO. This reaction is, in most cases, calcium and calmodulin dependent and requires numerous cofactors including FAD, FMN, BH_4, and NADP[97]. Vertebrates have three genes that encode NOS: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). NOS1 and NOS3 are considered the constitutive forms of NOS and are dependent on calcium whereas NOS2 is inducible and not dependent on calcium[97]. In vertebrates NO has many functions, some including acting as a neurotransmitter, being involved in blood vessel regulation[97], and is involved in the development of many human disease states such as Parkinson's disease, and Alzheimer's[98].

Drosophila melanogaster has a single ortholog and is most highly conserved with NOS1 (DIOPT score 13[89]), with the other two vertebrate forms of Nos falling after, NOS3 (DIOPT score 12[89]) and NOS2 (DIOPT score 10[89]). RNA-Seq expression data has fly Nos primarily located in the head, which is consistent with high levels of protein expression of NOS1 in human neurons and oligodendrocytes[99]. Fly Nos has been shown to be involved in numerous processes in the fly, such as imaginal disc regeneration[100], axon pruning and regrowth[101], and immune responses[102]. Very little

evidence has previously supported a role for NO signaling in ethanol-related behaviors, although, there have been a few studies in rodents[103-105]. Therefore this gap in knowledge of how NO signaling may play in ethanol-related behaviors is understudied.

5. Relevance

As mentioned in Section 1.1, alcohol use disorder has many negative influences on people that spans across the globe - from premature and/or preventable deaths, to a loss of productivity, mental health effects, or development of disease states[1]. Currently, there are not many treatment options available and those that are available are not always effective[106]. These treatment options include prescription drug treatment or individual/group therapy. One study found that when a person chose to undergo any treatment (either going to Alcoholics Anonymous (AA) or getting professional help) at a three year follow-up, had ~38% rate of relapse, whereas those that did not get any help had a relapse rate of ~57%[107]. This suggests that treatment, when options are available, are possible for decreasing the rate of relapse. It is this gap in knowledge of the mechanisms/factors influencing alcohol abuse, dependence, and alcohol use disorder that needs to be filled to allow for more effective treatment options to help, or help prevent, the development of the disease.

Studies utilizing model organisms, such as *Drosophila melanogaster*, are imperative for identification of gene/mechanism/environmental factors that could be translated to influencing the risk for humans to develop alcohol use disorder. Importantly, if flies could help us to understand the different genetic and environmental factors influencing alcohol-related behaviors, it could potentially lead to the ability of

multi therapeutic action in humans (treatment with both drugs and diet). This could potentially allow for more successful outcomes for those who suffer from alcohol use disorder, alcohol abuse, or alcohol dependence.

Chapter 2: Materials & Methods

Drosophila materials

Table sugar (sucrose; multiple brands from Richmond Restaurant Service, Richmond, VA), yellow cornmeal (enriched degerminated, 62-101, Genesee Scientific, San Diego, CA), saf-instant yeast (Lesaffre Yeast Corp., Milwaukee, WI), *Drosophila* agar type II (66-104, Apex BioResearch Products, Genesee Scientific, San Diego, CA) were used to make fly media along with ampicillin sodium salt (A9518), chloramphenicol (C0378), methyl 4-hydroxybenzoate (tegosept, H5501), and tetracycline (T3383) from Sigma-Aldrich, St. Louis, MO. For studies involving different yeast brands, MP Bakers (101400) and MP Brewers (903312) yeast, MP Biomedicals (Solon, OH) were used. Polypropylene fly culture bottles (AS-335) and cotton plugs (22-456-882) were from Fisher Scientific, Waltham, MA; polystyrene narrow fly vials (89092-722) were from VWR International, Radnor, PA; fly vial cotton plugs (51-101) were from Genesee Scientific, San Diego, CA. Flugs (49-102) were from Genesee Scientific, San Diego, CA. RU486 (M8046) was from Sigma Aldrich, St. Louis, MO; Ethanol (200 proof, 89125-172) was from VWR International, Radnor, PA.Gas drying tube caps (199610000), Bel-Art Products (Wanye, NJ); feeder caps for Con-Ex studies (FCS13/16NA1), MOCAP (Park Hills, MO); 200 (41-6304) and 400 (41-6140) μm mesh, Ted Pella, Inc. (Redding, CA); methyl 4-hydroxybenzoate, chloramphenicol, tetracycline, ampicillin and xylene cyanol, Sigma-Aldrich, St. Louis, MO; FD and C Blue No. 1, Blue No. 2, Red No. 40,

Green No. 5, Red No. 4, Red No. 6 and Yellow No. 5, Spectrum Chemical Manufacturing Corp., Gardena, CA were used for investigation of consumption-excretion and media physical interaction. For quantifying knockdown of Mef2 in adult fly brains, the following was used: Calbiochem normal goat serum (80000-994) was from VWR International, Radnor, PA; rabbit-anti *Mef2* was from Dr. Bruce M. Paterson, NIH; chicken anti-rabbit Alexa 647 (A-21443) was from Thermo Fisher, Waltham, MA; Invitrogen Molecular Probes SlowFade Gold Antifade Mountant (S36936) and Fisherbrand Premium Superfrost Microscope Slides (12-544-7) were from Fisher Scientific, Waltham, MA; paraformaldehyde powder, 95% (158127), sodium chloride (S7653), and Triton X-100 (T8787) were from Sigma Aldrich, St. Louis, MO; and cover glass no.1 (89239-692) was from VWR International, Radnor, PA. Sodium phosphate, dibasic, 7-hydrate, crystal (3824-01), potassium chloride, crystal (3040-01), and potassium phosphate, monobasic, crystal (3246-01) were all J.T.Baker brand from Avantor, Center Valley, PA. For sequencing of Mef2 point mutations the following was used: Proteinase K (P2308) was from Sigma-Aldrich, St. Louis, MO; DreamTaq Green DNA Polymerase (EP0712) was from Thermo Scientific, Waltham, MA; ExoSAP-IT (782000) Affymetrix, Santa Clara, CA. Plastics for molecular biology and other liquid handling were from Genesee Scientific, San Diego, CA and USA Scientific, Ocala, FL. Oligonucleotides were from Fisher Scientific, Waltham, MA. For sequencing of nitric oxide synthase point mutations, the following was used: Taq DNA polymerase (M0273S) was from New England Biolabs, Ipswich, MA; dNTP set (BIO-39025) was from Bioline, Memphis, TN; ExoSAP-IT (782000) Affymetrix, place.

Fly husbandry and stocks

The w^{1118} (*w[A]*), Laussane-S (LS), Oregon-R (OR) and Samarkand (Sam) strains (stock numbers 5905, 4268, 25211 and 4270, respectively), UAS-Tetanus Toxin Light Chain (stock number 28837), two Trh-Gal4s (stock numbers 38388 and 38389), *elav*^{c155} (*elav*-Gal4), and the *Mef2* RNAi line, JF03115, were obtained from the Bloomington *Drosophila* stock center (BDSC, Bloomington, IN). The r[A] strain was generated by backcrossing the w^{+} allele in Canton-S (supplied by Ron Davis, Scripts, Florida) into *w[A]* for 7 generations. The w^{1118} (*w[VDRC]*) strain and two *Mef2* RNAi stocks v15540 and v15550 (construct ID 5039) were was obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria). UAS-Kir2.1 was backcrossed to w^{1118} for 5 generations. UAS-Kir2.1 and genetic background controls were from Dr. Scott Pletcher. Con-Ex ExVial and INT volumes were not different when feeding studies were initiated with anesthetized versus awake flies (data not shown). All studies used age-matched flies reared, collected and tested side-by-side.

All RNAi lines for candidate genes in follow up from human SRE GWAS are in table 4.2, and were obtained from BDSC. *Mef2*^{65}, *Mef2*$^{30\text{-}5}$ (from Richard Cripps, The University of New Mexico) and *Mef2*$^{25\text{-}34}$ (from BDSC, stock #9861) were backcrossed for seven generations to *Mef2*$^{EP\text{-}321}$ (*Mef2*$^{EP\text{-}321}$, BDSC, stock #43412, an insertion marked with mini-*w* in the first intron of *Mef2*) that we previously backcrossed for seven generations to w[A]. The *Mef2*$^{EP\text{-}321}$ insertion did not alter ethanol sedation (not shown). The *Mef2*^{65}, *Mef2*$^{30\text{-}5}$ and *Mef2*$^{25\text{-}34}$ alleles were confirmed by PCR/sequencing using standard methods before and after being backcrossed to *Mef2*$^{EP\text{-}321}$. Primers for PCR (forward/reverse) and sequencing were as follows: *Mef2*^{65}, 5'-

AGATGCTGAATGTCCGAGTGT-3'/5'-GTGATGTGGCTTGTAGTGGC-3' and 5'-CCTTAATGCAGGTGCGCC-3'; *Mef2*$^{30\text{-}5}$, 5'-CAGTCAGCAGGAATCAGCCA-3'/5'-TTGTTGGTGAGGGACTCGTG-3' and5'-TGAGCATGAGCAGTAATTGAAC-3'); *Mef2*$^{25\text{-}34}$, 5'-CAGTCAGCAGGAATCAGCCA-3'/5'-TTGTTGGTGAGGGACTCGTG-3' and 5'-CCACCATCTCCGTTTCCATC-3'.

All stocks that were used in the investigation of nitric oxide synthase (NOS) and its effect on ethanol sedation is listed in Table 5.1 (labels, stock numbers, genotypes, chromosomal insertion location, where acquired, and any notes). Primers for Nos^{1} PCR and sequencing (5'-AGAATGCCCAAACCAGTCTAAATCG-3'/5'-GGTAGCACCACCAAAGGTCGC-3') and sequencing -5'-ACATCGCTTGCCGTTGCTGAAATC-3'. Sequencing for was performed via Eurofins.

Flies were grown to adulthood at 25°C/65% relative humidity with a 12-hour light/dark cycle on standard food medium (2Y10S3C: 2% saf-instant yeast, 10% sugar, 3.3% cornmeal, 1% agar, 2 g/L Tegosept, 0.125 g/L chloramphenicol, 0.02 g/L tetracycline and 0.1 g/L ampicillin) supplemented with live yeast. Flies (3 to 5 d-old) were collected under light CO_2 anesthesia, sex separated, and placed in fresh food vials containing the media indicated in the main text prior to the described studies.

In studies using yeast paste (live or heat-killed saf-instant yeast (35% w/v) in water), flies were collected and placed into fresh food vials (containing 2Y10S3C as described above) and provided yeast paste (1 g/vial) via inverted caps from 50 mL conical tubes placed in the open ends of the vials. For studies using nylon mesh barriers, caps from gas drying tubes were bored out, circular pieces of nylon mesh were

melted into the caps, and the cap-nylon mesh inserts were placed in the vials to provide an ~2 cm gap between the flies and the yeast paste.

The media in vials were 2Y10S3C (described above); 2Y10S3C missing antimicrobials, missing one or two nutrient components, or with all components diluted as described in the main text; 2Y10S3C supplemented with additional yeast, sugar or cornmeal as described in the main text; or 2Y, 10Y, 20Y or 30Y (2, 10, 20 or 30% yeast w/v in 1% agar). Unless otherwise noted, yeast indicates saf-instant bakers yeast.

Ethanol sedation, locomotor behavior, and ethanol rapid tolerance

Flies were collected, stored, and tested for ethanol sedation and rapid tolerance essentially as described [58, 108]. Sedation time 50 (ST50) values (i.e. the time required for 50% of flies in individual vials to become sedated) were determined by exposing flies to vapor from 85% ethanol in our standard protocol or from other ethanol concentrations mentioned in the figure legends. Locomotor behavior was assessed with this same process, without the addition of alcohol. For rapid tolerance, flies were sedated during an initial exposure to ethanol, allowed to recover for 4 hours at 25°C/65% relative humidity, and then sedated during a second exposure to ethanol [108]. Development of rapid tolerance was quantitated as fold increase in ST50 (i.e. ST50 from sedation 2 ÷ ST50 from sedation 1) or change in ST50 (i.e. ST50 from sedation 2 - ST50 from sedation 1).

Drosophila GeneSwitch

Flies harboring neuronal synaptobrevin-GeneSwitch (nsyb-GS) along with UAS-*Mef2* RNAi transgenes or controls with either transgene alone were fed food topped with 100 μl of 100 μM RU486 or vehicle (ethanol) for 6 days and used to determine alcohol sedation or β-galactosidase activity. Flies that contained Tubulin-GeneSwitch (tub-GS) and a transgene, or both controls, were fed 1 mM RU486 or vehicle (ethanol) food topped with 100 μl solution, for 6 days, unless specified otherwise in legend, and alcohol sedation was determined.

Internal Ethanol Measurement

Frozen flies were homogenized in 200 μl of distilled H_2O and centrifuged for 20 minutes at 4°C [58, 108]. Ethanol content in the supernatants was determined as previously described via a spectrophotometric method [58, 59] (all panels except internal ethanol experiments in Chapter 4 – Mef2) or by individual 20 μl supernatant sample aliquots were pipetted into 20 ml headspace gas chromatography (internal ethanol in Chapter 4 – Mef2) vials containing 960 μl deionized water, 500 mg NaCl and 20 μl of 5 mM 1-propanol internal standard. Samples were tested for ethanol concentration using an Agilent model 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID), 0.53 mm ID Rtx BAC-1 capillary column (Restek, Bellefonte, PA) and CTC CombiPal headspace autosampler (Leap Technologies, Carrboro, NC). Samples were incubated and agitated for 10 min at 70°C prior to automated injection. The GC parameters were as follows: 1.5 mL headspace injection volume, 5/1 split ratio, 3 min sample run time, injector temp 200°C, oven temp isothermal 150°C, detector temp

200°C, helium carrier gas flow rate 40 mL/min, nitrogen makeup gas flow rate 18 mL/min, hydrogen flame flow rate 25 mL/min and FID air flow rate 300 mL/min. Data were collected and analyzed by Clarity GC software (Apex Data Systems, Prague, CZ) using a linear regression analysis with no weighting. Ethanol concentrations were calculated by the internal standard method. A seven point calibration curve preceded the analysis of supernatant ethanol concentrations. Quality control ethanol standards at concentrations similar to those found in the test samples were interspersed at regular intervals with test samples (Fig 4.3 E & F).

Total, dry and water weight

Adult flies were reared and collected as above and weighed to determine total weight in groups of 11 while anesthetized in tared 1.5 ml snap-cap tubes with perforated lids. Tubes of flies were incubated at 50˚C (ambient humidity) for 24 or more h to volatilize water content and weighed to obtain dry weight. Water weight was determined as the difference between total and dry weight. Total, dry and water weights for each tube were expressed as mg/fly. Each tube of 11 flies generated a single datum

Media and nutrient consumption

Intake of food medium was measured using consumption-excretion of 1% FD&C Blue 1 in the indicated media using the sum of the dye excreted in the vial (ExVial) and the internal dye (INT) to reflect the volume of media consumption as described [109]. Flies were reared and collected as described above, placed on the indicated food medium containing 1% FD&C Blue 1 for 24 h, and then ExVial and INT were determined.

Nutrient consumption (fold of 2Y) was estimated as ([ExVial+INT] x [yeast concentration]) ÷ ([mean 2Y ExVial+INT] x [yeast concentration]).

Brain 5-HT levels

r[A] females, reared and collected as described above, were fed 2Y or 30Y media for 2 days. Brains were dissected from flies and 5-HT was measured essentially as previously described [110-112]. In brief, single fly brains were dissected, homogenized, diluted with 10 µL 20 µM perchloric acid (to prevent transmitter degradation) and then tissue content determined with capillary electrophoresis with fast scan cyclic voltammetry detection.

Consumption-excretion studies overview

Agar-based food medium containing dye (dissolved in media prior to solidifying) was poured into plastic feeder caps (Fig. 1A) and allowed to cool at room temperature to solidify, placed in a humidified plastic box, and stored at 4°C overnight. The following day, feeder caps containing media were warmed to room temperature for 1 h, inverted and placed in the open end of vials containing adult flies (Fig. 1B). The feeder caps used in these studies hold ~4.5 mL of medium (many-fold more than flies consume), have flanges that prevent them from falling into the vials and fit in the vials used so that condensation does not build-up, yet flies cannot escape. Adult flies (typically 15/vial, but see Fig. 6) in the vials consumed medium from the feeder caps (the only food source) and then excreted waste over time (Fig. 1C). A single feeder cap was used in each vial over the duration of each experiment. Feeder caps were discarded at the conclusion of

feeding (Fig. 1D). The dye inside the flies (internal dye, INT) was collected via homogenization of animals in 1.5 ml of water followed by centrifugation to pellet debris. The dye excreted by flies on the walls of the vials (excreted vial dye, ExVial) was collected by addition of 3 ml of water to vials followed by vortexing (Fig. 1D). Definitions of abbreviations for all Con-Ex measures are provided in Table S1. Absorbance of the INT and ExVial dye in water extracts was determined in a spectrophotometer (Pharmacia Biotech Ultraspec 2000) (Fig. 1E) at wavelengths appropriate for each dye (Blue 1, 630 nm; xylene cyanol, 615 nm; Red 40, 504 nm; Green 5, 608 nm; Blue 2, 608 nm; Red 4, 500 nm; Red 6, 442 nm; Yellow 5, 425 nm). Absorbance values were converted to volumes of medium consumed by interpolation from standard curves of pure dyes (Fig. 1F). Extracts of flies fed medium without dye controlled for background absorbance. The standard amount of time flies were allowed to consume-excrete in the vials was 24 h, but the consumption-excretion time was varied in some studies as described. When used, starvation of flies was achieved by housing them in empty vials for 17 h. In studies that assessed mating status on Con-Ex, virgin flies were collected under brief CO_2 and then housed at 15 flies per bottle in the presence of 15 males (mated) or in the absence of males (virgin) for 2 d prior to the initiation of Con-Ex. Flies in all studies had a single water/food source (the feeder cap) and were housed undisturbed at 25°C and 65% relative humidity under a 12-hour light/dark cycle while consuming media from feeder caps.

Excretion on food medium

Flies (20/vial) consumed standard medium containing 3% w/v Blue 1 from a feeder cap for 4 h (to accumulate INT dye). Flies were transferred into a new, second vial. A feeder cap containing the same medium, but no dye, was placed in the second vial and flies were allowed to excrete the previously consumed dye for 24 h (at which time all or virtually all INT dye has been excreted). The concentration of Blue 1 was increased to 3% for adequate dye signal. ExVial in the second vial was collected by adding 3 ml of water to the vials followed by vortexing. Excreted dye on the medium (ExMedium) was captured by melting the medium in each feeder cap in 25 ml of water in a microwave followed by centrifugation to pellet debris. Absorbance values of the ExVial and ExMedium dye were measured as described above. Extracts of food medium without dye were used to control for background absorbance. Absorbance values for dye collected from the vial walls and from the food medium were converted to volumes via interpolation from standard curves of pure dye dissolved in food medium. Due to low dye signal from groups with low dye concentrations, lower number of flies and after shorter periods of feeding, determining ExMedium in these studies was not possible.

CAFE-excretion studies

Studies on CAFE-excretion of liquid medium were performed as described [67] with modifications to capture excretion products. Adult flies (10/vial) were placed into empty food vials and prevented from escaping by foam plugs. One capillary feeding tube per vial was held in place by a plastic pipette tip placed in the foam plug such that the

capillary tube extended beyond the bottom of the plug by 3-5 mm. Flies were allowed to consume-excrete liquid medium (5% sucrose and 1% Blue 1 in water) from the capillary feeding tubes for 8 hours. The original capillary tubes containing sucrose and dye were removed, the amount of liquid medium consumed from each tube was recorded, and flies were then provided with fresh capillary feeding tubes containing 5% sucrose medium without Blue 1 for 18 h. Flies were housed in the same vials while consuming medium from both the first (containing Blue 1) and second (without Blue 1) capillary tubes, thereby allowing the excreted waste from each group of animals to accumulate in the vials and on the plugs. Excreted Blue 1 on the interior surface of vials (ExVial) was collected by adding 3 ml of water to each vial followed by vortexing. Excreted Blue 1 on foam plugs (ExPlug) was collected in 3 ml of water. Absorbance of Blue 1 was measured in a UV-vis spectrophotometer at 630 nm and absorbance values were converted to volumes by interpolation from standard curves of Blue 1. Flies were either fully fed or starved for 17 h by housing them in vials with 1% agar (wet starvation) or empty vials (starvation). Starvation with either protocol led to comparable amounts of medium consumption and excretion (not shown). Flies were housed undisturbed (except for replacing capillary tubes) in an opaque polycarbonate box at 25°C and 65% relative humidity under a 12-hour light/dark cycle throughout CAFE-excretion studies. Vials without flies were used to control for evaporation of liquid medium from capillary tubes.

Pletcher laboratory methods

For the studies reported, an equal amount of Canton-S embryos were dispensed and reared on conventional sugar, yeast, cornmeal, and agar medium (CT) to control for density of developing larvae/pupae and synchronize the emergence of adults. Newly emerged adults were placed on fresh CT diet to allow for fly mating. 8-9 day old mated female and male flies were sorted under light CO_2 anesthetization into groups of 15 same-sex flies and transferred onto SY10 dietary medium containing 10% (w/v) sucrose, 10% (w/v) yeast, 2% (w/v) agar, 0.3% (w/v) tegosept, 0.3% (v/v) propionic acid, 0.002% (w/v) tetracycline, and 0.005% (w/v) kanamycin. Eight replicate vials were created for each sex and time point. After keeping flies on SY10 medium for three days, flies were then transferred onto SY10 diet containing 1% FD&C Blue No. 1 dispensed into the Con-Ex MoCaps for the interval specified in the figures. Time course measurement studies were started 2 hours after lights-on. All larvae/pupae/adults were maintained at 25°C, 12-hour light/dark, and 50-60% humidity throughout the experiment. Processing of flies/vials, absorbance measurements, and data analysis were performed as described in the Grotewiel laboratory methods.

Human sample and phenotype

The Avon Longitudinal Study of Parents and Children (ALSPAC) initially recruited 14,541 pregnant women residing in Avon, UK, with expected dates of delivery April 1, 1991, to December 31, 1992; 15,247 is the initial number of pregnancies for which the mothers enrolled in the ALSPAC study and had either returned at least 1 questionnaire or attended a “Children in Focus” clinic by July 19, 1999. Of these initial pregnancies,

there was a total of 14,973 live births and 14,899 children who were alive at 1 year of age. Subsequent phases of enrollment increased the sample size over time. The phases of enrollment are described in more detail elsewhere [113, 114]. For the current analyses, full or partial phenotypic data were available for 5,626 offspring participants, in part reflecting the need for a subject to have had experience with alcohol to complete items related to alcohol sensitivity. The study website contains details of all the data that is available through a fully searchable data dictionary (http://www.bristol.ac.uk/alspac/researchers/our-data/). Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

Initial alcohol sensitivity in ALSPAC was assessed using the Self-Rating of the Effects of Alcohol scale (SRE) [18]. Participants, aged 15.5, 16.5, and 17.5, were asked to consider the first five or so times they consumed alcohol and to report the number of standard drinks (defined in the questionnaire/interview) they consumed before they experienced signs of intoxication. As described previously [115], responses were winsorized to account for outliers and total scores were derived according to recommendations by Schuckit and colleagues [116]. The total score was used as a continuous outcome in subsequent GWAS. See Edwards, Deak (115) for additional details.

Human genetic analyses

We used summary statistics from the SRE GWAS in ALSPAC, which contributed to our prior meta-analysis [115] but have not been independently reported, to identify

candidate genes of interest for potential follow-up. Summary statistics were uploaded to FUMA [117], which utilizes MAGMA [118] to conduct gene-based analyses. We selected the 1000 Genomes EUR subset to adjust for linkage disequilibrium [described in [119]].

Identification of Drosophila orthologues of candidate SRE genes

Of the human genes that were nominally implicated (p_{gene}<0.001) in SRE variation, we identified genes for investigation in *Drosophila* based on several criteria: (i) prior evidence of involvement in phenotypes of potential relevance to alcohol response (e.g. substance use, psychopathology, or fatty liver disease) as suggested by searches of the Public Health Genomics Knowledge Base and PubMed; (ii) human-*Drosophila* orthology as determined using DIOPT [78], an online tool available via the DRSC/TRiP Functional Genomics Resources at Harvard University; and (iii) public availability of *Drosophila* RNAi reagents for manipulating expression of genes in flies.

Drosophila immunohistochemistry

Whole brains from adult female flies were dissected in PBT (100 mM Phosphate buffer, pH 7.2, with 0.03% (v/v) Triton X-100) under a dissecting microscope and fixed in 0.5 mL snap cap tubes containing 4% paraformaldehyde for 20 minutes at room temperature on a tube rotator. Fixed brains were washed with PBT, blocked with 5% normal goat serum (NGS), and incubated in primary antiserum (anti-*Mef2*, diluted 1:5000 in NGS) on a tube rotator at 4°C for 48 hrs. Brains were washed with 0.3% PBT and exposed to the secondary antibody (chicken anti-rabbit Alexa 647, diluted 1:1000 in NGS) at 4°C for 48 hrs. Brains were then washed with PBT and mounted onto glass

slides in SlowFade mounting medium. Images were collected using a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) housed in the VCU Department of Anatomy and Neurobiology Microscope Facility. Confocal z-stack images using a pin hole of 1 Airy disc unit and Nyquist sampling were collected from each adult brain. Images were captured with a 10X objective using a numerical aperture of 0.3. The microscopy settings were optimized for the mushroom body regions of the brains from RNAi transgene control flies to (i) avoid oversaturation of images and (ii) allow comparisons in pixel intensity between brains expressing the *Mef2* RNAi in neurons and controls. The same settings (gain, offset, power) and z-stack slice thickness were used in all images. All images were processed using Image J (National Institutes of Health [120]). A threshold value was individually set for each z-stack so only pixels in the brains were analyzed. *Mef2* detection was determined as the mean pixel intensity for each brain [121].

Statistical analysis of Drosophila results

Statistical analyses (student's t-test, one- and two-way ANOVAs, Bonferroni's multiple comparisons (BMC), and normality tests) were performed using Prism 6.07 (GraphPad Software Inc., San Diego, CA). P values, reported in the legend, < 0.05 were considered statistically significant. All numerical data are reported as mean ± SEM.

Chapter 3: Dietary yeast influences ethanol sedation in *Drosophila* via serotonergic neuron function

A. Introduction

Consumption of alcohol (ethanol) has a wide range of pleasurable effects including psychomotor stimulation[122, 123], general improvement in mood and relief of stress[124]. Additionally, however, abuse of alcohol has far-reaching, negative health consequences[125, 126]. Alcohol abuse contributes to 3-4% of all preventable deaths worldwide, increases the risk for specific forms of cancer, and is responsible for hundreds of billions of dollars in costs annually within the United States alone[125]. Both environmental and heritable genetic factors contribute to the risk for abusing alcohol[14-16, 127]. A better understanding of these environmental and genetic risk factors could ultimately facilitate prevention and treatment of alcohol abuse.

Drosophila melanogaster (fruit fly or fly) is a leading invertebrate model for investigating molecular-genetic mechanisms that influence alcohol-related behaviors[71, 72, 128, 129]. Behavioral responses of flies to alcohol include locomotor stimulation at low doses[63, 130], sedation at higher doses[58, 59, 62, 77, 131-133], development of seizures upon withdrawal of alcohol[66], and development of tolerance after prior exposure to the drug[56, 60, 134]. Additionally, flies will voluntarily consume alcohol[67] and they develop exposure-dependent alcohol preference[135-138]. All of the behavioral responses to ethanol in flies are also found in other species including humans[71], strongly suggesting that alcohol

likely has conserved effects on nervous system function. Consistent with this possibility, many genes or genetic pathways that influence behavioral responses to alcohol in flies have also been implicated in various aspects of alcohol-related behaviors in other model organisms (e.g. *Clic* [132], GABA signaling[73, 139], *slo* potassium channels[140] and NPF/NPY signaling[75, 141]) as well as various aspects of alcohol consumption and abuse in humans (e.g. *Adh*[138, 142-144], *Rsu1*[79], *AUTS2*[78], *Ryr*[77]). Thus, at least some of the mechanisms underlying alcohol-related behavior in model organisms might also impact alcohol abuse in humans.

In addition to genetic factors, ~50% of the risk for abusing alcohol is influenced by environment[14-16, 127]. Diet is possibly one of the key—but largely underappreciated—environmental factors that influences alcohol phenotypes in humans. Supplementation of the diet with tryptophan decreases alcohol craving in human binge drinkers exposed to stress[49]. Additionally, patients with higher body mass indexes (BMI) are at an increased risk for heavy alcohol intake[42], development of alcohol dependence[44] and alcohol abuse[43]. Diet also influences multiple alcohol-related behaviors in rodents[84-86, 145] and *C. elegans* [87]. Furthermore, variants in the genes *FTO* and *CPNE5* are associated with both obesity and multiple alcohol phenotypes in humans[46, 47, 146] and several genes in flies might regulate both food intake and behavioral responses to alcohol[83]. These studies collectively suggest that diet and diet × genotype interactions might play important roles in multiple aspects of alcohol-related behavior in animals and impact risk for alcohol-related phenotypes in humans.

Several studies suggest that the serotonin (5-hydroxytryphtophan, 5-HT) system might modulate or mediate the effects of diet on behavioral responses to alcohol. In

flies, for example, dietary yeast influences brain 5-HT levels[147], serotonergic neurons regulate feeding[148-150], the 5-HT2A receptor impacts dietary protein consumption[147], and 5-HT is implicated in ethanol sedation[74]. Additionally, there is a large literature linking 5-HT to alcohol problems in humans (e.g.[151-155]). Despite the insights of the studies summarized here, the possibility that 5-HT signaling underlies diet-induced changes in behavioral responses has not been formally addressed.

In this report, we describe results from our studies on the role of diet in alcohol sedation in *Drosophila*. We chose flies for these studies because of their conserved alcohol-related behaviors[56, 58-60, 62, 63, 66, 67, 71, 77, 130-138], the powerful tools available for performing genetic analyses in this model[156], the ability to measure both ethanol sedation (see above) and food intake[109], the ability to control food composition[109, 147, 157], and the known genetic connections between fly alcohol behavior and human alcohol abuse[77-79, 138, 142-144]. We report that dietary yeast significantly impacts ethanol sedation in flies, possibly by influencing ethanol uptake/elimination. We also report that the effect of dietary yeast on ethanol sedation and uptake/elimination depends on serotonergic neuron function. Our studies establish flies as a model for exploring diet-induced changes in alcohol sedation and suggest that the serotonergic system might be a conserved regulator of the underlying processes.

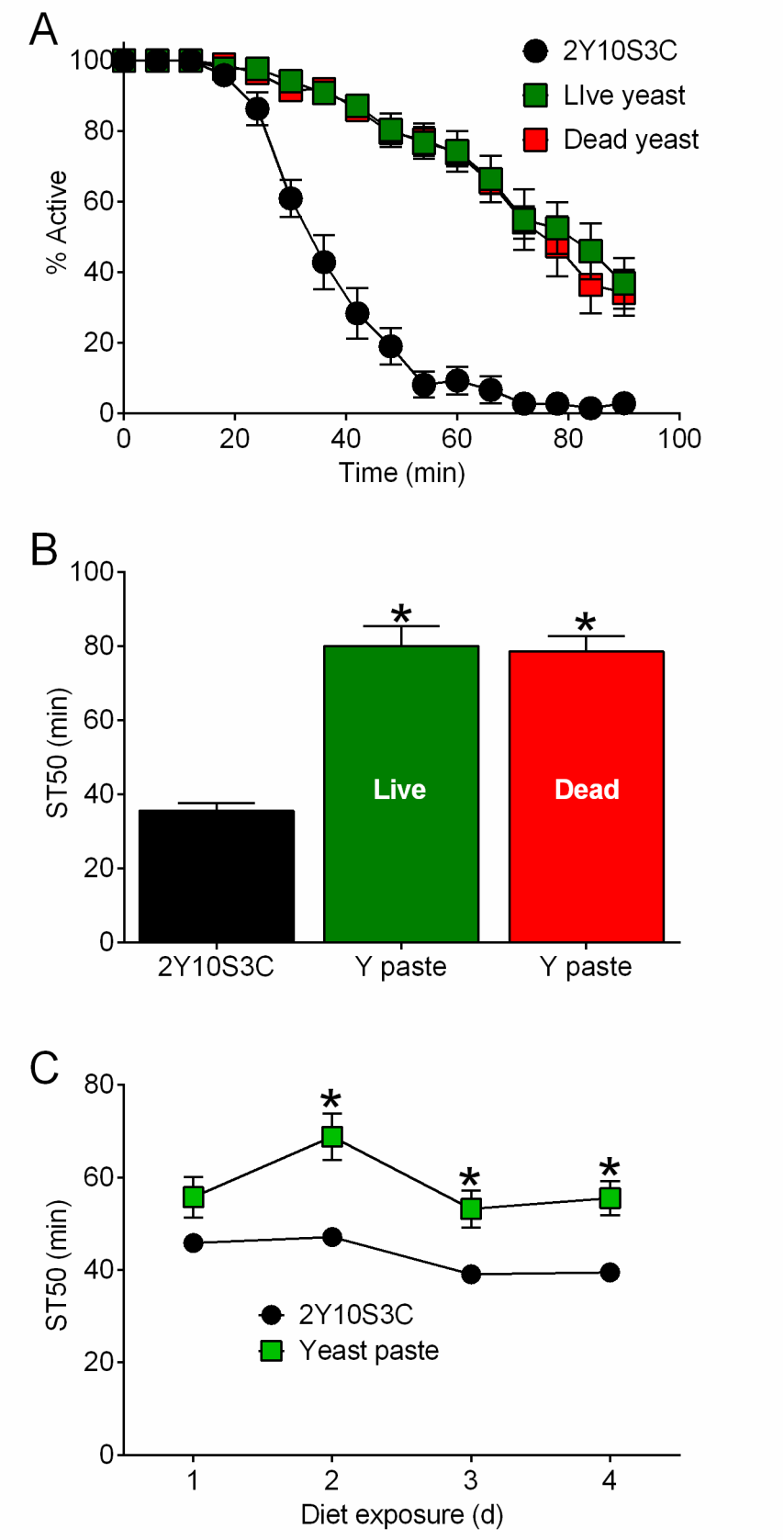
B. Results

Drugs, enzyme inhibitors and other molecules can be administered (i.e. fed) to flies via a simple paste made of yeast (*Saccharomyces cerevisiae*) and water (e.g.[158-160]). While establishing this drug treatment regimen for investigating alcohol behavior in flies, we found that flies exposed to a standard food medium supplemented with a paste

made from live yeast and water were substantially resistant to ethanol sedation (Fig. 3.1A, time-courses; Fig. 3.1B, sedation-time 50 (ST50) values) compared to flies provided only a standard medium containing 2% yeast, 10% sucrose and 3.3% cornmeal (hereafter 2Y10S3C). The resistance to ethanol sedation was evident by 2 d of exposure to yeast paste and persisted during at least 4 d of continuous exposure (Fig. 3.1C).

Yeast produce ethanol via fermentation[161-164], including under conditions used to rear *Drosophila*[58]. To address the possibility that the ethanol resistance in flies fed yeast paste reflected tolerance in response to ethanol produced by the supplemented yeast, we fed flies paste made of heat-killed yeast (which would be incapable of fermentation) and then assessed ethanol sedation. Flies fed heat-killed yeast paste were resistant to ethanol sedation compared to flies fed standard food, and ethanol sedation in flies fed heat-killed and live yeast paste were indistinguishable (Fig. 3.1A, time-courses; Fig. 3.1B, ST50 values). Therefore fermentation and ethanol production by supplemented yeast is not required for the yeast-induced change in resistance to ethanol sedation.

Flies were provided with supplemental yeast paste in the studies reported in Fig. 3.1. To address the possibility that increasing the concentration of yeast incorporated into agar-based fly media (versus supplementation with yeast paste) was capable of altering ethanol sedation, we assessed ST50 values in flies fed our standard fly medium (2% yeast, 2Y10S3C) and in media containing 10% (10Y10S3C), 20% (20Y10S3C) and 30% (30Y10S3C) yeast. Increasing the yeast concentration increased ST50 values in males (Fig. 3.2A) and females (Fig. 3.2B). Flies fed 20% yeast medium had increased ST50s after exposure to the diet for 2 or more d, whereas flies fed medium with 30%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Diet exposure (d)

Figure 3.1. Exposure to dietary yeast paste alters ethanol sedation sensitivity. (A) *w[A]* females fed a paste of live or dead yeast (35% w/v) for 2 d took longer to become sedated compared to flies fed 2Y10S3C (standard) medium (two-way ANOVA; time, $p<0.0001$; yeast, $p<0.0001$; interaction, $p<0.0001$; *Bonferroni's multiple comparisons (BMC) versus 2Y10S3C; $p<0.0001$; n=7-8 per data point) (Performed by Ian Hines). (B) ST50 values derived from panel A. Yeast (Y) paste had a significant overall effect on ST50s (one-way ANOVA, $p<0.0001$, n=7-8). ST50s were greater in flies fed live or dead yeast versus 2Y10S3C medium (*BMC, $p<0.0001$). ST50s were not distinguishable between flies fed live or dead yeast paste (BMC, $p=0.9682$) (Performed by Ian Hines). (C) Dietary yeast paste increased ST50 values in r[A] females (two-way ANOVA; yeast, $p<0.0001$; time, $p=0.0029$; interaction, $p=0.3486$; *BMC, $p=0.0136$ to <0.0001; n=8) (Performed by Monica Messick).

yeast had greater ST50 values after 1 or more d on the diet (Fig. 3.2). Increasing the yeast concentration in agar-based medium, like supplemental yeast paste, is therefore capable of eliciting resistance to ethanol sedation. Rearing flies on 2Y10S3C and 30Y10S3C promoted comparable patterns of adult emergence over time (Fig. 3.3A) and comparable total numbers of progeny (Fig. 3.3B), suggesting that our standard 2Y01S3C medium is not nutrient deficient and therefore the yeast-driven changes in ST50 (Fig. 3.2) are likely to be related to nutrient supplementation versus restoration of sufficient nutrients. The data in Fig. 3.2 also suggest that dietary yeast did not need to be alive to elicit resistance to ethanol sedation since the agar-based media were generated by boiling.

It seemed possible that increasing nutrient components other than yeast in dietary media might also influence ethanol sedation. We therefore fed flies standard agar-based media supplemented with sucrose or cornmeal and then measured their ST50s. We found that increasing these other nutrient components of dietary media for 1-3 d of feeding did not systematically or substantially alter ST50 values in males (Figs. 3.4A and 3.4C) or females (Figs. 3.4B and 3.4D). Although these experiments do not formally rule out a potential role for dietary sucrose or cornmeal in fly ethanol sedation resistance, they do indicate that altering these two components of the diet likely has a much more modest (if any) effect on ethanol sedation compared to yeast.

It also seemed possible that omitting other components of the fly media could affect ethanol sedation. We therefore measured ST50 values in male and female flies fed 2Y10S3C media with (+ATC) or without (-ATC) the antibiotics ampicillin, chloramphenicol, and tetracycline (Fig. 3.5A), and with (+TEG) and without (-TEG) the

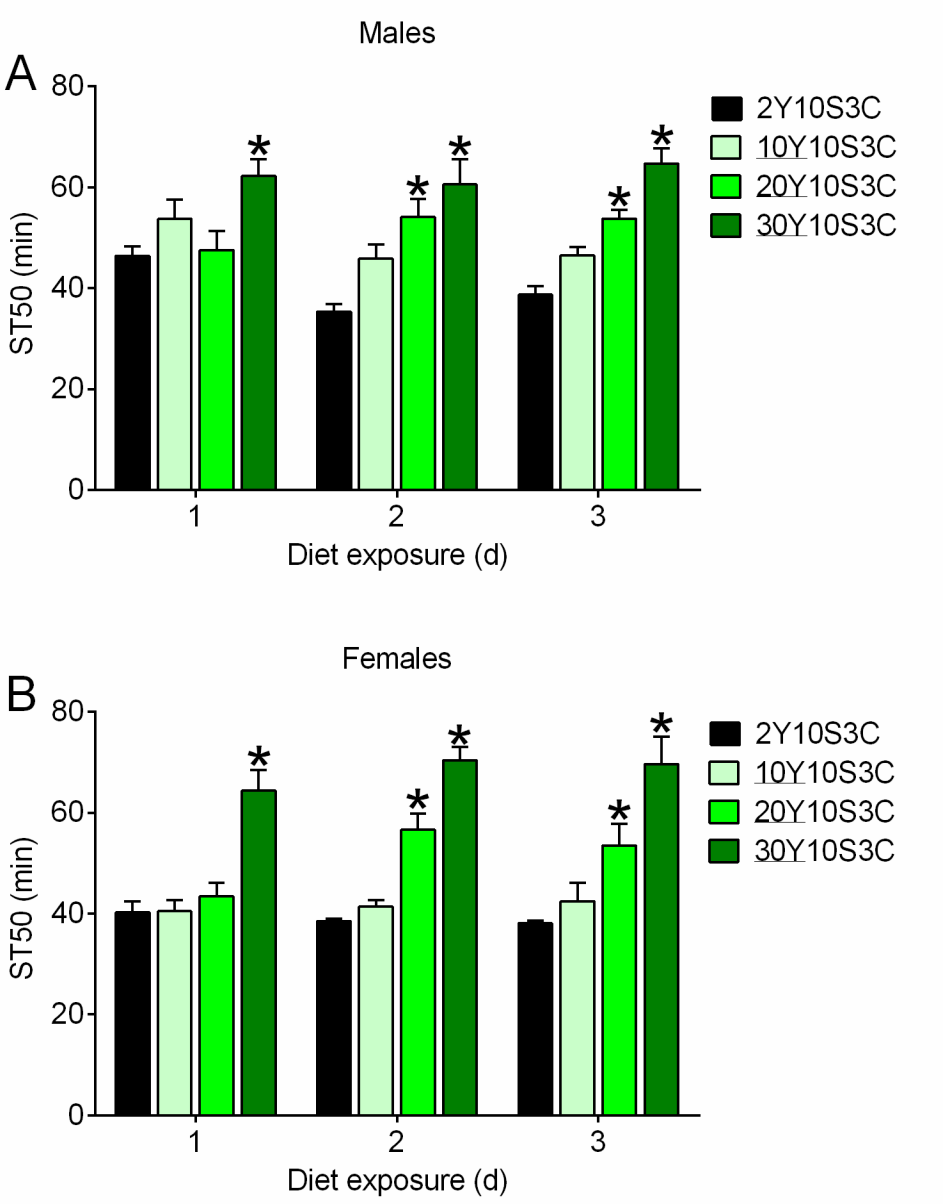


Figure 3.2. Supplementation of dietary media with yeast alters ethanol sedation. Flies were fed the indicated media for 1-3 d. Concentrations (w/v) of yeast (Y) used are underlined. ST50s in r[A] males (A) and females (B) were influenced by supplementing the diet with yeast (two-way ANOVA; yeast, $p<0.0001$; diet exposure time in males, $p=0.2665$; diet exposure time in females, $p=0.0852$; interaction in males, $p=0.0681$; interaction in females, $p=0.2749$; $n=6$). Compared to flies fed 2Y10S3C medium, ST50s were increased in flies fed media supplemented with yeast (*BMCs, $p=0.068$ to <0.0001). (Performed by Monica Messick).

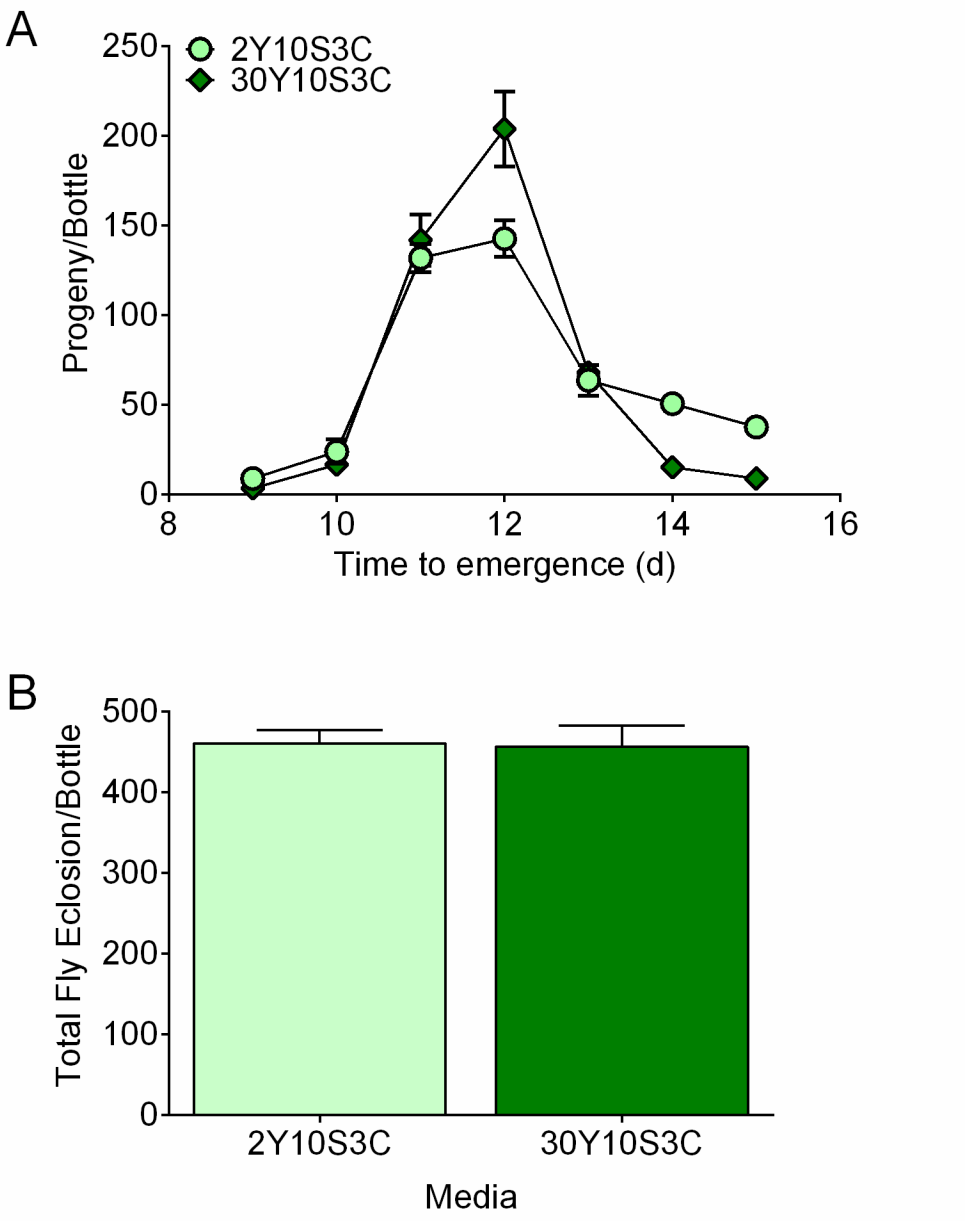


Figure 3.3. Time to emergence of adult progeny on standard food medium. (A, B) Mated adult females were introduced into bottles containing 2Y10S3C or 30Y01S3C media and newly emerged adult flies were collected and counted daily. (A) Time course of emerging adult flies starting on day 9 and peaking on day 12. (B) Total number of adult flies eclosed from day 9 to day 15 (two-tailed t test; p=0.4607; n=4 bottles/media).

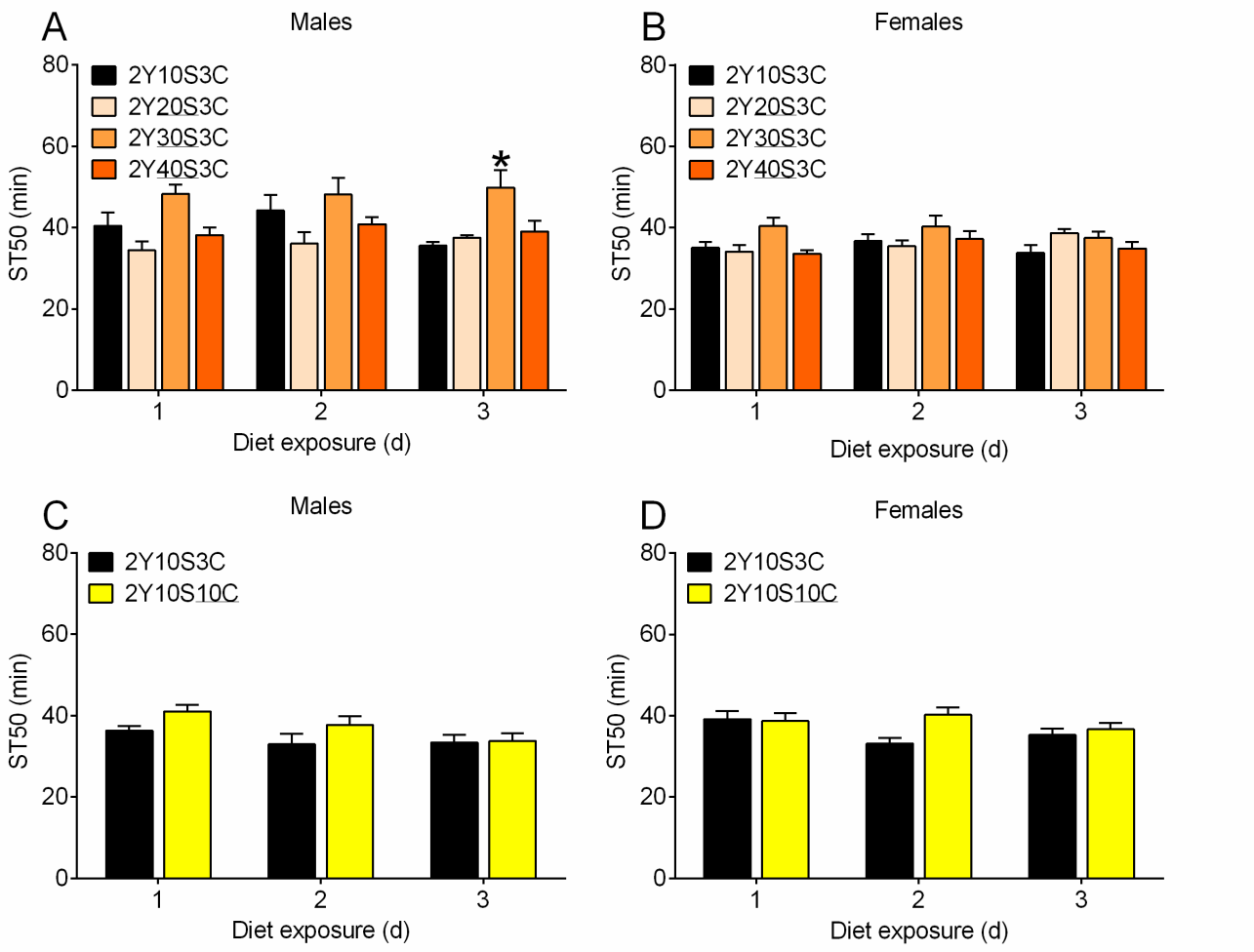


Figure 3.4. Increasing sugar or cornmeal in dietary media does not substantially alter ST50 values. Flies were fed the indicated media for 1-3 days. Supplementation of dietary media with sugar (A and B) or cornmeal (C and D) did not robustly alter ST50s. Sugar supplementation influenced ST50s in r[A] males (panel A; two-way ANOVA; sugar, $p<0.0001$; diet exposure time, 0.5328; interaction, $p=0.5471$; *BMC versus 2Y10S3C, $p=0.0047$) and females (panel B; two-way ANOVA; sugar, $p=0.0103$; diet exposure time, $p=0.3757$; interaction, $p=0.2862$). Overall, there was a significant effect of cornmeal supplementation on ST50s in males (panel C; two-way ANOVA; cornmeal, $p=0.0418$; diet exposure time, $p=0.0354$; interaction, $p=0.4242$), but not in females (panel D; two-way ANOVA; cornmeal, $p=0.0670$; diet exposure time, $p=0.2063$; interaction, $p=0.0833$). N=6 in all panels. (Performed by Monica Messick).

antifungal Tegosept (Fig. 3.5B). Additionally, to test whether omission of one or more of the nutrient components of 2Y10S3C medium could alter ethanol sedation, we assessed ST50 values in flies fed media that did not contain yeast, sucrose, orcornmeal individually (Fig. 3.6A), lacked combinations of yeast and sucrose, sucrose and cornmeal, or yeast and cornmeal (Fig. 3.6B), contained diluted media components (0.5X and 0.25X, Fig. 3.6C), or contained no yeast, sucrose or cornmeal (0X, Fig. 3.6C). Ethanol sedation was not significantly affected by the omission of antibiotics from the media (Fig. 3.5A), consistent with a previous report from our group[58], nor by omitting or reducing Tegosept (Fig. 3.5B), yeast, sugar, or cornmeal (Figs. 3.6A and 3.6B), or all nutrient components (Fig. 3.6C).

The results in Figs. 3.1-3.6 collectively indicate that increasing dietary yeast is capable of increasing resistance to ethanol sedation. To more directly test this possibility, we assessed ST50 values in males and females fed our standard 2Y10S3C medium, a medium with 2% yeast as the only nutrient (2Y), or a medium with 30% yeast as the only nutrient (30Y). ST50 values were indistinguishable in flies fed 2Y10S3C and 2Y media (Fig. 3.7A; left, males; right, females), consistent with our previous studies using media lacking sucrose or cornmeal (Fig. 3.6C). As expected, ST50 values were significantly greater in male and female flies fed a 30Y diet compared to both 2Y10S3C and 2Y (Fig. 3.7A). These results confirm that manipulating the concentration of dietary yeast in the absence of other nutrients is sufficient for altering ethanol sedation.

The studies reported in all figures discussed thus far used saf-instant bakers (SI Bak) yeast. To address whether SI Bak yeast was unique in its ability to elicit resistance

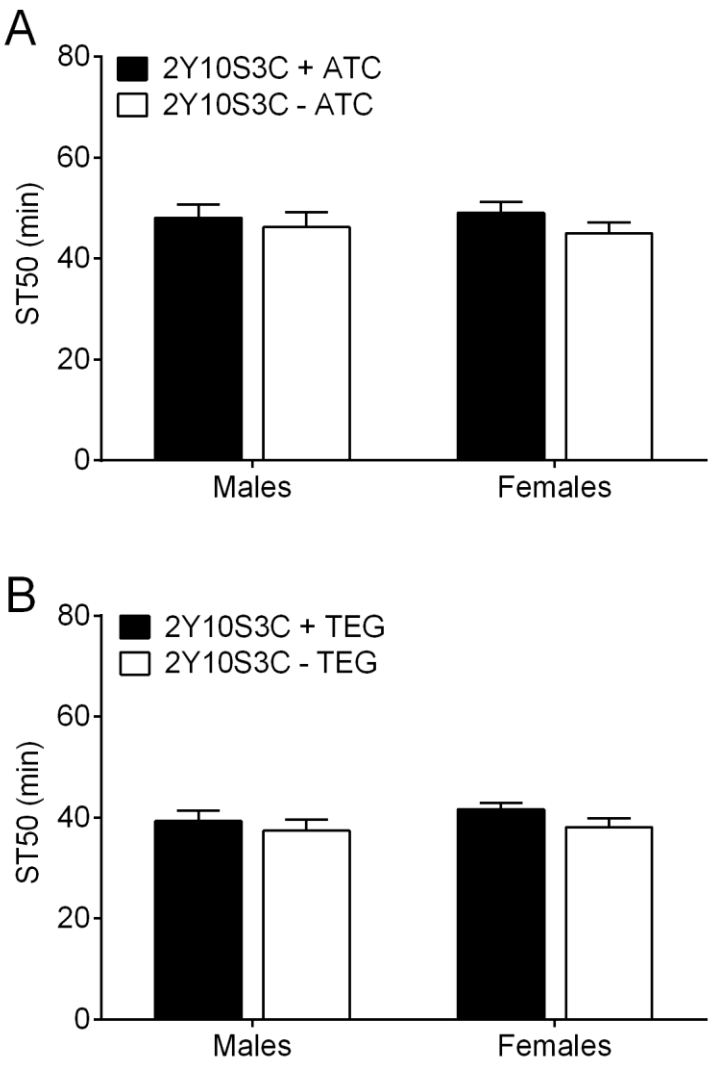
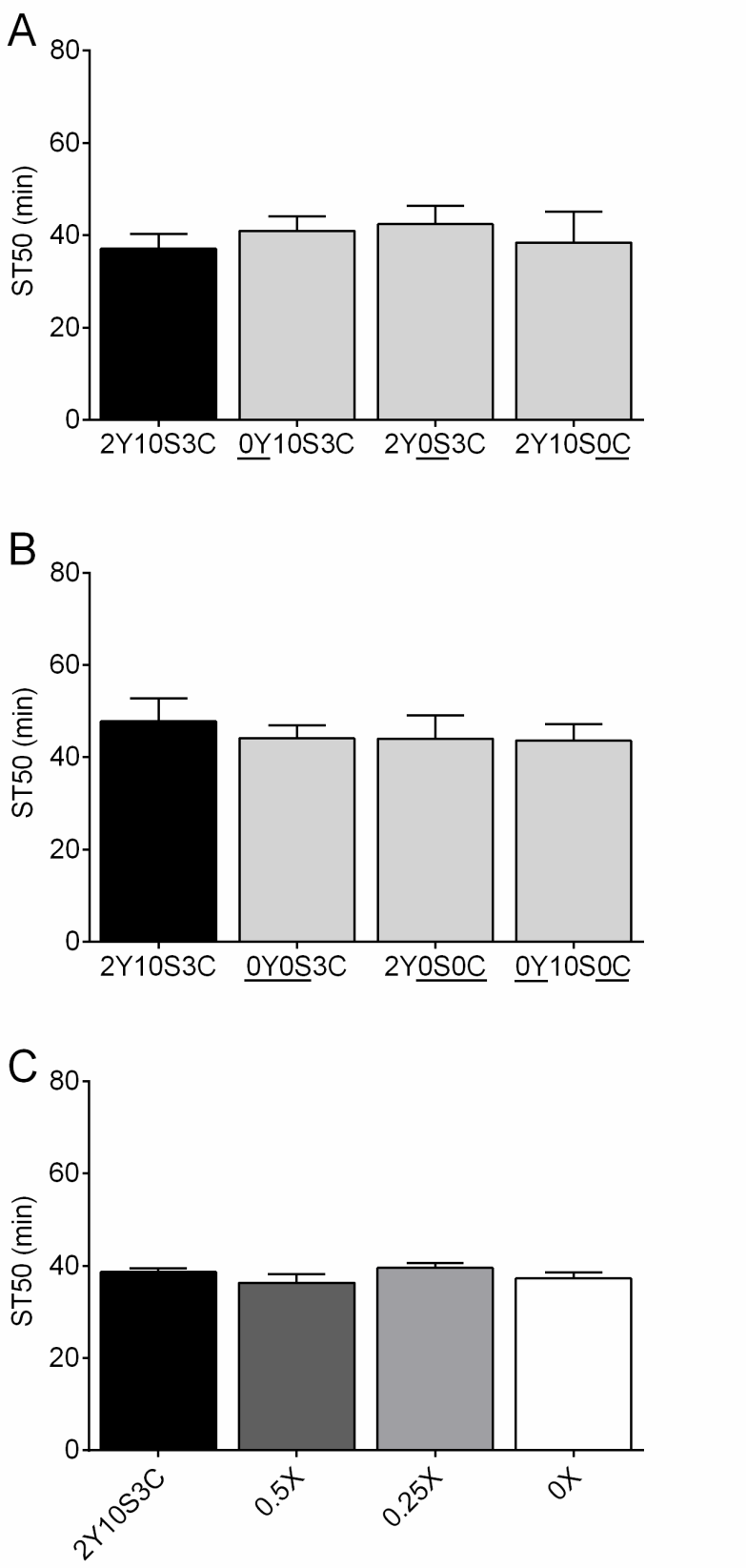


Figure 3.5. Dietary antimicrobials do not alter ST50 values. Flies were fed the indicated media for 2 d. (A) ST50s were indistinguishable in r[A] males and females fed media with (+ATC) or without (-ATC) ampicillin, tetracycline and chloramphenicol (two-way ANOVA; ATC, p=0.2452; sex, p=0.9481; interaction, p=0.6529; n=8). (B) Dietary media with (+TEG) or without (-TEG) Tegosept had no effect on ST50s in r[A] males and females (two-way ANOVA; TEG, p=0.1523; sex, p=0.4214; interaction, p=0.6527; n=8). (Performed by Monica Messick)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2Y10S3C
0.5X
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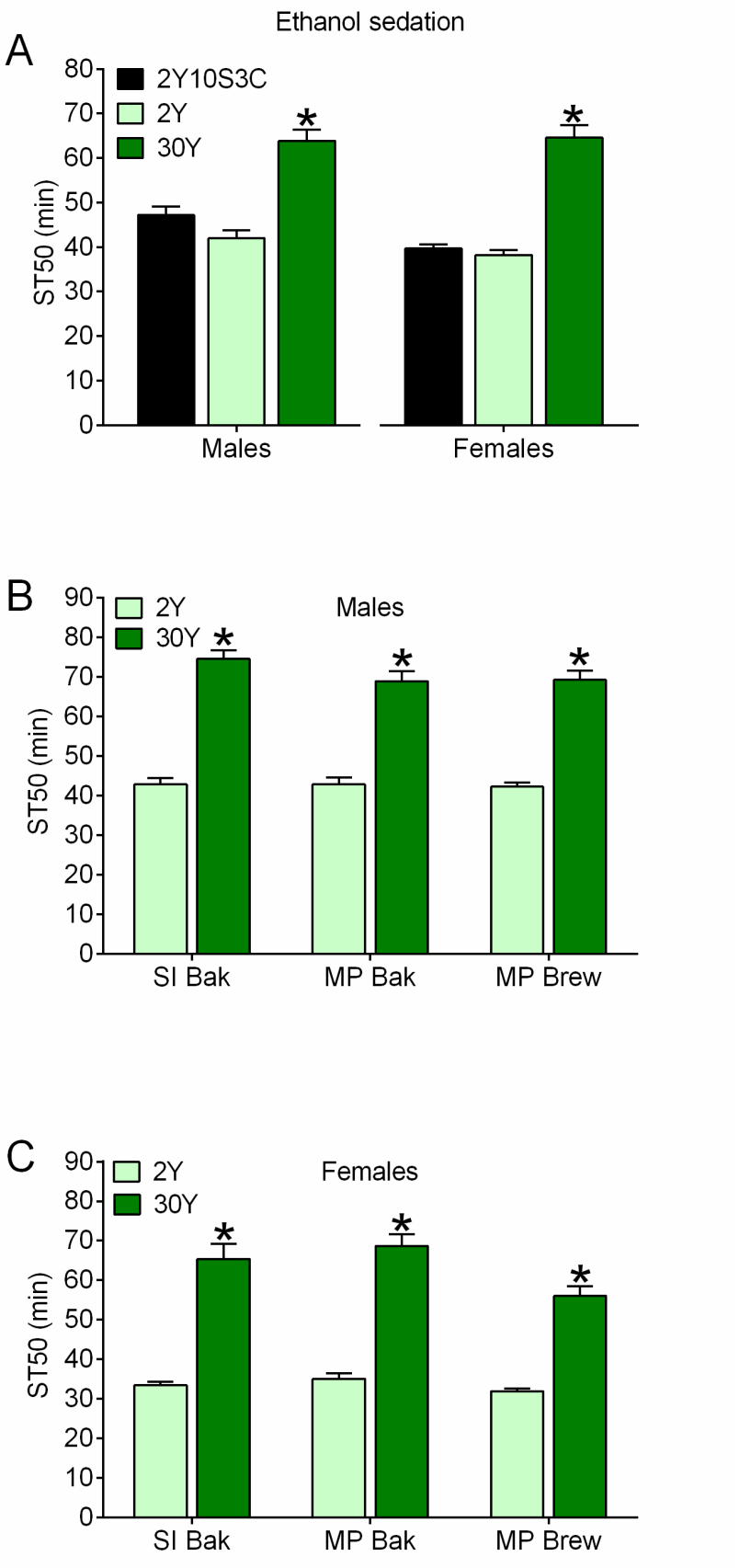
0X

Figure 3.6. Removal or dilution of media nutrients does not impact ST50 values. Flies were fed the indicated media for 2 d. (A) Omitting yeast (0Y), sugar (0S), or cornmeal (0C) from dietary media did not alter ST50s (one-way ANOVA, p=0.1989, n=6). (B) Removing 2 nutrient components from dietary media did not alter ST50s (one-way ANOVA, p=0.3001, n=6). (C) Dilution of 2Y10S3C medium (0.5X, 0.25X) and removal of yeast, sugar and cornmeal from the medium (0X) did not influence ST50s (one-way ANOVA, p=0.3364; n=8). (Performed by Monica Messick).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MP Brew
*

Figure 3.7. Effects of multiple types of dietary yeast influences ethanol sedation. (A) Flies were fed the indicated media for 2 d. ST50s were greater in r[A] males and females fed 30% yeast (30Y) compared to 2Y10S3C or 2% yeast (2Y) media (one-way ANOVAs; males, $p<0.0001$; females, $p<0.0001$; *BMC versus other groups, $p<0.0001$; n=8). ST50s were increased in male (B) and female (C – performed by Monica Messick) flies fed 30Y versus 2Y media for 2 d (individual two-way ANOVAs: males—yeast concentration, $p<0.0001$; yeast source, $p=0.2509$; interaction, $p=0.3232$; females—yeast concentration, $p<0.0001$; yeast source, $p=0.0048$; interaction, $p=0.1087$; *BMC versus 2Y, $p<0.0001$; n=8 for all groups). ST50s in females were lower on 30Y MP Brew than in 30Y SI Bak and MP Bak (BMC, $p=0.0202$ and 0.0012, respectively).

to ethanol sedation, we tested whether yeast from other sources could alter ST50 values (Figs. 3.7B, 3.7C). Males (Fig. 3.7B) and females (Fig. 3.7C) fed media containing 30% (30Y) SI Bak, MP bakers (MP Bak) or MP brewers (MP Brew) yeast were resistant to ethanol sedation compared to their sex-matched counterparts fed media with 2% yeast (2Y) from each source. Media with 30% of all three yeast sources had comparable effects on resistance to ethanol sedation in males (Fig. 3.7B), whereas 30% MP Brew yeast had a smaller effect than the other 2 yeast sources in females (Fig. 3.7C). The ability to induce resistance to ethanol sedation appears to be a common property of yeast. Additionally, our studies suggest that there could be subtle yeast x sex effects on ethanol sedation.

Like mammals, flies develop rapid ethanol tolerance, quantified as the change in resistance to ethanol during a second ethanol challenge after recovery from an initial exposure to the drug[56]. To determine whether a high yeast diet altered rapid tolerance in flies, we fed flies 2Y or 30Y media, measured their ethanol-naive ST50 values, allowed them to recover for 4 h, and then measured their ST50 values during a second ethanol exposure. Males (Fig. 3.8A) and females (Fig. 3.8B) fed 2Y and 30Y media developed robust rapid tolerance, but the development of rapid tolerance to ethanol was not altered by diet in either sex (Figs. 3.8A and 3.8B). This suggests that high concentrations of dietary yeast influence initial ethanol sedation, but not the development of rapid tolerance.

Flies from different genetic backgrounds can vary substantially in their feeding[109, 165], alcohol[62, 166], and other behaviors[167, 168]. To determine whether the effect of dietary

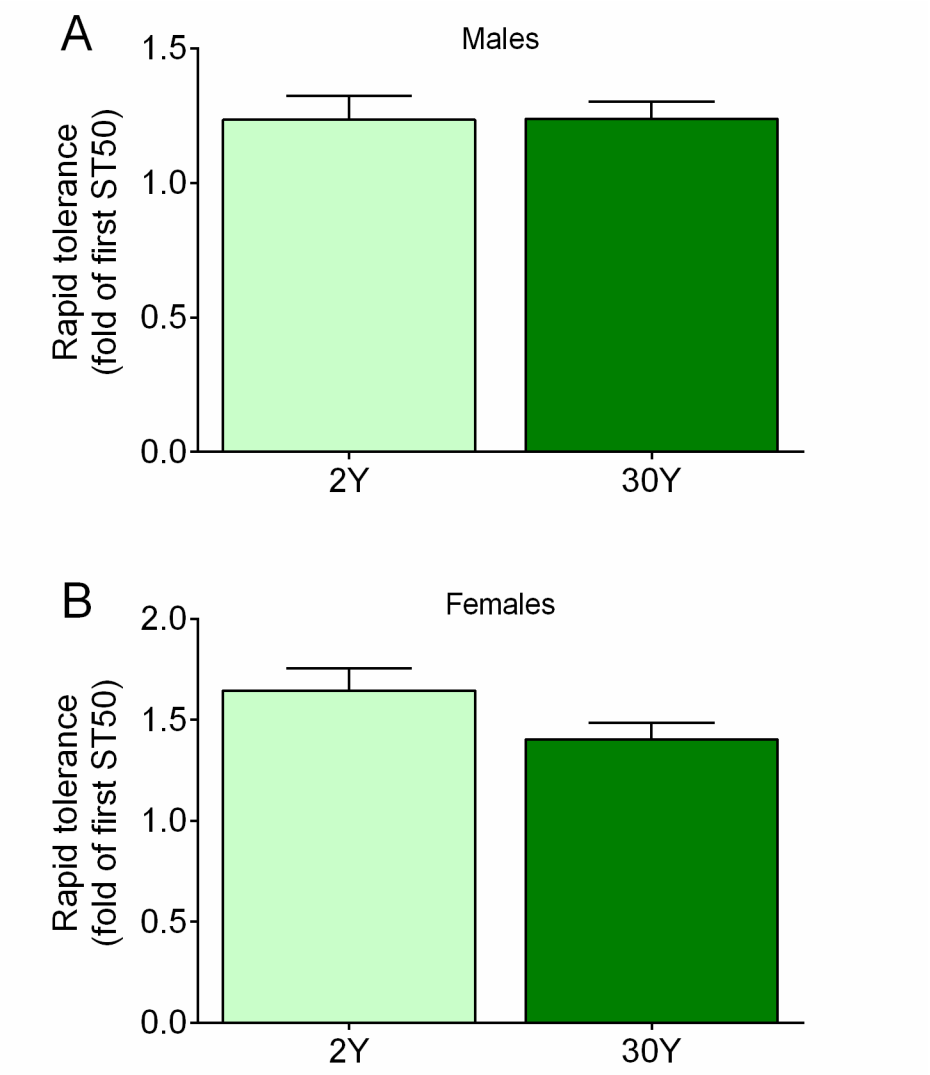


Figure 3.8. Yeast supplementation does not impact rapid tolerance to ethanol. Rapid tolerance was not significantly different in r[A] males (A) or females (B – performed by Monica Messick) fed 2Y or 30Y media for 2 d (individual two-tailed t tests; males, p=0.9773, n=8; females, p=0.0970; n=8). The ST50s during the second ethanol exposure were greater than during the first exposure (paired two-tailed t tests; 2Ymales, p=0.0218; 30Y males, p=0.0059; 2Y females, p<0.0001; 30Y females, p=0.0003).

yeast supplementation on resistance to ethanol sedation was a common property of flies, we measured ST50 values in four additional control strains (*w[A]*, Lausanne-S (LS), Oregon-R (OR) and Samarkand (SAM)) after feeding them 2Y or 30Y media for two days (Fig. 3.9) or one day (Fig. 3.10). Males and females fed 30Y medium had elevated ST50 values compared to flies fed 2Y medium in all cases. The magnitude ofthe supplemental yeast effect on ST50 values varied across the additional control strains tested (e.g. compare *w[A]* and SAM in Fig. 3.9A and 3.9D), consistent with widely appreciated genetic background effects on behavior. Although the effect of dietary yeast on ST50 values varied across the control strains tested, these data indicate that the increased resistance to ethanol sedation in response to supplemental dietary yeast is a common feature of flies. Additionally, these data confirm that providing flies with an elevated yeast diet for 1 or more days is sufficient to increase their resistance to ethanol sedation.

Altering the diet can lead to changes in the body mass of flies[169, 170]. To determine if yeast supplementation altered body mass in our experiments, we measured total, dry and water weight in several different control flies fed 2Y and 30Y media for 1 d. Compared to flies fed 2Y medium, flies on 30Y had increased total body mass in 9 of 10 cases, increased dry mass in 7 of 10 cases, and increased water weight in 8 of 10 cases (Table 3.1). To address if body mass might impact ethanol sedation, we explored whether total, dry, or water weight correlated with ST50 values in flies on 30Y vs 2Y media. Total, dry, and water weight did not correlate with ST50s in males or

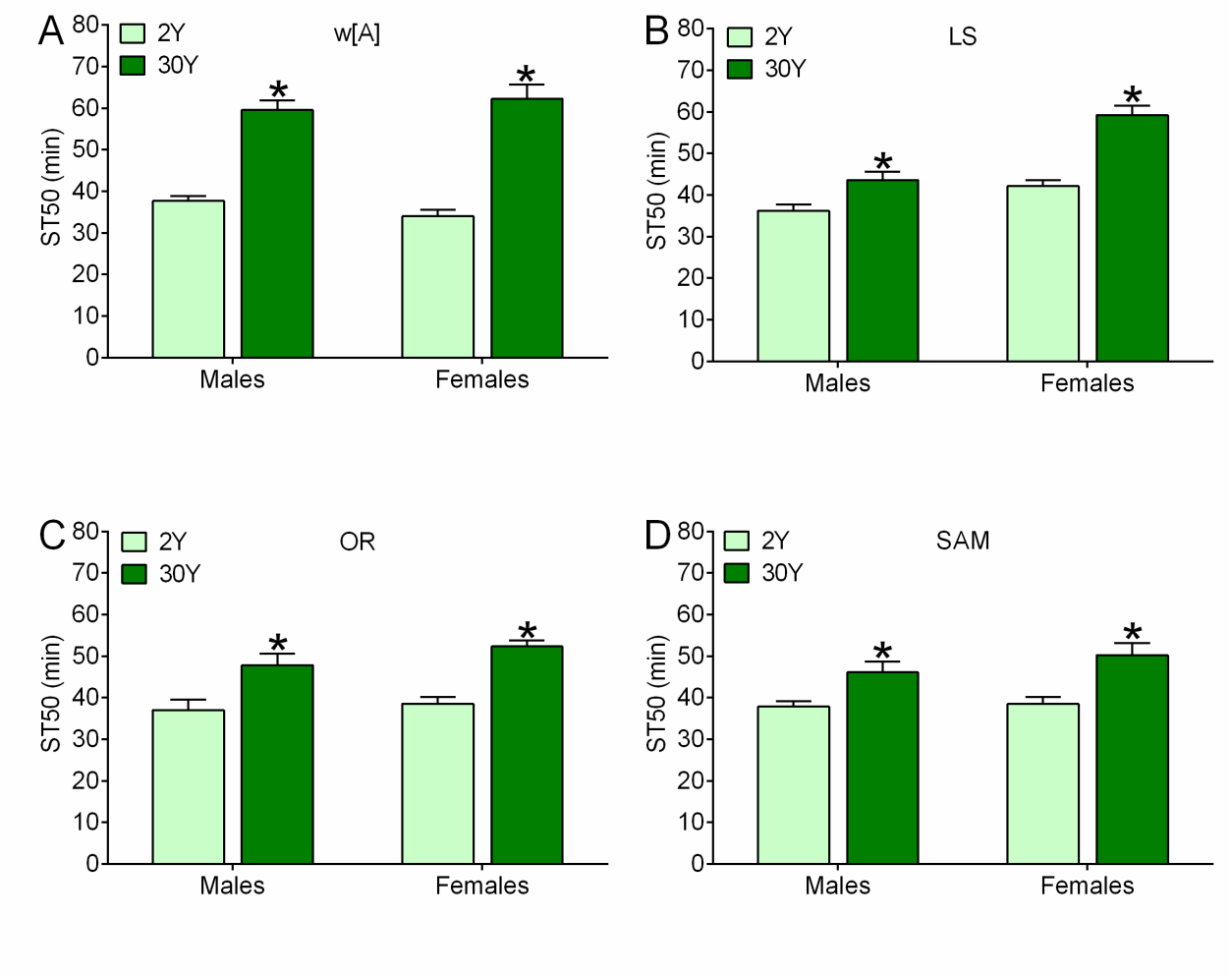


Figure 3.9. Dietary yeast impacts ethanol sedation in flies from several different genetic backgrounds after 2 day media exposure. Compared to flies fed 2Y medium, ST50s were increased in male and female *w[A]* (A), LS (B), OR (C) and Sam (D) after 2 d of feeding on 30Y medium (individual two-way ANOVAs; *w[A]*—yeast concentration, p<0.0001; sex, p=0.8266; interaction, p=0.1857; LS— yeast concentration, p<0.0001; sex, p<0.0001; interaction, p=0.0137; OR— yeast concentration, p<0.0001; sex, p=0.1756; interaction, p=0.4990; Sam— yeast concentration, p=0.0002; sex, p=0.2905; interaction, p=0.4390; *BMC versus 2Y, p=0.0299 to <0.0001; n=6 for all groups in all panels). (Performed by Monica Messick

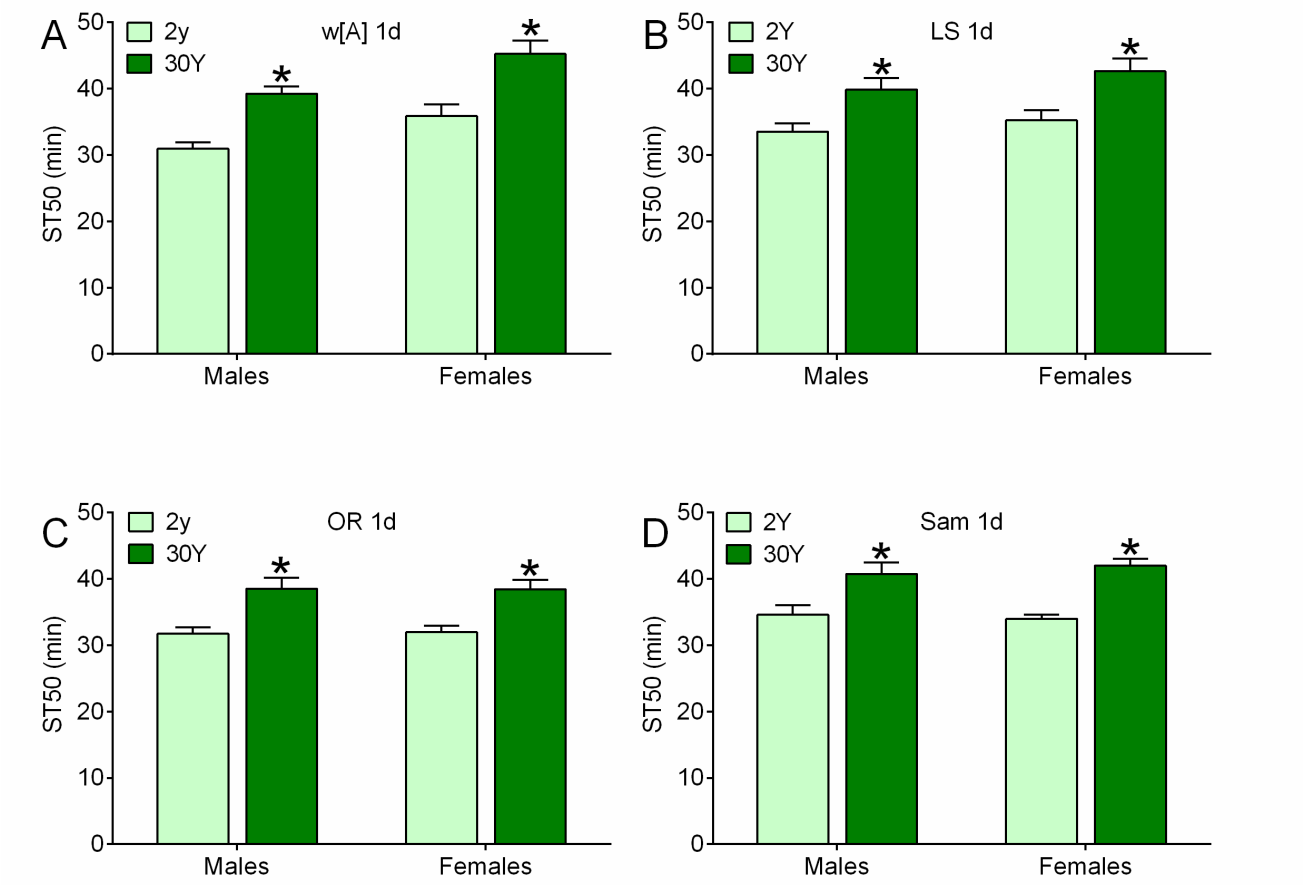


Figure 3.10. Dietary yeast impacts ethanol sedation in 24 hours of media exposure in flies from several different genetic backgrounds. Compared to flies fed 2Y medium, ST50s were increased in male and female *w[A]* (A), LS (B), OR (C) and Sam (D) after 1 d of feeding on 30Y medium (individual two-way ANOVAs; *w[A]*—yeast concentration, $p<0.0001$; sex, $p=0.0012$; interaction, $p=0.7528$; LS— yeast concentration, $p=0.0002$; sex, $p<0.1779$; interaction, $p=0.7468$; OR— yeast concentration, $p<0.0001$; sex, $p=0.9658$; interaction, $p=0.8976$; Sam— yeast concentration, $p<0.0001$; sex, $p=0.7948$; interaction, $p=0.4659$; *BMC versus 2Y, $p=0.0188$ to 0.0003; $n=8$ for all groups in all panels). (Performed by Monica Messick)

Table 3.1. Total, dry, and water weight (mg/fly) for multiple control strains and sex fed either 2Y or 30Y media.

			Total weight			Dry weight			Water weight		
Strain	**Sex**	**N**	**2Y**	**30Y**	**p**	**2Y**	**30Y**	**p**	**2Y**	**30Y**	**p**
r[A]	males	16	0.749 ± 0.018	0.814 ± 0.019	0.0166	0.151 ± 0.010	0.206 ± 0.010	0.0007	0.598 ± 0.009	0.608 ± 0.012	0.5214
	females	16	1.016 ± 0.026	1.373 ± 0.018	<0.0001	0.241 ± 0.013	0.355 ± 0.010	<0.0001	0.775 ± 0.022	1.018 ± 0.012	<0.0001
w[A]	males	8	0.752 ± 0.009	0.873 ± 0.017	<0.0001	0.188 ± 0.004	0.221 ± 0.013	0.0300	0.577 ± 0.015	0.652 ± 0.010	0.0004
	females	8	0.990 ± 0.014	1.283 ± 0.017	<0.0001	0.243 ± 0.010	0.344 ± 0.011	<0.0001	0.747 ± 0.011	0.939 ± 0.018	<0.0001
LS	males	8	0.681 ± 0.010	0.703 ± 0.022	0.3829	0.199 ± 0.003	0.179 ± 0.013	0.1448	0.482 ± 0.009	0.522 ± 0.016	0.0381
	females	8	0.991 ± 0.028	1.296 ± 0.015	<0.0001	0.279 ± 0.011	0.373 ± 0.009	<0.0001	0.712 ± 0.020	0.923 ± 0.013	<0.0001
OR	males	8	0.841 ± 0.0183	0.893 ± 0.008	0.0162	0.179 ± 0.020	0.216 ± 0.012	0.1287	0.662 ± 0.007	0.677 ± 0.008	0.1805
	females	8	1.148 ± 0.034	1.608 ± 0.035	<0.0001	0.300 ± 0.024	0.433 ± 0.031	0.0040	0.848 ± 0.023	1.176 ± 0.017	<0.0001
Sam	males	8	0.768 ± 0.018	0.851 ± 0.018	0.0036	0.200 ± 0.010	0.222 ± 0.011	0.1402	0.568 ± 0.013	0.630 ± 0.010	0.0013
	females	8	1.100 ± 0.011	1.432 ± 0.017	<0.0001	0.301 ± 0.011	0.416 ± 0.010	<0.0001	0.798 ± 0.007	1.016 ± 0.013	<0.0001

females (Table 3.2). Additionally, feeding 30Y medium for 1 d increased ST50 values in males and females of all genotypes tested (Figs. 3.1 and 3.10), even though some groups of animals did not have changes in total, dry, or water weight (Table 3.1). Thus, flies fed 30Y medium had increased total, dry, and water weight in most cases, but these changes were not required for altered ethanol sedation and body mass did not predict ST50 values.

To determine whether the effect of a high yeast diet on resistance to ethanol sedation was reversible, we fed flies 30Y medium for two days, switched them to 2Y for two days, then assessed their ST50 values. Flies fed 30Y food for two days were resistant to ethanol sedation compared to flies fed 2Y for two days as expected (Fig. 3.11A, males; Fig. 3.11B, females). In contrast, flies fed 30Y medium for two days and then switched to 2Y food for two days had ST50 values that were indistinguishable from flies fed 2Y medium only (Fig. 3.11A, males; Fig. 3.11B, females). The resistance to ethanol sedation driven by supplemental dietary yeast is therefore readily reversible in both males and females.

Flies are well known to adjust the volume of media they consume in response to changes in nutrient concentration in their diet[109, 165, 171]. This compensatory feeding is thought to help maintain steady total nutrient intake[165, 171], although this phenomenon does not always occur[109]. To address whether flies provided with 30Y medium consumed more nutrients than flies fed 2Y medium, we performed consumption-excretion (Con-Ex) studies using FD&C Blue 1 as a food tracer[109]. Males and females both consumed decreased volumes of 30Y versus 2Y media as anticipated (Fig. 3.12A). Given that 30Y medium has 15-fold the yeast concentration of 2Y medium, the level of

Table 3.2. ST50 values and weights do not correlate across multiple control strains and sex.

	ST50	
	Male	Female
Total Weight	0.1318	0.4580
Dry Weight	0.0734	0.3537
Water Weight	0.1054	0.4921

Pearson Correlation, p=0.1485-0.8031; n=10-14.

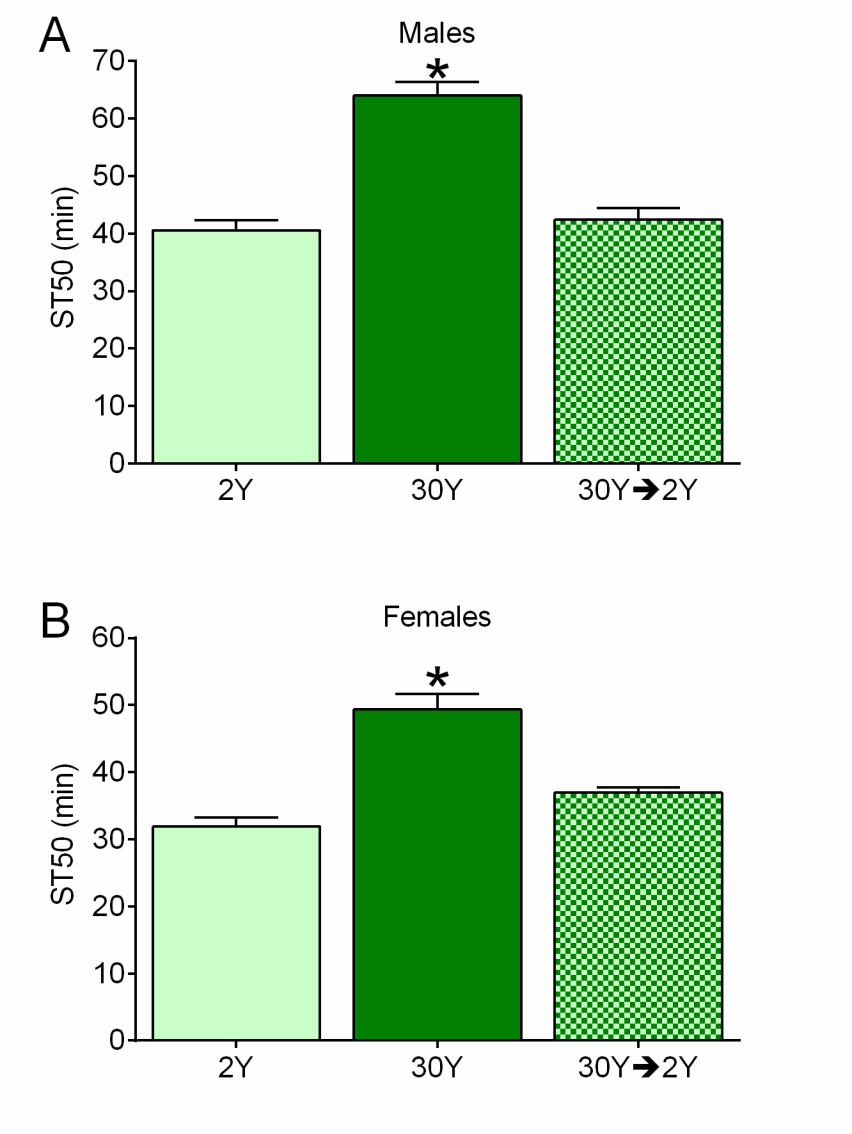


Figure 3.11. Reversible effects of dietary yeast on ethanol sedation. Dietary regimen impacted ST50 values in males (A) and females (B – Performed by Ellyn Dunbar) (individual one-way ANOVAs for effect of diet; males, $p<0.0001$; females, $p<0.0001$; n=8). Compared to flies fed only 2Y medium, ST50 values were increased in males and females fed 30Y medium for 2 d (*BMC, $p<0.0001$; n=8), but not in flies fed 30Y for 2d and then switched to 2Y for an additional 2d (BMC; males, $p>0.9999$; females, $p=0.1097$).

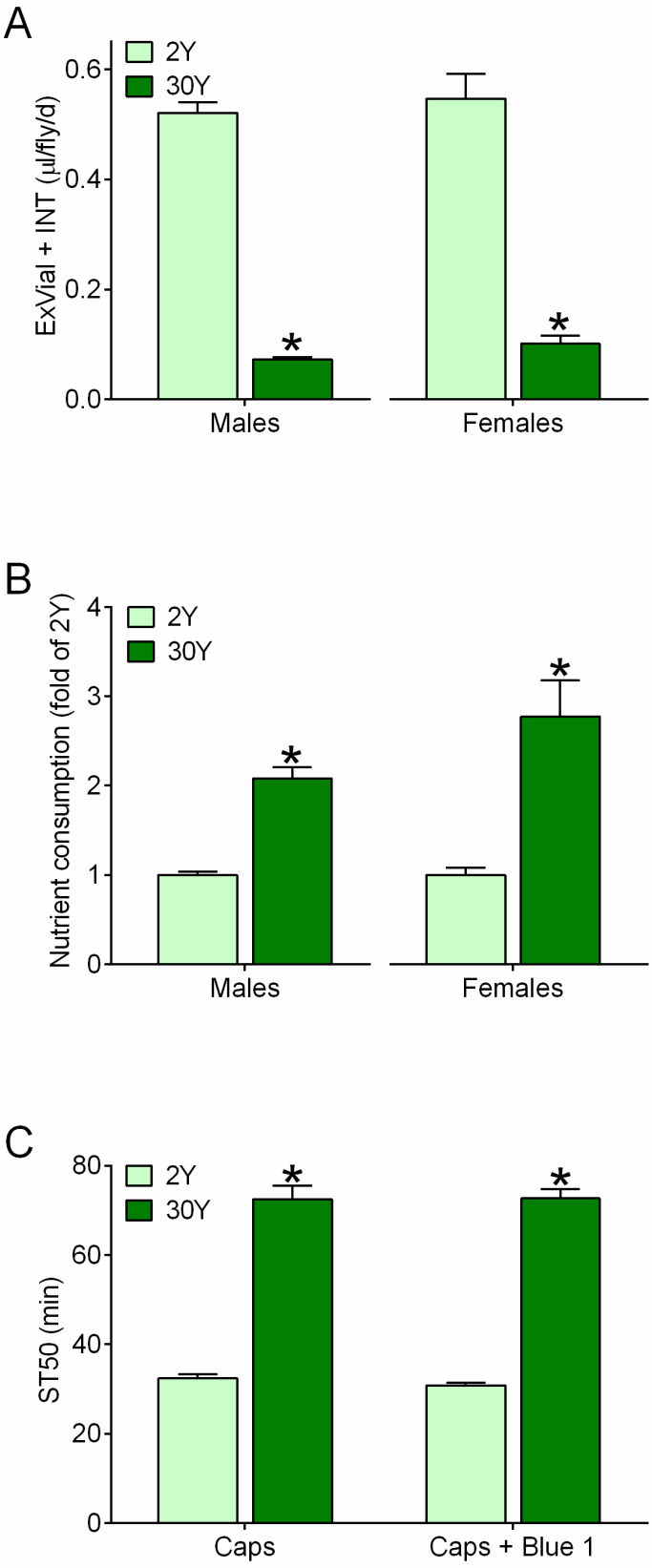

A
2Y
30Y
ExVial + INT (µl/fly/d)
0.6
0.4
0.2
0.0
*
*
Males
Females
B
2Y
30Y
Nutrient consumption (fold of 2Y)
4
3
2
1
0
*
*
Males
Females
C
2Y
30Y
ST50 (min)
80
60
40
20
0
*
*
Caps
Caps + Blue 1

Figure 3.12. Control r[A] flies consume more nutrients from 30Y versus 2Y media. (A) Flies consumed-excreted lower volumes (ExVial+INT) of 30Y medium compared to 2Y medium during 24 h Con-Ex studies (*two-tailed t tests, $p<0.0001$; males, n=8; females, n=6) (Performed by Brandon Shell). (B) Flies consumed more nutrients (relative to 2Y, calculated from panel A) from 30Y medium compared to 2Y (*two-tailed t tests; males, $p<0.0001$, n=8; females, $p=0.0016$; n=6). (C) Flies fed 30Y medium had increased ST50 values compared to flies fed 2Y medium when all media were provided in feeder caps (Caps) for 2 d. Including Blue 1 in the media had no effect on ST50 values (two-way ANOVA; yeast concentration, $p<0.0001$; Blue 1, $p=0.7200$; interaction, $p=0.6652$; *BMCs versus 2Y, $p<0.0001$; n=12).

consumption represented in Fig. 3.12A results in 30Y-fed flies ingesting at least 2-fold the total nutrients as flies fed 2Y (Fig. 3.12B; males, left; females; right). Importantly, consumption of media from the feeder caps in Con-Ex experiments and the presence of FD&C Blue 1 in the media did not have discernable effects on yeast-induced resistance to ethanol sedation (Fig. 3.12C). These data show that increased yeast nutrient consumption accompanies the increase in resistance to ethanol sedation, suggesting that they are causally linked.

Olfactory cues from yeast influence life span in flies[172]. To determine if olfactory cues from supplemental yeast are sufficient to elicit resistance to ethanol sedation, we assessed whether mesh barriers that prevented flies from directly contacting the yeast paste blocked the change in ST50 values. We used barriers with two different mesh sizes to test this possibility because (i) we reasoned that barriers of both sizes would eliminate the ability of flies to contact the food surface and (ii) the lager mesh size would be more porous to olfactory cues from the yeast paste. Compared to flies fed standard medium, flies that physically contacted supplemental yeast paste were resistant to ethanol sedation (Fig. 3.13A and 3.13B) as expected (Fig. 3.1). In contrast, ST50 values in flies that could not contact the supplemental yeast due to mesh barriers were indistinguishable from flies fed a standard diet only (Fig. 3.13A and 3.13B). The yeast-induced resistance to ethanol sedation therefore requires physical contact with, and presumably consumption of, the supplemental yeast to produce resistance to ethanol sedation.

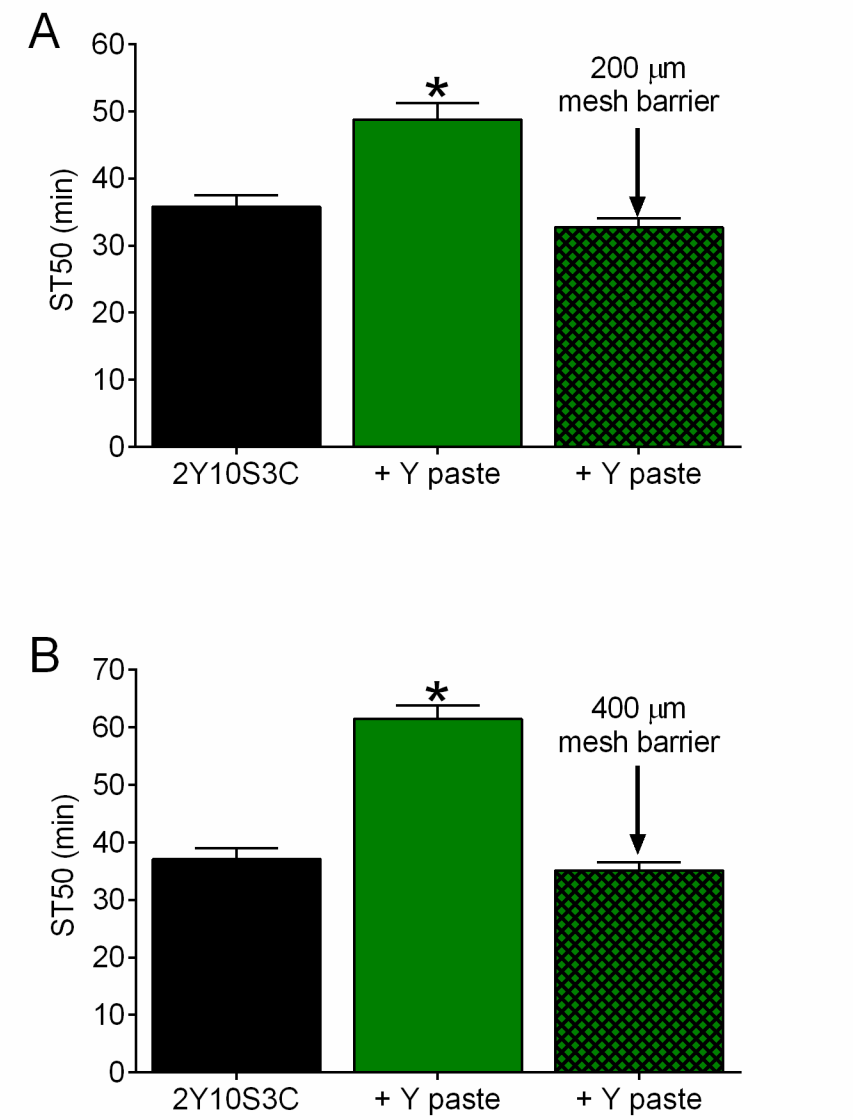


Figure 3.13. Effect of dietary yeast paste on ST50 values requires physical contact. Flies had access to the indicated media for 2 d. (A, B) Compared to flies fed 2Y10S3C medium, ST50 values were increased in flies that had access to yeast paste (green bars), but not in flies that were prevented from physically contacting the yeast paste by a mesh barrier (green hatched bars). There was an overall effect of treatment group in *w[A]* (panel A) and r[A] (panel B) females (one-way ANOVAs, $p<0.0001$, $n=8$ in A and B). ST50s were greater in flies with access to yeast paste compared to the other groups (*BMC, $p=0.0003$ to <0.0001). ST50s were indistinguishable in flies fed 2Y10S3C and in flies prevented from physically contacting the yeast paste (BMC; panel A, $p=0.8415$; panel B, $p>0.9999$).

The mechanism by which dietary yeast influences ethanol sedation in flies is of obvious interest. Intriguingly, a high yeast diet increases brain 5-HT levels in flies [[147], confirmed here (30Y: 439.8 ± 89.0 fmol/brain, n=11; 2Y: 231.7 ± 36.9 fmol/brain, n=14; t test, p=0.0282)]. Additionally, serotonergic neuron function is important for regulating food consumption in larval and adult flies[148-150], the 5-HT2A receptor plays a role in preference for dietary protein consumption in flies[147], and 5-HT has been implicated in fly ethanol sedation[74]. Furthermore, there is a large literature linking 5-HT to alcohol problems in humans (e.g. [151-155]). These findings collectively suggested that there could be mechanistic connections between serotonergic neurons and the effect of dietary yeast on ethanol sedation. To address this possibility, we determined if suppression of serotonergic neurons influenced the effect of dietary yeast on ST50 or the consumption of high yeast medium.

Compared to 2Y medium, ST50 values were increased by 30Y diet in control flies with the *Trh*-Gal4.long3 or the *Trh*-Gal4.long2 driver alone, a UAS-Kir2.1 transgene alone, or a UAS-TeTxLC(E2) transgene alone (first four bars, Figs. 3.14A, 3.15A, 3.16A, 3.17A). These control flies also consumed more nutrients when fed 30Y medium (first four bars, Figs. 3.14B, 3.15B, 3.16B, 3.17B). Inhibition of serotonergic neurons by expression of UAS-Kir2.1 (which hyperpolarizes neurons[173]) via *Trh*-Gal4.long3 (Fig. 3.14A, hatched bars) or via *Trh*-Gal4.long2 (Fig. 3.15A, hatched bars) blocked the effect of 30Y medium on ST50 values. Similarly, *Trh*-Gal4-driven expression of tetanus toxin light chain (UAS-TeTxLC(E2)), which inhibits vesicle release[174]) in serotonergic neurons blocked the effect of yeast supplementation on ST50 values (Figs. 3.16A, 3.17A,

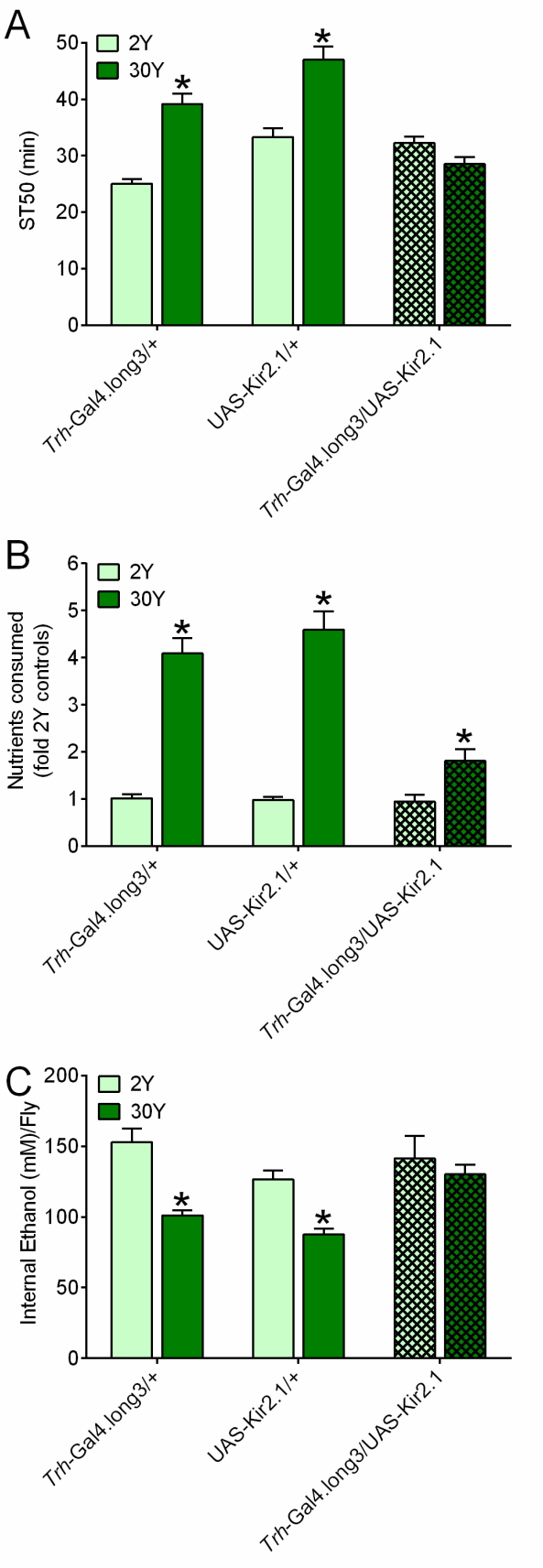

A
2Y
30Y
ST50 (min)
50
40
30
20
10
0
*
*
Trh-Gal4.long3/+
UAS-Kir2.1/+
Trh-Gal4.long3/UAS-Kir2.1
B
2Y
30Y
Nutrients consumed
(fold 2Y controls)
6
5
4
3
2
1
0
*
*
*
Trh-Gal4.long3/+
UAS-Kir2.1/+
Trh-Gal4.long3/UAS-Kir2.1
C
2Y
30Y
Internal Ethanol (mM)/Fly
200
150
100
50
0
*
*
Trh-Gal4.long3/+
UAS-Kir2.1/+
Trh-Gal4.long3/UAS-Kir2.1

Figure 3.14. Inhibition of serotonergic neurons with Kir2.1 blunts the effect of a high yeast diet on ethanol sedation, nutrient consumption and internal ethanol levels. Male flies of the indicated genotypes consumed 2Y or 30Y media for 1 d prior to determination of ST50s, nutrient consumption, and internal ethanol. (A) There were overall effects of yeast concentration and genotype on ST50s, and an interaction between the two factors (two-way ANOVA; yeast, $p<0.0001$; genotype, $p<0.0001$; interaction, $p<0.0001$; n=8). Compared to flies fed 2Y medium, ST50s were greater in control flies (*Trh*-Gal4.long3/+ and UAS-Kir2.1/+) on 30Y (*BMC, $p<0.0001$), but not in flies with inhibition of serotonergic neurons (*Trh*-Gal4.long3/+; UAS-Kir2.1/+; hatched bars; BMC, $p=0.3174$). (B) Overall, yeast concentration and genotype influenced nutrient consumption and there was an interaction between yeast and genotype (two-way ANOVA; yeast, $p<0.0001$; genotype, $p<0.0001$; interaction, $p<0.0001$; n=8). All genotypes consumed more nutrients from 30Y than 2Y (*BMC, $p\leq<0.001$). (C) Overall, the concentration of dietary yeast and genotype influenced internal ethanol levels after exposure to vapor from 85% ethanol for 36 minutes (two-way ANOVA; yeast, $p<0.0001$; genotype, $p=0.0072$; interaction, $p=0.0733$; n=8). Internal ethanol was decreased in control flies (*Trh*-Gal4.long3/+ and UAS-Kir2.1/+) fed 30Y versus 2Y media (*BMC, $p\leq0.0094$), but yeast concentration had no effect on internal ethanol in *Trh*-Gal4.long3/+; UAS-Kir2.1/+ flies (hatched bars; BMC, $p>0.9999$).

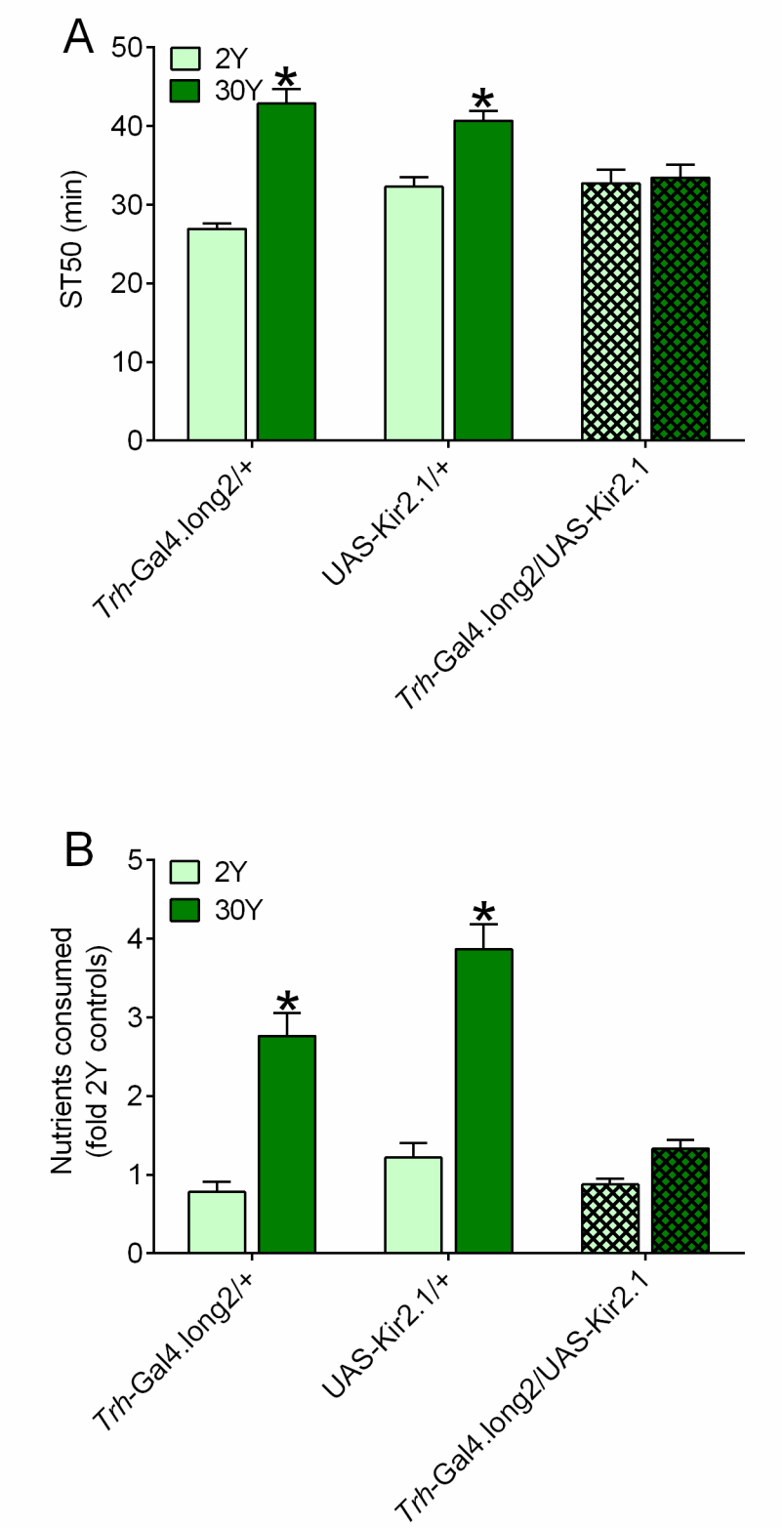

A
2Y
30Y
ST50 (min)
0
10
20
30
40
50
*
*
Trh-Gal4.long2/+
UAS-Kir2.1/+
Trh-Gal4.long2/UAS-Kir2.1
B
2Y
30Y
Nutrients consumed
(fold 2Y controls)
0
1
2
3
4
5
*
*
Trh-Gal4.long2/+
UAS-Kir2.1/+
Trh-Gal4.long2/UAS-Kir2.1

Figure 3.15 Inhibition of serotonergic neurons with Kir2.1 blunts the effect of a high yeast diet on ethanol sedation and nutrient consumption: replication with a second *Trh*-Gal4 driver. Male flies of the indicated genotypes consumed 2Y or 30Y media for 1 d prior to determination of ST50s and nutrient consumption. (A) Overall, yeast concentration, but not genotype, impacted ST50s and there was an interaction between the two factors (two-way ANOVA; yeast, $p<0.0001$; genotype, $p=0.0724$; interaction, $p<0.0001$; $n=8$). ST50s were greater in control flies (*Trh*-Gal4.long2/+ and UAS-Kir2.1/+) on 30Y versus 2Y media (*BMC, $p\leq0.0005$), but yeast concentration did not alter ST50s in flies with inhibition of serotonergic neuron function (*Trh*-Gal4. long2/+; UAS-Kir2.1/+; hatched bars; BMC, $p>0.9999$). (B) Overall, yeast concentration and genotype influenced nutrient consumption and there was an interaction between yeast and genotype (two-way ANOVA; yeast, $p<0.0001$; genotype, $p<0.0001$; interaction, $p<0.0001$; $n=8$). Control (*Trh*-Gal4. long2/+ and UAS-Kir2.1/+) flies consumed more nutrients from 30Y than 2Y (*BMC $p<0.0001$), but nutrient consumption from 2Y and 30Y was indistinguishable in *Trh*-Gal4. long2/+; UAS-Kir2.1/+ flies (hatched bars; BMC, $p=0.3767$).

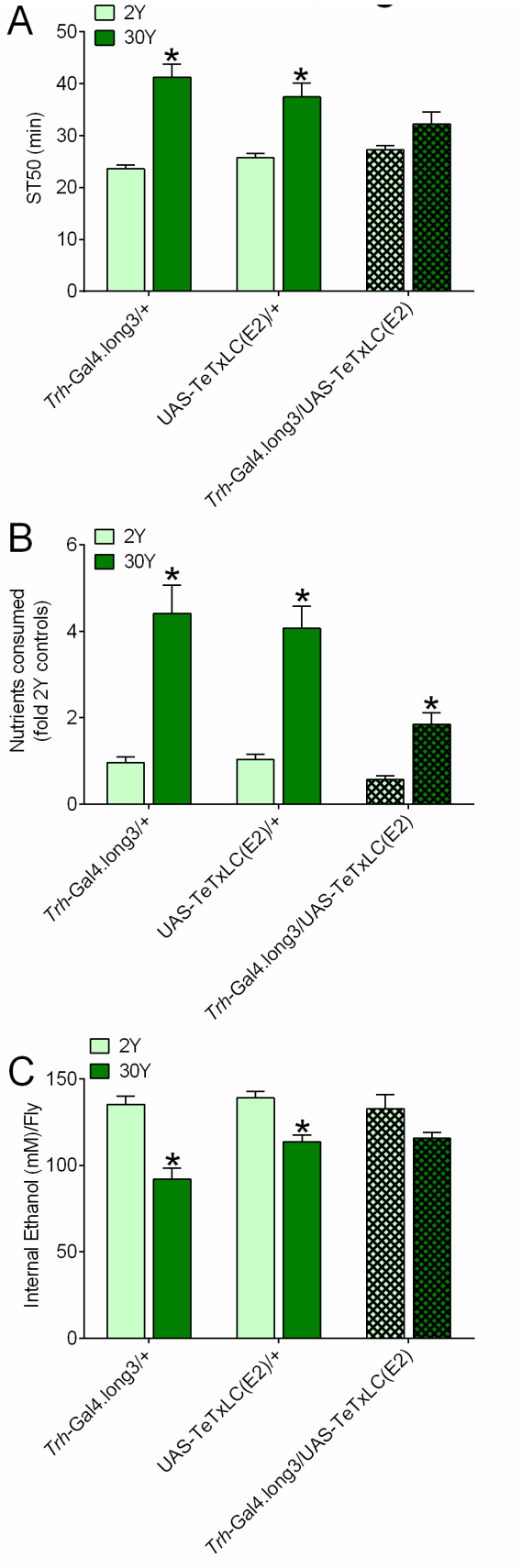

A
2Y
30Y
ST50 (min)
50
40
30
20
10
0
*
*
Trh-Gal4.long3/+
UAS-TeTxLC(E2)/+
Trh-Gal4.long3/UAS-TeTxLC(E2)
B
2Y
30Y
Nutrients consumed
(fold 2Y controls)
6
4
2
0
*
*
*
Trh-Gal4.long3/+
UAS-TeTxLC(E2)/+
Trh-Gal4.long3/UAS-TeTxLC(E2)
C
2Y
30Y
Internal Ethanol (mM)/Fly
150
100
50
0
*
*
Trh-Gal4.long3/+
UAS-TeTxLC(E2)/+
Trh-Gal4.long3/UAS-TeTxLC(E2)

Figure 3.16. Expression of tetanus toxin in serotonergic neurons dampens the effect of dietary yeast on ethanol sedation, nutrient intake and internal ethanol levels. Male flies were fed 2Y or 30Y media for 1 d. (A) Overall, yeast concentration, but not genotype, influenced ST50s, and there was an interaction between yeast and genotype (two-way ANOVA; yeast, $p<0.0001$; genotype, $p=0.3451$; interaction, $p=0.0058$; n=8). Compared to flies fed 2Y medium, control *Trh*-Gal4.long3/+ and UAS-TeTxLC(E2)/+ flies fed 30Y had greater ST50s (*BMCs, $p\leq0.0002$), but dietary yeast had no discernable effect on ST50s in flies expressing Tetanus Toxin Light Chain in serotonergic neurons (*Trh*-Gal4.long3/+; UAS-TeTxLC(E2)/+; hatched bars; BMC, $p=0.1996$). (B) Yeast and genotype had significant overall effects on nutrient consumption and there was an interaction between the factors (two-way ANOVA; yeast, $p<0.0001$; genotype, $p<0.0001$; interaction, $p=0.0053$; n=6-8). All genotypes consumed more nutrients on 30Y versus 2Y (*BMCs, $p\leq0.0257$). (C) Overall, internal ethanol was affected by yeast concentration and genotype, but there was no interaction between the factors (two-way ANOVA; yeast, $p<0.0001$; genotype, $p=0.0472$; interaction, $p=0.0524$; n=8). Compared to flies fed 2Y, internal ethanol was decreased in control *Trh*-Gal4.long3/+ and UAS-TeTxLC(E2)/+ flies fed 30Y (*BMCs, $p\leq0.0045$), but not in *Trh*-Gal4.long3/+; UAS-TeTxLC(E2)/+ flies (BMC, $p=0.0807$).

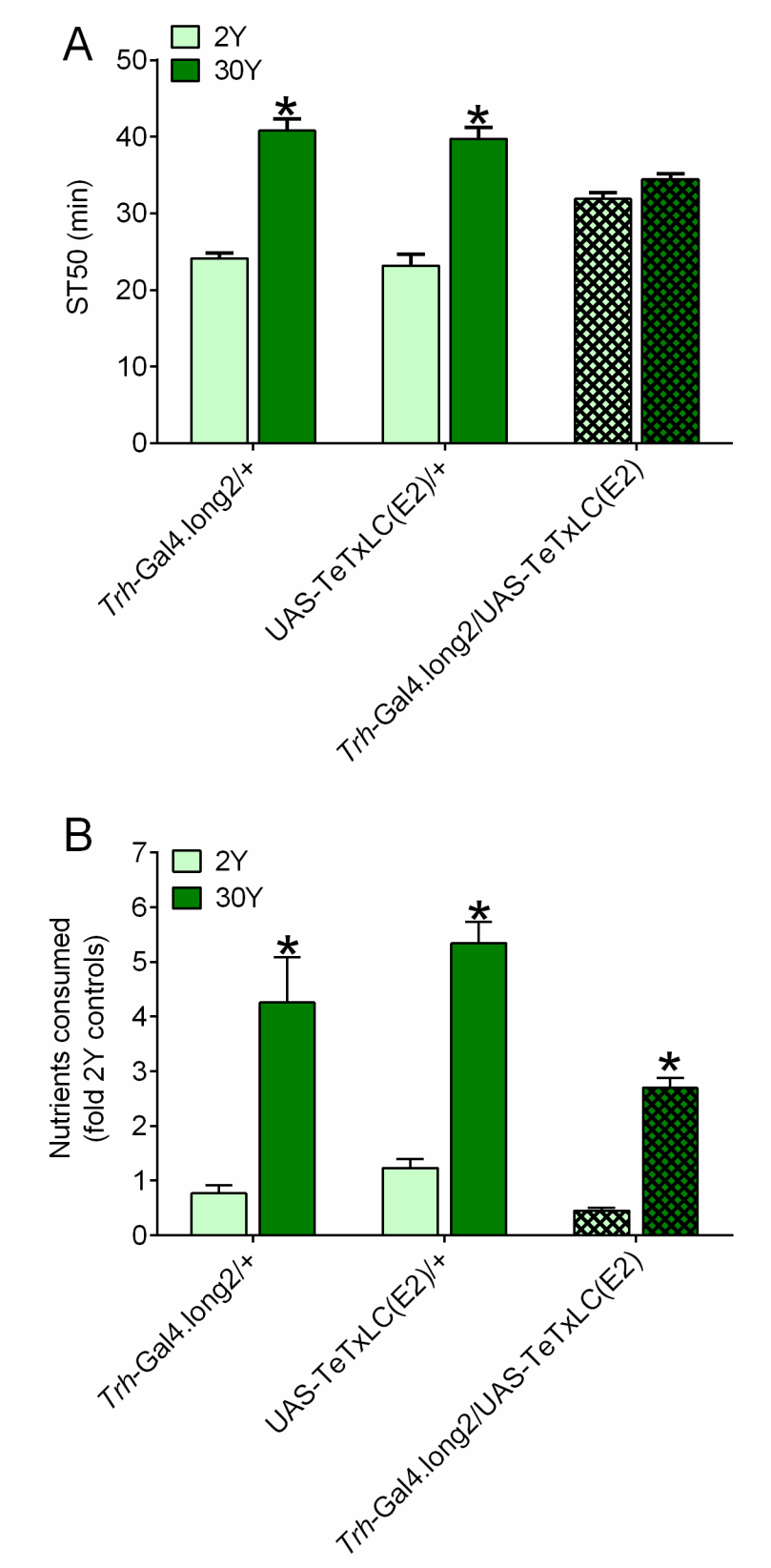

A
2Y
30Y
ST50 (min)
50
40
30
20
10
0
*
*
Trh-Gal4.long2/+
UAS-TeTxLC(E2)/+
Trh-Gal4.long2/UAS-TeTxLC(E2)
B
2Y
30Y
Nutrients consumed
(fold 2Y controls)
7
6
5
4
3
2
1
0
*
*
*
Trh-Gal4.long2/+
UAS-TeTxLC(E2)/+
Trh-Gal4.long2/UAS-TeTxLC(E2)

Figure 3.17. Expression of tetanus toxin in serotonergic neurons dampens the effect of dietary yeast on ethanol sedation and nutrient intake: replication with a second *Trh*-Gal4 driver. Male flies of the indicated genotypes consumed 2Y or 30Y media for 1 d prior to determination of ST50s and nutrient consumption. (A) Overall, yeast concentration, but not genotype, impacted ST50s and there was an interaction between the two factors (two-way ANOVA; diet, $p<0.0001$; genotype, $p=0.3555$; interaction, $p<0.0001$; $n=8$). ST50s were greater in control flies (*Trh*-Gal4.long2/+ and UAS-TeTxLC(E2)/+) on 30Y versus 2Y media (*BMC, $p<0.0001$), but yeast concentration did not alter ST50s in flies with inhibition of serotonergic neuron function (*Trh*-Gal4. long2/+; UAS-TeTxLC(E2)/+; hatched bars; BMC, $p=0.3990$). (B) There were main effects of yeast concentration and genotype on nutrient consumption, but there was not an interaction between yeast and genotype (two-way ANOVA; yeast, $p<0.0001$; genotype, $p=0.0004$; interaction, $p=0.0621$; $n=8$). All genotypes consumed more nutrients on 30Y versus 2Y media (*BMC; $p\leq0.0006$).

hatched bars). The effect of a high yeast diet on ethanol sedation therefore requires functional serotonergic neurons.

Regarding media consumption, flies expressing Kir2.1 via *Trh*-Gal4.long3 had greater intake of nutrients when fed 30Y vs 2Y media (Fig. 3.14B), but not when Kir2.1 was expressed by *Trh*-Gal4.long2 (Fig. 3.15B). Flies expressing tetanus toxin via both *Trh*-Gal4 drivers consumed significantly more nutrients from 30Y versus 2Y media (Figs. 3.16B, 3.17B). Thus, inhibition of serotonergic neurons did not consistently blockthe increase in nutrient intake on 30Y medium, but these flies appeared to consume fewer nutrients than control genotypes when on 30Y.

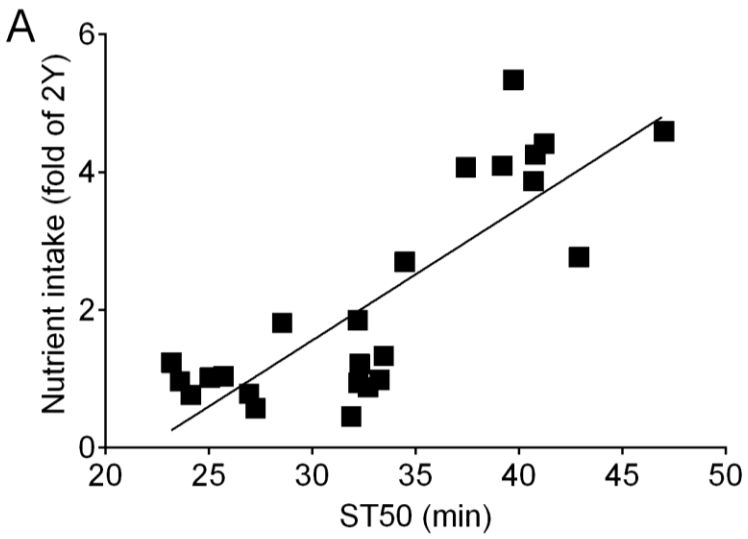
We postulated that a high yeast diet might impact net uptake/elimination of ethanol and, if true, that suppression of serotonergic neurons might influence internal ethanol levels in flies on a high yeast diet. We therefore measured internal ethanol in control flies and in flies expressing either UAS-Kir2.1 or UAS-TeTxLC(E2) in serotonergic neurons when fed 2Y or 30Y media. Internal ethanol concentrations during sedation from exogenous ethanol were decreased in control flies on 30Y vs 2Y media (Figs. 3.14C, 3.16C, first four bars), indicating that a high yeast diet influences ethanol uptake and/or elimination. Interestingly, the effect of 30Y diet on internal ethanol levels was blocked by inhibition of serotonergic neurons via expression of UAS-Kir2.1 (Fig. 3.14C, hatched bars) or UAS-TeTxLC(E2) (Fig. 3.16C, hatched bars).

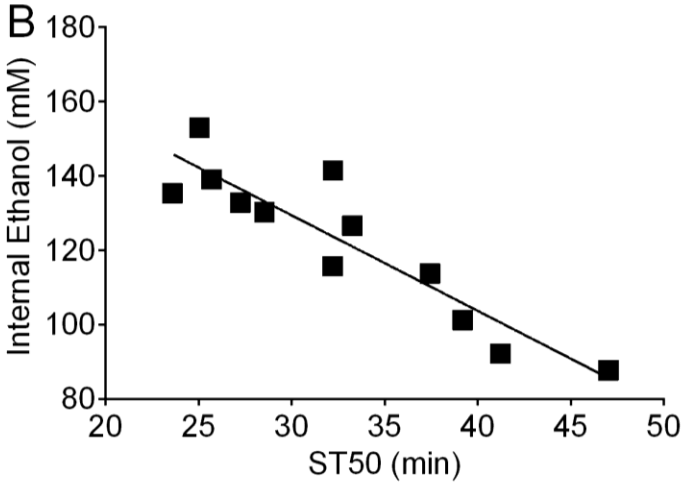
The data in Figs. 3.14-3.17 raised the possibility that serotonergic neurons drive yeast consumption which in turn drives internal ethanol levels and ethanol sedation. To further explore this possibility, we determined whether there were correlations between nutrient consumption, internal ethanol levels, and ST50 values using data from Figs.

3.14, 3.15, 3.16, and 3.17. We found strong, significant correlations between all pairs of measures (Fig. 3.18). ST50 and nutrient intake exhibited a positive correlation (Fig. 3.18A), while ST50 and internal ethanol (Fig. 3.18B) as well as nutrient intake and internal ethanol (Fig. 3.18C) exhibited negative correlations. These results support a model in which a high yeast diet leads to serotonergic neuron-dependent increases in nutrient intake, and that increased nutrient intake subsequently alters the uptake or elimination of ethanol resulting in lower internal ethanol levels, ultimately leading to increased ST50 values (Fig. 3.19A). Alternatively, when serotonergic neurons are inhibited, flies consume a similar amount of nutrients on 30Y as 2Y, there no changes in internal ethanol levels, and subsequently, no changes in ST50s (Fig. 3.19B).

C. Discussion

Fruit flies are an important genetic model organism for investigating the molecular basis of a plethora of physiological outputs including alcohol-related behaviors[56, 58-60, 62, 63, 66, 67, 71, 77, 130-138], food consumption[109, 165], and responses to diet[157, 169, 175-183]. To the best of our knowledge, our studies are the first to integrate these three areas of biology in the fly. We find that increasing the concentration of yeast in the diet, but not increasing other dietary components or decreasing all components of our standard medium, makes flies resistant to ethanol sedation. The resistance to ethanol sedation requires physical access to dietary yeast, is a common property of yeast, is seen in both males and females of multiple control strains, is reversible, appears to be caused by a mechanism independent of rapid tolerance, and is associated with increased yeast nutrient consumption as well as decreased internal ethanol levels. Importantly, the effect of a


A
Nutrient intake (fold of 2Y)
0
2
4
6
20
25
30
35
40
45
50
ST50 (min)


B
Internal Ethanol (mM)
80
100
120
140
160
180
20
25
30
35
40
45
50
ST50 (min)

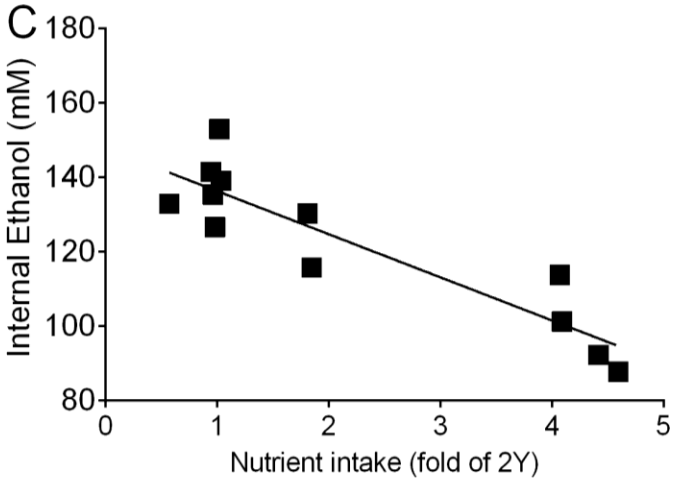
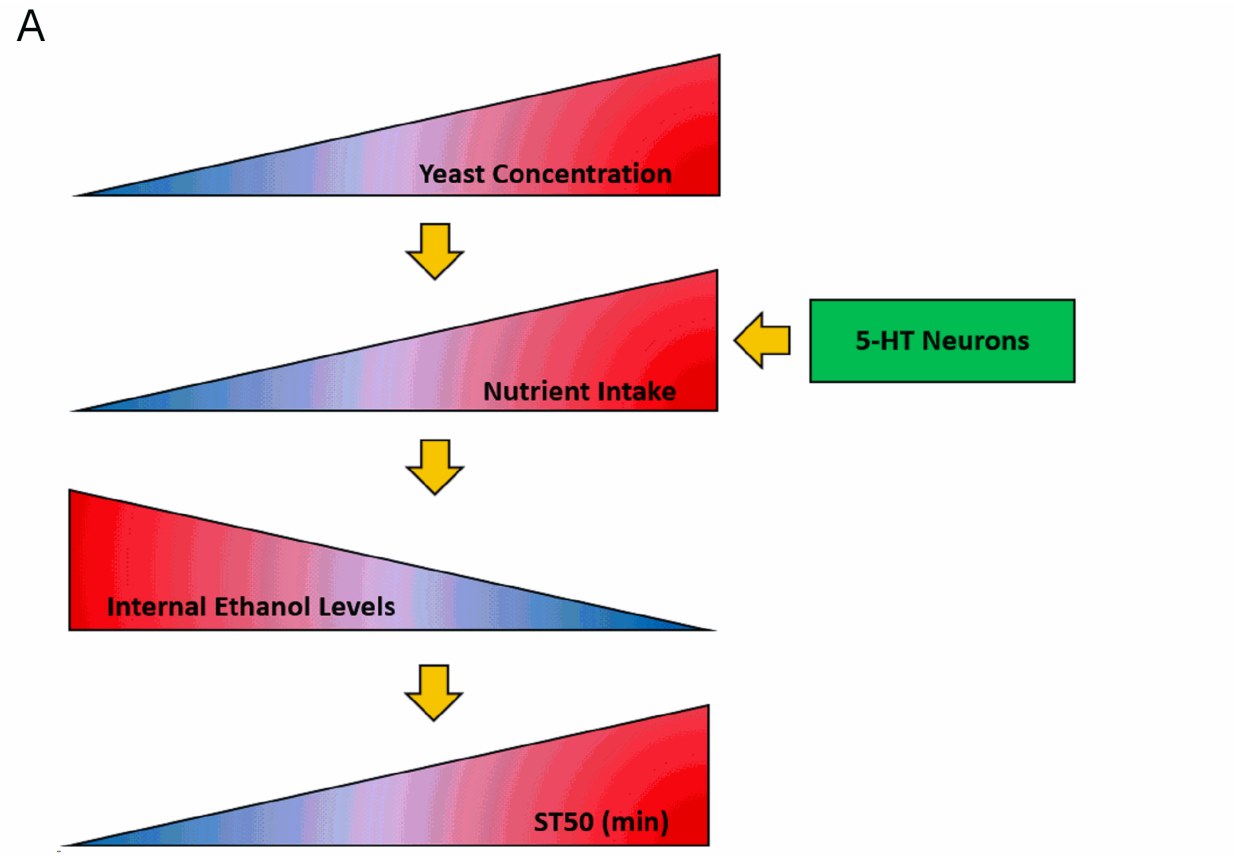

C
Internal Ethanol (mM)
80
100
120
140
160
180
0
1
2
3
4
5
Nutrient intake (fold of 2Y)

Figure 3.18. Correlations between ST50, nutrient intake, and internal ethanol levels. Data from figures 8, 9, S7, and S8 were combined to assess correlations between ST50, nutrient intake, and internal ethanol levels. (A) There was a positive correlation between ST50 and nutrient intake (Pearson $r=0.827$, $p<0.0001$, $n=24$). (B) ST50 values inversely correlated with internal ethanol levels (Pearson $r=-0.913$, $p<0.0001$, $n=12$). (C) Nutrient intake negatively correlated with internal ethanol levels (Pearson $r=-0.903$, $p<0.0001$, $n=12$). Lines are best fit linear regressions.


A
Yeast Concentration
Nutrient Intake
5-HT Neurons
Internal Ethanol Levels
ST50 (min)

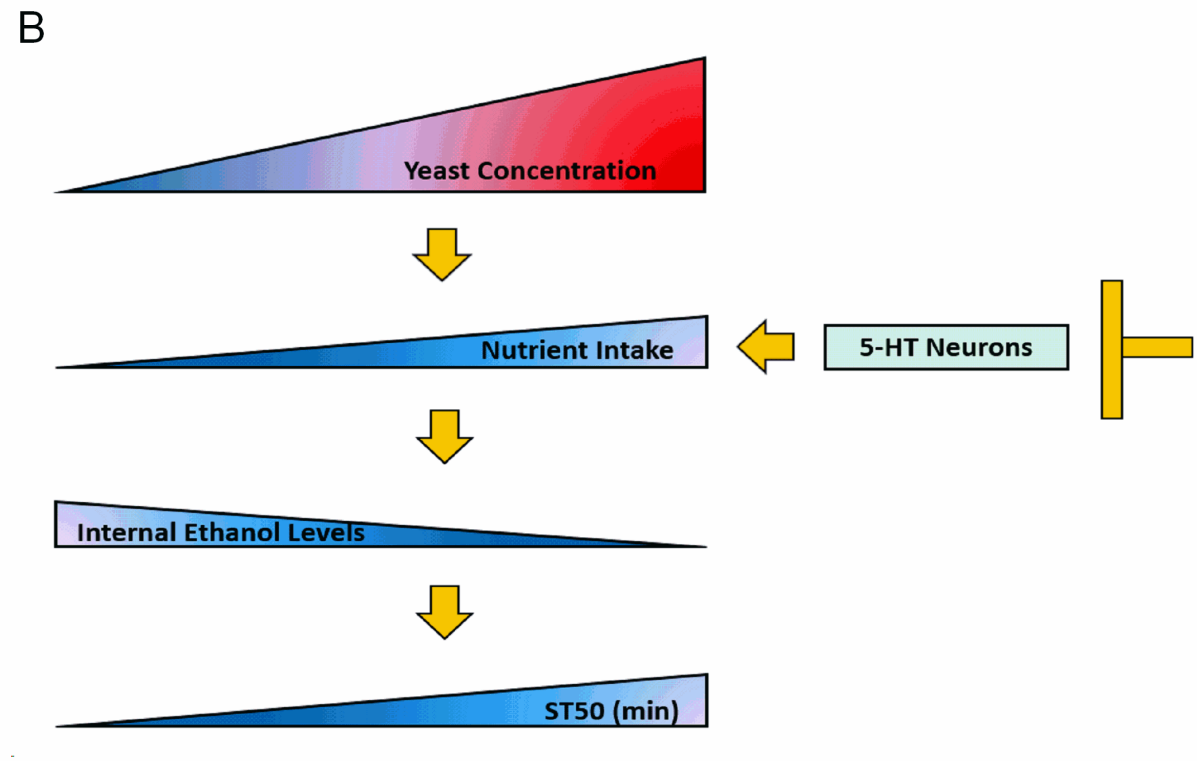

B
Yeast Concentration
Nutrient Intake
5-HT Neurons
Internal Ethanol Levels
ST50 (min)

Figure 3.19. Model for effect of dietary yeast on ethanol sedation. (A) As the yeast concentration in the diet increases, nutrient intake increases, internal ethanol levels decrease, and the time to sedation (ST50) is extended. 5-HT neurons positively regulate nutrient intake and thereby influence the effect of dietary yeast on internal ethanol and ST50. (B) Inhibition of serotonergic neuron function prevents the increase of nutrient intake when flies are fed high yeast media. This lack of increased intake of nutrients leads to there being no changes in internal ethanol, and subsequently, no difference in ST50 values when flies are fed high yeast media.

high yeast diet on ethanol sedation and internal ethanol levels is blunted by inhibition of serotonergic neurons.

In principle, our data on yeast supplementation and ethanol sedation could be interpreted in two ways. One interpretation is that yeast supplementation of a diet otherwise capable of supporting growth and normal behavior causes resistance to ethanol sedation. A second, alternative interpretation is that decreasing the concentration of dietary yeast below that required for normal growth and behavior leads to ethanol sedation sensitivity. We favor the former interpretation for several reasons. In previous studies, adult flies reared on our standard medium weigh approximately the same (e.g. ~1 mg for females[59, 62, 132]) as flies grown under routine conditions used in other labs (e.g. [184]). In the studies reported here, flies reared on our standard 2Y10S3C and supplemented 30Y10S3C media emerged with similar time-courses and in the same numbers. These results suggest that flies grown on 2Y10S3C are not nutrient-deprived. Additionally, the increased resistance to alcohol sedation in our studies requires yeast concentrations in excess of 10%, which is higher than yeast concentrations used in routine fly culture. Our interpretation of these observations is that yeast supplementation of a diet otherwise sufficient in nutrients is capable of increasing resistance to ethanol sedation. It is extremely challenging, however, to formally rule out the possibility that flies fed our standard medium are not at least somewhat nutrient-deprived. Thus, it is a matter of perspective whether our data are interpreted to mean that yeast-supplementation increases resistance to ethanol sedation or that yeast-restriction decreases resistance to ethanol sedation. Importantly, either interpretation

wholly supports the hypothesis that the concentration of yeast in the fly diet influences ethanol sedation.

Each *Drosophila* laboratory can and often does use a unique recipe for fly media. Differences in fly media composition could lead to variability in baseline ethanol sedation or potentially a lack of reproducibility of results across laboratories. We suggest that it become standard practice in the field to report all components and the concentrations used for fly media for studies on alcohol sedation as has been suggested previously for studies in other areas[157].

The ability to manipulate ethanol sedation by changing the yeast concentration in the fly diet expands the utility of the *Drosophila* model for investigating genes and genetic pathways that underlie alcohol-related behaviors. With our data as a backdrop, the fly model should be suitable for pursuing at least three major areas of research: molecular and cellular mechanisms like serotonergic signaling that drive nutrient consumption as it relates to ethanol sedation, nutrient-driven changes in ethanol uptake and/or elimination, and pathways downstream of nutrient intake that change behavioral responses to alcohol. It is interesting to speculate that at least some genetic manipulations known to influence resistance to ethanol sedation in flies or other species might relate to one or more of these three areas.

Dietary yeast influences brain 5-HT content in flies[147], 5-HT likely plays a role in fly ethanol sedation[74], and 5-HT is connected to problematic alcohol consumption in humans (e.g. [151-155]). Additionally, serotonergic neurons and serotonin signaling are important for hunger/satiety and feeding behavior in both larval and adults flies[148-150, 185]. Our studies in flies suggest that serotonergic neurons might influence ethanol sedation

via effects on nutrient consumption and ethanol uptake/elimination, raising the possibility that there could be a link between 5-HT, diet, and alcohol-related behavior in other species.

The effect of diet on alcohol-related behavior is not unique to flies. In *C. elegans*, mutations that disrupt synthesis of eicosapentaenoic acid (EPA, an omega-3 polyunsaturated fatty acid) blunt the development of acute functional tolerance to alcohol and dietary supplementation with this fatty acid facilitates acute functional tolerance[87]. Reduced caloric intake in rats enhances the alcohol-deprivation effect and reinstatement of ethanol-seeking behavior[86] and food deprivation decreases alcohol drinking in mice[145]. Furthermore, providing mice with different, but otherwise routinely used, laboratory diets influences ethanol drinking, ethanol consumption, and ethanol-induced locomotion[84], and altering EPA in the diet of mice influences both ethanol sensitivity and consumption[85]. These results indicate that diet-induced changes in alcohol-related behavior are a common feature of metazoans. Therefore, identification of the underlying mechanisms via studies like those described here has the potential to be valuable for both prevention and treatment of AUD.

Chapter 4: Convergent evidence from humans and *Drosophila melanogaster* implicates the transcription factor *MEF2B*/*Mef2* in alcohol sensitivity

A. Introduction

Alcohol consumption is common in the United States and elsewhere, with rates in 2014 as high as 87.6% of individuals 18 or older reporting lifetime use and 71.0% reporting use within the last year[186]. Although many individuals use alcohol without developing problems, over a quarter of those who abuse alcohol use progress to abuse[187]. The World Health Organization estimates that 283 million people over the age of 15 years across the globe have alcohol use disorder (AUD) and that alcohol usage contributes to 3 million premature deaths annually[126]. Problematic alcohol consumption also leads to a substantial public health burden in the United States. For example, excessive consumption of alcohol cost the United States an estimated $249 billion in 2010 and caused over 88,000 deaths between 2006 and 2010[188, 189]. Despite the substantial, multi-layered consequences of AUD and other forms of problematic alcohol consumption, we currently have a rather incomplete understanding of the mechanisms that drive their genesis.

It is clear, though, that the risk for developing AUD is under genetic influence. Twin studies show that AUD is moderately heritable (h^2=0.49[190]) indicating genetic factors account for ~50% of the underlying risk. Additionally, outcomes such as frequency or quantity of alcohol use[191, 192] and intoxication[193] exhibit comparable levels

of heritability. Genome-wide association studies (GWAS) have been used to identify genetic variance that influences problematic alcohol consumption. Such studies have implicated several genes in alcohol consumption including *AUTS2* and *KLB* (which encode a transcriptional repressor and a receptor for fibroblast growth factor 21 (FGF21), respectively) as well as genes that encode alcohol- and aldehyde dehydrogenases[78, 194-196]. Other studies have suggested that the gene *RYR3* (which encodes a ryanodine receptor) and multiple genes that encode proteins in the SWI/SNF complex influence the development of alcohol dependence[77, 197]. Despite these and other advances[22, 23, 198-200], our understanding of the genetic etiology of alcohol outcomes is relatively limited.

Genetic model organisms, principally *Drosophila melanogaster* (fruit flies), *Caenorhabditis elegans* (worms), and *Mus musculus* (mice), have been used extensively to identify molecular-genetic mechanisms underlying many different alcohol-related behaviors. These model organisms have conserved behavioral responses to alcohol including (collectively) locomotor activation at low doses, sedation at high doses, tolerance during prolonged or after repeated doses of alcohol, and withdrawal after discontinuation of the drug[56, 201-204]. Additionally, flies and mice voluntarily consume alcohol and this consumption can escalate with continuing exposure[68, 205, 206]. Given that humans exhibit comparable behavioral responses to alcohol, it is possible that many of the molecular targets for alcohol or the signaling pathways required for modulating the effects of alcohol on behavior might be conserved across species. There are data that support this possibility. For example, *AUTS2, KLB, RYR, RSU1, SWI/SNF* and other genes implicated in various alcohol behaviors in humans also influence

behavioral responses to acute alcohol in flies, worms, and mice[77-79, 194, 196, 197].These findings suggest that the initial response to alcohol rating scale in humans might be driven—at least in part—by changes in behavioral responses to initial alcohol exposure revealed by studies in genetic model organisms.

We previously reported a meta-analysis of GWAS on the Self-Rating of the Effects of Alcohol (SRE) across two adolescent to young adult samples, Avon Longitudinal Study of Parents and Children (ALSPAC) and Spit for Science (S4S)[115]. The SRE [18] captures the number of alcoholic drinks needed for respondents to experience intoxication when they first began drinking (i.e., initial sensitivity). Higher scores on the SRE have been associated with the development of alcohol use and misuse[17, 207], suggesting that it has predictive validity for risk of later problematic alcohol consumption. The meta-analytic SNP-based heritability estimate (h^2_{SNP}) in our previous report[115] was modest (h^2_{SNP}=0.19, SE=0.10) in the combined ALSPAC and S4S samples. This modest heritability was driven, however, by the ALSPAC sample, (h^2_{SNP}=0.36, SE=0.14, p=0.04); the heritability estimate did not differ from 0 in S4S. While our previous report focused on primary and secondary analyses of the meta-analytic results, the moderate h^2_{SNP} in ALSPAC suggested that loci implicated in that sample might be particularly valuable in follow-up studies. We consequently (i) conducted gene-based analyses of SRE in the ALSPAC sample to identify promising loci; (ii) identified, among the loci most strongly implicated in ALSPAC, those genes with orthologues in *Drosophila*; and (iii) assessed ethanol sedation sensitivity and tolerance in flies expressing RNAi targeting candidate genes.

The studies reported here implicated 37 human genes in SRE responses in the ALSPAC sample. Of these genes, we focused on 6 (*APP, ATG5, GPD2, ISL1, MEF2B,* and *PCDH15*) because previous results suggested they might be involved in relevant phenotypes in humans, these 6 genes had orthologues in flies, and they were publicly available RNAi reagents to manipulate them. We found that neuronal expression of RNAi against the fly gene *Mef2* decreased ethanol sedation sensitivity. Subsequently, we found that flies with loss-of-function mutations in *Mef2* also had decreased ethanol sedation sensitivity. These alterations in ethanol sedation in flies with decreased *Mef2* expression were not accompanied by changes in internal ethanol levels or rapid tolerance to ethanol. Interestingly, another group recently reported that decreased *Mef2* function in flies alters rapid tolerance[208], suggesting that the consequences of *Mef2* action in alcohol-related behavior might be context-dependent. Our studies collectively implicate human *MEF2B* in SRE responses and indicate that flies with altered *Mef2* expression have parallel changes in ethanol sedation. Given that *MEF2B* and *Mef2* encode transcription factors, our data further suggest that proteins with *MEF2B*/*Mef2*-dependent expression might be targets of ethanol or be involved in the modulation of behavioral responses to the drug.

B. Results

Human genetic analyses and identification of loci for potential screening in *Drosophila*.

As reported previously[115], SRE scores were moderately heritable in the ALSPAC sample (h^2_{SNP}=0.36, p=0.04). To explore potential genetic contributions to SRE scores, we used summary statistics from the SRE GWAS in ALSAPC to conduct gene-based

analyses in Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA). This yielded results for 18,187 genes, of which 37 had p_{gene}<0.001 for SRE (Table 4.1). Of these 37 genes, 29 were orthologous to a total of 52 *Drosophila* genes (defined as having DIOPT score ≥ 2)[89] (Table 4.1). Additionally, of the 37 genes with p_{gene}<0.001 for SRE, nine had been previously associated with phenotypes (e.g., substance use, psychiatric findings, and others) we postulated might be related to alcohol use or misuse (Table 4.1). Thus, nine human genes (*APP, ATG5, BORCS8, BORCS8-MEF2B, GPD2, ISL1, MEF2B, PCDH15* and *SFSWAP*) had suggestive associations with SRE, had previously been connected to potentially relevant phenotypes in humans, and were orthologous to at least one gene in flies (Table 4.1). The 9 top candidate human genes noted above are orthologous to a total of 12 *Drosophila* genes (Table 4.1). We obtained RNAi reagents from public stock centers to manipulate expression of 6 of the most highly conserved fly genes (*Appl, Atg5, Cad99c, Gpo1, Mef2,* and *tup*). We did not explore the 2 remaining fly genes because (i) for *CG32590* no function has been ascribed to its gene product and (ii) for *su(w[a])* only a single RNAi reagent was available to manipulate it and it is a rather complex locus with several additional genes residing within its transcription unit[209], greatly complicating genetic analyses.

Initial screening of *Drosophila* orthologues in ethanol sedation and rapid tolerance

To explore the role of the 6 *Drosophila* genes in ethanol-related behavior, we

Table 4.1. Candidate loci for follow-up in *Drosophila*.

Human Gene Symbol	Pgene	Previously associated human phenotypes of interest[1]	Drosophila Gene Symbol	DIOPT Score
MEF2B	0.0007412	Obsessive compulsive disorder	Mef2	4
APP	0.00050488	Cognition, neuropsychological tests, psychiatric status rating scales, psychotic disorders, tobacco use disorder	Appl	13
ATG5	0.00058657	Drug hypersensitivity, tobacco use disorder	Atg5	14
BORCS8[2]	0.00050716	Tobacco use disorder, obsessive compulsive disorder	CG32590	13
BORCS8-MEF2B[3]	0.0007412	Tobacco use disorder	Mef2	4
GPD2	0.00028942	Fatty liver	Gpo-1	13
ISL1	0.00038948	ADHD	Tup	11
PCDH15	0.00012322	Alcoholism, tobacco use disorder	Cad99C	9
SFSWAP	0.00038044	Tobacco use disorder	su(w[a])	13
ANKRD6	0.00035656	N/A	dgo	4
ASPG	0.00081699	N/A	CG6428, CG8526	13
BHLHE40	0.00074851	N/A	Several	2
BIRC6	0.00053743	N/A	Bruce	15
C1QTNF5	0.00037187	N/A	N/A	N/A

DEPDC7	9.33E-05	N/A	N/A	N/A
DSG1	1.27E-05	N/A	N/A	N/A
EXOC1	2.97E-05	N/A	Sec3	14
IKZF1	0.00015571	N/A	N/A	N/A
LGSN	0.00034442	N/A	Gs1	2
LRRTM1	0.00031065	N/A	caps, trn	2
MFRP	0.00037187	N/A	CG42255	2
MMP17	0.00013157	N/A	Mmp2	7
PPP2R5E	0.00059389	N/A	wdb	13
PRAMEF11	0.00044431	N/A	N/A	N/A
PRB1	0.00036335	N/A	N/A	N/A
PSMD6	0.00062142	N/A	Rpn7	15
PXMP2	0.00076238	N/A	CG7970	7
SCGN	0.00078013	N/A	Cbp53E	8
SCN3B	0.00026792	N/A	N/A	N/A
SET	0.00023109	N/A	Set	13
STRN3	0.00051438	N/A	Cka	15

TCP11L1	0.00014072	N/A	CG16721	13
THAP2	0.00030577	N/A	CG14860	2
TMEM185B	0.00083227	N/A	CG14194	12
TMEM53	0.00069173	N/A	CG8245	14
USP2	0.00047617	N/A	Usp2	9
ZBTB44	5.54E-06	N/A	N/A	N/A

Listed orthologs have a DIOPT score ≥2.

[1]Primary resource was the Public Health Genomics Knowledge Base, supplemented by NCBI literature searches.

[2]This locus is also known as MEF2BNB.

[3]This locus is also known as MEF2BNB-MEF2B

constitutively expressed RNAi targeting each gene (22 RNAi transgenes total) in neurons using elav-Gal4 and then assessed ethanol sedation and ethanol rapid tolerance in adults. Expression of 3 different RNAi transgenes targeting *Mef2*, two of which have independent target sequences (Figure 4.1), increased ST50 values relative to both genetic controls (Table 4.2 and see below). In contrast, expression of *Mef2* RNAi transgenes in neurons did not alter the development of rapid tolerance (Table 4.2). Thus, we focused our subsequent fly studies on the role of *Mef2* in ethanol sedation. Expression of RNAi targeting the other 5 *Drosophila* genes did not consistently alter either ethanol sedation or rapid tolerance compared to controls (Table 4.2). We note, however, that neuronal expression of 2 RNAi transgenes against *Cad99C* with unique target sequences (HMS01451 and JF02660) made flies resistant to ethanol sedation (Table 4.2), suggesting that this gene might also warrant further study.

Mef2 in *Drosophila* ethanol sedation

Human *MEF2B* is orthologous to *Drosophila Mef2* (Table 4.1). Both genes encode transcription factors with three domains: MADS (MCM1, agamous, deficiens, SRF), MEF2, and transcriptional activation (Fig 4.2)[88, 92]. Using the longest isoforms for both human MEF2B (isoform 1) and fly Mef2 (isoform H), we found that the proteins are 46% similar/37% identical overall at the amino acid level[210]. Importantly, the MADS and MEF2 domains in the human and *Drosophila* gene products are 85.5% identical/96.4% similar and 82.8% identical/93.1% similar, respectively (Fig 4.2)[88, 210]. The

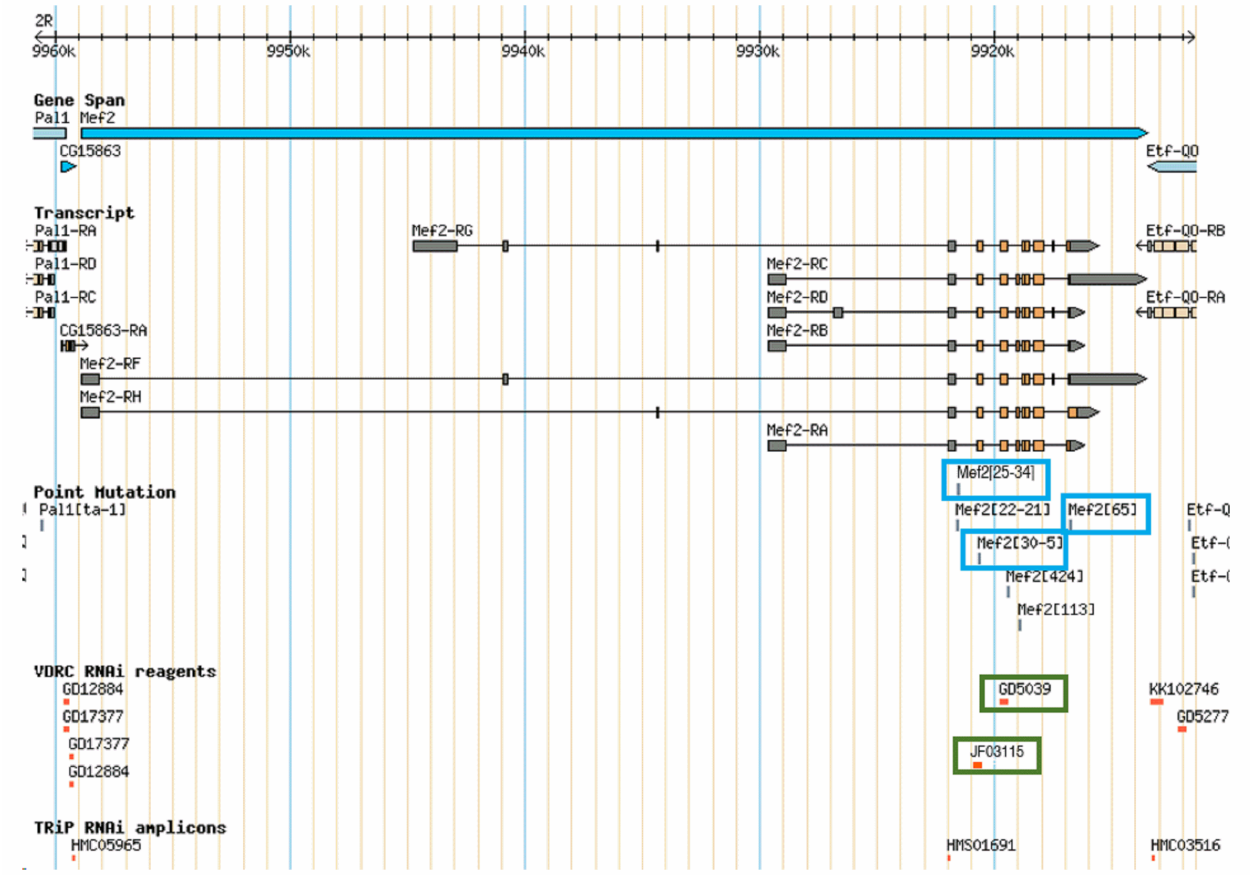


Figure 4.1. The *Mef2* transcriptional region. Image is the *Mef2* region from FlyBase with additional manual annotations. All *Mef2* reagents used in this study are shown below the gene span and transcripts. *Mef2* RNAi target sequences are boxed in green (GD5039 (corresponding to v15550 and v15549) and JF03115). The locations of the *Mef2* mutations are boxed in blue (*Mef2*$^{30\text{-}5}$, *Mef2*^{65}, and *Mef2*$^{25\text{-}34}$).

Table 4.2. Ethanol sedation results from top candidates from human GWAS with *Drosophila* orthologs.

Experimental set	Target gene	Gal4	RNAi	ST50 E1	RapTol (E2/E1)	n	Statistical test	ST50 E1 p values	RapTol p values
1	*Appl*	elav	JF02878	28.16 ± 1.37	1.92 ± 0.135	8	ANOVA	0.0155	0.0896
	Control	--	JF02878	25.26 ± 1.47	2.31 ± 0.14	7	BMC	0.3304	0.1315
	Control	elav	--	21.90 ± 1.38*	1.89 ± 0.14	8	BMC	0.0087	> 0.9999
1	*Atg5*	elav	HMS01244	42.19 ± 1.39	1.27 ± 0.06	8	ANOVA	< 0.0001	0.0002
	Control	--	HMS01244	29.40 ± 2.74*	2.33 ± 0.24*	8	BMC	0.0001	< 0.0001
	Control	elav	--	23.54 ± 0.51*	1.83 ± 0.07*	8	BMC	< 0.0001	0.0258
2	*Atg5*	elav	JF02661	34.90 ± 1.97	2.11 ± 0.13	8	ANOVA	0.0075	0.8988
	Control	--	JF02661	32.63 ± 1.37	2.06 ± 0.11	8	BMC	0.5776	> 0.9999
	Control	elav	--	27.69 ± 0.90*	2.14 ± 0.12	8	BMC	0.0048	> 0.9999
3	*Atg5*	elav	VSH330300	25.39 ± 1.47	2.00 ± 0.17	8	ANOVA	0.0002	0.0061
	Control	--	VSH330300	19.99 ± 0.69*	2.52 ± 0.09*	8	BMC	0.0238	0.0375
	Control	elav	--	29.85 ± 1.77	1.80 ± 0.16	8	BMC	0.0670	0.6998
4	*Atg5*	elav	KK108904	34.90 ± 3.57	1.36 ± 0.18	8	ANOVA	0.0060	0.0400
	Control	--	KK108904	42.28 ± 2.03	1.36 ± 0.09	8	BMC	0.1041	> 0.9999
	Control	elav	--	29.31 ± 1.55	1.82 ± 0.12	8	BMC	0.2672	0.0520
1	*Cad99c*	elav	HMS01451	46.44 ± 2.39	1.51 ± 0.10	8	ANOVA	0.0007	0.0804
	Control	--	HMS01451	33.24 ± 3.45*	2.41 ± 0.43	8	BMC	0.0044	0.0650
	Control	elav	--	30.26 ± 1.96*	1.73 ± 0.18	8	BMC	0.0007	> 0.9999
2	*Cad99c*	elav	JF02660	34.51 ± 2.59	2.02 ± 0.17	8	ANOVA	0.0007	0.1720

	Control	--	JF02660	24.81 ± 0.88*	2.28 ± 0.14	8	BMC	0.0012	0.3777
	Control	elav	--	25.26 ± 1.07*	1.91 ± 0.09	8	BMC	0.0018	> 0.9999
3	*Cad99c*	elav	JF02761	27.53 ± 1.00	2.02 ± 0.11	8	ANOVA	0.0073	0.0227
	Control	--	JF02761	25.84 ± 0.99	2.01 ± 0.11	8	BMC	0.4010	> 0.9999
	Control	elav	--	23.05 ± 0.68*	1.67 ± 0.045*	8	BMC	0.0042	0.0293
4	*Cad99c*	elav	v27215	23.74 ± 0.95	1.90 ± 0.10	8	ANOVA	0.9960	0.0462
	Control	--	v27215	23.85 ± 0.70	1.60 ± 0.10	8	BMC	> 0.9999	0.1393
	Control	elav	--	23.84 ± 1.2	2.00 ± 0.13	8	BMC	> 0.9999	> 0.9999
5	*Cad99c*	elav	v27216	52.88 ± 1.21	1.48 ± 0.04	8	ANOVA	< 0.0001	0.0813
	Control	--	v27216	48.45 ± 0.96	1.59 ± 0.05	8	BMC	0.0798	0.7097
	Control	elav	--	40.10 ± 1.94*	1.76 ± 0.13	8	BMC	< 0.0001	0.0554
6	*Cad99c*	elav	v27212	41.54 ± 2.02	1.66 ± 0.07	8	ANOVA	0.0170	0.0425
	Control	--	v27212	48.96 ± 1.12*	1.39 ± 0.05	8	BMC	0.0172	0.0562
	Control	elav	--	42.49 ± 2.12	1.44 ± 0.10	8	BMC	> 0.9999	0.1482
7	*Cad99c*	elav	v3739	37.50 ± 1.64	1.65 ± 0.11	8	ANOVA	0.0812	0.2700
	Control	--	v3739	39.30 ± 1.89	1.58 ± 0.10	8	BMC	0.9974	> 0.9999
	Control	elav	--	43.56 ± 2.00	1.34 ± 0.15	8	BMC	0.0612	0.2512
1	*Gpo1*	elav	v19565	36.08 ± 1.08	1.25 ± 0.05	8	ANOVA	0.1106	0.0080
	Control	--	v19565	39.86 ± 1.65	1.35 ± 0.06	8	BMC	0.1711	0.5090
	Control	elav	--	35.64 ± 1.65	1.55 ± 0.08*	8	BMC	> 0.9999	0.0049

2	*Gpo1*	elav	KK107425	41.30 ± 1.61	1.36 ± 0.07	8	ANOVA	< 0.0001	0.0013
	Control	--	KK107425	29.44 ± 1.05*	1.88 ± 0.07*	8	BMC	< 0.0001	0.0013
	Control	elav	--	30.24 ± 1.20*	1.80 ± 0.12*	8	BMC	< 0.0001	0.0054
3	*Gpo1*	elav	HMC04006	24.19 ± 0.99	2.32 ± 0.18	8	ANOVA	< 0.0001	< 0.0001
	Control	--	HMC04006	20.36 ± 0.65*	2.29 ± 0.10	8	BMC	0.0134	> 0.9999
	Control	elav	--	39.95 ± 1.01*	1.38 ± 0.047*	8	BMC	< 0.0001	< 0.0001
1	*Mef2*	elav	JF03115	46.90 ± 2.12	1.51 ± 0.07	8	ANOVA	< 0.0001	0.2099
	Control	--	JF03115	37.89 ± 0.96*	1.63 ± 0.05	8	BMC	0.0006	0.3923
	Control	elav	--	33.49 ± 1.09*	1.67 ± 0.07	8	BMC	< 0.0001	0.1868
2	*Mef2*	elav	v15550	57.25 ± 1.80	1.39 ± 0.05	8	ANOVA	< 0.0001	0.6152
	Control	--	v15550	44.81 ± 1.31*	1.49 ± 0.09	8	BMC	< 0.0001	0.6738
	Control	elav	--	39.98 ± 1.71*	1.43 ± 0.06	8	BMC	< 0.0001	> 0.9999
3	*Mef2*	elav	v15549	48.04 ± 2.53	-	8	ANOVA	*< 0.0001*	-
	Control	--	v15549	35.26 ± 1.66*	-	8	BMC	*0.0003*	-
	Control	elav	--	33.15 ± 1.58*	-	8	BMC	*< 0.0001*	-
1	*tup*	elav	HMC03317	47.66 ± 0.96	1.38 ± 0.06	5	ANOVA	0.0002	0.1069
	Control	--	HMC03317	44.64 ± 1.76	1.41 ± 0.08	5	BMC	0.3628	> 0.9999
	Control	elav	--	34.84 ± 1.66*	1.62 ± 0.09	5	BMC	0.0001	0.1076
2	*tup*	elav	v45859	38.51 ± 1.54	1.47 ± 0.09	8	ANOVA	0.0414	0.9583
	Control	--	v45859	41.13 ± 0.95	1.46 ± 0.03	8	BMC	0.3801	> 0.9999
	Control	elav	--	35.86 ± 1.52	1.49 ± 0.08	8	BMC	0.3681	> 0.9999
3	*tup*	elav	KK101489	40.06 ± 2.11	1.57 ± 0.12	8	ANOVA	0.0009	0.2162
	Control	--	KK101489	44.78 ± 2.40	1.47 ± 0.10	8	BMC	0.3577	> 0.9999
	Control	elav	--	32.03 ± 1.54*	1.74 ± 0.088	8	BMC	0.0344	0.7602

P values from one-way ANOVA tests (to assess overall effects of genotype) and BMCs (for planned comparisons between flies expressing RNAi and their respective controls) are shown. *BMC shown in red for significance. (Performed by Brandon Shell).

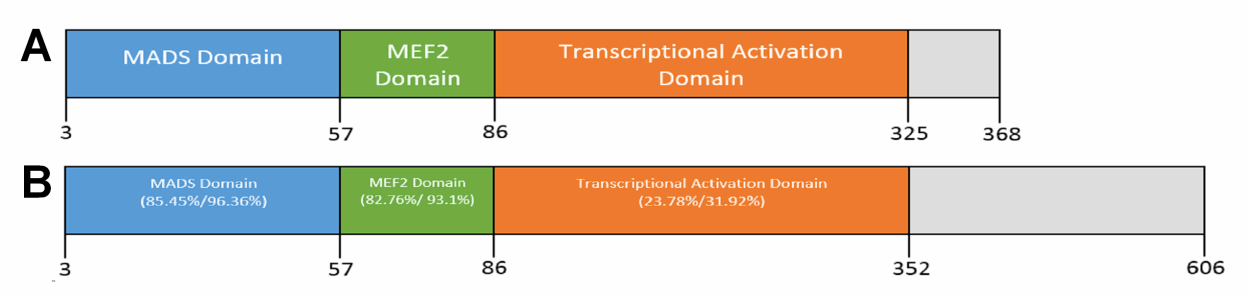


Figure 4.2. Human *MEF2B* and *Drosophila Mef2* protein identity/similarity. Depiction of the three (MADS, *MEF2*, and transcriptional activation) domains constituting human (A) and *Drosophila* (B) *MEF2B* and *Mef2* and their corresponding amino acid identity and similarity. Transcriptional activation domain amino acid identity and similarity is compared up to amino acid 325 for human *MEF2B* and amino acid 352 for *Drosophila Mef2.*

transcriptional activation domain is not as well conserved as the other two domains[88, 210] (Figure 4.2). The overall conservation in domain structure of the proteins, along with the conservation of the primary amino acid sequences of these domains, suggests that *MEF2B* and *Mef2* might have conserved functions in humans and *Drosophila*. We note that the *MEF2B* chromosomal region in humans has two non-overlapping genes (*MEF2B* and *BORCS8*), each of which produces its own transcript. This region also produces a read-through transcript, *BORCS8-MEF2B* (a.k.a. *MEF2BNB-MEF2B*). Given that *BORCS8* has no known function, *BORCS8* and *BORSCS8-MEF2B* were not considered further in the current study.

As noted above, pan-neuronal expression of the *Mef2* RNAi v15550 increased sedation time 50 (ST50) values compared to controls (Figure 4.3A, ethanol sedation time-course; Figure 4.3C, ST50 values). Similarly, pan-neuronal expression of a second *Mef2* RNAi (v15549) with the same target sequence as v15550 also increased ST50 values compared to controls (Figure 4.4). More importantly, flies with pan-neuronal expression of another RNAi transgene with a distinct target sequence in *Mef2* (JF03115) also had increased ST50 values (Figure 4.3B, ethanol sedation time-course; Figure 4.3D, ST50 values). Therefore pan-neuronal expression of *Mef2* RNAi lines affects ethanol sedation in flies.

To determine if the altered ethanol sedation in flies expressing *Mef2* RNAi could be due to a net change in uptake or metabolism of ethanol, we measured internal ethanol concentrations in the same genotypes assessed in ethanol sedation. We found that expression of *Mef2* in neurons had no significant effect on internal ethanol in flies

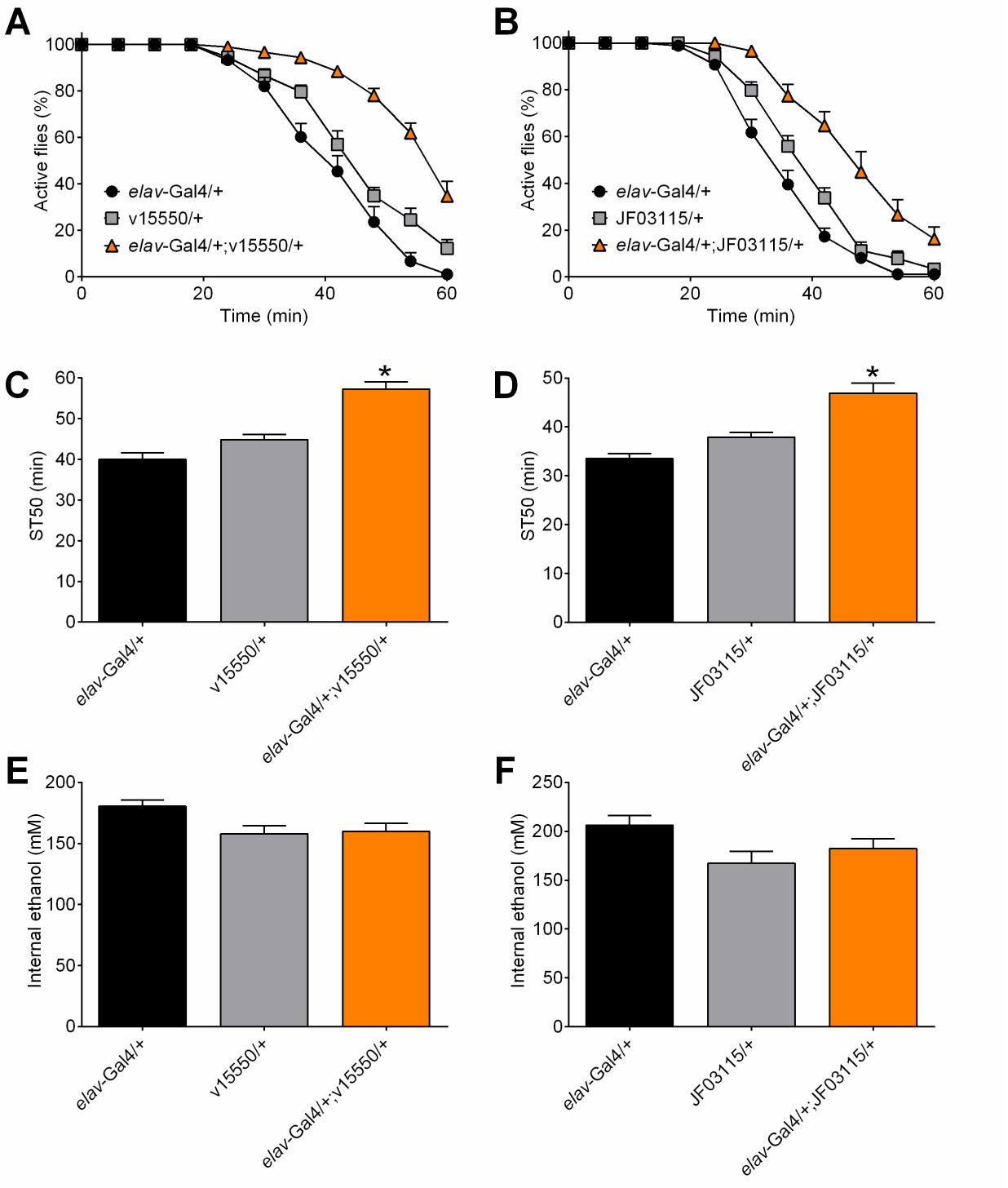

A
Active flies (%)
Time (min)
elav-Gal4/+
v15550/+
elav-Gal4/+;v15550/+
B
Active flies (%)
Time (min)
elav-Gal4/+
JF03115/+
elav-Gal4/+;JF03115/+
C
ST50 (min)
*
elav-Gal4/+
v15550/+
elav-Gal4/+;v15550/+
D
ST50 (min)
*
elav-Gal4/+
JF03115/+
elav-Gal4/+;JF03115/+
E
Internal ethanol (mM)
elav-Gal4/+
v15550/+
elav-Gal4/+;v15550/+
F
Internal ethanol (mM)
elav-Gal4/+
JF03115/+
elav-Gal4/+;JF03115/+

Figure 4.3. Ethanol sedation and internal ethanol levels in flies with pan-neuronal expression of *Mef2* RNAi. (A and B) Ethanol sedation time courses of flies with pan-neuronal *Mef2* RNAi expression. Expression of *Mef2* RNAi transgenes v15550 (A, *elav*-Gal4/+;v15550/+) and JF03118 (B, *elav*-Gal4/+;JF03115/+) in neurons extended sedation time-courses compared to controls (*elav*-Gal4/+, v15550/+, JF03118/+). (C, D) ST50 values derived from the data in panels A and B. Pan-neuronal expression of v15550 © and JF03115 (D) increased ST50 values compared to controls (individual one-way ANOVAs, $p<0.0001$; *Bonferroni's multiple comparisons (BMCs), $p<0.0001$ ©, $p\leq0.006$ (D); n=8). (E, F) Internal ethanol levels in flies exposed to 85% ethanol vapor for 42 minutes. © In studies with v15550, genotype had a significant overall effect on internal ethanol concentrations (one-way ANOVA, $p=0.0296$, n=8), but internal ethanol in flies expressing v15550 was not significantly different than controls (BMCs, $p=0.0541$-0.9999). (F) Genotype did not have a significant overall effect on internal ethanol concentrations in studies with JF03115 (one-way ANOVA, $p=0.0574$, n = 8).

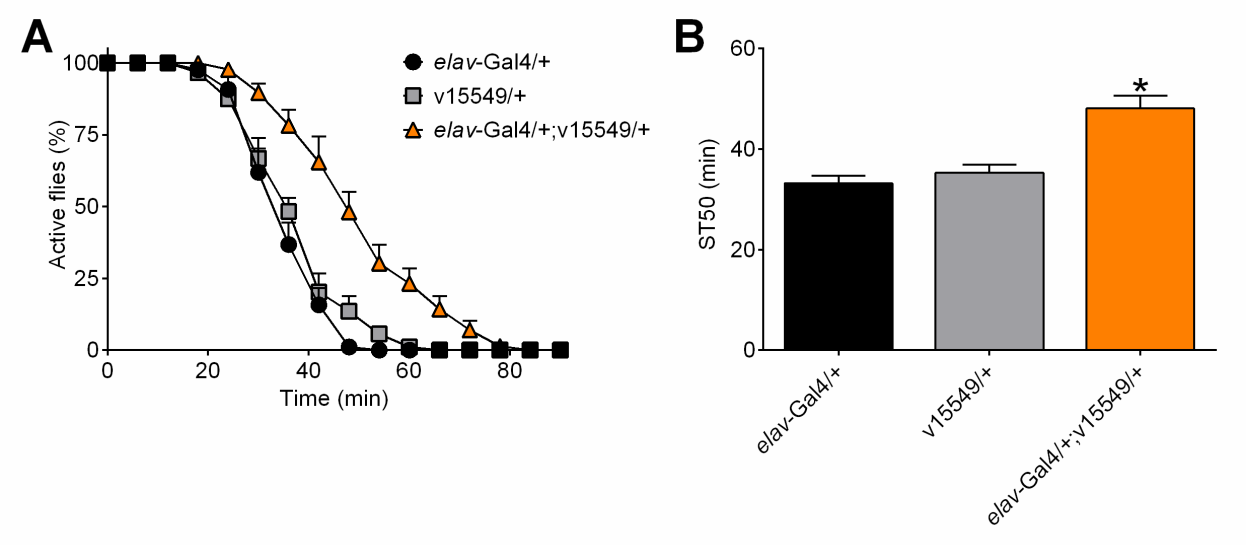


Figure 4.4. Expression of v15549, an RNAi transgene with the same target sequence as v15550, alters ethanol sedation when expressed pan-neuronally. (A) Ethanol sedation time courses from control flies (*elav*-Gal4/+ and v15550/+) and flies expressing *Mef2* RNAi v15549 in neurons (*elav*-Gal4/+;v15549/+). (B) ST50 values were greater in *elav*-Gal4/+;v15549/+ flies than in controls (one-way ANOVA, $p<0.0001$; *BMCs vs controls, $p\leq0.0003$; $n = 8$).

(Figures 4.3E and 4.3F). Expression of *Mef2* RNAi in neurons therefore affects ethanol sedation without having a measurable impact on the overall disposition of ethanol.

While completing our studies on human *MEF2B* and fly *Mef2*, Adhikari and colleagues[208] reported that ethanol sedation was increased (evidenced by decreased ST50 values) in flies with pan-neuronal expression of one of the same *Mef2* RNAi transgenes used in our studies, although pan-neuronal expression of a *Mef2* dominant-negative protein did not impact ethanol sedation sensitivity in this study[208]. The ST50 values from control flies in the experiments conducted by Adhikari et. al were ~15 minutes, which is shorter than ST50 values from our studies (30-40 minutes when using vapor from 85% ethanol). This difference in ST50 values raised the possibility that knocking down *Mef2* might decrease ST50 values when they are relatively short (as in Adhikari et. al), while knocking down *Mef2* might increase ST50 values when they are relatively long (as in our studies). To explore this possibility, we expressed *Mef2* RNAi transgenes pan-neuronally in flies and then assessed their sedation in response to vapor from 100% ethanol. Exposure of flies to vapor from 100% ethanol appeared to decrease overall ST50 values as expected (Figure 4.5). More importantly, though, flies with pan-neuronal expression of *Mef2* RNAi transgenes v15550 (Figure 4.5A and C) and JF03115 (Figure 4.5B and D) continued to have significantly increased ST50 values compared to controls even when exposed to vapor from the highest possible concentration of ethanol in our behavioral paradigm. Although additional studies would be required to fully explore the differences between the phenotypes seen in flies with altered *Mef2* reported here and by Adhikari and colleagues[208], our data suggest that

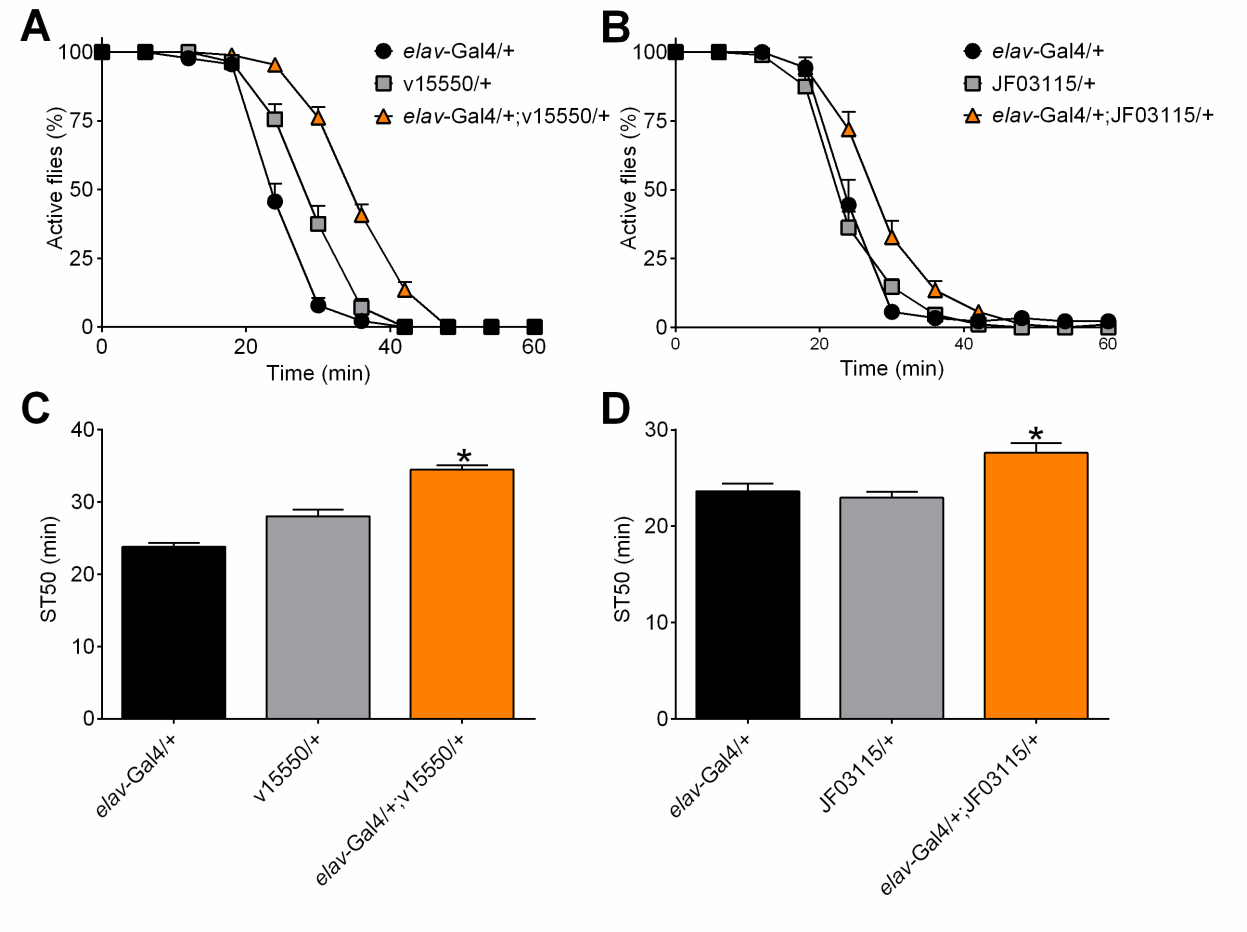


Figure 4.5. Sedation by high dose ethanol in flies expressing *Mef2* RNAi. (A and B) Sedation time courses in response to vapor from 100% ethanol for flies with pan-neuronal expression of *Mef2* RNAi (A, *elav*-Gal4/+;v15550/+; B, *elav*-Gal4/+;JF03115/+) and controls (*elav*-Gal4/+, v15550/+, JF03115/+). (C, D) ST50 values from the data in panels A and B, respectively. © Pan-neuronal expression of v15550 © and JF03115 (D) increased ST50 values (individual one-way ANOVAs; panel C, $p<0.0001$; panel D, $p=0.0012$; panel C, *BMCs vs controls, $p<0.0001$; panel D, BMCs vs controls, $p=0.0012$-0.0049; $n=8$).

these differences are not due to the overall magnitude of ST50 values obtained from the behavioral paradigms used in the respective studies.

Adhikari and colleagues also reported that pan-neuronal expression of *Mef2* RNAi or a Mef2 dominant negative protein decreased the development of rapid tolerance to ethanol. We therefore also assessed ethanol rapid tolerance, calculated as either the relative (Fig 4.6) or absolute (Fig 4.7) change in ST50 using vapor from 85% (Figure 4.6A, 4.6B, 4.7A, 4.7B) or 100% (Figures 4.6C, 4.6D, 4.7C, 4.7D) ethanol. In contrast to Adhikari and colleagues, expression of *Mef2* RNAi in neurons did not alter rapid tolerance under the conditions of our experiments (Figures 4.6 and 4.7). Additional studies would again be required to fully explore the differences between our data and the data from Adhikari et. al[208], but as we found for sedation (see above) these differences were not readily resolved by increasing the ethanol concentration used for exposure.

We used immunofluorescence to assess whether pan-neuronal expression of v15550 and JF03115 decreased *Mef2* levels in whole fly brains (Figure 4.8). We focused our histological analyses at the level of the mushroom bodies because neurons in these structures are known to express readily detectable levels of *Mef2*[211]. Control flies harboring the *Mef2* RNAi transgenes without a Gal4 driver (Figure 4.8A, v15550/+; Figure 4.8B, JF30015/+) had strong expression of *Mef2* in the mushroom bodies (arrowheads) as expected. Visual inspection of representative images suggested that this *Mef2* signal was decreased in flies with pan-neuronal expression of *Mef2* RNAi transgenes (Figure 4.8C: elav-Gal4/+;v15550/+; Figure 4.8D: elav-Gal4/+;JF30115/+).

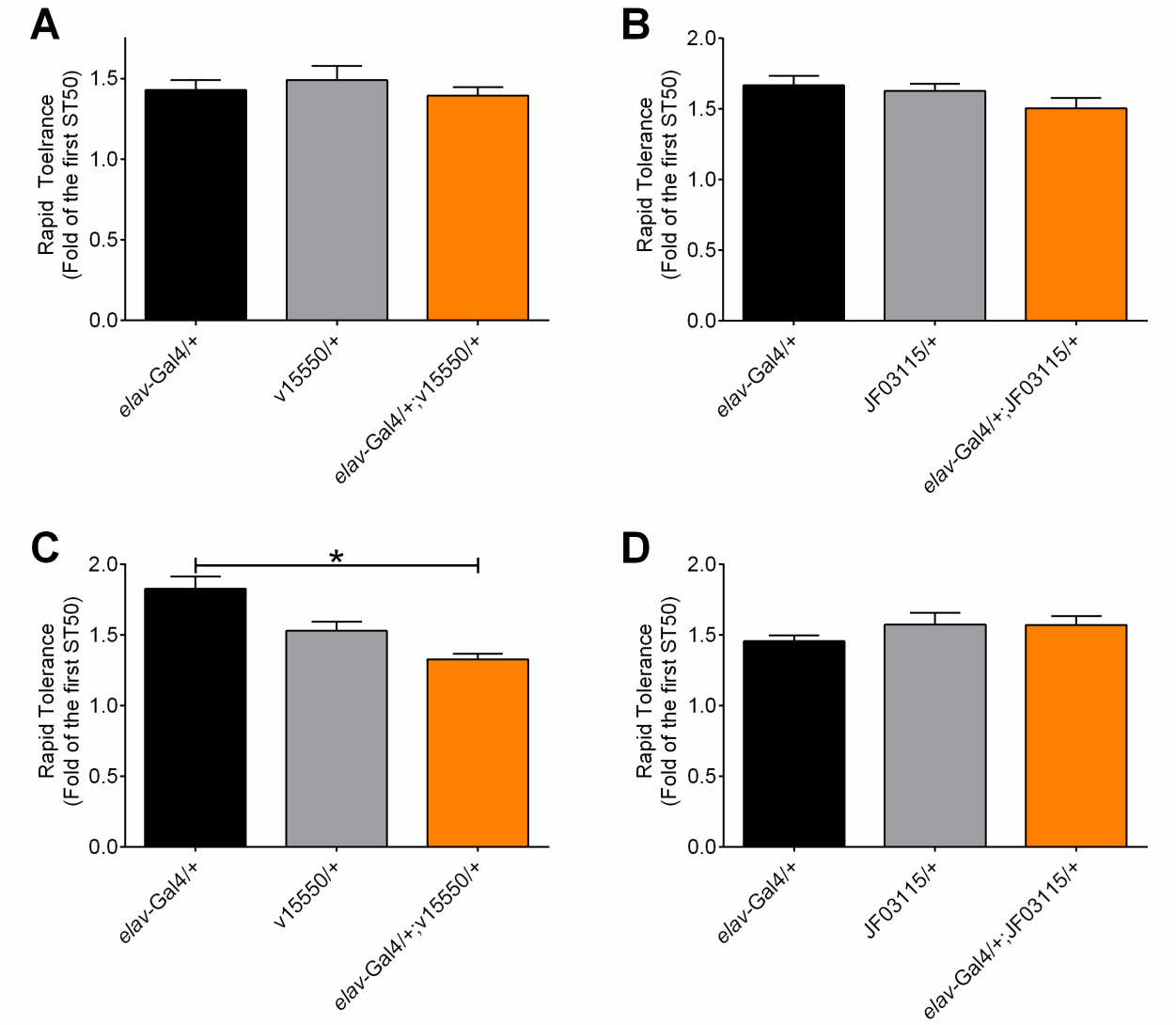


Figure 4.6. Rapid tolerance and pan-neuronal expression of *Mef2* RNAi. Development of rapid tolerance was not significantly altered by expression of *Mef2* RNAi. (A, B) Rapid tolerance in response to exposure of vapor from 85% ethanol. Expression of v15550 (A) or JF03115 (B) in neurons (individual one-way ANOVAs; panel A, p=0.6152; panel B, p=0.2099; n=8). (C, D) Rapid tolerance to sedation from vapor from 100% ethanol. In experiments with v15550, there was a significant overall effect of genotype (one-way ANOVA, p=0.0001, n=8) and *elav*-Gal4/+;v15550/+ flies were different than *elav*-Gal4/+ controls (BMC, p<0.0001), but not different than v15550/+ controls (p=0.0860). (D) In studies with JF03115, genotype had no overall effect (one-way ANOVA, p=0.3530, n=8).

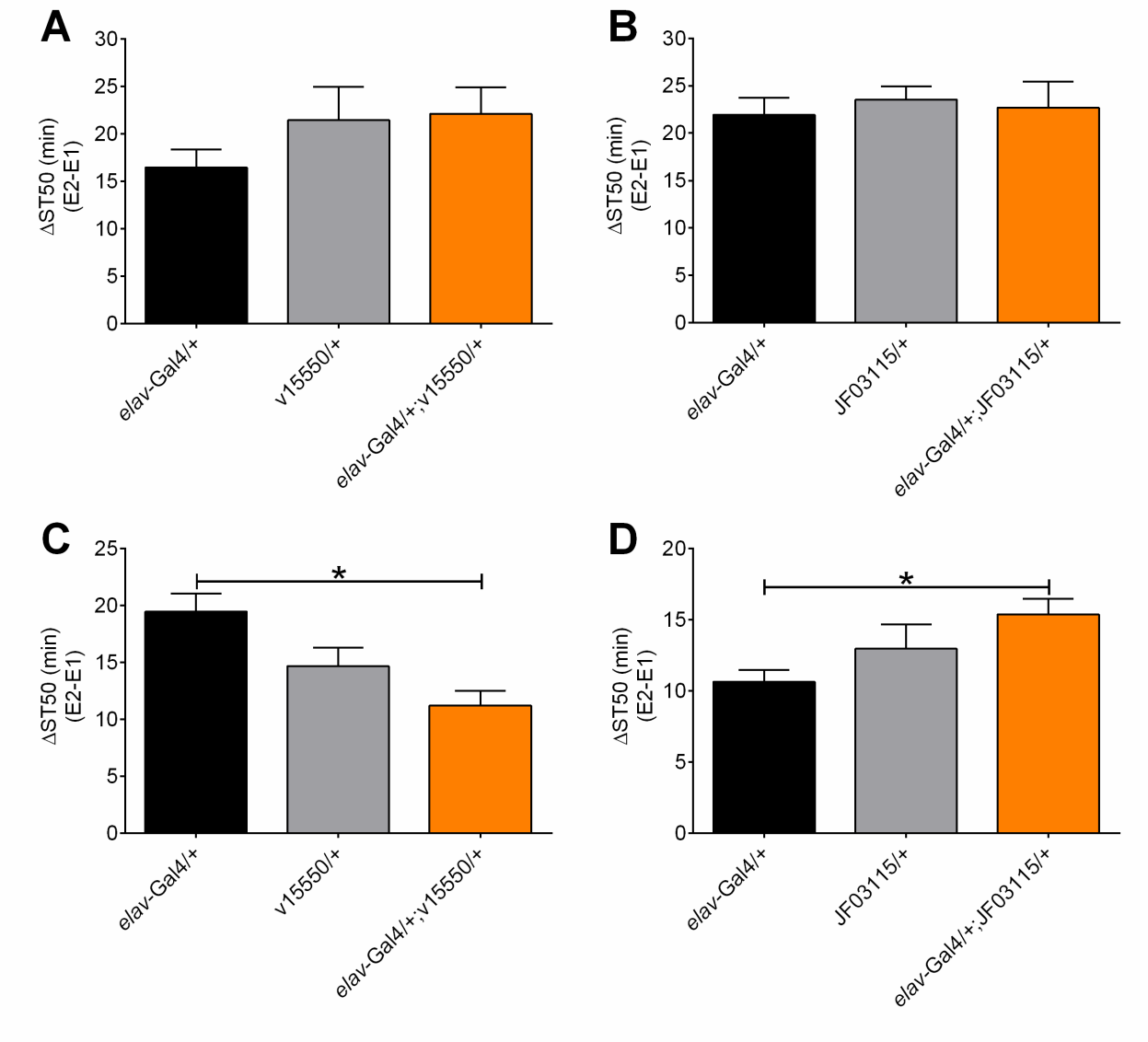


Figure 4.7. Changes in ST50 values, used as a measure of rapid tolerance, are not consistently altered in flies with pan-neuronal expression of *Mef2* RNAi.
Development of rapid tolerance, as measured by the absolute change in ST50 values from E1 to E2, was not significantly altered by expression of *Mef2* RNAi. (A, B) Rapid tolerance in response to exposure of vapor from 85% ethanol. Expression of v15550 (A) or JF03115 (B) in neurons (individual one-way ANOVAs; panel A, p=0.3246; panel B, p=0.8582; n=8). (C, D) Rapid tolerance to sedation from vapor from 100% ethanol. In experiments with v15550, there was a significant overall effect of genotype (one-way ANOVA, p=0.0034, n=8) and *elav*-Gal4/+;v15550/+ flies were different than *elav*-Gal4/+ controls (BMC, p=0.0018), but not different than v15550/+ controls (p=0.2422). (D) In studies with JF03115, genotype also had an overall effect (one-way ANOVA, p=0.0499, n=8) and *elav*-Gal4/+;JF03115/+ flies were different than *elav*-Gal4/+ controls (BMC, p=0.0310), but not different than JF03115/+ controls (p=0.3850).

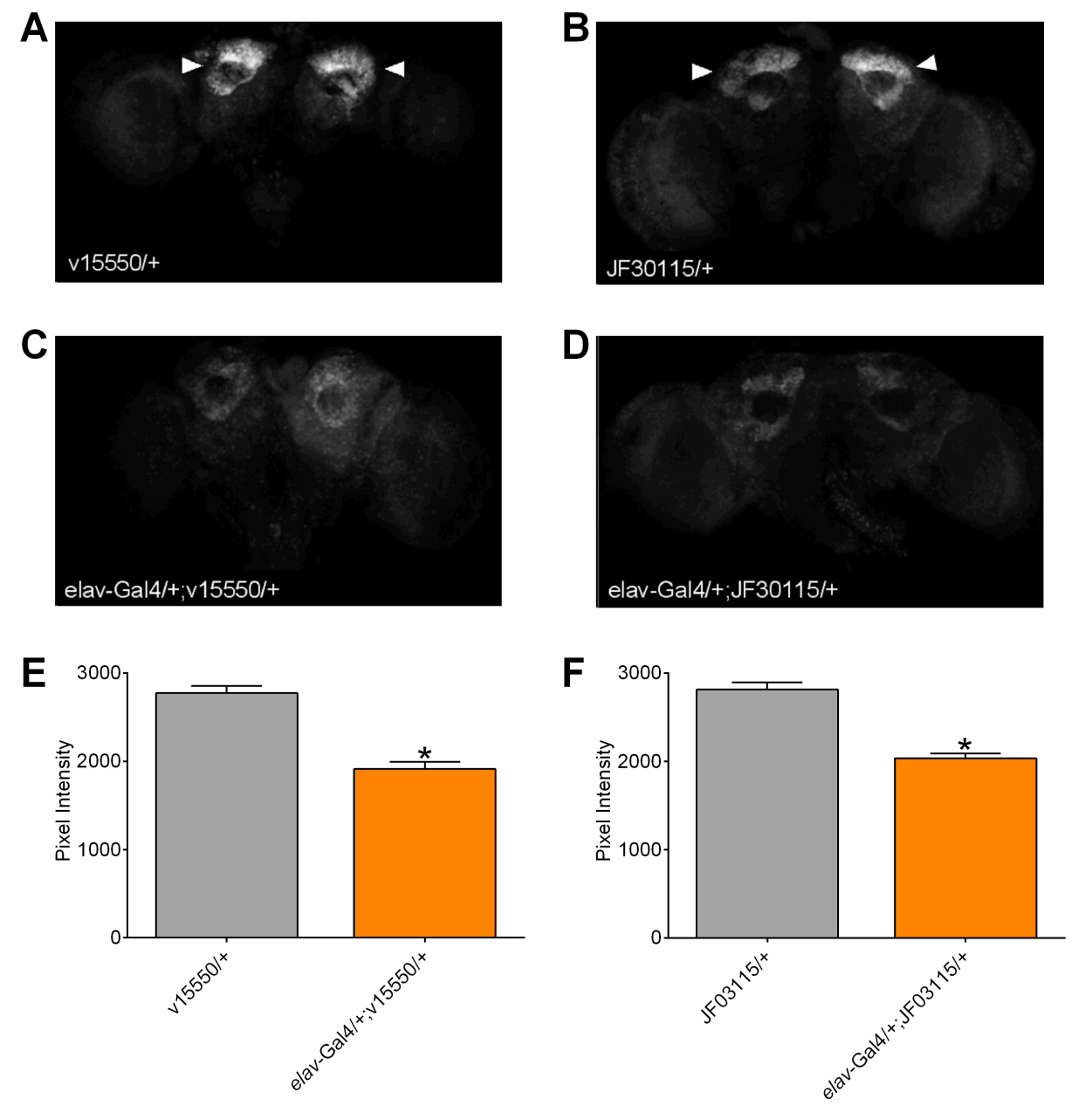


Figure 4.8. Whole brain *Mef2* expression and validation of *Mef2* RNAi transgenes. (A-D) representative (10X) confocal images of whole mount brains immunolabeled with anti-*Mef2*. In control flies (A, v15550/+; B, JF03115/+), expression of *Mef2* was prominent in the calyx of the mushroom bodies (arrowheads) and at lower levels throughout the brain. Detection of *Mef2* appeared to be reduced in flies pan-neuronally expressing *Mef2* RNAi transgenes (C, *elav*-Gal4/+;v15550/+; D, *elav*-Gal4/+;JF03115/+) compared to brains from RNAi transgene control animals (A, B). (E and F) Quantitation of *Mef2* immunolabeling. Pixel intensity was significantly reduced in flies expressing *Mef2* RNAi transgenes (E, *elav*-Gal4/+;v15550/+; F, *elav*-Gal4/+;JF03115/+) compared to their respective RNAi transgene controls (students t-tests; panel E, $p=0.0002$; panel F, $p<0.0001$, $n = 4\text{-}5$).

Quantitation of the *Mef2* signal from all optical sections representing whole brains confirmed that pan-neuronal expression of *Mef2* RNAi transgenes significantly decreased *Mef2* expression (Figure 4.8E and 4.8F). Both of the *Mef2* RNAi transgenes used throughout our studies therefore knockdown expression of *Mef2* in neurons.

To further explore the possibility that *Mef2* plays a role in ethanol sedation, we obtained several previously characterized loss-of-function mutant *Mef2* alleles (*Mef2*$^{25\text{-}34}$ [212], *Mef2*$^{30\text{-}5}$ [213, 214], and *Mef2*^{65} [215] (Fig 4.1), backcrossed them to a control stock (see Methods), and then performed genetic complementation analyses. Flies heterozygous for *Mef2*$^{30\text{-}5}$, *Mef2*^{65}, and *Mef2*$^{25\text{-}34}$, had no detectable change in ST50 values (Figure 4.9). Importantly, *Mef2*$^{30\text{-}5}$/*Mef2*^{65} (Figure 4.9A, time-courses; Figure 4.9C, ST50 values) and *Mef2*$^{25\text{-}34}$/*Mef2*^{65} (Figure 4.9B, time-courses; Figure 4.9D, ST50 values) transheterozygous flies had increased ST50 values compared to all other groups tested. These data confirm that flies with reduced *Mef2* function have increased ST50 values under the conditions used in our experiments.

To identify whether *Mef2* expression in neurons is necessary for normal ethanol sedation during adulthood, v15550 (Fig 4.10A and B) or JF03115 (Fig 4.10C and D) was expressed by neuronal synaptobrevin GeneSwitch (*nsyb*-GS). Expression of the Mef2 RNAi, v15550, by *nsyb*-GS had increased ST50s when treated with RU486 compared to vehicle control (Fig. 4.10A, checkered bars). Importantly, driver and Mef2 RNAi controls did not have differences in ST50 values when treated with either vehicle or RU486 (Fig. 4.10A, first four bars). This result was not able to be reproduced (Fig. 4.10B). Additionally, two experiments testing flies containing the Mef2 RNAi JF03115 and *nsyb*-GS did not have altered ST50s when treated with RU486 or vehicle (Fig.

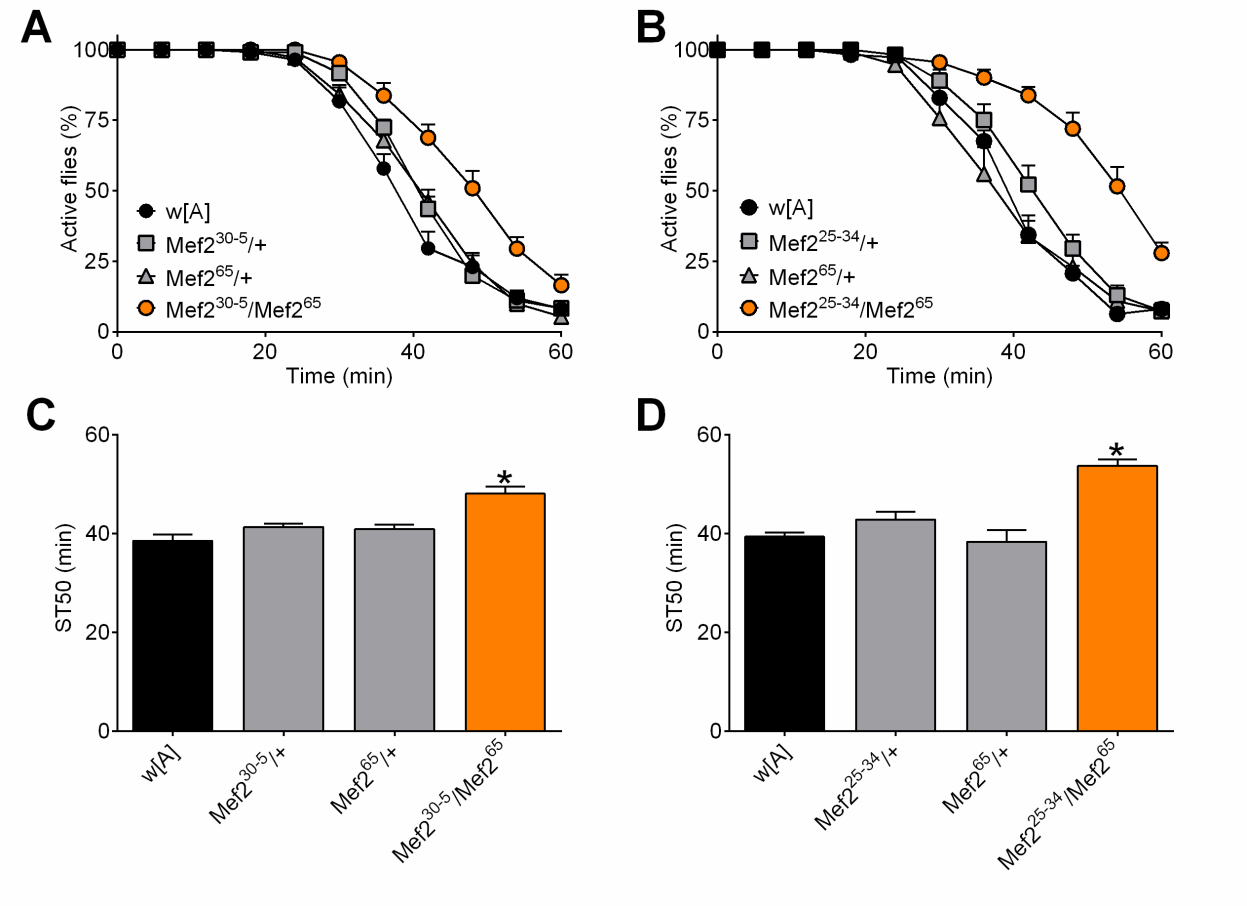


Figure 4.9. *Mef2* mutants are resistant to ethanol sedation. (A and B) Ethanol sedation time courses of control flies (w[A]) and the indicated *Mef2* genotypes. Compound heterozygous *Mef2* mutants *Mef2*$^{30\text{-}5}$/*Mef2*^{65} (A) and *Mef2*$^{25\text{-}34}$/*Mef2*^{65} (B) took longer to become sedated than flies heterozygous for these alleles. (C, D) ST50 values, derived from the data in panels A and B, respectively. There was a significant overall effect of *Mef2* genotype on ST50 values (individual one-way ANOVAs; panel C, $p<0.0001$; panel D, $p<0.0001$; $n=10$). ST50 values were greater in *Mef2*$^{30\text{-}5}$/*Mef2*^{65} (C) and *Mef2*$^{25\text{-}34}$/*Mef2*^{65} (D) flies than in all other genotypes (*BMCs; panel C, $p\leq0.0003$; panel D, $p<0.0001$)

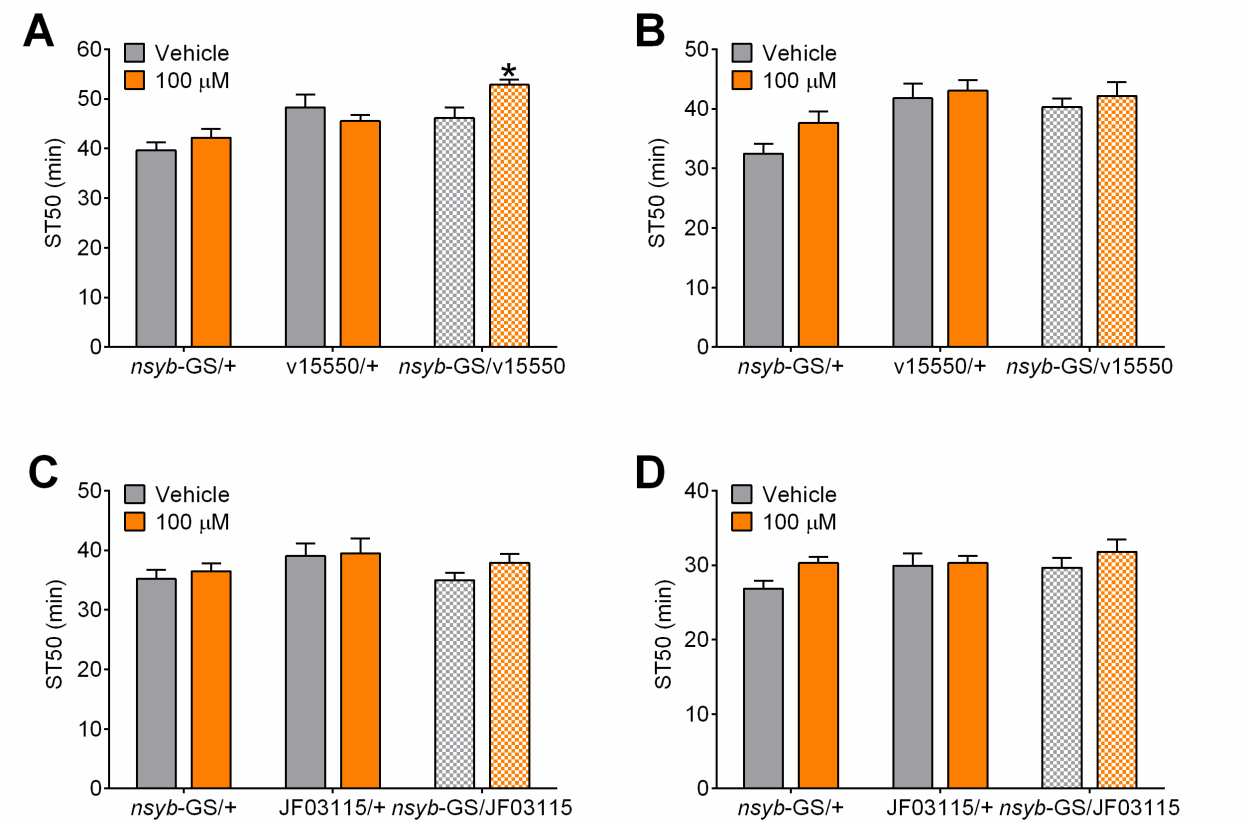


Figure 4.10. *Mef2* RNAi adulthood expression by neuronal synaptobrevin GeneSwitch does not alter ethanol sedation. Female flies were treated with vehicle or 100 μM for 6 days. (A) ST50 values of flies with *nsyb*-GS expression of Mef2 RNAi, (*nsyb*-GS/v15550) specifically during adulthood, had increased values compared to vehicle control, whereas controls (nsyb-GS/+ and v15550/+) did not have a difference in ST50 when treated with vehicle or RU486 (two-way ANOVA; treatment---genotype, p=0.0403; genotype, p<0.0001; *BMC 100 μM versus Vehicle; *nsyb*-GS/+, p=0.9768; v15550/+, p=0.8676; *nsyb*-GS/v15550, p=0.0344; n=8). (B) Results from panel A did not reproduce. ST50 values of flies with *nsyb*-GS expression of Mef2 RNAi, (*nsyb*-GS/v15550) and controls (nsyb-GS/+ and v15550/+) did not have a difference in ST50 when treated with vehicle or RU486 (two-way ANOVA; genotype, p=0.0009; *BMC 100 μM versus Vehicle; *nsyb*-GS/+, p=0.1986; v15550/+, p>0.9999; *nsyb*-GS/v15550, p>0.9999; n=8). (C) ST50 values of flies with *nsyb*-GS expression of Mef2 RNAi, (*nsyb*-GS/JF03115) and controls (nsyb-GS/+ and JF03115/+) did not have a difference in ST50 when treated with vehicle or RU486 (two-way ANOVA; n.s.; n=8). (D) Results from panel C were reproduced where expression of Mef2 RNAi in neurons during adulthood did not alter ethanol sedation. ST50 values of flies with *nsyb*-GS expression of Mef2 RNAi, (*nsyb*-GS/JF03115) and controls (nsyb-GS/+ and JF03115/+) did not have a difference in ST50 when treated with vehicle or RU486 (two-way ANOVA; n.s.; n=8) (sedation performed by B.C.S.)

4.10C and 4.10D, checkered bars). These data suggest that Mef2 expression during adulthood is not required for ethanol sedation.

C. Discussion

The overarching goal of this study was to explore molecular-genetic underpinnings of behavioral responses to alcohol by (i) using human genetic analyses to identify candidate genes that might influence SRE and then (ii) testing the role of *Drosophila* orthologues of those human candidate genes in fly ethanol sedation and rapid tolerance. We chose to focus our studies on human SRE scores and fly ethanol sedation/rapid tolerance in part because these measures are well established responses to ethanol exposure in the respective species[56, 116, 201, 216] and both measures have demonstrated value for identifying genes that influence alcohol abuse, dependence or other aspects of alcohol abuse[17, 77, 78, 116, 216-221]. Additionally, both the SRE in humans and ethanol sedation/rapid tolerance in flies are behavioral responses to acute ethanol exposure, raising the possibility that our approach could uncover evolutionarily conserved mechanisms driving the behaviors in humans and flies.

Our SRE GWAS in the ALSPAC sample found 37 candidate genes with $p_{gene} < 0.001$. We prioritized our examination of fly orthologues of these genes based on 3 criteria: reported roles of the candidate genes in human phenotypes that might predict roles in ethanol behavior, presence of obvious orthologous genes in the fly genome, and availability of *Drosophila* RNAi reagents to manipulate gene expression. A total of 6 human and 6 orthologous fly genes met these criteria: *APP/Appl, ATG5/Atg5, GPD2/Gpo1, ISL1/tup, MEF2B/Mef2* and *PCDH15/Cad99C*. We consequently explored the role of the fly orthologues in ethanol sedation and rapid tolerance.

Flies with constitutive expression of RNAi targeting *Mef2* in neurons, as well as flies with loss of function mutations in *Mef2*, were resistant to ethanol sedation in our experiments. Decreased function of *Mef2* did not impact internal ethanol levels, indicating that *Mef2* influences the pharmacodynamic response to ethanol. Interestingly, decreased function of *Mef2* did not alter rapid tolerance to ethanol, suggesting that *Mef2* might influence acute sedation, but not tolerance, under the conditions used in our studies. Importantly, our studies on *Mef2* used 3 different RNAi transgenes with 2 different target sequences and 3 independent loss-of-function alleles, all confirmed at the protein or DNA level, collectively demonstrating a role for *Mef2* in fly ethanol sedation.

Another group also recently reported that *Mef2* plays a role in *Drosophila* alcohol-related behavior. In contrast to our results, their studies indicate that constitutive neuronal expression of RNAi against *Mef2* or expression of a *Mef2* dominant negative alters rapid tolerance to ethanol, but not necessarily ethanol sedation[208]. We noted that the ST50 values were somewhat shorter in the previous report when compared to our results, but increasing the ethanol concentration to the maximum possible in our studies (thereby shortening the ST50) did not meaningfully alter our conclusions. Additional experiments will therefore be required to address whether the differences between the previously published and our results might be explained by differences in behavioral paradigms, genetic backgrounds, or environmental conditions. Importantly, though, both studies demonstrate that *Mef2* influences behavioral responses to acute ethanol exposure in flies, thereby mutually reinforcing the other.

The human *MEF2* family of genes has 4 members (*MEF2*A, *MEF2B*, *MEF2*C and *MEF2*D) whereas the fly genome contains only a single orthologue (*Mef2*). The primary amino acid sequence of fly *Mef2* protein has similar overall levels of conservation (32 to 43 percent identical, 43 to 56 percent similar[89]) relative to all of the human *MEF2* proteins. All gene products are known or predicted to be transcription factors[88, 222] and are involved in numerous biological processes (e.g., muscle differentiation[90, 91], human disease/cancer[92, 93], neuronal differentiation[94-96], and others. Of the *MEF2* family members within our ALSPAC SRE gene-based analyses, *MEF2B* was the most robustly implicated (p_{gene}=0.0007). Additionally, though, our analyses suggested that *MEF2A* might also be involved (p_{gene}=0.02), whereas *MEF2C* and *MEF2D* were not associated with SRE scores in our studies (p>0.05). None of the *MEF2* family members have, to our knowledge, met stringent genome-wide significance criteria for associations with alcohol-related outcomes in other human GWAS. Interestingly, though, using the recently developed online tool GWAS ATLAS (atlas.ctglab.nl[223]), which processes GWAS results through MAGMA to derive gene-based summary statistics, we identified nominal associations (p<0.05) between all four human *MEF2* genes and alcohol outcomes. Furthermore, *MEF2B* is associated with alcohol consumption in a GWAS study[196] and a secondary analysis of GWAS summary statistics using the PheWAS option in the online GWAS ATLAS (http://atlas.ctglab.nl/PheWAS) revealed that aggregate variation in *MEF2B* is associated with changes in alcohol use from 10 years prior in UK Biobank data. The PheWAS tool and literature searches reveal associations between alcohol-related outcomes and other human MEF2 genes as well: *MEF2A* was associated with alcohol intake frequency in UK Biobank, *MEF2C* was associated with

alcohol dependence symptom counts in COGA[224], and both *MEF2C* and *MEF2D* were associated with a range of alcohol-related outcomes in UK Biobank including alcohol intake frequency, drinking status, and amount of alcohol consumed on drinking days. These diverse associations—in conjunction with the results of the current study—suggest that multiple or perhaps even all members of the *MEF2* family might impact variation in alcohol outcomes. Together, our studies in humans and flies, in combination with previous studies in humans, suggest that *MEF2* family members might impact initial sensitivity to ethanol, thereby influencing the risk for abusing the drug.

As transcription factors, *MEF2* family members presumably influence alcohol-related behaviors principally by regulating expression of other genes. Adhikari and colleagues recently reported that *Mef2* induces the expression of the immediate early gene Hr38, thereby regulating rapid tolerance in flies [208]. Additional *Mef2* target genes might also be important for ethanol behaviors. For example, Sivachenko and colleagues identified 342 genes with *Mef2*-dependent expression in flies[225]. Fifteen of these 342 genes (~3.5-fold more than expected by chance, Table 4.3) are known to influence behavioral responses to ethanol in *Drosophila*, suggesting that genes regulated by *Mef2* family members might be enriched for genes that influence alcohol-related behavior. A more comprehensive characterization of *Mef2*-regulated gene expression could therefore be a gateway for better understanding mechanisms underlying a variety of behaviors in response to alcohol exposure.

Beyond studies on alcohol, *Mef2* genes are important for the effects of cocaine on neuronal morphology and behavioral responses. Cocaine increases dendritic spine density in the mouse nucleus acumbens via a process that requires reductions in *Mef2*

protein activity, and behavioral sensitization to cocaine requires an increase in *Mef2* protein activity[226]. Combined with our findings, these results raise the possibility that *Mef2* proteins and their target genes might have conserved roles in behavioral and other types of responses to multiple drugs of abuse. A more comprehensive understanding of *Mef2* in alcohol behavior could therefore have important implications for risk assessment for—and potentially treatment of—substance abuse more broadly.

Table 4.3. Overlapping genes from previously indicated genes involved in *Drosophila* alcohol behavior and *Mef2* target genes in adult brains.

Fly Gene Symbol[1]	Fly Gene Identifier	Fly Annotation Symbol	Orthologous Human Gene Symbol[2]	Human Gene ID	DIOPT Score	Weighted Score
dorsal	FBgn0260632	CG6667	RELA	9955	9	8.73
			REL	9954	8	7.7
			RELB	9956	6	5.93
DopEcR	FBgn0035538	CG18314	GPR52	4508	2	1.93
			GPR21	4476	2	1.88
Mef2	FBgn0011656	CG1429	MEF2A	6993	12	11.79
			MEF2C	6996	11	10.89
			MEF2D	6997	11	10.84
			BORCS8-MEF2B	39979	4	4.04
			MEF2B	6995	4	3.93
Bx	FBgn0265598	CG44425	LMO1	6641	9	8.84
			LMO3	6643	8	7.89
			LMO2	6642	4	4
Fas2	FBgn0000635	CG3665	NCAM2	7657	11	10.8
			NCAM1	7656	10	9.65
InR	FBgn0283499	CG18402	IGF1R	5465	12	11.83
			INSR	6091	10	9.88
			INSRR	6093	9	8.85
mys	FBgn0004657	CG1560	ITGB1	6153	13	12.84
			ITGB2	6155	9	8.85
			ITGB7	6162	9	8.85
			ITGB3	6156	8	7.89
			ITGB5	6160	6	5.84
			ITGB6	6161	5	4.91
			ITGB4	6158	5	4.85
			ITGB8	6163	4	3.94

			ITGBL1	6164	2	2
Pdk1	FBgn0020386	CG1210	PDPK1	8816	11	10.74
			PDPK2P	49897	5	5.04
puc	FBgn0243512	CG7850	DUSP10	3065	9	8.73
			DUSP8	3074	4	3.99
			DUSP16	17909	4	3.99
			DUSP14	17007	3	3.04
			DUSP18	18484	3	3.04
			DUSP28	33237	3	2.97
			SSH3	30581	3	2.94
			SSH2	30580	3	2.94
			DUSP4	3070	3	2.94
			DUSP1	3064	3	2.94
			DUSP6	3072	2	2.04
			DUSP7	3073	2	2.04
			DUSP21	20476	2	2.01
			DUSP2	3068	2	1.91
			DUSP27	25034	2	1.91
			DUSP26	28161	2	1.91
			SSH1	30579	2	1.91
			STYX	11447	2	1.91
			DUSP22	16077	2	1.91
			DUSP5	3071	2	1.91
			DUSP12	3067	2	1.91
			DUSP3	3069	2	1.91
			DUSP19	18894	2	1.91
			DUSP15	16236	2	1.91
			DUPD1	23481	2	1.91
			DUSP13	19681	2	1.91
w	FBgn0003996	CG2759	ABCG2	74	6	5.89
			ABCG4	13884	5	4.84

			ABCG1	73	4	3.83
			ABCG8	13887	4	3.81
			ABCG5	13886	3	2.91
aret	FBgn0000114	CG31762	CELF2	2550	11	10.89
			CELF1	2549	11	10.84
			CELF6	14059	4	3.94
			CELF4	14015	3	2.91
			CELF5	14058	3	2.91
			CELF3	11967	2	1.9
bun	FBgn0259176	CG42281	TSC22D1	16826	10	9.88
			TSC22D2	29095	5	4.95
			TSC22D4	21696	5	4.87
			TSC22D3	3051	4	3.97
CG42389	FBgn0259735	CG42389	FNDC3A	20296	12	11.79
			FNDC3B	24670	10	9.76
cpo	FBgn0263995	CG43738	RBPMS	19097	8	7.75
			RBPMS2	19098	7	6.8
dunce	FBgn0000479	CG32498	PDE4D	5144	11	10.74
			PDE4B	5142	11	10.69
			PDE4A	5141	10	9.72
			PDE4C	5143	8	7.78

[1] Mef2 target genes were elucidated in the following article: “The transcription factor Mef2 links the Drosophila core clock to Fas2, neuronal morphology, and circadian behavior” Sivachenko, et al, 2013.
[2] Human orthologous genes and scores come from DIOPT (https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl).

Chapter 5: Nitric oxide synthase, and nitric oxide signaling, in *Drosophila* ethanol sedation

A. Introduction

Nitric oxide synthase (Nos) is an enzyme that converts L-Arginine to Citrulline and NO. Mammals contain neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) and its product, NO, has been demonstrated be involved in signaling as a neurotransmitter, regulation of blood vessels[97], and regulating human disease states[98]. Signaling of NO to activate soluble guanaylyl cyclase (sGC) is the canonical NO signaling pathway. NO is able to readily able to diffuse through cell membranes to subsequently activate sGC. Once sGC is activated, cyclic GMP (cGMP) is produced, resulting in downstream signaling effects [227, 228]. Other signaling pathways (S-nitrosation) via NO are also important for proper regulation of cellular processes and the development of human disease states[229].

A few rodent studies have demonstrated the ability of NOS to influence alcohol-related behaviors[103-105]. These studies substantiate the need for further investigation into the mechanism NO signaling in alcohol-related behaviors. The long term goal would be to use the NO signaling mechanistic aspects as potential new treatment options for those who suffer from alcohol use disorder. We therefore used *Drosophila melanogaster* to address multiple questions about Nos, and NO signaling, in fly alcohol-related behaviors. *Drosophila melanogaster* has a single ortholog and is most highly

conserved with NOS1 but shares similarity to NOS3 and NOS2[89]. Additionally, fly Nos has been shown to be involved in numerous processes in the fly, such as imaginal disc regeneration[100], axon pruning and regrowth[101], and immune responses[102].

We initially set out to determine if Nos plays a role in ethanol-related behaviors, address what tissue and timing of interest in which Nos expression is required, and to determine the mechanism of action that NO signaling is influencing to regulate ethanol sedation. Our preliminary research in flies suggest that knockdown of Nos results in increased ethanol sensitivity. RNAi results have not elucidated a tissue of interest, but expression of Nos RNAi ubiquitously during adulthood also results in increased ethanol sedation. These data suggest a specific role for Nos and NO signaling in ethanol sedation and specifically for a role during an adulthood process in flies.

B. Results

The *Drosophila* Nos gene is located on chromosome 2L, where there are two genes closely flanking the Nos transcriptional region and two genes located within Nos (Fig. 5.1). We obtained multiple reagents to assess the effect of the reagents targeting Nos on ethanol-related behaviors. Fig. 5.1A highlights the locations of the Nos transposon insertions or null lines (blue boxes) that are used in the study, while Fig. 5.1B highlights the targeted sequences by Nos RNAi lines (purple boxes). Reagents used are all listed in Table 5.1. A previous study in our lab generated Nos as a possible candidate gene for influencing ethanol sedation when in interaction with another gene of interest. Results from this previous study did not suggest that Nos and the gene of

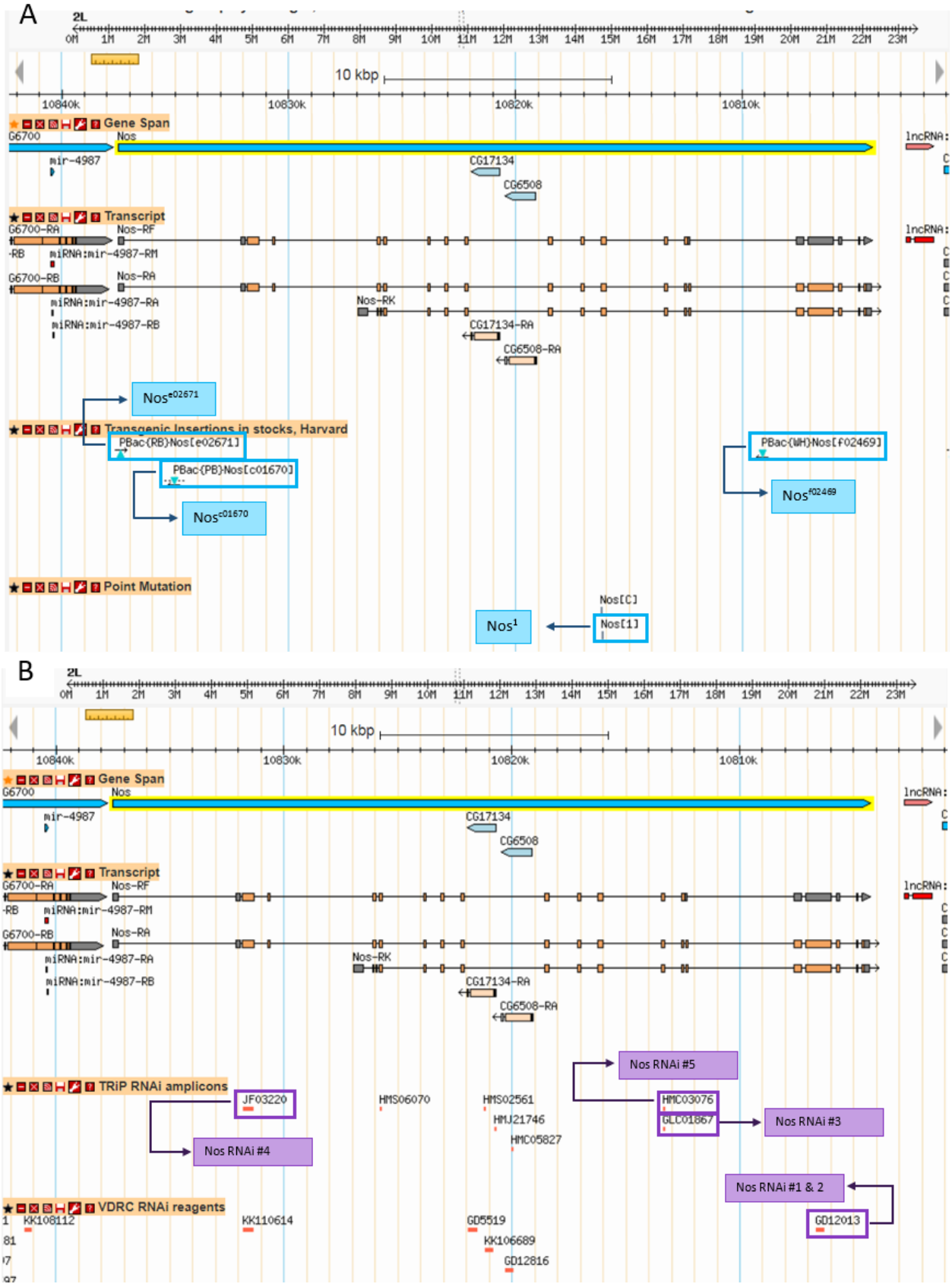

A
2L
10 kbp
10840k
10830k
10820k
10810k
Gene Span
G6700
Nos
mir-4987
CG17134
CG6508
lncRNA:
Transcript
G6700-RA
Nos-RF
-RB
miRNA:mir-4987-RM
G6700-RB
Nos-RA
miRNA:mir-4987-RA
miRNA:mir-4987-RB
Nos-RK
CG17134-RA
CG6508-RA
Nos^e02671
Transgenic Insertions in stocks, Harvard
PBac{RB}Nos[e02671]
PBac{PB}Nos[c01670]
Nos^c01670
PBac{WH}Nos[f02469]
Nos^f02469
Point Mutation
Nos[C]
Nos[1]
Nos^1
B
2L
10 kbp
10840k
10830k
10820k
10810k
Gene Span
G6700
Nos
mir-4987
CG17134
CG6508
lncRNA:
Transcript
G6700-RA
Nos-RF
-RB
miRNA:mir-4987-RM
G6700-RB
Nos-RA
miRNA:mir-4987-RA
miRNA:mir-4987-RB
Nos-RK
CG17134-RA
CG6508-RA
Nos RNAi #5
TRiP RNAi amplicons
JF03220
HMS06070
HMS02561
HMJ21746
HMC05827
HMC03076
GLC01867
Nos RNAi #3
Nos RNAi #4
Nos RNAi #1 & 2
VDRC RNAi reagents
KK108112
KK110614
GD5519
KK106689
GD12816
GD12013

Figure 5.1. Nos transcriptional region (FlyBase). Image shows the location of the Nos gene and the subsequent transcripts of the gene. The Nos mutants used in this study are boxed in blue (A). Locations of Nos RNAi target sequences are boxed in purple (B).

Table 5.1. Reagents used to investigate Nos in *Drosophila* ethanol-related behaviors.

Label	Stock Number	Chromosome Insertion	Genotype	Acquired	Notes
Nos RNAi Stocks					
Nos RNAi #1	v27722	X	w[1118] P{GD12013}v27722	VDRC	Lethal when expressed by *repo*-Gal4
Nos RNAi #2	v27725	2	w[1118]; P{GD12013}v27725	VDRC	Lethal when expressed by *repo, da, & Mef2*-Gal4
Nos RNAi #3	57700	2	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01867}attP40	BDSC	
Nos RNAi #4	28792	3	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03220}attP2	BDSC	
Nos RNAi #5	50675	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03076}attP2	BDSC	
Nos RNAi #6	v27722[wA]	X	w[1118] P{GD12013}v27722	Schmitt BX	
Nos RNAi #7	v27725[wA]	2	w[1118]; P{GD12013}v27725	Schmitt BX	
Nos RNAi #8	57700 [1495]	2	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01867}attP40	Schmitt BX	
Nos RNAi #9	28792 [1495]	3	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03220}attP2	Schmitt BX	
Nos RNAi #10	50675 [1495]	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03076}attP2	Schmitt BX	
Nos RNAi #11	NEW 50675	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03076}attP2	BDSC	
Nos Transposon Elements and Null Stocks					
Nos^{e02671}	e02671(wA)	2	PBac{RB}Nose02671	EHMS/LL BX	
Nos^{c01670}	c01670(wA)	2	PBac{PB}Nosc01670	EHMS/LL BX	
Nos^{f02469}	f02469(wA)	2	PBac{WH}Nosf02469	EHMS/LL BX	
Nos^{1}	Nos^{1} (wA)	2	w[1118]; Nos[1]/CyO, P{w[+mC]=ActGFP}JMR1	BDSC/Schmitt BX	
UAS-Nos Stocks					
UAS-Nos #1	56829	X	P{UAS-Nos.L}1, w*	BDSC	
UAS-Nos #2	56823	2	w^{1118}; P{UAS-Nos.L}2	BDSC/Schmitt BX	
UAS-Nos #3	UAS-Nos3;UAS-Nos5	II on 2 & I on 3		Yakubovich	

sGC RNAi Stocks					
sGC RNAi #1	v107074	2	P{KK102774}VIE-260B	VDRC	Lethal when expressed by *da, elav, & Mef2*-Gal4
sGC RNAi #2	v100706	2	P{KK108015}VIE-260B	VDRC	
sGC RNAi #3	64009	2	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ30322}attP40	BDSC	
sGC RNAi #4	60876	2	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22589}attP40	BDSC	
sGC RNAi #5	28748	3	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03176}attP2	BDSC	
sGC RNAi #6	28786	3	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03214}attP2	BDSC	
sGC RNAi #7	36817	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01038}attP2	BDSC	
Miscellaneous Stocks					
E75 RNAi	35780 (1495)	2	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01530}attP40	BDSC	
Fdh RNAi	34937	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01268}attP2	BDSC	
UNF RNAi	39032 (1495)	3	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01951}attP2	BDSC	
y^1w^1	1495		y[1]w[1]	BDSC	

BX = Backcrossed for 7 generations. LL BX = Lara Lewellyn performed backcross

interest were genetically interacting to influence ethanol sedation. The reagents that were obtained and used to investigate Nos caused an increased ethanol sedation, and subsequently, became a candidate gene of interest for further investigation. The reagents that were originally obtained were three individual Nos piggyBac transposon insertions and were assessed for their ability to alter Nos expression and their ability to influenced ethanol sedation. Nos^{e02671}, females (left panel) and males (right panel), had decreased ST50 values, or increased sensitivity to alcohol, compared to their genetic background controls (Fig. 5.2A). The two additional transposon insertions, Nos^{c01670} and Nos^{f02469}, also had significantly decreased ST50s compared to control (Fig. 5.2B). Additionally, as part of the screen it was determined that the three Nos transposons insertions significantly decreased the relative Nos expression as found by qRT-PCR results (Fig. 5.2C).To further characterize how the Nos transposon insertions influenced ethanol sedation, locomotor behavior was assessed. All three of the Nos transposon insertions did not differ in locomotor behavior compared to their genetic background control, but all groups were different from the positive locomotor defect control (w[A] 4 min Vortex) (Fig. 5.2D). Also, it is possible that the Nos transposon insertions alter ethanol sedation via changes in ethanol uptake/metabolism, therefore internal ethanol was measured in the three Nos transposon insertion lines and control flies. There were no consistent changes in internal ethanol levels of the Nos transposon insertions and their genetic background control (Fig. 5.2E). These studies suggest that Nos is important for normal ethanol sedation in *Drosophila melanogaster* and that locomotor defects or alterations in internal ethanol uptake/metabolism are not the cause for the Nos transposon insertions increased ethanol sensitivity phenotype.

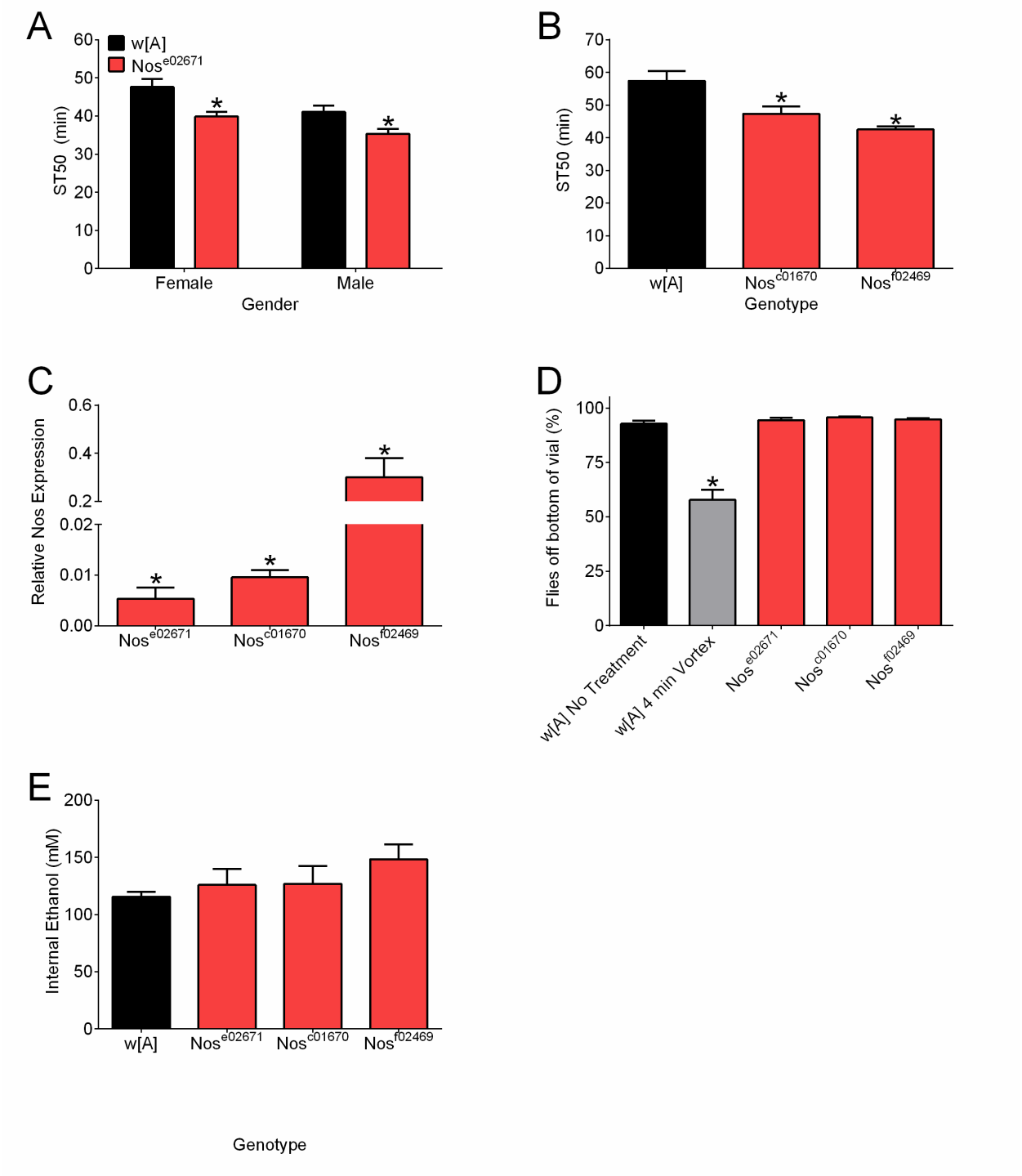

A
w[A]
Nos e02671
ST50 (min)
Female
Male
Gender
B
ST50 (min)
w[A]
Nos c01670
Nos f02469
Genotype
C
Relative Nos Expression
Nos e02671
Nos c01670
Nos f02469
D
Flies off bottom of vial (%)
w[A] No Treatment
w[A] 4 min Vortex
Nos e02671
Nos c01670
Nos f02469
E
Internal Ethanol (mM)
w[A]
Nos e02671
Nos c01670
Nos f02469
Genotype

Figure 5.2. Characterization of Nos p-element insertions. Sedation was performed using 70% ethanol. (A) Overall, genotype and gender affected ST50s but not its interaction (two-way ANOVA; genotype, p=0.0002; gender, p=0.0016; interaction, p=0.5357). Female and male Nos^{e02671} flies had decreased ST50s when compared to their corresponding female and male genetic background control, w[A] (*BMC versus w[A]; Female, p=0.0035; Male, p=0.0345; n=10). (B) Genotype caused an overall effect on ST50 values determining ethanol sedation with additional Nos mutants (one-way ANOVA; p=0.0003). Female Nos^{c01670} and Nos^{f02469} had increased ethanol sedation compared to controls (*BMC verses w[A]; Nos Mutant #2, p=0.0077; Nos Mutant #3, p=0.0001; n=10). (C) Nos transposon insertions did have significantly reduced levels of relative Nos expression (*individual two-tailed t tests, different from 1; Nos^{e02671}, p<0.0001; Nos^{c01670}, p<0.0001; Nos^{f02469}, p=0.0031; n=3-4) (Performed by Lara Lewellyn). (D) There was an overall effect of treatment on locomotor behavior (one-way ANOVA; p<0.0001). Positive locomotor defect control flies, w[A] 4 min vortex had a significant difference in locomotor values to all other groups (*BMC verses w[A] 4 minute vortex; w[A], p<0.0001; Nos^{e02671}, p<0.0001; Female Nos^{c01670}, p<0.0001; Nos^{f02469}, p<0.0001; n=8), whereas the Nos transposon insertions are not different from the w[A] no treatment group (Performed by Ian Hines). (E) Overall, internal ethanol levels were not affected by genotype when comparing Nos transposon insertions to control, w[A] (one-way ANOVA; p=0.1182; n=5-15) (Performed by Jena Butler).

One particularly challenging aspect of using piggyBac transposon insertions is that it has been previously demonstrated that the transposons are able to influence genes within a 100 kb range of that insertion[230]. Therefore the data above make it challenging to say that the effects on Nos gene expression is the sole cause of the altered ethanol sedation phenotype. To address this, we obtained a Nos null allele (Nos^{1})[101]. The point mutation changes the control sequence of TGG, encoding tryptophan (Fig. 5.3A) to TGA, encoding a premature stop codon (Fig. 5.3B). The premature stop codon occurs in the reductase domain NOS, which is required for the enzyme to convert L-Arginine to L-Citruline and NO[97]. Nos^{1} was backcrossed to Nos^{e02671} (which had been backcrossed to w[A]). Using Nos^{e02671}, marked with w^{+} (red eyes) allowed for tracking of the Nos^{1} allele (no marker) via eye color throughout the generations. After backcrossing, the ability to maintain the point mutation was confirmed via sequencing (Fig. 5.3A and 5.3B). To determine whether the Nos^{1} influenced ethanol sedation, an ethanol dose response was performed. In three of the five concentrations of ethanol used (55%, 70%, and 85%), Nos^{1} homozygous flies had decreased ST50s compared to controls (Fig. 5.3C). These results strongly implicate Nos as playing an important role in normal ethanol sedation behavior in flies.

To further characterize Nos^{1}, homozygous Nos^{1} male and female flies were exposed to 55% ethanol vapor and their ethanol sedation determined. Both males and females had a decrease in ST50 values compared to controls (Fig. 5.4A, time course and Fig 5.4B, ST50s obtained from panel A). To determine whether Nos^{1} had locomotor defects, locomotor behavior was assessed. There were no differences in locomotor behavior of the Nos^{1} flies compared to control flies, but the positive control flies had a

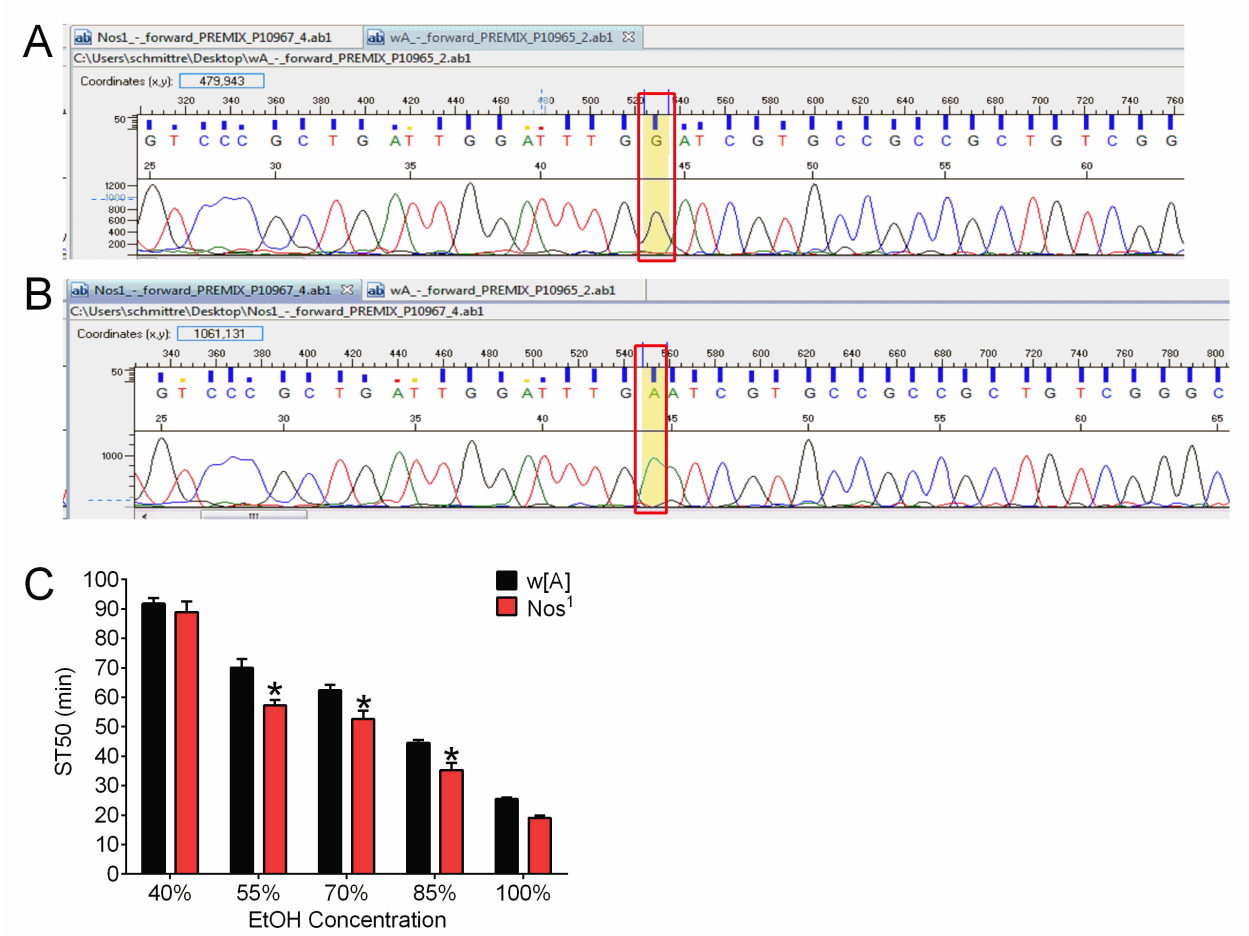


Figure 5.3. Sequencing confirmation of Nos null mutation and its effect on ethanol sedation. (A) Control, w[A] (Nos Mutant #4 was backcrossed to Nos^1 for tracking of eye color; Nos^1 was previously backcrossed to w[A]), sequencing of region containing location of the Nos null point mutation; TG**G** > Tryptophan (red box). (B) Confirmation of retained Nos null point mutation through 7 generations of backcrossing; TG**A** > Stop (red box). (C) For the dose response of Nos^1 and controls, w[A], there was a significant overall effect of genotype and ethanol concentration on ST50 values, but not for the interaction (two-way ANOVA; genotype, $p<0.0001$; ethanol concentration, $p<0.0001$; interaction, $p=0.2086$). There was a significant decrease in ST50 values of Nos^1 when compared to control, w[A], flies with most ethanol concentrations except when 40% and 100% ethanol concentrations were used (*BMC versus w[A]; 40%, $p>0.9999$; 55%, $p=0.0004$; 70%, $p=0.0120$; 85%, $p=0.0174$; 100%, $p=0.2137$; n=8).

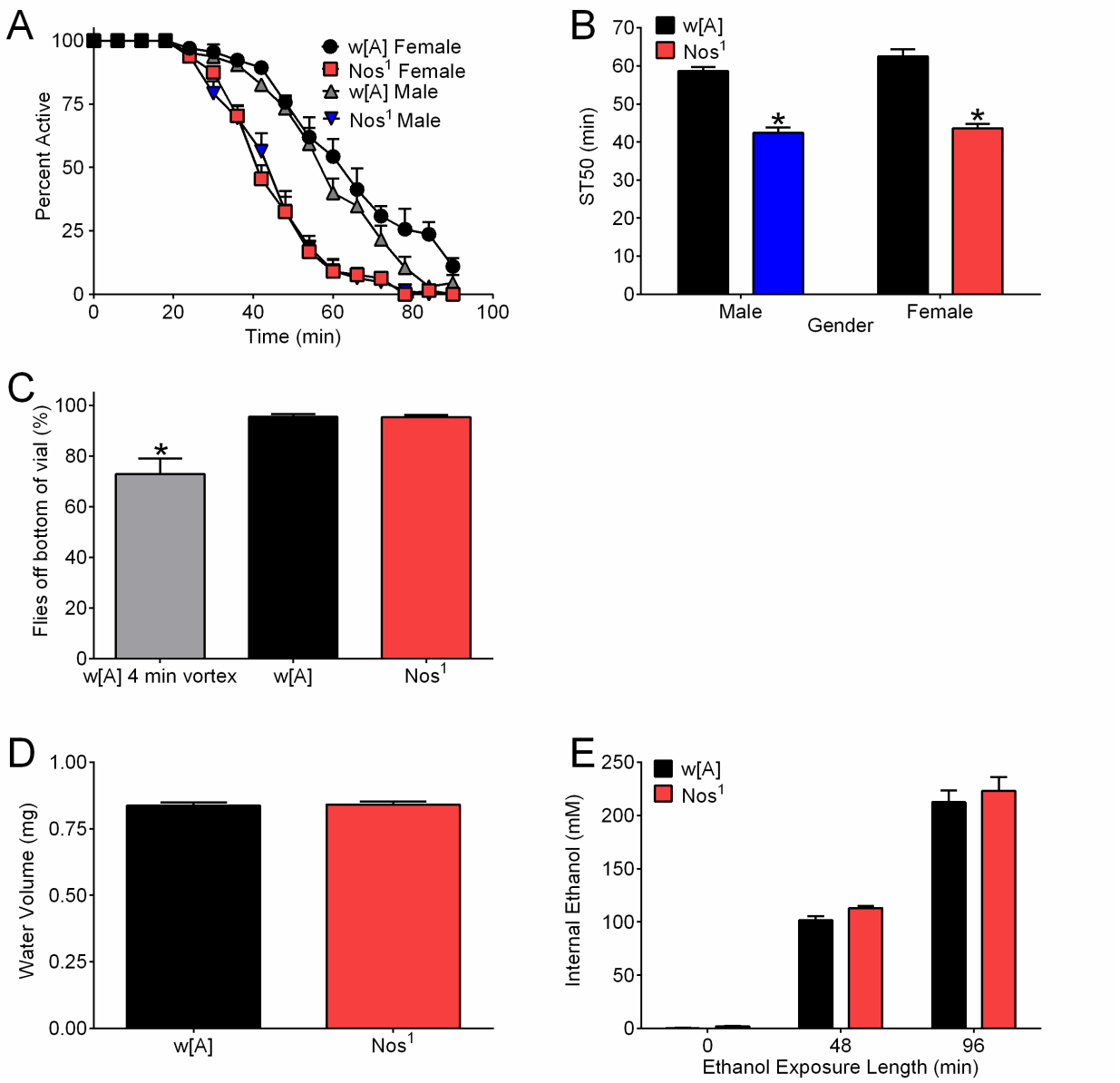

A
Percent Active
Time (min)
w[A] Female
Nos1 Female
w[A] Male
Nos1 Male
B
ST50 (min)
w[A]
Nos1
Male
Female
Gender
C
Flies off bottom of vial (%)
w[A] 4 min vortex
w[A]
Nos1
D
Water Volume (mg)
w[A]
Nos1
E
Internal Ethanol (mM)
w[A]
Nos1
Ethanol Exposure Length (min)

Figure 5.4. Ethanol sedation, locomotor, and internal ethanol characterization of Nos null. Flies were exposed to 55% ethanol. (A) Time-course of Nos^1 male and female flies and control, w[A], male and female flies. (B) ST50 values derived from panel A. Genotype had an overall effect on ST50 values, but there was no effect of gender or interaction (two-way ANOVA; genotype, $p<0.0001$; gender, $p=0.0743$; interaction, $p=0.3369$). Nos^1 flies, both male and female, had decreased ST50s compared to controls, w[A] (*BMC versus w[A]; male, $p<0.0001$; female, $p<0.0001$; n=12). (C) An overall effect of treatment was seen in flies exposed to locomotor testing (one-way ANOVA; $p=0.0002$). w[A] and Nos^1 females are significantly different from the locomotor positive control flies, w[A] 4 min vortex, but not each other (*BMC versus w[A] 4 min vortex; w[A], $p=0.0006$; Nos^1, $p=0.0007$; versus w[A]; Nos^1, $p>0.9999$; n=8). (D) The water content of Nos^1 female flies was not different from control, w[A], female flies (t-test; $p=0.8211$; n=8). (E) There is an overall effect of the ethanol exposure length on significant locomotor defect compared to control and Nos^1 (Fig. 5.4C). Internal ethanol was assessed in Nos^1 flies at multiple time points of exogenous ethanol exposure (Fig. internal ethanol levels, but no effect of genotype or interaction (two-way ANOVA; genotype, $p=0.1735$; ethanol exposure, $p<0.0001$; interaction, $p=0.7537$; n=8). There were no difference in internal ethanol levels between the Nos^1 and control, w[A], female flies when measured at multiple ethanol exposure time points.

significant locomotor defect compared to control and Nos^1 (Fig. 5.4C). Internal ethanol was assessed in Nos^1 flies at multiple time points of exogenous ethanol exposure (Fig. 5.4E) and values were corrected for their corresponding water volume (Fig. 54D), Nos^1 flies did not have differences in water volume (Fig. 5.4D) or internal ethanol levels when exposed to ethanol for 0, 48, or 96 minutes (Fig. 5.4E). These data suggest that Nos is required for normal ethanol sedation and that locomotor defects and ethanol uptake/metabolism are most likely not the cause of the increased ethanol sedation.

Another initial response to alcohol that can be assessed in flies is rapid tolerance. Therefore we assessed whether the Nos null fly, Nos^1, influenced the development of rapid tolerance. Rapid tolerance is determined by exposing flies to ethanol during a first exposure, allowing the flies to recover for four hours, and reassessing their ethanol sedation during a second ethanol exposure. Nos^1 flies were sensitive to alcohol in the first exposure (E1, left panel), as expected, but there was no difference in ST50s between the Nos null and control flies in the second ethanol exposure (E2, right panel) (Fig. 5.5A). Rapid tolerance can be quantified by as the ST50 from the second ethanol exposure (E2) divided by the first ethanol exposure ST50 (E1). Nos^1 flies have an increase in the development of rapid tolerance compared to controls (Fig. 5.5B). These data suggests that Nos may not only affect initial ethanol sedation, but also the development of rapid tolerance.

The model of how Nos influences ethanol sedation is of important interest, different tissues or timing could have very different implications for the role of NO signaling. We utilized the UAS-Gal4 system[69] to express Nos RNAi lines in different tissues. The target locations of the Nos RNAi lines are highlighted in Fig. 5.1B (purple

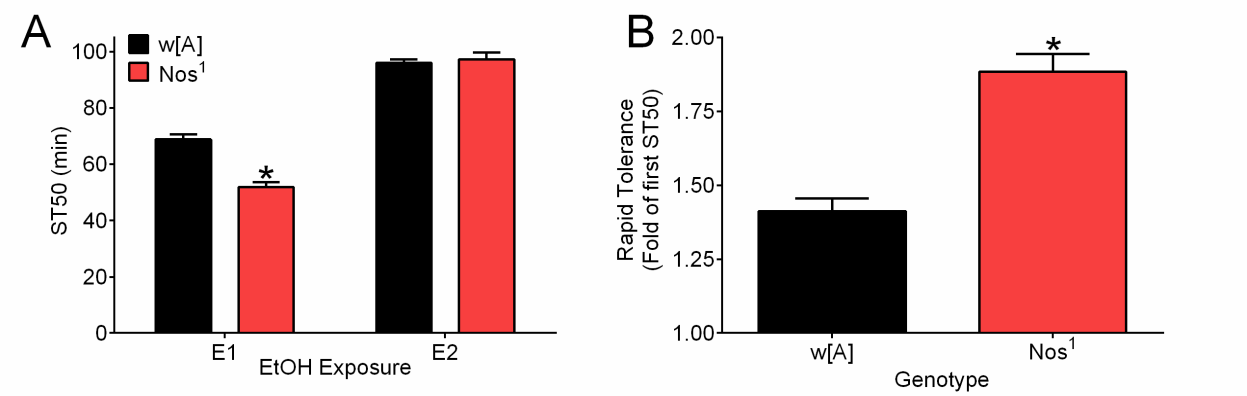


Figure 5.5. Nos null effect on rapid tolerance. For determining ethanol sedation, flies were exposed to 55% ethanol vapor. (A) Genotype, ethanol exposure, and subsequent interaction between the factors had a significant effect on ST50 values (two-way ANOVA; genotype, $p<0.0001$, ethanol exposure, $p<0.0001$; interaction, $p<0.0001$). Nos^1 flies have a decreased ST50 compared to control flies, w[A] during the first ethanol exposure (E1), but not the second ethanol exposure (E2) (*BMC versus w[A]; E1, $p<0.0001$; E2, $p>0.9999$; n=11-12). (B) Rapid tolerance, calculated as ST50 of E2 ÷ ST50 of E1 (data from panel A), is significantly higher in Nos^1 compared to control, w[A] (t-test; $p<0.0001$; n=11-12).

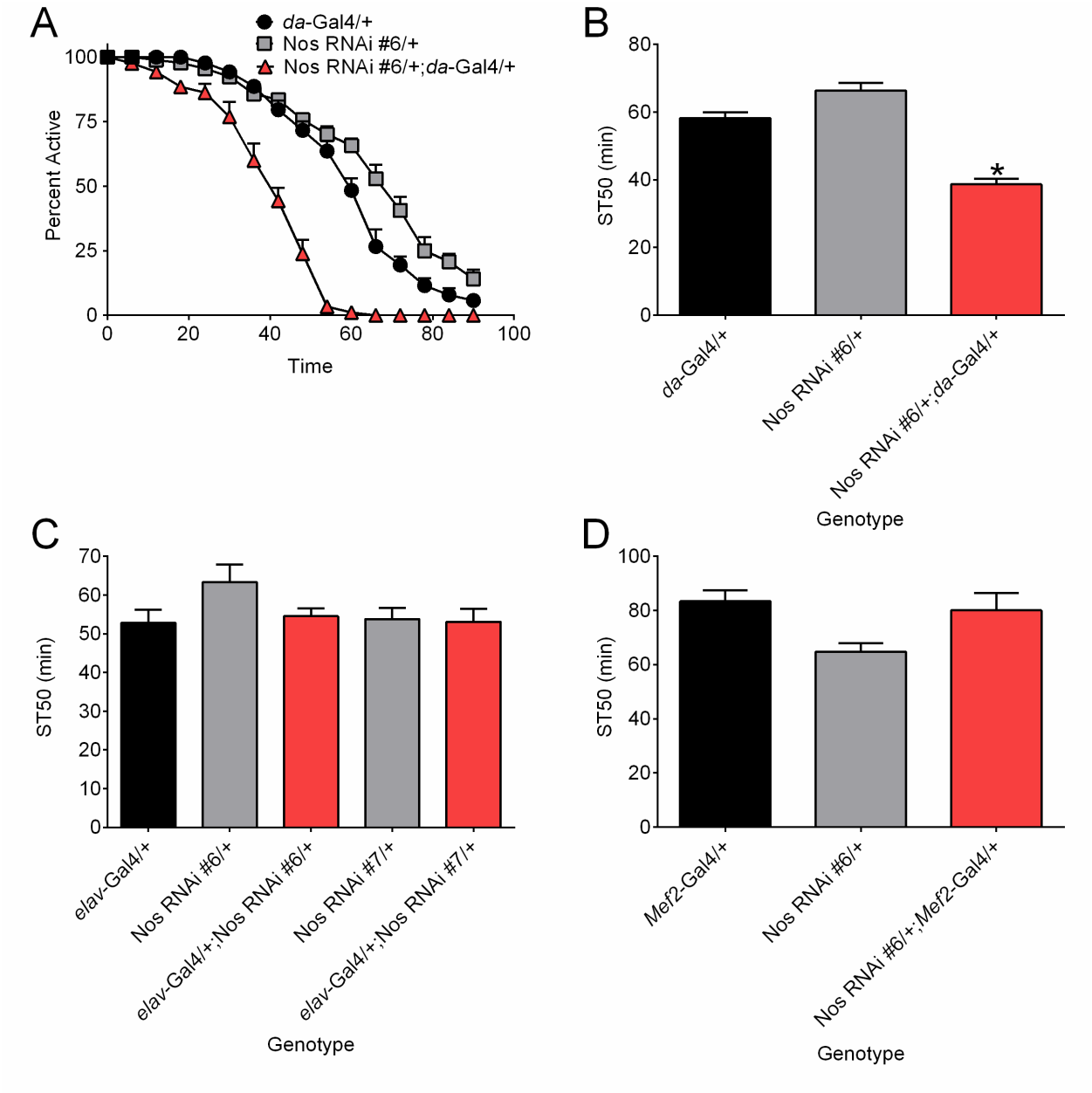

A
da-Gal4/+
Nos RNAi #6/+
Nos RNAi #6/+;da-Gal4/+
Percent Active
Time
B
ST50 (min)
*
da-Gal4/+
Nos RNAi #6/+
Nos RNAi #6/+;da-Gal4/+
Genotype
C
ST50 (min)
elav-Gal4/+
Nos RNAi #6/+
elav-Gal4/+;Nos RNAi #6/+
Nos RNAi #7/+
elav-Gal4/+;Nos RNAi #7/+
Genotype
D
ST50 (min)
Mef2-Gal4/+
Nos RNAi #6/+
Nos RNAi #6/+;Mef2-Gal4/+
Genotype

Figure 5.6. Broad expression of Nos RNAi #6 alters ethanol sedation, but not when expressed pan-neuronally or in muscles. Female flies exposed to 70% ethanol vapor during sedation. (A) Ethanol sedation time-course of flies containing *da*-Gal4 and Nos RNAi #6 (red triangles) and controls (black circles and gray squares). (B) ST50 values, from panel A, are significantly affected by genotype (one-way ANOVA; $p<0.0001$). When Nos RNAi #6 is expressed broadly (red bar) there is a decrease in ST50s compared to both driver, *da*-Gal4/+, and RNAi transgene, Nos RNAi #6/+, controls (black and gray bars, respectively) (*BMC versus Nos RNAi #6/+;*da*-Gal4/+; *da*-Gal4/+, $p<0.0001$; Nos RNAi #6/+, $p<0.0001$; n=8). (C) ST50 values are not altered when Nos RNAi #6 or #7 is expressed pan-neuronally (red bars) compared to both driver, *elav*-Gal4/+, and RNAi transgene, Nos RNAi #6 or #7/+, controls (black and gray bars, respectively) (one-way ANOVA; $p=0.1584$; n=8). (D) There is an overall genotype effect on ST50 values (one-way ANOVA; $p=0.0274$; n=8), but they are not significantly altered when Nos RNAi #6 is expressed in muscles (red bar) and compared to both driver, *Mef2*-Gal4/+, and RNAi transgene, Nos RNAi #6/+, controls (black and gray bars, respectively).

boxes). Expression of Nos RNAi #6 throughout the fly results in a decreased ethanol sedation compared to both the driver (*da*-Gal4/+) and RNAi (Nos RNAi #6) controls (Fig. 5.6A, time course and 5.6B, ST50s obtained from panel A). Expression of Nos RNAi #6 and #7 pan-neuronally (Fig. 5.6C), or Nos RNAi #6 in muscles (Fig. 5.6D) did not alter ethanol sedation. Furthermore, expression of other Nos RNAi lines in different tissues did not affect ethanol sedation or the development of rapid tolerance (Table 5.2).

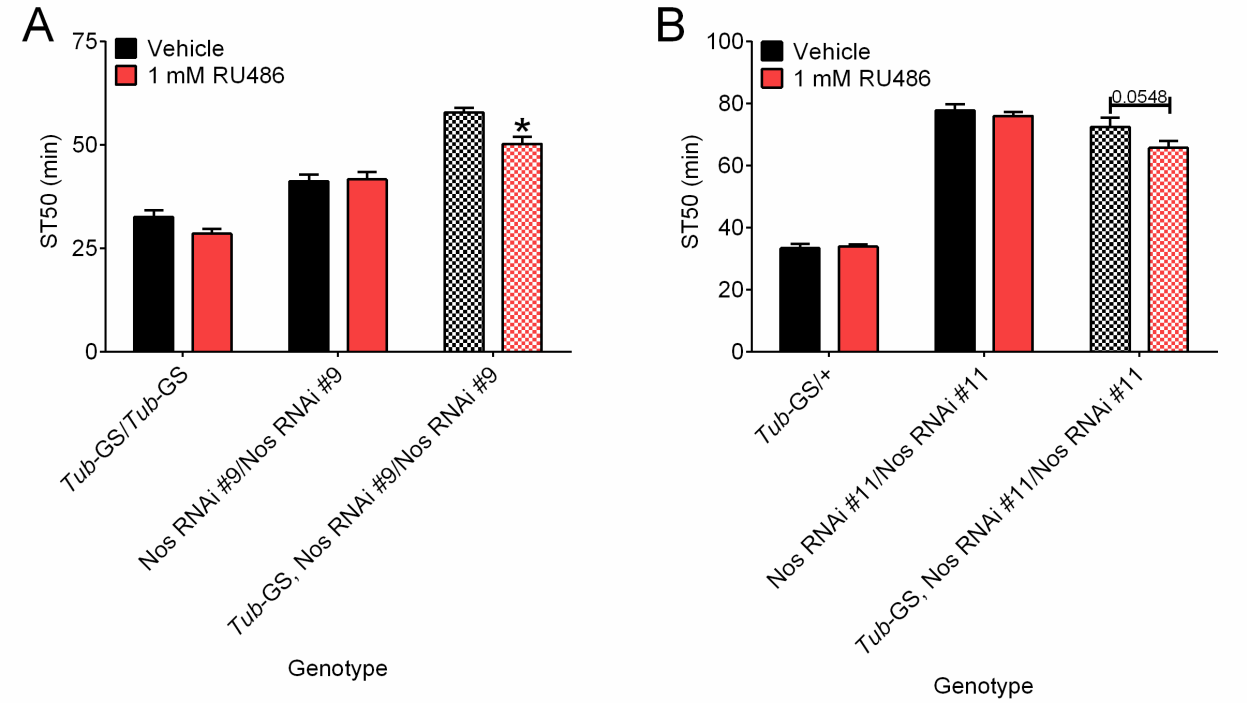
To determine whether Nos expression during adulthood is important for ethanol sedation, we utilized the GeneSwitch system[70]. GeneSwitch is a steroid inducible Gal4 system, which allows for temporal control of expression of a transgene by introducing a steroid to flies. We expressed a single copy of Nos RNAi lines, driven by a ubiquitous GeneSwitch driver and compared the ST50 values between vehicle treated (no expression of transgene) and RU486 (steroid, expression of transgene) for each genotype. There were no consistent effects on Nos RNAi expression during adulthood on ethanol sedation (Table 5.3). It is possible that expression of a single copy of Nos RNAi alone is not sufficient to cause a robust knockdown of Nos. To factor in this possible limitation, we created a double transgenic fly that would contain both the ubiquitous GeneSwitch Driver and two copies of a Nos RNAi line. When flies expressed one copy of the ubiquitous GeneSwitch driver and two copies of Nos RNAi #9, they had a decrease in ST50 when treated with RU486 compared to vehicle treated flies (Fig. 5.7A, checkered bars). Importantly, controls containing the GeneSwitch driver or two copies of the Nos RNAi #9 transgene did not have differences in ST50 values when treated with either vehicle or RU486 (Fig. 5.7A, first four bars). Additionally, ubiquitous

Table 5.2. Expression of multiple Nos RNAi lines do not alter ethanol sedation or rapid tolerance when expressed broadly, pan-neuronally, in muscles, or in glial cells.

Groups	Gal4	Nos RNAi Line	ST50 E1	RapTol (E2/E1)	n	Statistical test	ST50 E1 p values	RapTol p values
RNAi	da	GLC01867	65.33 ± 4.796	1.724 ± 0.1115	8	ANOVA	0.0315	0.0298
Control	--	GLC01867	75.74 ± 4.447	1.532 ± 0.1014	8	BMC	0.1779	0.2897
Control	da	--	81.85 ± 2.886	1.358 ± 0.0363	8	BMC	0.0200*	0.0176*
RNAi	elav	GLC01867	70.68 ± 3.806	1.641 ± 0.0611	8	ANOVA	0.2866	0.1257
Control	--	GLC01867	77.91 ± 3.958	1.470 ± 0.0801	8	BMC	0.4043	0.1689
Control	elav	--	78.86 ± 3.895	1.462 ± 0.0570	8	BMC	0.3025	0.1439
RNAi	repo	GLC01867	83.26 ± 5.736	1.388 ± 0.0924	8	ANOVA	0.5591	0.3527
Control	--	GLC01867	74.95 ± 6.495	1.484 ± 0.1438	8	BMC	0.6494	>0.9999
Control	repo	--	76.09 ± 5.180	1.243 ± 0.1057	8	BMC	0.7878	0.7758
RNAi	da	JF03220	78.80 ± 3.256	1.230 ± 0.0549	8	ANOVA	0.0005	0.1494
Control	--	JF03220	87.64 ± 1.952	1.299 ± 0.0529	8	BMC	0.0428*	0.7259
Control	da	--	70.23 ± 2.159	1.374 ± 0.0530	7	BMC	0.0597	0.1511
RNAi	Mef2	JF03220	78.83 ± 3.929	1.297 ± 0.0456	8	ANOVA	0.1360	0.8995
Control	--	JF03220	95.55 ± 7.820	1.253 ± 0.0870	8	BMC	0.0969	>0.9999
Control	Mef2	--	86.73 ± 4.360	1.269 ± 0.0594	8	BMC	0.6673	>0.9999
RNAi	elav	JF03220	79.34 ± 1.632	1.343 ± 0.0291	8	ANOVA	0.0261	0.0564
Control	--	JF03220	78.23 ± 1.540	1.345 ± 0.0394	8	BMC	>0.9999	>0.9999
Control	elav	--	70.98 ± 3.025	1.486 ± 0.0613	8	BMC	0.0257*	0.0713
RNAi	repo	JF03220	77.84 ± 2.336	1.341 ± 0.0345	8	ANOVA	0.0002	0.8468
Control	--	JF03220	75.51 ± 3.175	1.329 ± 0.0680	8	BMC	>0.9999	>0.9999
Control	repo	--	60.51 ± 1.940	1.379 ± 0.0793	8	BMC	0.0002	>0.9999

Table 5.3. Ubiquitous adulthood expression of one copy of Nos RNAi lines does not consistently alter ethanol sedation.

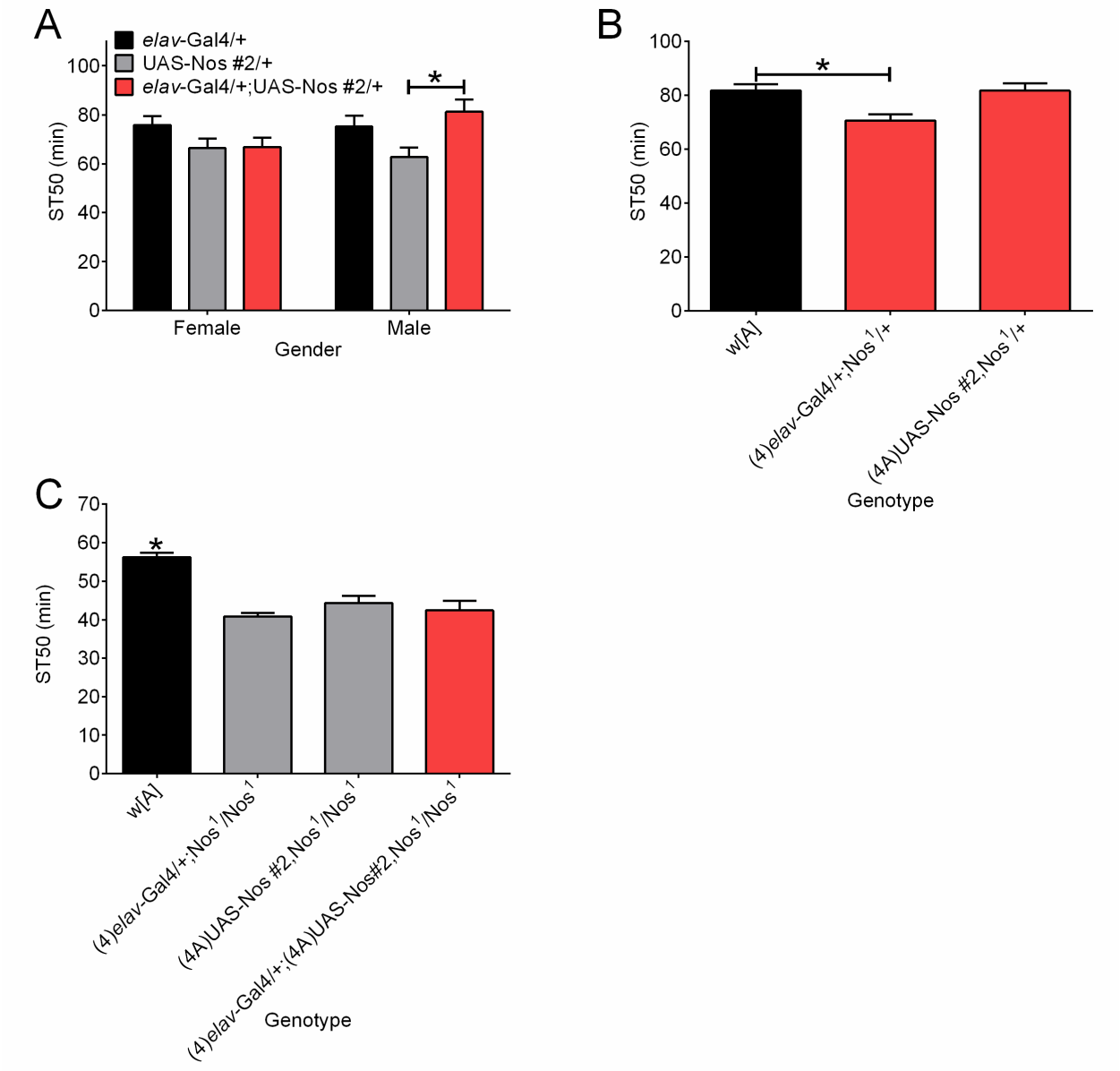
Groups	GS	Nos RNAi Line	Vehicle ST50	RU486 ST50	n	Statistical test	Treatment
RNAi	Tub	v27722	61.33 ± 2.764	75.41 ± 2.867	8	ANOVA – BMC	<0.0001*
RNAi	Tub	v27722	78.53 ± 4.210	77.27 ± 4.122	3	t-test	0.5367
RNAi	Tub	GLC01867	32.65 ± 0.814	32.36 ± 1.870	8	ANOVA - BMC	>0.9999
RNAi	Tub	JF03220	54.56 ± 1.197	48.83 ± 1.278	8	ANOVA - BMC	0.1314
RNAi	Tub	JF03220	53.31 ± 1.746	48.18 ± 0.641	8	ANOVA - BMC	0.0671
RNAi	Tub	HMC03067	29.48 ± 1.480	29.89 ± 1.320	8	ANOVA - BMC	>0.9999
RNAi	Tub	HMC03067	28.81 ± 2.297	31.95 ± 1.085	8	ANOVA - BMC	0.4647



5.7. Nos expression during adulthood may be important for normal ethanol sedation. Female flies were treated with 1 mM RU486 or vehicle for 6 days before determining ethanol sedation with 55% ethanol. (A) Overall, there was an effect of genotype, treatment, and an interaction between the factors on ST50 values (two-way ANOVA; genotype, $p<0.0001$; treatment, $p=0.0042$; interaction, $p=0.0391$). Flies containing one copy of *Tub*-GS and two copies of Nos RNAi #9, or ubiquitous induced expression of Nos RNAi #9, had a significant decrease in ST50 in flies that were treated with RU486 compared to vehicle treated, whereas controls (*Tub*-GS/*Tub*-GS and Nos RNAi #9/Nos RNAi #9) did not have a difference in ST50s compared between RU486 and vehicle treated flies (*BMC versus vehicle; *Tub*-GS/*Tub*-GS, $p=0.1977$; Nos RNAi #9/Nos RNAi #9, $p>0.9999$; *Tub*-GS, Nos RNAi #9/Nos RNAi #9, $p=0.0035$; $n=8$). (B) Genotype significantly altered ST50 values whereas treatment and the factor interaction did not (two-way ANOVA; genotype, $p<0.0001$; treatment, $p=0.0993$; interaction, $p=0.1740$). There was no difference in ST50 in either controls (*Tub*-GS/+ and Nos RNAi #11/Nos RNAi #11) or flies with induced expression of Nos RNAi #11(*Tub*-GS, Nos RNAi #11/Nos RNAi #11) when comparing RU486 and vehicle treated (BMC versus vehicle; *Tub*-GS/+, $p>0.9999$; Nos RNAi #11/Nos RNAi #11, $p>0.9999$; *Tub*-GS, Nos RNAi #11/Nos RNAi #11, $p=0.0548$; $n=8$).

adulthood GeneSwitch expression of two copies of a second Nos RNAi (#11), was trending sensitive when treated with RU486 compared to vehicle (Fig. 5.7B, checkered bars). Again, there was no difference in ST50s of the driver and Nos RNAi controls when treated with either vehicle or RU486 (Fig. 5.7B, first four bars). These data suggest that Nos expression, and subsequently NO signaling, specifically during adulthood may be important for normal ethanol sedation in the fly.

As the approach to investigate the tissue of interest was not fruitful when using the Nos RNAi lines, we looked determine the answers to these questions by utilizing the potential rescue the Nos null, Nos^1, phenotype. We obtained three UAS-Nos transgenes and determined whether they were able to increase Nos expression. All three lines obtained were able to significantly increase relative Nos expression when expressed ubiquitously compared to both the driver and their individual UAS-Nos transgene controls (data not shown). We moved forward with the UAS-Nos transgene that could induce the highest amounts of Nos and the one inducing the lowest amounts of Nos (UAS-Nos #1 and UAS-Nos #2, respectively). Next we determined whether expression of either of the UAS-Nos transgenes could influence ethanol sedation when expressed pan-neuronally. Pan-neuronal expression of UAS-Nos #1 caused a decrease in ST50s compared to controls (data not shown), whereas pan-neuronal expression of backcrossed UAS-Nos #2 did not cause a consistent difference in ST50s compared to controls (Fig. 5.8B). Therefore, backcrossed UAS-Nos #2 was used to determine whether the Nos^1 ethanol sensitivity phenotype could be rescued. We created a fly containing the pan-neuronal Gal4 driver (*elav*-Gal4) and Nos^1 as well as a fly containing UAS-Nos #2 and Nos^1. Control sedation was determined in flies containing one copy of


A
elav-Gal4/+
UAS-Nos #2/+
elav-Gal4/+;UAS-Nos #2/+
ST50 (min)
Female
Male
Gender
B
ST50 (min)
w[A]
(4)elav-Gal4/+;Nos1/+
(4A)UAS-Nos #2,Nos1/+
Genotype
C
ST50 (min)
w[A]
(4)elav-Gal4/+;Nos1/Nos1
(4A)UAS-Nos #2,Nos1/Nos1
(4)elav-Gal4/+;(4A)UAS-Nos#2,Nos1/Nos1
Genotype

5.8. Nos overexpression in neurons does not alter ethanol sedation or rescue the Nos null ethanol sedation phenotype. Female flies were exposed to 55% ethanol to determine ethanol sedation. (A) There was an overall effect of genotype on ST50s but not gender or their interaction (two-way ANOVA; genotype, p=0.0259; gender, p=0.3319; interaction, p=0.0820). There were no differences in ST50 values in female flies (left) pan-neuronally expressing UAS-Nos #2 (red bar) compared to the driver control (black bar) or UAS-Nos transgene controls (gray bars). Male flies (right) pan-neuronally expressing UAS-Nos #2 (red bar) was significantly different from the UAS transgene control (gray bar), but not from the driver control (black bar) (*BMC versus *elav*-Gal4/+;UAS-Nos #2/+; *elav*-Gal4/+, p=0.9141; UAS-Nos #2/+, p=0.0106; n=6). (B) Genotype significantly affected ST50s (one-way ANOVA; p=0.0055). Flies containing *elav*-Gal4 and a single Nos^1 allele had a significant decrease in ST50 values compared to control, w[A], whereas flies containing UAS-Nos #2 and one allele of Nos^1 did not (*BMC versus w[A]; (4)*elav*-Gal4/+;Nos^1/+, p=0.0090; (4A) UAS-Nos #2, Nos^1/+, p>0.9999; n=8). (C) Overall, genotype affected ST50 values (one-way ANOVA; p<0.0001). Positive control flies ((4)*elav*-Gal4/+;Nos^1/Nos^1 and (4A) UAS-Nos #2, Nos^1/Nos^1, gray bars) were sensitive to ethanol compared to control, w[A] (black bar). Rescue flies, (4)*elav*-Gal4/+;(4A)UAS-Nos #2, Nos^1/Nos^1 (red bar), was also sensitive to alcohol compared to control (*BMC versus w[A]; (4)*elav*-Gal4/+; Nos^1/Nos^1, p<0.0001; (4A) UAS-Nos #2, Nos^1/Nos^1, p=0.0005; (4)*elav*-Gal4/+;(4A)UAS-Nos #2, Nos^1/Nos^1, p=0.0001; n=5).

the pan-neuronal driver and one copy of Nos^1 and of flies containing one copy of UAS-Nos and one copy of Nos^1. In both these control flies, the result anticipated would be no differences in ST50s because Nos^1 needs to be present in two copies to alter ethanol sedation. This was the case for the second control containing the UAS-Nos transgene, but not the first control containing the pan-neuronal driver (Fig. 5.8C). The rescue experiment was performed, and the two positive control flies (containing two copies of the pan-neuronal Gal4 driver and two copies of Nos^1 and flies containing one copy of UAS-Nos and two copies of Nos^1) were significantly sensitive to alcohol compared to control as anticipated (Fig. 5.8D, two gray bars versus black bar). The rescue fly, containing the pan-neuronal Gal4 driver, UAS-Nos #2, and two-copies of Nos^1 had a decrease in ST50s compared to control (Fig. 5.8D, red bar versus black bar). These data suggest that the reagents used in this study were not capable of answering the question at hand, and therefore, the rescue experiment failed.

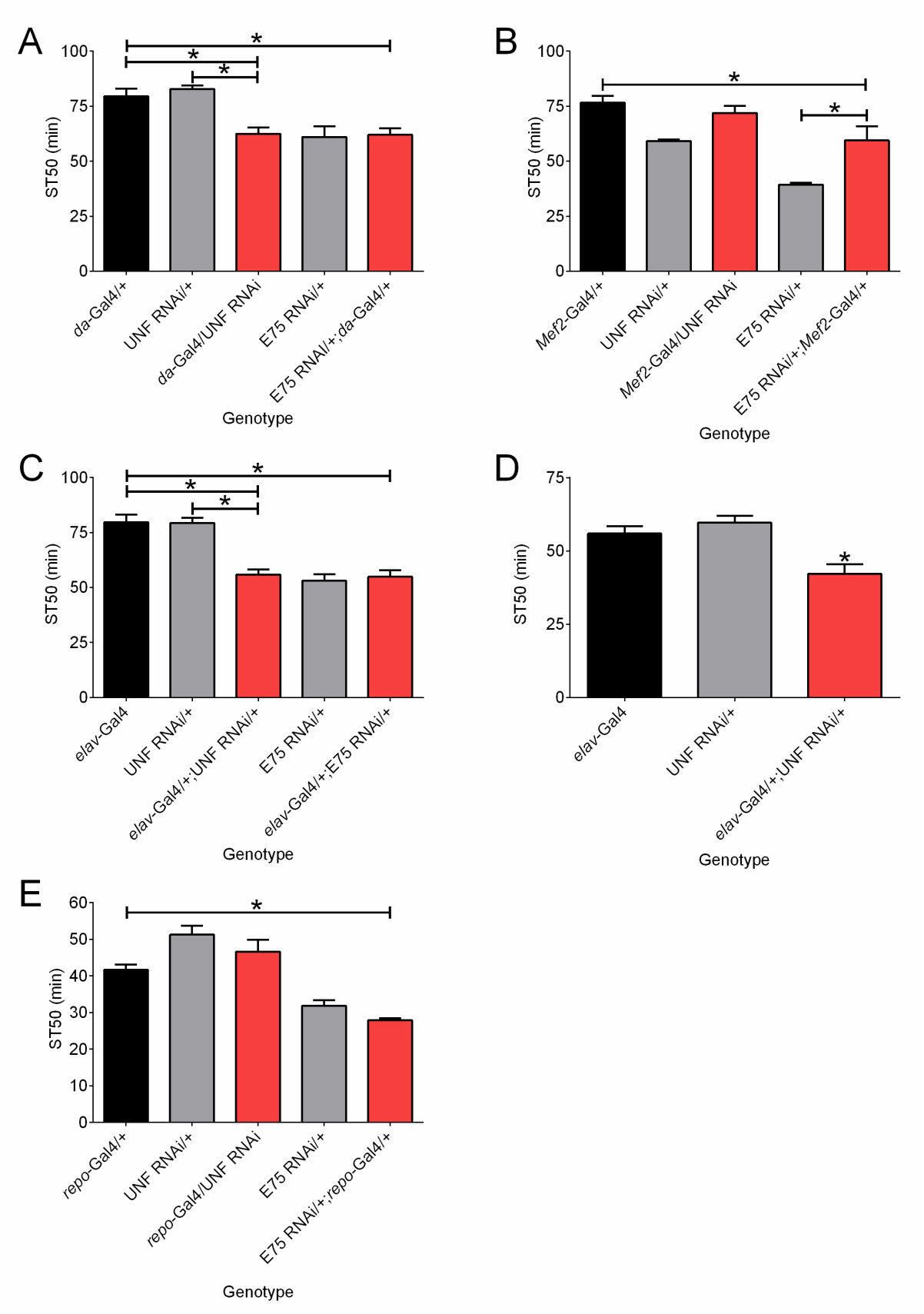
To address the mechanism that Nos, and subsequently NO signaling, may be influencing ethanol sedation, downstream targets of NO were elucidated via literature searches. One of the canonical NO signaling pathways in vertebrates is NO activation of soluble guanylyl cyclase (sGC), which has also been demonstrated to occur in invertebrates[228]. We therefore obtained multiple RNAi reagents targeting both the guanylyl cyclase α-subunit at 99B and guanylyl cyclase β-subunit at 100B, which are specific subunits that make up the conventional sGC in flies[231]. Broad expression of RNAi lines against the subunits of sGC did not alter ethanol sedation (Table 5.4). A second set of candidates that could underlie the effect of NO signaling on ethanol sedation is E75 and UNF. NO levels mediate the interaction between E75 and UNF to

Table 5.4. sGC, a downstream signaling target of NO, does not have consistent evidence for involvement in ethanol sedation.

Groups	Gal4	sGC RNAi Line	ST50	n	Statistical test	P value	
RNAi	da	KK108015	53.80 ± 2.985		ANOVA	<0.0001	Run with experiment HMJ30322
Control	--	KK108015	52.00 ± 1.758		BMC	>0.9999	
Control	da	--	50.84 ± 3.296		BMC	>0.9999	
RNAi	da	HMJ30322	33.53 ± 4.845		ANOVA	<0.0001	Run with experiment KK108015
Control	--	HMJ30322	34.10 ± 2.536		BMC	>0.9999	
Control	da	--	59.00 ± 1.106		BMC	0.0002*	
RNAi	da	JF03176	50.62 ± 3.888	6	ANOVA	0.1085	Run with experiments JF03214 & HMC03067 – same driver control
Control	--	JF02176	56.98 ± 1.962	6	BMC	>0.9999	
Control	da	--	57.40 ± 3.948	6	BMC	0.4978	
RNAi	da	JF03214	50.62 ± 3.888	6	ANOVA	0.1085	Run with experiments JF03176 & HMC03067 – same driver control
Control	--	JF03214	59.90 ± 1.609	6	BMC	>0.9999	
Control	da	--	62.65 ± 1.800	6	BMC	0.0192*	
RNAi	da	HMC03067	50.62 ± 3.888	6	ANOVA	0.1085	Run with experiments JF03176 & JF03214 – same driver control
Control	--	HMC03067	59.08 ± 1.859	6	BMC	>0.9999	
Control	da	--	58.38 ± 2.591	6	BMC	0.2915	

switch between axon pruning and regrowth[101]. Flies broadly expressing a UNF RNAi had decreased ST50s compared to the driver and RNAi transgene controls, whereas an E75 RNAi had no effect (Fig. 5.9A). When RNAi targeting E75 or UNF was expressed in muscles or glia, there was no effect on ethanol sedation in either cases (Fig. 5.9B and 5.9E). Significantly, pan-neuronal expression of UNF RNAi had decreased ST50s compared to controls, but the RNAi targeting E75 did not (Fig. 5.9C). Ethanol sensitivity in flies with UNF RNAi expression pan-neuronally was able to be reproduced (Fig. 5.9D). This data suggests that UNF may be important for ethanol sedation and would be a good candidate to study further in regards to being a mechanism underlying NO signaling influences on ethanol sedation.

An additional mechanism of NO signaling is called S-nitrosation. NO has a very short half-life of a few seconds[232], therefore additional mechanisms are needed to allow for NO to be stable for longer periods of time. S-nitrosation is the process of adding NO to cysteine thiols of proteins to result in post translation modifications and occurs in both vertebrates[233] and flies[234]. This process is highly regulated via S-nitrosoglutathione reductase (GSNOR)[233] in vertebrates or by the fly ortholog, fdh[234]. When an Fdh RNAi is expressed broadly or pan-neuronally, there is a trend of increased ST50s of the pan-neuronal expressed Fdh RNAi compared to controls whereas that was not the case for the broad expression of the Fdh RNAi (Fig. 5.10A). In a separate experiment, pan-neuronal expression of the Fdh RNAi had increased ST50s compared to both controls (Fig. 5.10B). These results suggest that Fdh, and subsequently S-nitrosation, may be important for ethanol sedation and could be a good


A
ST50 (min)
100
75
50
25
0
*
*
*
da-Gal4/+
UNF RNAi/+
da-Gal4/UNF RNAi
E75 RNAi/+
E75 RNAi/+;da-Gal4/+
Genotype
B
ST50 (min)
100
75
50
25
0
*
*
Mef2-Gal4/+
UNF RNAi/+
Mef2-Gal4/UNF RNAi
E75 RNAi/+
E75 RNAi/+;Mef2-Gal4/+
Genotype
C
ST50 (min)
100
75
50
25
0
*
*
*
elav-Gal4
UNF RNAi/+
elav-Gal4/+;UNF RNAi/+
E75 RNAi/+
elav-Gal4/+;E75 RNAi/+
Genotype
D
ST50 (min)
75
50
25
0
*
elav-Gal4
UNF RNAi/+
elav-Gal4/+;UNF RNAi/+
Genotype
E
ST50 (min)
60
50
40
30
20
10
0
*
repo-Gal4/+
UNF RNAi/+
repo-Gal4/UNF RNAi
E75 RNAi/+
E75 RNAi/+;repo-Gal4/+
Genotype

5.9. Potential candidate mechanisms underlying NO signaling effects on ethanol sedation. Female flies were exposed to 55% ethanol in panels A-C and 85% ethanol in panels D-E. (A) Genotype played a role in ST50 values (one-way ANOVA; $p<0.0001$). Flies broadly expressing UNF RNAi (*da*-Gal4/UNF RNAi, red bar) had reduced ST50s compared to driver (*da*-Gal4/+, black bar) and UNF RNAi control (UNF RNAi/+, gray bar) (*BMC versus *da*-Gal4/UNF RNAi; *da*-Gal4/+, $p=0.0039$; UNF RNAi/+, $p=0.0008$). Flies broadly expressing E75 RNAi (E75 RNAi/+;*da*-Gal4/+, red bar) was only different from the driver (*da*-Gal4/+, black bar) control but not its RNAi control (E75 RNAi, gray bar) (*BMC versus E75 RNAi/+;*da*-Gal4/+; *da*-Gal4/+, $p=0.0029$; E75 RNAi/+, $p>0.9999$; n=7-8). (B) Genotype played an overall role in ST50 values (one-way ANOVA; $p<0.0001$). Flies expressing UNF RNAi (*Mef2*-Gal4/UNF RNAi, red bar) in muscle had no differences in ST50 when compared to driver (*Mef2*-Gal4/+, black bar) and UNF RNAi control (UNF RNAi/+, gray bar). Flies expressing E75 RNAi (E75 RNAi/+;*Mef2*-Gal4/+, red bar) in muscle had a decrease in ST50 compared to the driver (*Mef2*-Gal4/+, black bar) control but an increased ST50 compared to its RNAi control (E75 RNAi, gray bar) (*BMC versus E75 RNAi/+;*Mef2*-Gal4/+; *Mef2*-Gal4/+, $p=0.0069$; E75 RNAi/+, $p=0.0012$; n=8). (C) Genotype significantly influenced ST50s (one-way ANOVA; $p<0.0001$). Pan-neuronal expression of UNF RNAi (*elav*-Gal4/+;UNF RNAi/+, red bar) had reduced ST50s compared to driver (*elav*-Gal4/+, black bar) and UNF RNAi control (UNF RNAi/+, gray bar) (*BMC versus *elav*-Gal4/+;UNF RNAi/+; *elav*-Gal4/+, $p<0.0001$; UNF RNAi/+, $p<0.0001$). Flies pan-neuronally expressing E75 RNAi (*elav*-Gal4/+;E75 RNAi/+, red bar) had a decreased ST50 value compared to the driver (*elav*-Gal4/+, black bar) control, but not its RNAi control (E75 RNAi, gray bar) (*BMC versus *elav*-Gal4/+;E75 RNAi/+; *elav*-Gal4/+, $p<0.0001$; E75 RNAi/+, $p>0.9999$; n=8). (D) Reproduction of UNF RNAi expression pan-neuronally, genotype still affected ST50 values (one-way ANOVA; $p=0.0004$). UNF RNAi expressed pan-neuronally (*elav*-Gal4/+;UNF RNAi/+, red bar) had reduced ST50s compared to driver (*elav*-Gal4/+, black bar) and UNF RNAi control (UNF RNAi/+, gray bar) (*BMC versus *elav*-Gal4/+;UNF RNAi/+; *elav*-Gal4/+, $p=0.0033$; UNF RNAi/+, $p=0.0003$; n=8). (E) Genotype significantly affected ST50 values (one-way ANOVA; $p<0.0001$). Flies expressing UNF RNAi (*repo*-Gal4/UNF RNAi, red bar) in all glial cells had no differences in ST50 when compared to driver (*repo*-Gal4/+, black bar) and UNF RNAi control (UNF RNAi/+, gray bar). Flies expressing E75 RNAi (E75 RNAi/+;*repo*-Gal4/+, red bar) throughout glia had a decrease in ST50 compared to the driver (*repo*-Gal4/+, black bar) control but no difference when compared to its RNAi control (E75 RNAi, gray bar) (*BMC versus E75 RNAi/+;*repo*-Gal4/+; *repo*-Gal4/+, $p=0.0001$; E75 RNAi/+, $p=0.7590$; n=8).

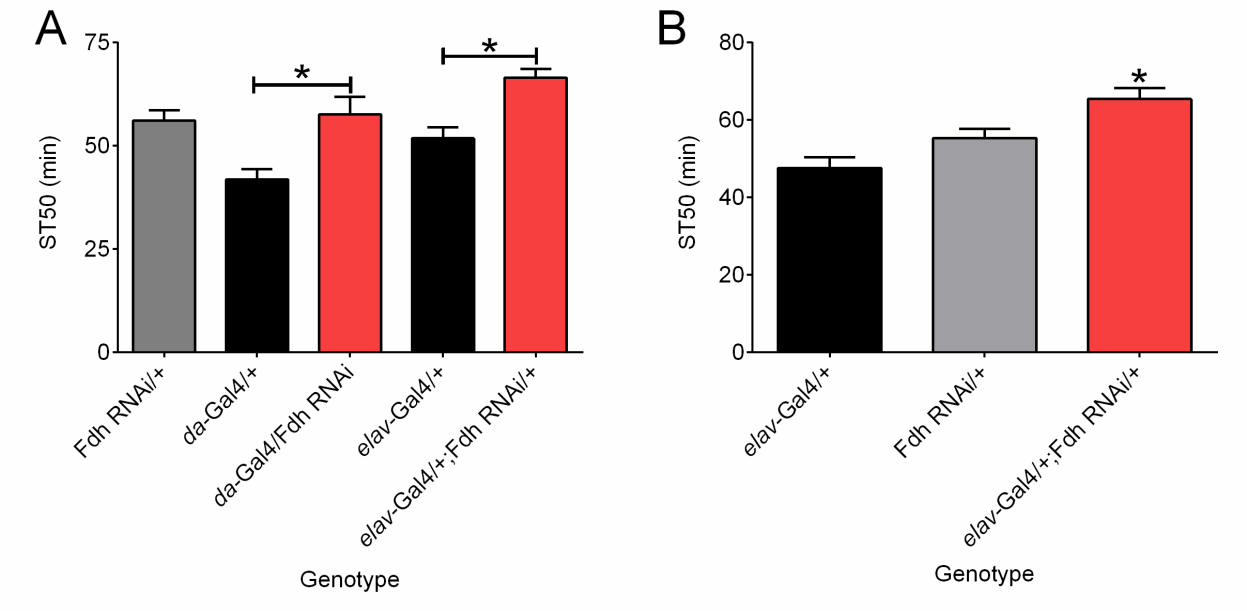


Figure 5.10. Second possible mechanism of how NO signaling may influence ethanol sedation, via the post-translational modification of S-nitrosation. Female flies were exposed to 85% ethanol. (A) Genotype had an overall effect on ST50 values (one-way ANOVA; $p<0.0001$). In both cases, flies expressing Fdh RNAi in either a broad manner (*da*-Gal4/Fdh RNAi, first red bar) or pan-neuronally (*elav*-Gal4/+;Fdh RNAi/+, second red bar) were significantly different from their corresponding driver controls (*da*-Gal4/+ or *elav*-Gal4, black bars), but not the Fdh RNAi control (Fdh RNAi/+, gray bar) (*BMC versus *da*-Gal4/Fdh RNAi; Fdh RNAi/+, $p>0.9999$; *da*-Gal4/+, $p=0.0018$; versus *elav*-Gal4/+;Fdh RNAi/+; Fdh RNAi/+, $p=0.0622$; *elav*-Gal4/+, $p=0.0040$; $n=8$). (B) Repeat sedation of Fdh RNAi being expressed pan-neuronally, genotype had an overall effect on ST50 (one-way ANOVA; $p=0.0005$). Flies expressing Fdh RNAi pan-neuronally (*elav*-Gal4/+;Fdh RNAi/+, red bar) had an increase in ST50s compared to both the driver (*elav*-Gal4/+, black bar) and the RNAi transgene control (Fdh RNAi/+, gray bar) (*BMC versus *elav*-Gal4/+;Fdh RNAi/+; *elav*-Gal4/+, $p=0.0003$; Fdh RNAi/+, $p=0.0291$; $n=8$).

candidate for investigation as to the mechanism of action for NO signaling in ethanol sedation.

C. Discussion

NO is an important molecule with wide-ranging effects on human disease states[229]. Importantly, Nos has been shown to be important in some rodent alcohol-related behaviors. Specifically, one study found that treatment of rats with NOS inhibitors resulted in an increase in the anxiolytic effects of alcohol as demonstrated by increased time spent in the open arms of the elevated plus maze[103]. A second study found that treatment of rats with sodium nitroprusside (SNP), a NO donor, caused an increase in the development of tolerance during a second day of ethanol exposure[104]. Additionally, in a third investigation, it was found that neuronal Nos (nNos) null mice consumed larger amounts of increased concentrations of alcohol and were able to recover from doses of alcohol more quickly than their control counterparts by recovering faster from the loss of righting[105].

Our studies here demonstrate that Nos may play a role in ethanol sedation in flies. Multiple transposon insertion flies were consistent in having an increase in ethanol sedation compared to controls. One important note is that the piggyBac transposon insertion flies (Nos^{e02671}, Nos^{c01670}, and Nos^{f02469}) not only significantly alter Nos gene expression, but all significantly influence numerous others genes expression that are located near Nos (data not shown). This suggests that the increased ethanol sedation seen in these three mutants may not be specific to Nos and could be due to the alteration of one of the other genes, or even a combined effect of multiple genes being

altered at once. Notably, the results obtained from using the Nos null fly (Nos^1), demonstrates the role for Nos in ethanol sedation and supports that the results seen in the Nos transposon insertion flies is due to Nos expression changes.

Results using Nos^1 suggest Nos may play a role in the development of rapid tolerance, but there is a second way of interpreting the data. Nos^1 flies are sensitive in E1, but have no difference in ST50 during E2. It is possible that whatever effect the null mutation in the Nos null caused is specific to the first ethanol exposure, and the effect is able to be either ineffective or recovered by the second ethanol sedation. This would suggest that the appearance of increased rapid tolerance in Nos^1 flies is actually not an increase in rapid tolerance, rather the Nos null flies regaining control levels of response to alcohol.

Overall, the use of Nos RNAi lines to investigate the tissue of importance of Nos expression was inconclusive. It is possible that there is a specific threshold that Nos has to be knocked down to have effects on ethanol sedation and the RNAi lines may not be capable of robustly knocking down Nos to these levels. Although Nos RNAi #6, when expressed broadly, appears to influence ethanol sedation, it has an off-target prediction. It is possible that the effect seen by this RNAi is really due to the alteration of the off-target gene and not the effect on Nos. Additionally, the RNAi data suggesting Nos may not be important for ethanol sedation is because it is plausible we have not targeted the correct tissue of importance. The Gal4 driver with broad expression may not have a strong enough expression pattern in the tissue of importance for a phenotype to be seen.

On the other hand, expressing two copies of Nos RNAi lines ubiquitously during adulthood did cause a change in ethanol sedation. Not only does this data suggest that Nos is important for ethanol sedation, and specifically during adulthood, but gives substance to the idea that one copy of an RNAi alone is not causing a robust enough knockdown to produce a phenotypic effect. Future experiments would be useful for creating double transgenic lines containing more than one copy of an RNAi, more than one copy of a Gal4 driver, or further combinations of more copies of RNAi and Gal4 driver, to potentially increase the Nos knockdown.

The Nos null rescue experiments did not produce results that are able to be interpreted for or against a role of Nos in ethanol sedation. Flies containing the pan-neuronal driver and only one copy of Nos^{1} were sensitive to alcohol, suggesting that the reagents used were not adequate for the rescue experiment. A fly containing only one copy of Nos^{1} should not have ST50s different from controls (data not shown) because two copies of Nos^{1} is needed to produce changes in ethanol sedation. With this fly having an increase in ethanol sedation, the rescue fly containing the pan-neuronal Gal4 driver, UAS-Nos, and two copies of Nos^{1} cannot readily be assessed for its ability to rescue the null phenotype. Further investigation and production of sufficient Nos reagents is needed to address whether Nos^{1} can be rescued in either a tissue/timing specific manner by UAS-Nos.

The mechanism that NO signaling is influencing to alter ethanol sedation is of significance to understand the role of NO in this phenotype. In our studies, the canonical signaling pathway, activation of sGC, by NO does not have any support for being involved in regulating ethanol sedation responses. This is not to say that sGC is not

involved, we just do not have data to support that it does. Two other candidate mechanisms, the process of switching between axon pruning and regrowth[101] and that of posttranslational modifications via S-nitrosation[234], could be used for further investigation. In both these instances, RNAi expression against key players in the pathways resulted in an ethanol sedation effect that would be consistent with the ethanol sedation sensitivity seen in Nos Mutant flies. Moreover, additional RNAi lines or mutants would be needed to confirm the candidate genes as having a role in ethanol sedation. Further investigation, via genetic interaction studies would be important to identify whether these candidate mechanisms are indeed regulated by NO to influence ethanol sedation.

These studies suggest that Nos, and NO signaling, play a role in the development of normal ethanol sedation in flies. More work is needed to elucidate mechanism of action, but it appears that Nos expression during adulthood is important for ethanol sedation and would make a good starting point for future investigations.

6. Discussion

A. Diet, serotonin, and alcohol-related behaviors in mammals and flies

Some evidence suggests that serotonin and food consumption are connected and that serotonin and alcohol behaviors are connected. To my knowledge, the experiments performed in our laboratory are some of the first to address the connection between serotonin, food consumption, and alcohol-related behaviors.

Previous studies have shown that there is an inverse relationship between the levels of brain serotonin and food intake in humans[235], as well as in *Drosophila melanogaster.* One study found that activation of specific subsets of serotonergic neurons in flies resulted in increased food seeking behavior even when fully fed. Conversely, when serotonergic neurons were inhibited, flies that were starved did not increase their food seeking behavior as would be expected[149]. Additionally, the authors found that different serotonin receptors on different subsets of dopaminergic neurons regulate the response of hunger or satiety[149]. A second group demonstrated that activation of a subset of serotonergic neurons in adult flies caused an increase in food consumption even when fed[150]. Furthermore, a third study addressing food consumption in larvae, found that the drug Metitepine causes decreased feeding by specifically blocking the serotonin 2A receptor[148]. These studies suggests that there is an effect of serotonergic neuron signaling in regulating consumption of food in flies and that the different effects and cellular targets may be diverse. Therefore, further investigation is

needed to fully understand the role of serotonergic neuron function, and presumably serotonin signaling, in fly feeding behaviors.

The relationship between serotonin and alcohol behaviors has been investigated in a few human and rodent studies. In one study, the authors found that a binge drinker on a tryptophan low diet undergoes a stress condition (public speaking), they experience an increased desire to drink alcohol[49]. Oppositely, when non-binge drinkers intake a tryptophan high diet were exposed to a stressful event, they had an increased desire for consumption of alcohol[49]. Additionally, alcohol dependent rats were treated with an inhibitor of the serotonin transporter, fluoxetine or other anti-depressants, and the treatment caused a significant decrease in the amount of withdrawal symptoms exhibited once the alcohol was removed[236]. Short term fluoxetine treatment allows for an increase in serotonin signaling in the synaptic cleft due to the inhibition of the serotonin reuptake transporter located on the presynaptic membrane. Therefore this last result suggests that increased serotonin signaling may be able to dampen the effects of alcohol withdrawal. In a second rodent study, alcohol dependent rats were treated with either Phentermine (a dopamine agonist) or 5-HTP (serotonin precursor) and, when given separately, there were no effects on the amount of alcohol withdrawal seizures[237]. When Phentermine and 5-HTP were give together, there was a drastic decrease in the amount of rats that had withdrawal seizures and they observed a decrease in these rats alcohol consumption[237]. The authors found that serotonin levels increased with 5-HTP and Phentermine + 5-HTP in rat striatal and cortical tissues, dopamine levels did not change, but the dopamine metabolite increased suggesting (at least in part) that serotonin levels are involved in the decrease in withdrawal seizures and alcohol

intake[237]. In combination, these previous studies in rodents and humans demonstrate that there may be a connection between tryptophan levels, serotonin signaling, and alcohol dependence/consumption.

Our results demonstrate recapitulated findings of aspects seen in previous literature. We demonstrate that flies consume less volume, but intake more nutrients, when on a high yeast media compared to a low yeast media. This increase in nutrients is followed by decreased internal ethanol levels and subsequent increases in ST50 values. When serotonergic neurons are inhibited, by expression of the human Kir2.1 channel specifically driven by a serotonergic neuron Gal4 driver, there is a blunting of the increased nutrient intake effect on flies on a high yeast diet. With the previous literature, it is possible serotonin signaling to dopaminergic neurons is part of the mechanism for how nutrient intake influences internal ethanol levels and ST50 values. Inhibition of dopaminergic neurons in flies would be a beginning place to start addressing if they play a role in diet induced ethanol resistance. Secondly, as serotonin and dopamine have been implicated in influencing alcohol withdrawal seizures, it increases dopaminergic neuron function as a candidate, for being involved downstream of serotonin, in our model of diet induced ethanol resistance.

A second hypothesis that could be used to explain the connection between all of these key players (food consumption, serotonin signaling, and alcohol-related behaviors) is the role of ADH and ALDH in serotonin metabolism in humans. In humans, serotonin is metabolized into 5-hydroxyindole-3-acetaldehyde (5-HIAL) by monoamine oxidase (MAO), and under normal conditions, is further broken down into a primary metabolite (5-HIAA by ALDH) and secondary metabolite (5-HTOL by ADH)[238]. When

alcohol is consumed, the ratio of serotonin metabolites changes where there is an increase in 5-HTOL and a decrease in 5-HIAA levels[238]. It would be interesting to determine whether this switching of the primary serotonin metabolite in the presence of alcohol may have to do with the compensatory effects of ALDH and ADH activity needed to accommodate alcohol metabolism. The first step of alcohol metabolism is an oxidizing reaction while 5-HIAL metabolism is a reduction reaction. This could allow for the perfect set of conditions (circular loop of cofactors needed for each reaction) to allow for increased enzyme activity of ADH[239]. The second step of alcohol metabolism, via ALDH, and the other half of 5-HIAL metabolism are both oxidative reactions. In this case both reactions are requiring NAD+, which may become the limiting reagent and could explain why ALDH metabolism of 5-HIAL does not produce the primary metabolite when alcohol is consumed[239]. Subsequently, if this hypothesis were correct, it could explain the increase in negative alcohol side effects when 5-HTOL is made the primary metabolite[238]. ALDH would be competing between alcohol and 5-HIAL metabolism, potentially making the levels of the intermediate ethanol metabolite (acetaldehyde) to increase, thereby increasing negative alcohol side effects. This evidence that serotonin levels, either by metabolism as just described or by serotonin reuptake (5-HTTP), in combination with the genetic association data could suggest that the composition of diet is important for alcohol-related responses in humans. Specifically with serotonin as a diet component, leads to a very possible factor for influencing the effects of alcohol and subsequently a potential risk factor/mechanism for AUD that could be exploited.

Flies have many of the key components that would be needed to address this hypothesis of alcohol and serotonin metabolism being able to influence alcohol-related

responses. The limiting step with using flies is that they, to data, do not have an orthologous gene for MAO or a known gene to breakdown serotonin. Figuring out how serotonin is broken down, if it is, would need to be elucidated. Recently, a fly gene has been discovered that is involved in the degradation of tyramine[240], another monoamine. Therefore it is possible that flies contain a way to metabolize serotonin. Our results from studies in flies (serotonergic neurons being important for consumption of nutrients and subsequently altering internal ethanol levels and ST50s) are consistent with this hypothesis in humans.

B. Mef2 and its influence in ethanol-related behaviors in flies and humans

The overarching goal of performing GWAS and taking candidate genes and altering them in *Drosophila* was to explore the possible molecular-genetic underpinnings of behavioral responses to alcohol in humans. The ALSPAC sample contained phenotypic data, in the form of SRE scores, and genotyping data. We used this information to determine whether any genes were associated with SRE scores. Briefly, of the candidate genes found from the GWAS, knockdown of Mef2 appeared to be the genetic manipulation that most consistently altered ethanol sedation in the same direction, but not rapid tolerance, in flies.

Mammals have 4 members (*MEF2*A, *MEF2B*, *MEF2*C and *MEF2*D) and are all predicted to be transcription factors[88, 222] and are involved in numerous biological processes (e.g., muscle differentiation[90, 91], human disease/cancer[92, 93], neuronal differentiation[94-96], and others). In the ALSPAC SRE gene-based analyses, *MEF2B* was nominally associated (p_{gene}=0.0007), but in another online tool GWAS ATLAS

(atlas.ctglab.nl[223]), there was a small association (p<0.05) between *MEF2A, MEF2B, MEF2C,* and *MEF2D* and alcohol outcomes. This suggests that *MEF2,* as a family of transcription factors, may play important roles in alcohol behaviors in humans.

To address the possible downstream mechanism of Mef2 influencing alcohol sedation in flies, we utilized a previous study that performed a fly brain Mef2 chromatin immunoprecipitation followed by microarray to identify Mef2 targets. The authors identified 342 genes with *Mef2*-dependent expression in flies[225]. Impressively, fifteen of these 342 genes (~3.5-fold more than expected by chance, Table 4.3) were found to influence behavioral responses to ethanol in *Drosophila*. These overlapping genes would be a great start for looking at the downstream mechanism of action of Mef2. All fly genes listed have multiple reagents available through the Bloomington Drosophila Stock Center and/or the Vienna Drosophila Resource Center. It would be an important step to first determine the candidate genes ability to alter ethanol sedation using either mutants or RNAi lines, determine which genes may fit the hypothesis of being a downstream target of Mef2, and then further validation of the reagents. Once this initial screen is performed, genetic interaction studies would be needed to confirm their role in the Mef2 mechanism responsible for altering ethanol sedation in flies. Furthermore, an additional study of interest would be to look into more GeneSwitch reagents to identify a neuronal driver that is able to significantly induce transgene expression when treated with RU486. This would allow for determining whether Mef2 expression during development or adulthood is important for normal ethanol seadation and would help to elucidate the downstream mechanism.

C. Nitric oxide synthase and nitric oxide signaling in alcohol related behaviors

NO is small molecule that is able to diffuse across cell membranes, has a short half-life, and is implicated in numerous human disease[229]. There are some rodent studies with evidence suggesting a role for Nos in alcohol-related behaviors in mammals[103-105]. Briefly, our studies in *Drosophila* support the role for Nos in ethanol sedation. Multiple Nos transposon insertion flies and a Nos null fly had increased ethanol sensitivity and preliminary adulthood expression of two copies of a Nos RNAi line implicate a role of Nos during adulthood to influence this behavior.

As specific adulthood expression of Nos appears to be important, of the candidate mechanisms we found to influence ethanol sedation, it would be more likely that NO signaling is acting via the S-nitrosation pathway and not the E75/UNF axon pruning and regrowth developmental pathway. As the E75/UNF axon pathway is a developmental process, my data would support the role for a separate mechanism of which NO signaling is acting. This is not to say that Nos expression during development isn't important for ethanol sedation, or that the mechanism of NO cannot be via E75/UNF signaling. Further studies would need to be performed to rule in or out these possibilities. Importantly, S-nitrosation is important for protein function and cell signaling[229], making this pathway a very attractive target for further investigation.

D. Summary

Alcohol abuse is a disastrous disease that affects a significant portion of people around the world. Understanding the genetic and environmental factors that influence the risk are important for treatment, and prevention of, alcohol dependence, abuse, or

alcohol use disorder. Using *Drosophila melanogaster* as a model, we have found that media with increased yeast concentrations cause decreased internal ethanol levels and, subsequently, result in ethanol resistance. Our data also suggest a role for serotonergic neuron function in regulating nutrient intake on high yeast media, and when inhibited, there is a blunting of the yeast-induced ethanol resistant phenotype. Additionally, we have demonstrated evidence for both the transcription factor, Mef2, and the gene, Nos, as playing roles in ethanol sedation. Further elucidation of their mechanisms, and more information on serotonergic neuron function and nutrient intake, would allow for potential development of new therapies where both diet and drug treatments could be used. This would potentially allow for enhanced recovery for people suffering from alcohol abuse.

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Chapter 7: Appendices

7.1 - Measurement of solid food intake in *Drosophila* via consumption-excretion of a dye tracer

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A. Abstract

Although the *Drosophila melanogaster* (fly) model is a popular platform for investigating diet-related phenomena, it can be challenging to measure the volume of agar-based food media flies consume. We addressed this challenge by developing a dye-based method called Consumption-Excretion (Con-Ex). In Con-Ex studies, flies consume solid food labeled with dye, and the volume of food consumed is reflected by the sum of the dye inside of and excreted by flies. Flies consumed-excreted measurable amounts of FD&C Blue No. 1 (Blue 1) and other dyes in Con-Ex studies, but only Blue 1 was readily detectable at concentrations that had no discernable effect on consumption-excretion. In studies with Blue 1, consumption-excretion (i) increased linearly with feeding duration out to 24 h at two different laboratory sites, (ii) was sensitive to starvation, mating status and strain, and (iii) changed in response to alteration of media composition as expected. Additionally, the volume of liquid Blue 1 consumed from capillary tubes was indistinguishable from the volume of Blue 1 excreted by flies, indicating that excreted Blue 1 reflects consumed Blue 1. Our results demonstrate that Con-Ex with Blue 1 as a food tracer is a useful method for assessing ingestion of agar-based food media in adult flies.

B. Introduction

The fruit fly (*Drosophila melanogaster*) has emerged as a powerful model for investigating the effects of diet on both physiological and disease-like states. Studies in flies have revealed that diet has substantial effects on lifespan [177, 182, 241-243], egg-laying [157], metabolism [184], fat deposition [183], perceived nutritional value of food [147], food choice [244], sleep [175] and a host of other phenotypes [169, 170, 176-182, 245-255]. Flies are housed on solid, agar-based media for days at a time in most laboratory studies. Unfortunately, it can be challenging to determine the volume of media flies consume under routine housing conditions [256]. This lack of clarity greatly undermines the causal connections that can be drawn between diet, dietary intake and physiological measures in the fly model.

Approaches for measuring solid food consumption in flies have been described. Methods that rely solely on the accumulation of internal dye consumed from fly food can be highly problematic because the internal dye signal quickly plateaus with time [165]. Other previously described approaches record the proportion of flies extending the proboscis (mouth parts) into the food [257] or the proportion of flies feeding on food [258]. A limitation of these two other approaches is that it is unclear if proboscis extension into food media or the proportion of flies feeding on food media equates with the volume of media consumed. Another method called FlyPAD determines feeding behavior in individual flies based on their interaction with solid food medium [259]. This highly sophisticated method quantifies a number of key parameters associated with feeding in flies, but was not designed to measure consumption of solid food media under routine laboratory conditions.

Arguably the most promising approach for measuring solid food intake in flies under typical housing conditions described to date quantitates the consumption of dietary media labeled with a radioactive tracer. Studies using this approach revealed that mated females consume more media than virgin females [260], demonstrated [171] or confirmed [165] that flies adjust their intake of solid media to compensate for changes in nutrient concentrations, and that RU486 (the steroid inducer in a frequently used conditional expression strategy in flies [70, 261]) reduces food consumption [252]. Unfortunately, the prospect of radioactive flies being inadvertently released from a laboratory makes this approach impractical for some users. Additionally, the radioactive chemicals (e.g. leucine, ATP, CTP, etc.[165, 171, 260]) used in this approach must be metabolized and then incorporated into long-lived molecules within the fly to be detected. The requirement for metabolism and macromolecular incorporation, two processes that might change in response to altered dietary composition, is a potential confound for using radioactive tracers to measure ingestion of food media.

Here, we report the development of a dye-based method called Consumption-Excretion (Con-Ex) for assessing solid food intake in flies. Flies in Con-Ex studies consume solid media labeled with FD&C Blue No. 1 for hours to days at a time. The amount of food media consumed is reflected by the sum of the dye inside flies and the dye excreted from flies. Our studies show that Con-Ex is suitable for detecting the effects of starvation, fly strain, mating status and changes in the composition of the diet on media intake. Additionally, our studies show that Con-Ex has utility in multiple laboratory environments. Con-Ex is technically straightforward, inexpensive, and

requires equipment found in virtually any laboratory. The Con-Ex method should be suitable for assessing consumption of solid food media in flies in a wide range of laboratories, especially those in which the use of radioactive food labels might be challenging.

C. Results

The goal of these studies was to develop and validate a simple, reliable, inexpensive method for measuring consumption of solid food medium in *Drosophila*. Several related ideas influenced the method we developed: (1) many inexpensive food dyes are commercially available; (2) when flies consume food medium labeled with dye, accumulation of dye inside flies plateaus over time because dye intake is quickly balanced by excretion of the dye as waste [165]; (3) dye in excreted waste should accumulate over time within vials in which flies are housed; and (4) at the conclusion of a feeding experiment, the sum of the dye found inside flies and the dye excreted from flies should reflect food intake. Considering these ideas, we developed the Consumption-Excretion (Con-Ex) method. Major experimental steps in Con-Ex studies include (i) placing adult flies in empty food vials containing a single removable feeder cap that contains solidified food medium labeled with dye (Figs. 7.1A, 7.1B); allowing flies to consume food from the feeder caps and excrete waste in the vials for prescribed periods of time (Fig. 7.1C); (iii) at the conclusion of food exposure, extracting the dye inside flies (Internal dye, INT) and the excreted dye (ExVial, ExMedium) (Fig. 7.1D); (iv)

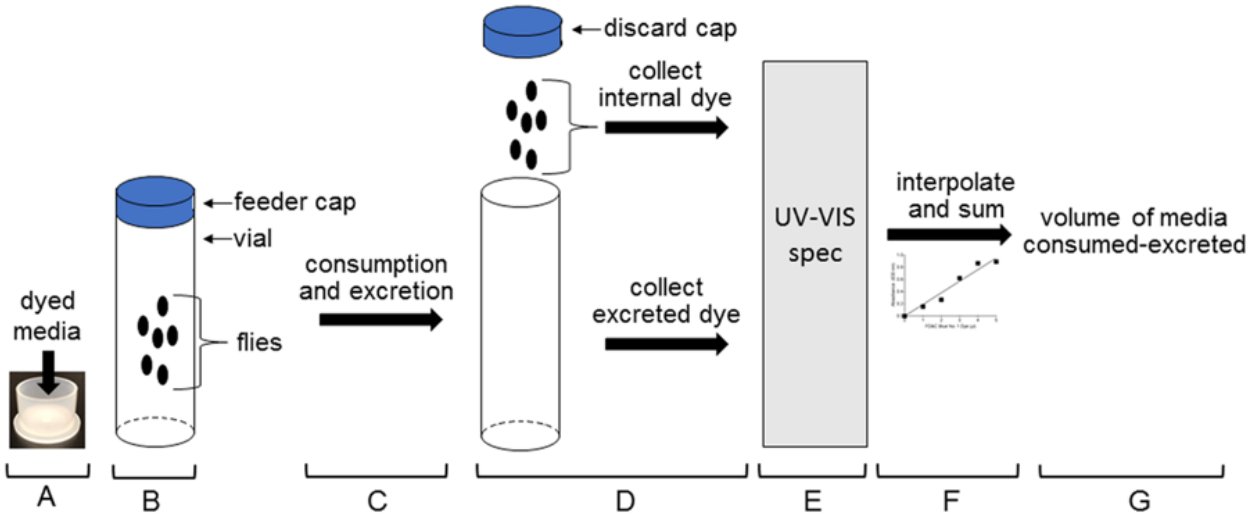

Figure 7.1. Con-Ex experimental process. Agar-based food medium containing dye (dissolved in media prior to solidifying) was poured into plastic feeder caps (Fig. 1A) and allowed to cool at room temperature to solidify, placed in a humidified plastic box, and stored at 4 °C overnight. The following day, feeder caps containing media were warmed to room temperature for 1 h, inverted and placed in the open end of vials containing adult flies (Fig. 1B). The feeder caps used in these studies hold ~4.5 mL of medium (many-fold more than flies consume), have flanges that prevent them from falling into the vials and fit in the vials used so that condensation does not build-up, yet flies cannot escape. Adult flies (typically 15/vial, but see Fig. 6) in the vials consumed medium from the feeder caps (the only food source) and then excreted waste over time (Fig. 1C). A single feeder cap was used in each vial over the duration of each experiment. Feeder caps were discarded at the conclusion of feeding (Fig. 1D). The dye inside the flies (internal dye, INT) was collected via homogenization of animals in 1.5 ml of water followed by centrifugation to pellet debris. The dye excreted by flies on the walls of the vials (excreted vial dye, ExVial) was collected by addition of 3 ml of water to vials followed by vortexing (Fig. 1D). Absorbance of the INT and ExVial dye in water extracts was determined in a spectrophotometer (Fig. 1E) at wavelengths appropriate for each dye. Absorbance values were converted to volumes of medium consumed by interpolation from standard curves of pure dyes (Fig. 1F). Extracts of flies fed medium without dye controlled for background absorbance.

quantitating the dye in these extracts via spectrophotometry (Fig. 7.1E); (v) converting the dye absorbance to volume via interpolation from standard curves of pure dye (Fig. 7.1F); and calculating the consumed-excreted volume of media (Fig. 7.1G). See Table 7.1 for definitions of all measures.

We initiated our Con-Ex studies by identifying a suitable dye (and its concentration) and then validated the method in a number of ways including (i) determining whether it could detect the effects of feeding duration, genetic background, starvation and media composition on food intake, (ii) exploring whether the method works in multiple laboratories, and (iii) determining whether consumed dye equated with excreted dye.

Identification of dyes and appropriate fly density for Con-Ex

To identify dyes suitable for Con-Ex studies, we provided flies (r[A], a standard control strain in the Grotewiel laboratory) with food containing 1% w/v of several different dyes for 24 h and then assessed the signal to noise (i.e. absorbance of ExVial+INT dye divided by the background absorbance). The dyes FD&C Blue No. 1, xylene cyanol and FD&C Red 40 (Fig. 7.2A), as well as the dyes FD&C Blue No. 2, FD&C Red 4 and FD&C Yellow 5 (Fig. 7.2B) all had significantly greater signal than noise. Based on their large signal to noise ratios and the absolute magnitudes of their signals, we chose to further explore the utility of FD&C Blue No. 1 (Blue 1), xylene cyanol (XC) and FD&C Blue No. 2 (Blue 2) in Con-Ex studies. The other dyes tested (Red 40, Green 5, Red 4, Red 6 and Yellow 5) might have had smaller signals in Con-

Table 7.1. Definitions of measures used in Con-Ex studies.

Measure	Definition
INT	The volume of internal dye
ExVial	The volume of dye excreted on the vial walls
ExMedium	The volume of dye excreted on the food medium
ExAdj	The total volume of dye excreted calculated as the ExVial volume adjusted for the ExMedium volume
ExVial+INT	The volume of internal dye plus the volume of dye excreted on the vial walls
ExAdj+INT	The total amount of dye consumed-excreted calculated as the internal volume of dye plus the volume of dye excreted in the vial adjusted for the volume excreted on the food medium
ExPlug	The volume of dye excreted on the foam plug

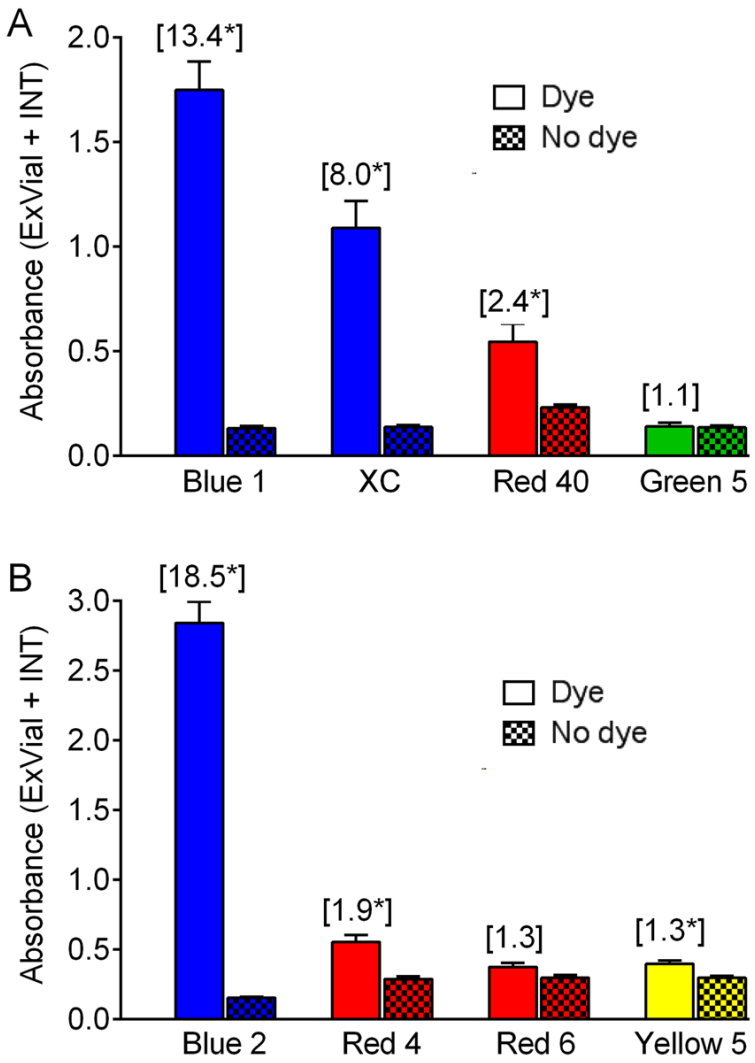

Figure 7.2. Signal-to-noise for candidate food dyes in Con-Ex. Data are the summed absorbance values of INT + ExVial. Dye (simple filled bars): absorbance values from flies fed media containing 1% of the indicated dyes (signal). No Dye (hatched bars): absorbance values from flies fed media without dye (noise). Control r[A] female flies (20/vial) consumed media and excreted waste products in vials for 24 h. Numbers in brackets indicate signal-to-noise ratios. Signal (Dye) was greater than noise (No dye) in experiments with (panel A) Blue 1, xylene cyanol (XC) and Red 40, and (panel B) Blue 2, Red 4 and Yellow 5 (*individual two-tailed t tests, $p = 0.007$ to <0.0001, $n = 5–6$).

Ex studies because they are aversive tastants, are metabolized after being consumed, have pharmacological properties that suppress medium consumption or excretion, or other reasons and were not considered further.

Ideally, the food tracers used in a feeding method should not significantly influence consumption of the media being consumed. To determine if Blue 1, XC or Blue 2 satisfied this criterion, we fed flies increasing concentrations of the dyes in either our standard fly medium (2% yeast, 10% sucrose, 3.3% cornmeal, 1% agar and antimicrobials (hereafter 2Y10S3C), Figs. 7.3A, 7.3C, 7.3E) or in a medium containing 1% agar only (Figs. 7.3B, 7.3D, 7.3F). We reasoned that using two different media might reveal food × dye interactions (i.e. food-dependent effects of dye). Note that unless indicated otherwise we hereafter report the volume of dyed media consumed determined from interpolation of standard curves of pure dye.

The concentration of Blue 1 (0.25-2% w/v) in either 2Y10S3C (Fig. 7.3A) or agar-only media (Fig. 7.3B) had no effect on the sum of INT and ExVial dye, consistent with a previous report showing that Blue 1 does not impact consumption [165]. The dose-effects of the other dyes were more complex. Consumption-excretion of 2Y10S3C was not affected by 0.25-2.0% XC (Fig. 7.3C), but this dye dose-dependently decreased consumption-excretion of media made with agar only (Fig. 7.3D). In contrast, increasing concentrations of Blue 2 increased consumption-excretion of both 2Y10S3C (Fig. 7.3E) and agar-only media (Fig. 7.3F). Since changes in consumption-excretion driven by

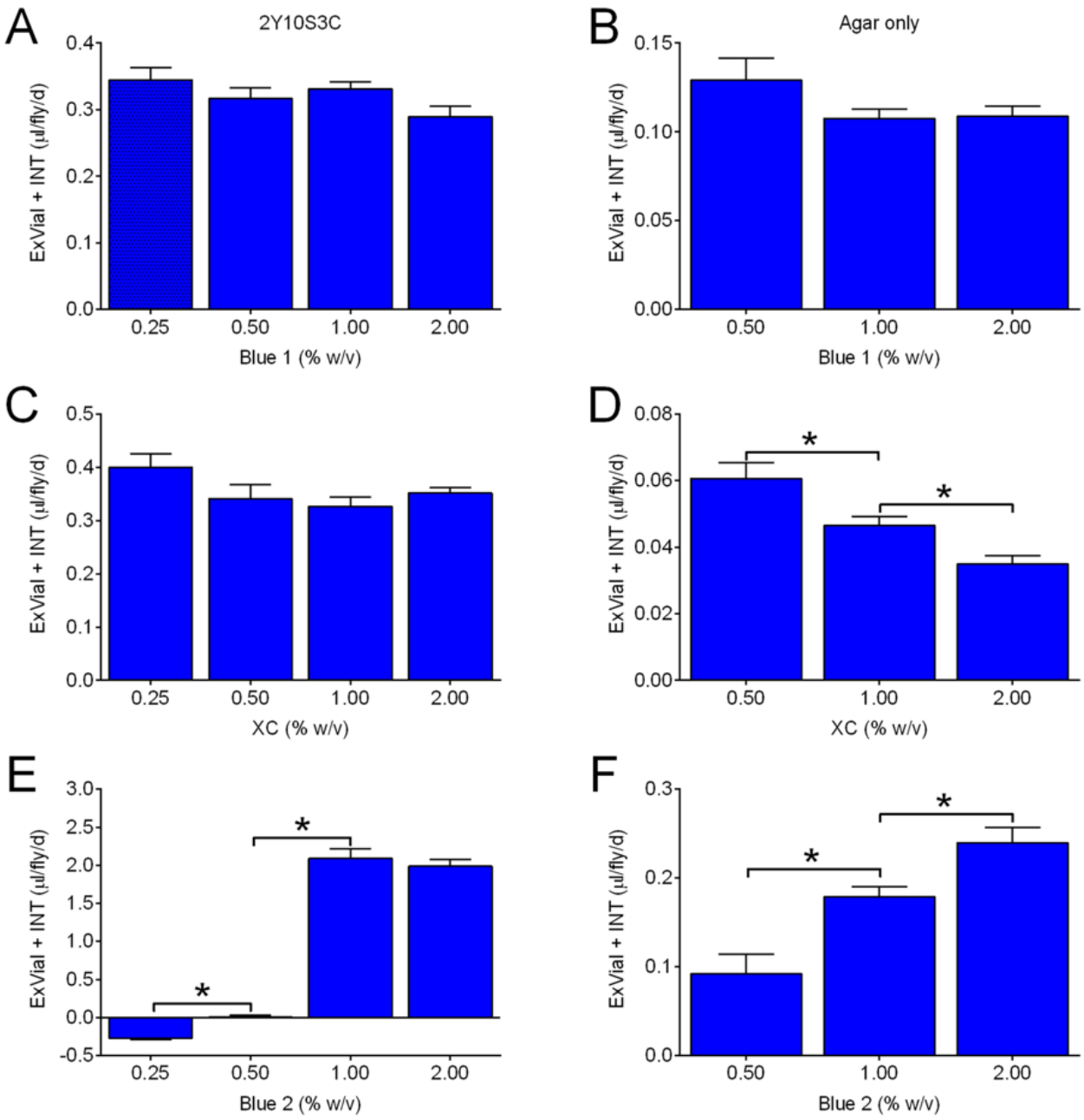
A
2Y10S3C
ExVial + INT (µl/fly/d)
0.4
0.3
0.2
0.1
0.0
0.25
0.50
1.00
2.00
Blue 1 (% w/v)
B
Agar only
ExVial + INT (µl/fly/d)
0.15
0.10
0.05
0.00
0.50
1.00
2.00
Blue 1 (% w/v)
C
ExVial + INT (µl/fly/d)
0.5
0.4
0.3
0.2
0.1
0.0
0.25
0.50
1.00
2.00
XC (% w/v)
D
ExVial + INT (µl/fly/d)
0.08
0.06
0.04
0.02
0.00
*
*
0.50
1.00
2.00
XC (% w/v)
E
ExVial + INT (µl/fly/d)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
*
*
0.25
0.50
1.00
2.00
Blue 2 (% w/v)
F
ExVial + INT (µl/fly/d)
0.3
0.2
0.1
0.0
*
*
0.50
1.00
2.00
Blue 2 (% w/v)

Figure 7.3. Effect of dye concentration on Con-Ex. Control r[A] females consumed 2Y10S3C (A,C,E) or media containing only 1% agar (B,D,F) with the indicated concentrations of Blue 1 (A,B), xylene cyanol (XC, panels C,D), or Blue 2 (E,F). Flies consumed media and excreted waste in the vials for 24 h. ExVial + INT dye was not affected by the concentration of Blue 1 in 2Y10S3C (A) or agar-only media (B) (individual one-way ANOVAs; 2Y10S3C, $p = 0.2255$; agar-only medium, $p = 0.1533$; $n = 8–20$ in panel A and 8 in panel B). ExVial + INT dye was not affected by the concentration of xylene cyanol in 2Y10S3C (C, one-way ANOVA, $p = 0.0839$, $n = 16$), but increasing concentrations of xylene cyanol in agar-only media decreased ExVial + INT dye (D; one-way ANOVA, $p < 0.0001$, $n = 8$). The concentration of Blue 2 in 2Y10S3C (E, one-way ANOVA, $p < 0.0001$, $n = 16$) and agar-only media (F, one-way ANOVA, $p < 0.0001$, $n = 16$) significantly increased ExVial + INT dye. Asterisks indicate significantly different pair-wise groups in D,E and F (*Bonferroni's multiple comparisons, $p = 0.0493$ to <0.0001).

food tracers such as XC and Blue 2 could be a significant experimental confound, we focused all subsequent studies on Blue 1.

To identify the appropriate density of animals to use in Con-Ex studies, we varied the number of flies/vial during 24 h feeding experiments. ExVial+INT measured with Blue 1 was comparable when 10-20 flies per vial were used, but it decreased significantly with 30 flies per vial (Fig. 7.4). Although we do not understand why consumption-excretion per fly decreased as the density of animals increased, these results indicate that using a consistent number of flies, and 20 or fewer flies, is an important practical consideration in Con-Ex studies with Blue 1 as a tracer under the conditions used in these experiments.

Con-Ex time-course studies under different laboratory conditions

The data in Figs. 2, 3 and S1 are from experiments in which flies consumed-excreted media for 24 h. To address how the duration of Con-Ex studies influenced the results, we performed time-course experiments with Blue 1 as a food label using r[A] (Fig. 7.5A) and Canton-S (Fig. 7.5B) females. Importantly, the data in Figs. 7.5A and 7.5B were generated in the Grotewiel and Pletcher laboratories, respectively. In both studies, the amount of INT Blue 1 plateaued or peaked by 4 (Fig. 7.5A) or 12 (Fig. 7.5B) hours after the initiation of feeding. There was an initial lag in appearance of ExVial Blue 1 (Fig. 7.6A and 7.6B), but thereafter the amount of ExVial dye accumulated with time (Fig. 7.5). ExVial and ExVial+INT dye accumulation approximated linear functions in both r[A] and Canton-S females (linear regression; R^2 = 0.8330 to 0.9387; $p<0.0001$ in all cases). The quantitative differences between the results in Figs. 7.5A and 7.5B are

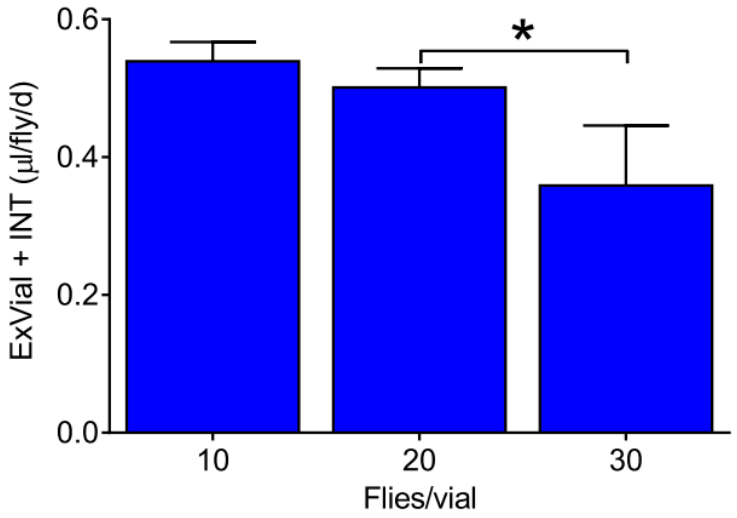

Figure 7.4.Fly density in Con-Ex. Control r[A] females at the indicated number/vial consumed 2Y10S3C with 1% Blue 1 for 24 h while excreting waste. The density of flies significantly influenced ExVial+INT (one-way ANOVA, $p<0.0001$, $n=16$). ExVial+INT was lower in flies housed in groups of 30/vial than other groups (*Bonferroni's multiple comparison test, $p=0.0010$ to <0.0001), but was not different in groups of 10 and 20 flies (Bonferroni's multiple comparison, $p=0.9255$).

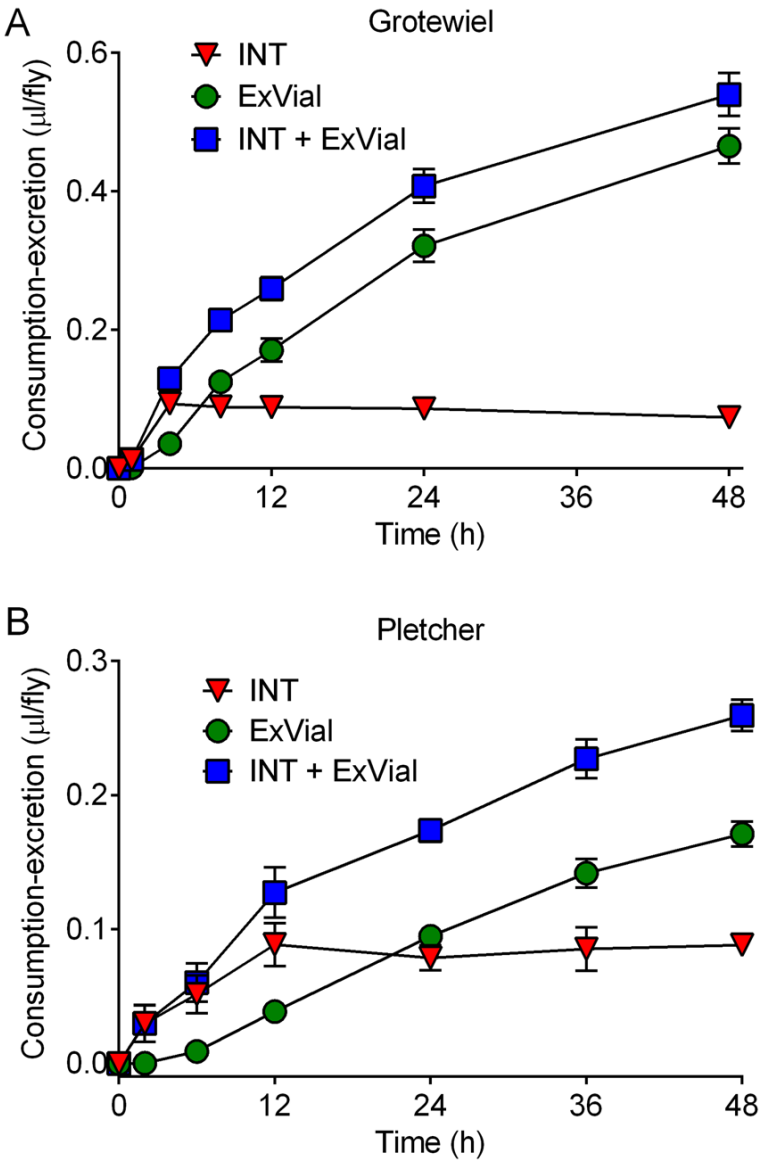

Figure 7.5. Con-Ex time-courses. Control r[A] (A) and Canton-S (B) females consumed medium labeled with 1% Blue 1 and excreted waste for the indicated times (X-axes). Data are INT, ExVial and ExVial + INT from the Grotewiel (A) and Pletcher (B) laboratories. Time influenced INT and ExVial (one-way ANOVAs; panel A, $p < 0.0001$; panel B, $p < 0.0001$; $n = 8$ at each time-point).

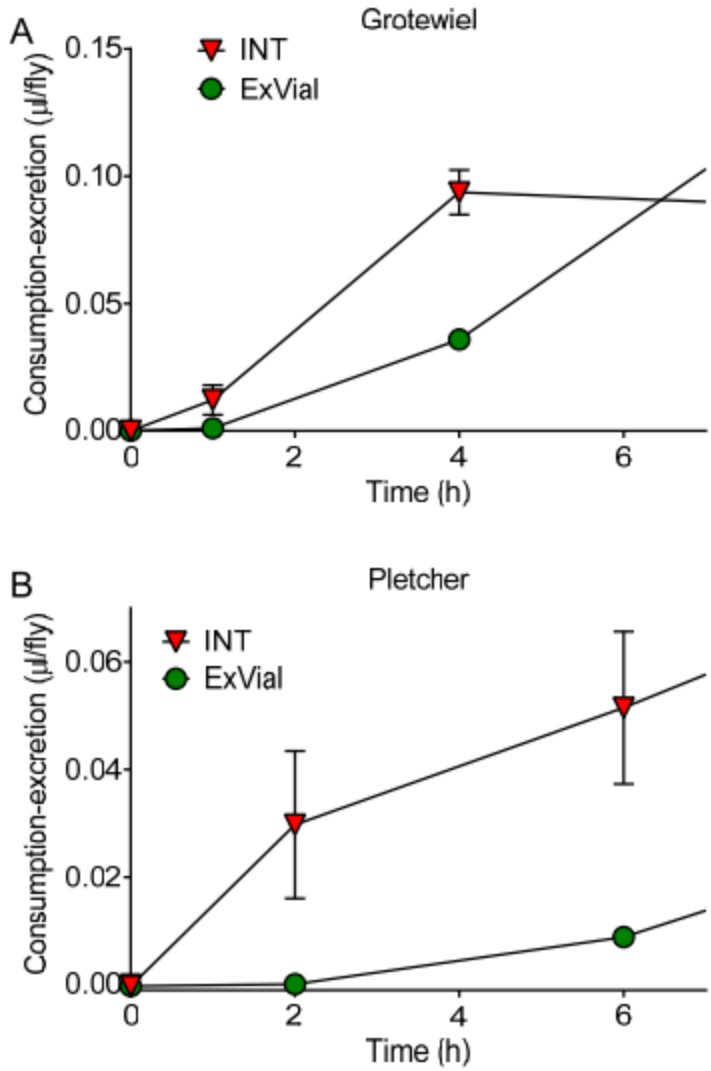

Figure 7.6. Early time-points of INT and ExVial. Detailed view of early time-points from the data in Fig. 7.5.

presumably driven by differences in environment including food media, strain and age of the animals (see Methods). Our results with different strains and ages of flies reared under different conditions suggest that accumulation of consumed-excreted Blue 1 can be measured out to 24 or possibly 48 h without major concerns regarding ceiling effects, that consumption of Blue 1 precedes its excretion, and that the Con-Ex method works well in different laboratories.

Detecting strain, starvation, mating status and media composition effects

To address the ability of the Con-Ex method to detect differences across fly strains, we assessed 24 h consumption-excretion in control r[A] and control Lausanne-S (LS) females fed 2Y10S3C medium. ExVial+INT in LS flies was ~twice that of r[A] (Fig. 7.7A), indicating that the Con-Ex method can readily detect the effects of strain (genetic background in this case) on consumption of food media. There was no difference in total body weight of r[A] and LS females (0.939±0.004 and 0.991±0.026 mg, respectively; t test, p=0.076; n=8) and therefore the differences in Con-Ex between these two strains in unrelated to body size. Although the effects of genetic background on *Drosophila* feeding behavior are widely appreciated (e.g. [165]), the difference in Con-Ex between the r[A] and LS strains we report is novel.

A period of starvation in flies increases their consumption of liquid food media [175, 244, 262, 263]. To determine whether starvation increases consumption of solid media, we compared 4 h consumption-excretion in fully fed and starved flies. We assessed

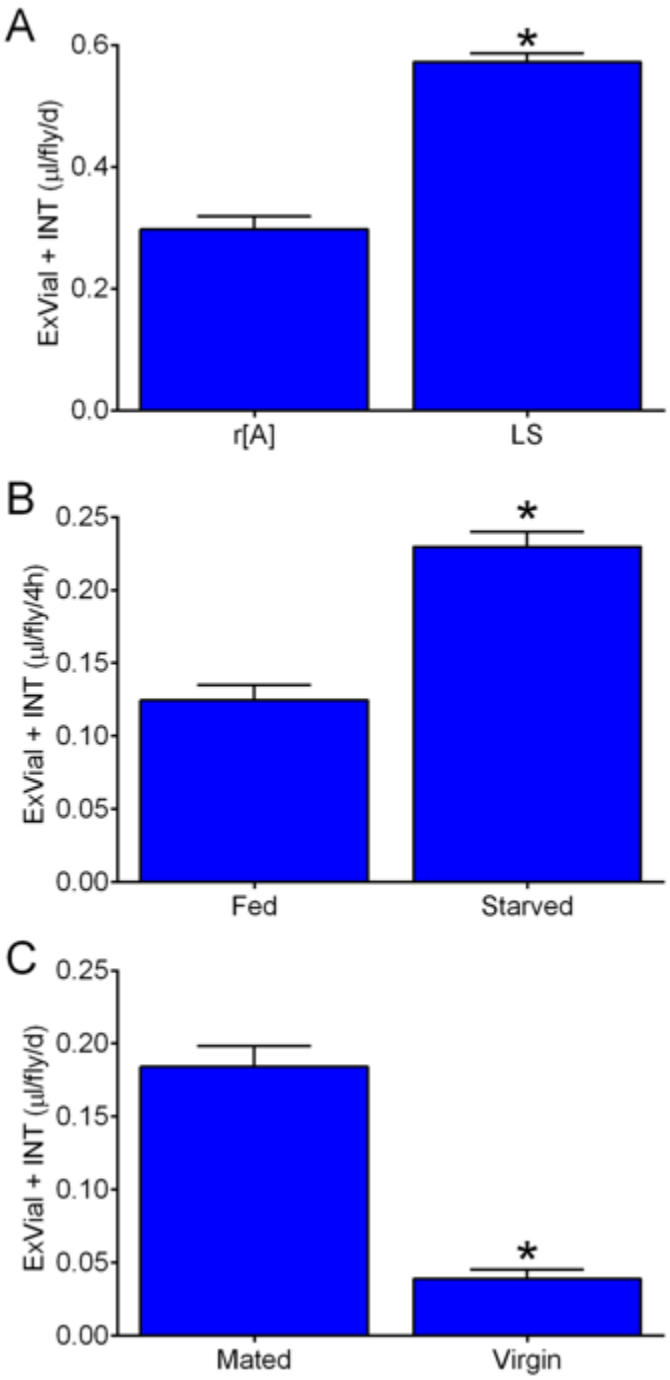

Figure 7.7. Genetic background, starvation and mating influence Con-Ex. Control females were fed 2Y10S3C containing 1% Blue 1 for 24 h (A,C) or refed this medium for 4 h (B). ExVial + INT was significantly greater in LS compared to r[A] (A; *two-tailed t test, $p < 0.0001$, $n = 8$), was greater in starved compared to fully fed r[A] females (B; *two-tailed t test, $p < 0.0001$, $n = 8$), and was greater in mated compared to virgin r[A] females (C; *two-tailed t test, $p < 0.0001$, $n = 10$).

consumption-excretion at 4 h (as opposed to a more typical 24 h) in these studies because the effects of starvation might wane once flies return to a fully fed state. Prior starvation substantially increased consumption-excretion of media labeled with Blue 1 in r[A] females (Fig. 7.7B). Con-Ex studies are therefore capable of detecting the consequences of short-term starvation on refeeding in flies.

Mated females consume more solid food media than virgin females when consumption is measured by a radioactive tracer [165]. To assess the utility of Con-Ex for detecting the effect of mating status on medium consumption, we compared 24 h consumption-excretion in mated and virgin females. Mated females consumed-excreted more medium than virgin females (Fig. 7.7C) in Con-Ex as expected [165].

Flies exhibit compensatory feeding (i.e. an increase in the volume medium consumed as the concentration of nutrients is decreased [165, 171]). As expected, decreasing the concentration of all components of 2Y10S3C (1.0X) medium to 0.25X led to an increase in ExVial+INT (Fig. 7.8A). Furthermore, increasing the concentration of yeast from 2% to 30% or increasing the sucrose concentration from 10% to 30% significantly reduced ExVial+INT (Figs. 7.8B and 7.8C, respectively). Increasing the concentration of corn meal from our standard 3.3% to 10% (the practical maximum), however, did not significantly alter ExVial+INT (Fig. 7.8D). Taken together, the data in Fig. 7.8 indicate that Con-Ex studies with Blue 1 can detect the expected changes in feeding behavior in response to alteration of food media composition. Our results also suggest that flies

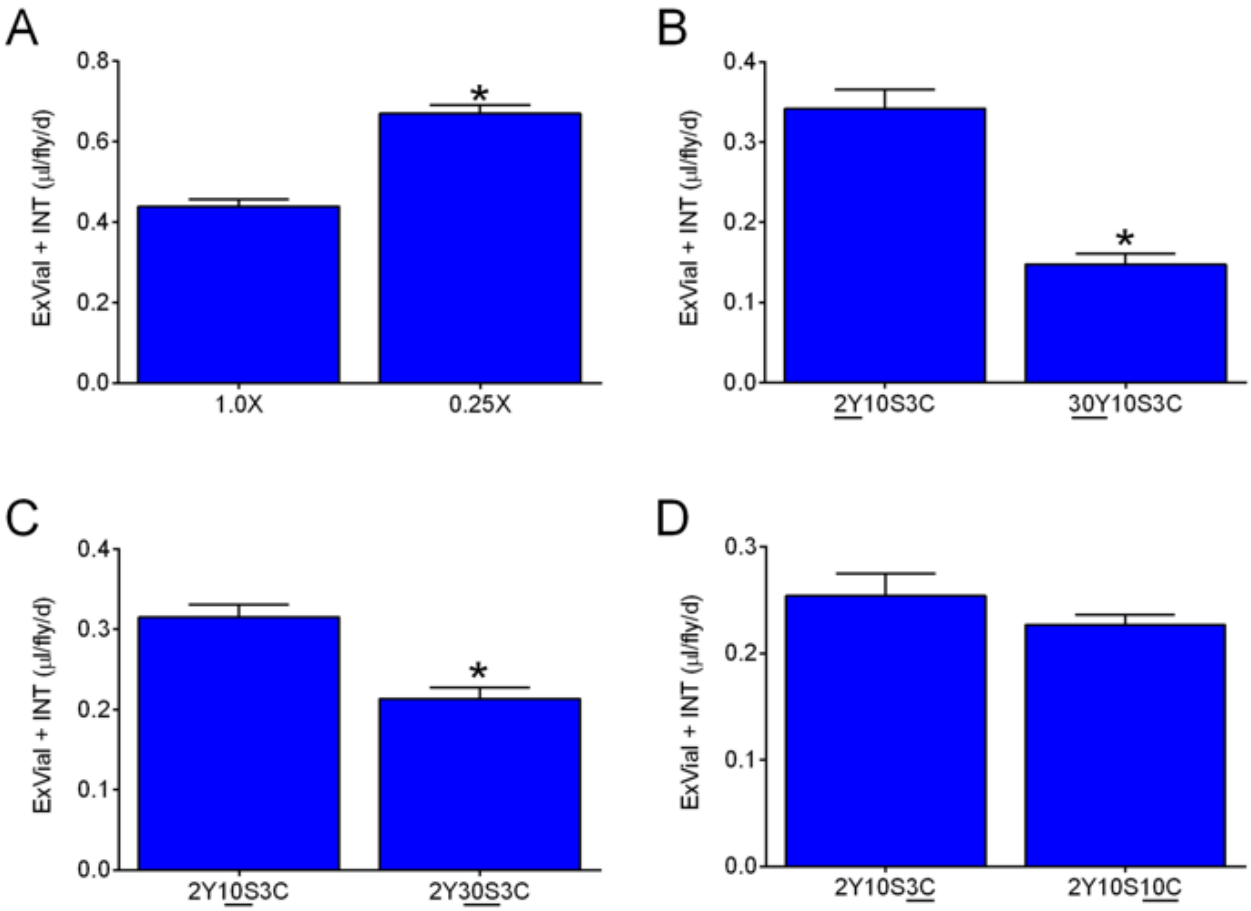

Figure 7.8. Composition of food media influences Con-Ex. Control r[A] females consumed the indicated media labeled with 1% Blue 1 and excreted waste for 24 h. ExVial + INT was greater in flies that consumed 0.25X medium than in flies fed 1.0 × 2Y10S3C medium (panel A, *two-tailed t test, $p < 0.0001$, $n = 16$), in flies fed medium containing 2% yeast (2Y10S3C) compared to 30% yeast (30Y10S3C) (panel B, *two-tailed t test, $p < 0.0001$, $n = 8$) and in flies fed medium containing 10% sucrose (2Y10S3C) compared to 30% sucrose (2Y30S3C) (panel C, *two-tailed t test, $p = 0.0003$, $n = 8$). (D) ExVial + INT was indistinguishable in flies fed medium containing 3% cornmeal (2Y10S3C) and 10% cornmeal (2Y10S10C) (two-tailed t test, $p = 0.2532$, $n = 8$).position of food media influences Con-Ex. Control r[A] females consumed the indicated media labeled with 1% Blue 1 and excreted waste for 24 h. ExVial + INT was greater in flies that consumed 0.25X medium than in flies fed 1.0 × 2Y10S3C medium (panel A, *two-tailed t test, $p < 0.0001$, $n = 16$), in flies fed medium containing 2% yeast (2Y10S3C) compared to 30% yeast (30Y10S3C) (panel B, *two-tailed t test, $p < 0.0001$, $n = 8$) and in flies fed medium containing 10% sucrose (2Y10S3C) compared to 30% sucrose (2Y30S3C) (panel C, *two-tailed t test, $p = 0.0003$, $n = 8$). (D) ExVial + INT was indistinguishable in flies fed medium containing 3% cornmeal (2Y10S3C) and 10% cornmeal (2Y10S10C) (two-tailed t test, $p = 0.2532$, $n = 8$).

might alter their dietary consumption in response to changes in some (yeast and sucrose), but not all (cornmeal), food components.

Coupling of CAFE and Con-Ex to assess Blue 1 as a tracer for consumption

The capillary feeding (CAFE) assay has been used extensively to measure consumption of liquid diets in flies [67, 165, 175, 244, 262]. We coupled CAFE and Con-Ex methods in a single experimental design to address whether the volume of Blue 1 excreted might reflect the amount of Blue 1 consumed. In pilot studies, we found that flies consuming liquid media (labeled with Blue 1) from capillary tubes excreted on both the vial wall and the foam plug holding the capillary tubes (not shown, but see below). Importantly, Blue 1 applied directly to foam plugs can be collected and quantitated (Fig. 7.9A), thereby allowing it to be measured as part of the excreted dye signal. Additionally, while flies fed medium containing Blue 1 have readily detectable INT dye after 24 h of access to the medium, flies contain no detectable INT dye 24 h after being switched to medium without dye (Fig. 7.9B). We therefore performed CAFE-excretion studies in which flies were provided access to liquid 5% sucrose media containing Blue 1 in capillary tubes for 8 h (to measure the amount of labeled media they consumed and also allow excreted dye to begin accumulating) and then were switched to capillary tubes containing liquid medium without dye for 24 h (to allow INT dye from prior consumption to be excreted). We used both fed and starved flies to generate a range of liquid medium consumption and excretion in these experiments in anticipation of correlation analyses (Fig. 7.10B).

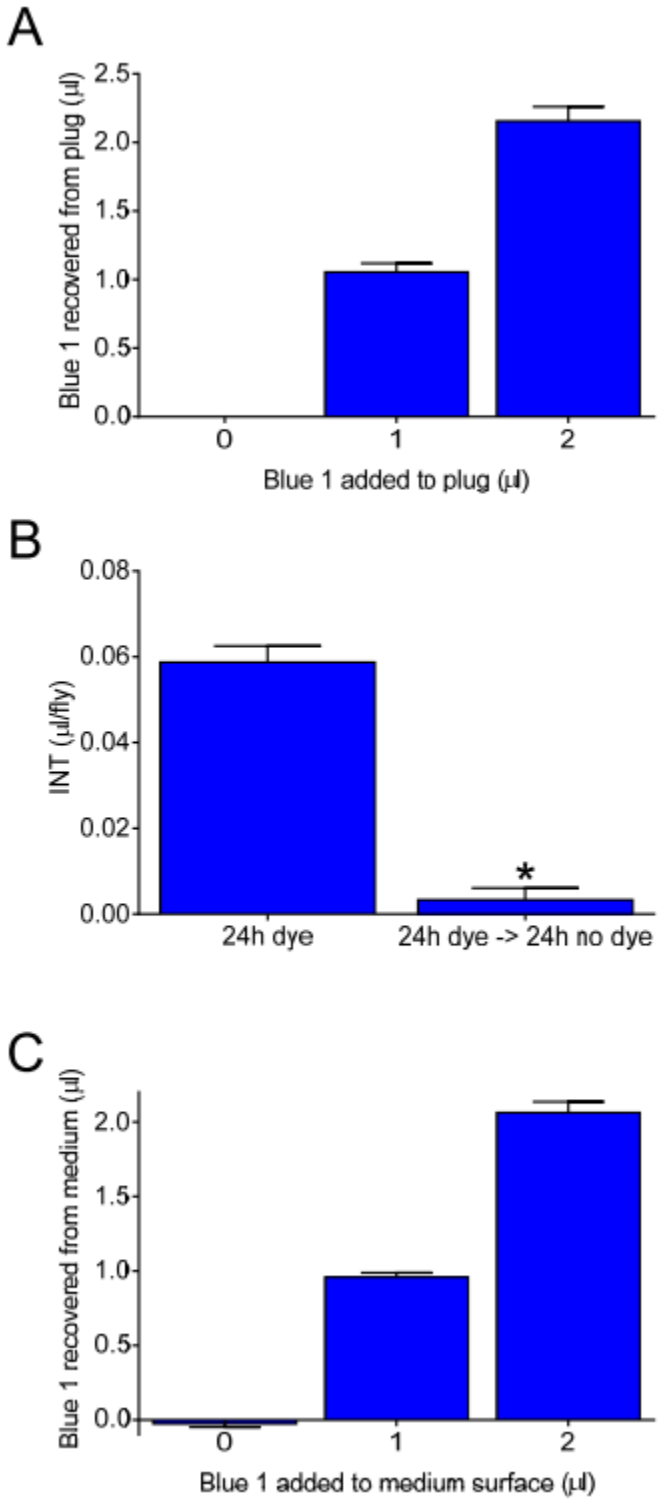
A
Blue 1 recovered from plug (µl)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
Blue 1 added to plug (µl)
B
INT (µl/fly)
0.08
0.06
0.04
0.02
0.00
*
24h dye
24h dye -> 24h no dye
C
Blue 1 recovered from medium (µl)
2.0
1.5
1.0
0.5
0.0
0
1
2
Blue 1 added to medium surface (µl)

Figure. 7.9. Methodological background for measuring excretion on foam plugs and food medium. (A) The amount of Blue 1 recovered from foam plugs were indistinguishable from the volume added (individual one sample t tests, p=0.2829 to 0.9999, n=3). (B) INT in r[A] females that had consumed dyed food for 24 h (24 h dye) was significantly greater than in flies after subsequently consuming media without dye for an additional 24 h (24 h dye -> 24 h no dye) (*two-tailed t test, p 24 h no dye flies was not distinguishable from zero (one sample two-tailed t test, p=0.2518). (C) The volume of Blue 1 extracted from the food medium in feeder caps was indistinguishable from the volumes added to the surface of the medium (individual one sample t tests, p=0.2607 to 0.4577, n=4).

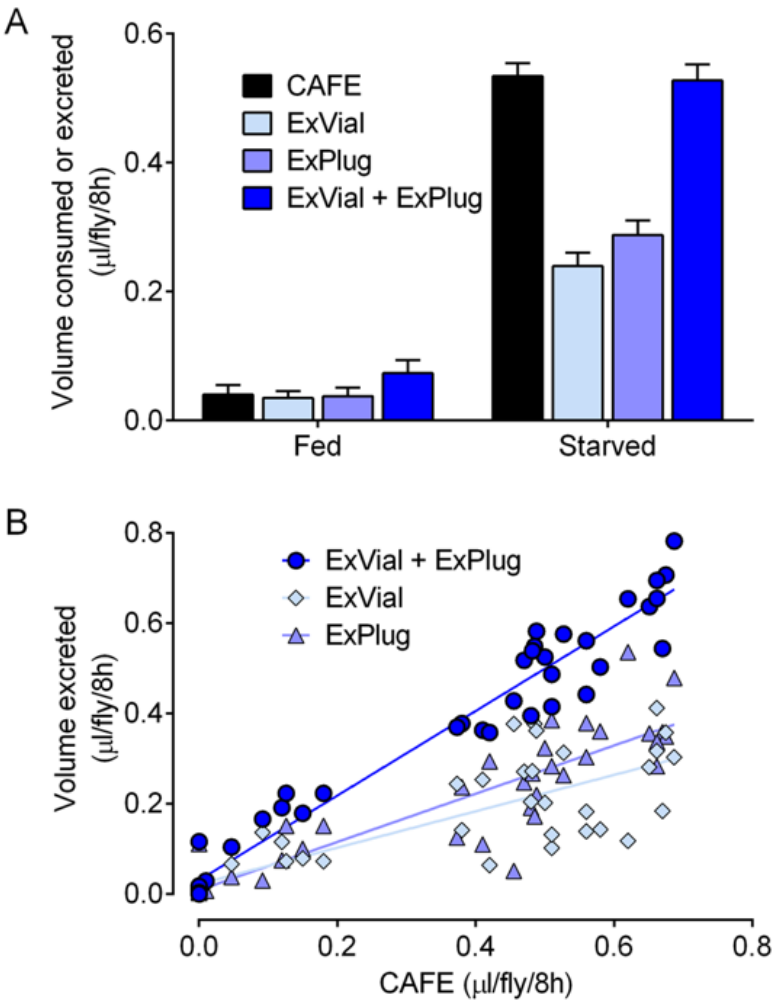

Figure 7.10. CAFE consumption of Blue 1 equates with excretion of Blue 1. (A) In control r[A] females, the volume of liquid medium consumed from capillary feeders (CAFE) and excreted (ExVial + ExPlug) was significantly increased by starvation, but the two measures were statistically indistinguishable (two-way ANOVA; starvation, $p < 0.0001$; measure, $p = 0.5302$, interaction, $p = 0.3537$; $n = 18–24$ per group). (B) Additional analysis of the data in panel A. The volume of liquid medium consumed from capillary feeders (CAFE) correlated with ExVial, ExPlug and ExVial + ExPlug (Pearson correlations: $R^2 = 0.6573$, $R^2 = 0.8165$, $R^2 = 0.9539$, $p < 0.0001$ for all, $n = 42$). Lines are best-fit linear regressions.

Previously fed flies consumed little liquid medium from capillary tubes while starved flies consumed considerably more as expected given the relatively short feeding period employed (Fig. 7.10A). Flies excreted a substantial amount of dye on the vial walls (ExVial) and on the foam plugs (ExPlug) (Fig. 7.10A). The amount of Blue 1-labeled liquid medium consumed by flies (CAFE) was indistinguishable from the amount of Blue 1 excreted (ExVial+ExPlug) in both fed and starved flies (Fig. 7.10A). Additionally, the amount of liquid Blue 1 consumed from capillary tubes (CAFE) correlated with (i) ExVial+ExPlug, (ii) ExVial and (iii) ExPlug (Fig. 7.10B). The volume of Blue 1 excreted therefore reflects, and is potentially equivalent to, the volume of Blue 1 consumed by flies under the conditions used in these studies.

Con-Ex in males

The results reported in Figs. 7.2-7.10 are from studies using females. We therefore performed an additional set of experiments to address whether Con-Ex is similarly suitable for studies in males. INT dye plateaued quickly and ExVial as well as ExVial+INT accumulated in a largely linear fashion out to ~48 h in both in r[A] and Canton-S males (Fig. 7.11A, Grotewiel lab; Fig. 7.11B, Pletcher lab). Additionally, ExVial+INT was greater in LS compared to r[A] males (Fig. 7.11C) and starvation increased ExVial+INT in r[A] males (Fig. 7.11D). The results from males and females are qualitatively similar overall, indicating that Con-Ex is suitable for studies in both sexes.

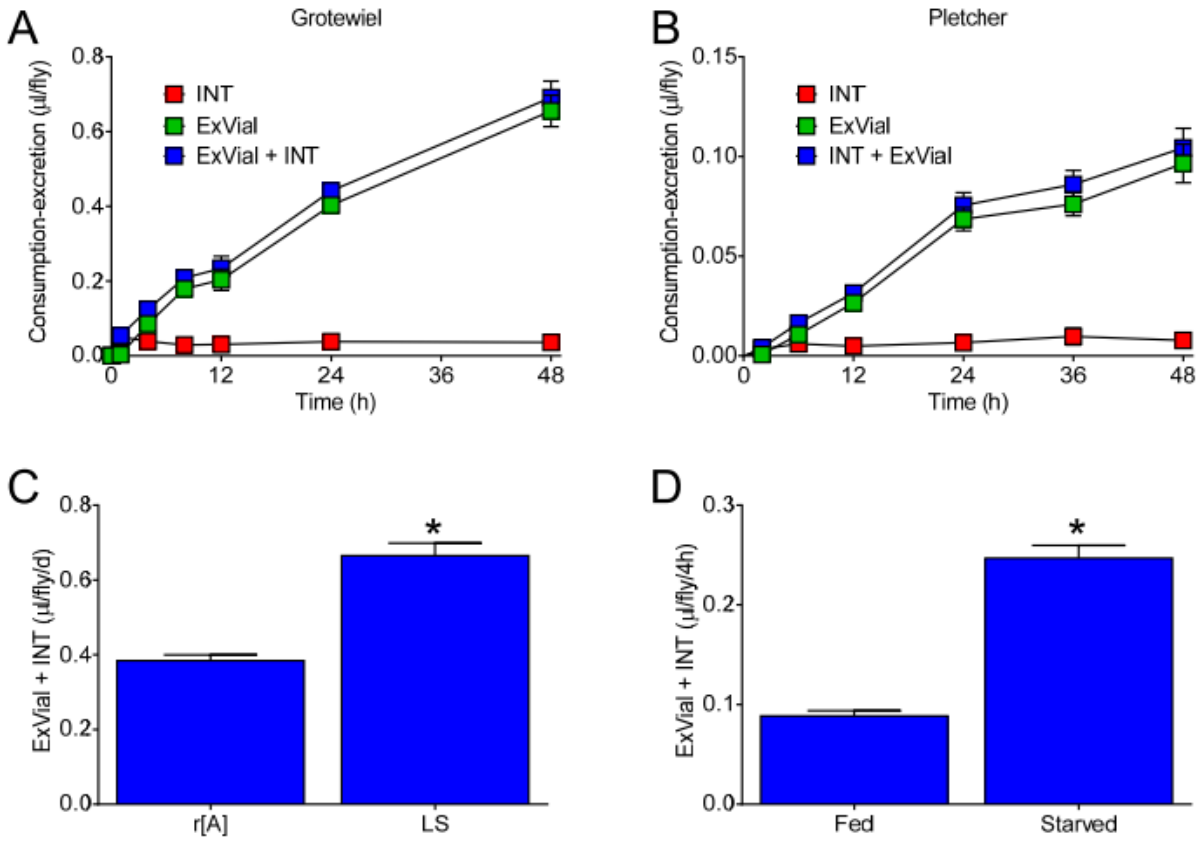

Figure 7.11. Targeted Con-Ex studies in males. (A and B) Time-dependent accumulation of INT, ExVial and ExVial+INT in r[A] (A, Grotewiel laboratory) and Canton-S (B, Pletcher 2 laboratory). ExVial and ExVial+INT increased in a linear fashion with time ($p<0.0001$, $R2 = 0.8450$-0.9108, $n=7$-8 per time-point). (C) ExVial+INT was greater in LS compared to r[A] males (two-tailed t test, $p<0.0001$, $n=8$). (D) Starvation increased ExVial+INT in r[A] males (two-tailed t test, $p<0.0001$, $n=8$).

Excretion of dye on the food medium

In addition to INT and ExVial dye, we anticipated that flies in Con-Ex studies would excrete dye on the food medium (excreted on medium, ExMedium). To address this possibility, we fed control flies 2Y10S3C food labeled with Blue 1 for 4 h (vial 1) and transferred the dye-fed flies to new vials containing feeder caps with medium without dye (vial 2). Flies then excreted the previously consumed dyed media for 24 h in vial 2 and we collected the resulting ExVial and ExMedium dye. Importantly, flies excreted the vast majority (if not all) of the Blue 1-labeled contents of their gastrointestinal tract in 24 h (Fig. 7.9B) and Blue 1 added to the surface of the food medium in feeder caps was readily recovered by water extraction (Fig. 7.9C).

When provided with 2Y10S3C medium, control r[A] and LS females excreted comparable percentages of waste dye on the food medium (ExMedium %, Fig. 7.12A). Interestingly, whereas diluting 2Y10S3C medium to 0.25X had no effect on ExMedium %, increasing the yeast concentration to 30% led to an increase in ExMedium % (Fig. 7.12B). Additionally, increasing the sucrose concentration to 30% and increasing the cornmeal concentration to 10% both decreased ExMedium % (Figs. 7.12B and 7.12C). The simplest interpretation of these data is that ExMedium % might not vary substantially across control strains fed the same diet, but it is influenced by media composition.

The most comprehensive approach when using Con-Ex would in principle be to measure INT, ExVial and ExMedium simultaneously. This is unfortunately impossible when using a single food tracer because the medium must be labeled to track it, the

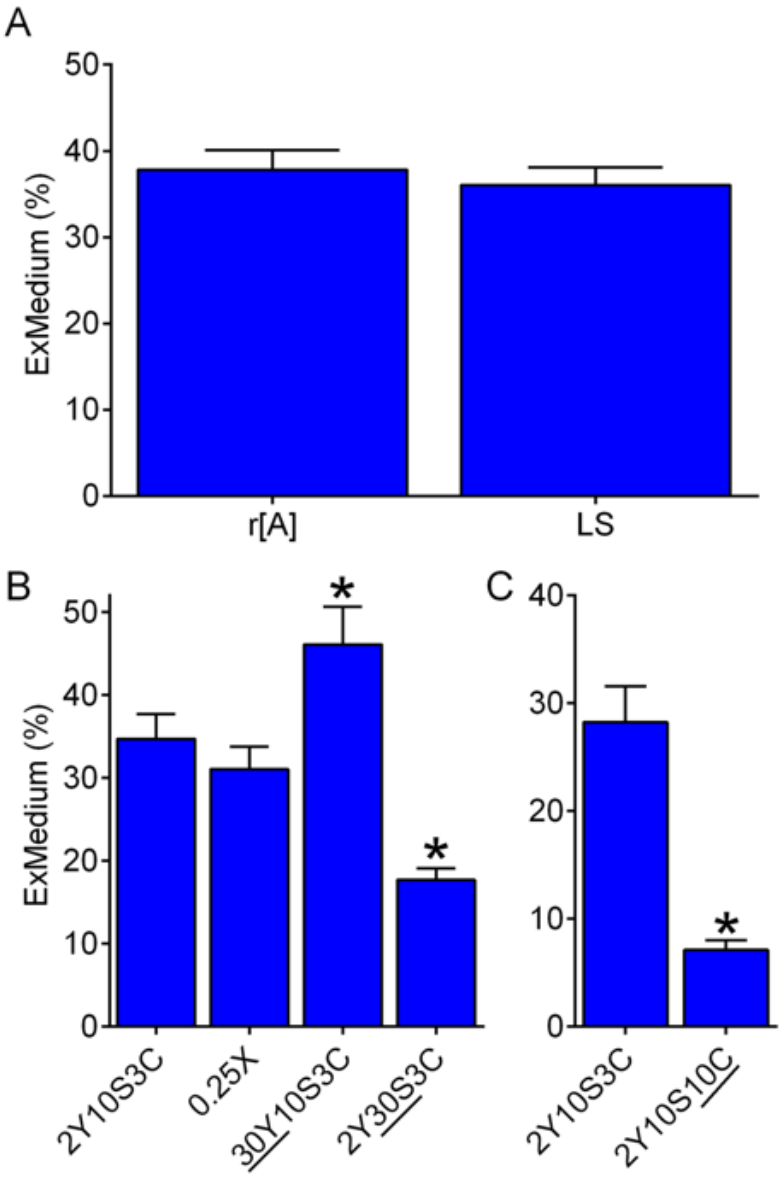

Figure 7.12. Excretion on the food medium in Con-Ex. Data are percentage of excreted dye found on the food medium. (A) The percentage of dye on 2Y10S3C medium (ExMedium %) was indistinguishable in r[A] and LS females (two-tailed t test, $p = 0.5738$, $n = 8$). (B) Food medium composition influenced ExMedium % (one-way ANOVA, $p < 0.0001$, $n = 6–10$). Compared to 2Y10S3C, ExMedium % was significantly greater on 30Y10S3C (*Bonferroni's multiple comparison, $p = 0.0366$) and lower on 2Y30S3C (*Bonferroni's multiple comparison, $p = 0.0009$. (C) ExMedium % was lower on 2Y10S10C than on 2Y10S3C (*two-tailed t test, $p < 0.0001$, $n = 8$).

flies must have physical access to the medium to consume it and access to the medium allows flies to deposit waste products on the already labeled medium. Given this experimental limitation, we explored whether including ExMedium measurements was necessary to detect changes in consumption-excretion by reanalyzing the data in Figs. 7.3A, 7.4, 7.5A, 7.7A, and 7.8A-D (data from experiments in which the relevant measurements were made). In these analyses, we calculated the adjusted volume excreted in the vial (ExAdj) as ExVial x [1/(1-fraction ExMedium)] (the final bracketed term accounts for the fraction of dye on feeder caps). In addition to ExMedium, we also explored whether including INT had a substantial impact on Con-Ex data interpretation.

Using the results of 8 independent studies shown in Figs. 7.3A, 7.4, 7.5A, 7.7A, and 7.8A-D, we performed a total of 19 statistical tests on the four different measures: ExVial alone, ExAdj alone, ExVial+INT and ExAdj+INT (Table 7.3). Note that due to low dye signal from groups with low dye concentrations (Fig. 7.3A), lower number of flies (Fig. 7.4) and after shorter periods of feeding (Fig. 7.5A), determining ExMedium in these studies was not possible and consequently we assumed that ExMedium (and therefore the calculation of ExAdj) was not affected by dye concentration, the number of flies per vial or time on the medium. In 17 of 19 tests, the statistical outcomes were indistinguishable across all four measures (Table 7.3). Of the two cases in which the statistical tests were not consistent across the four measures, one involved the percent change at early time-point in time-course studies during which INT is a substantial fraction of the total dye measured (Table 7.3, Fig. 7.5A, Bonferroni's: 1-4 h) and the other involved a food medium with the highest practical concentration of cornmeal which

Table 7.2. Components and calories in food media.

	Media						
	2Y10S3C	15Y10S3C	30Y10S3C	2Y20S3C	2Y30S3C	2Y40S3C	2Y10S10C
Yeast (g/L)	20	150	300	20	20	20	20
Protein (g/L)	10	75	150	10	10	10	10
Fiber (g/L)	5.4	40.5	81	5.4	5.4	5.4	5.4
Other Carbs (g/L)	1.2	9	18	1.2	1.2	1.2	1.2
Fats (g/L)	1.2	9	18	1.2	1.2	1.2	1.2
kCal/L	78	585	1170	78	78	78	78
Sugar (g/L)	100	100	100	200	300	400	100
Protein (g/L)	0	0	0	0	0	0	0
Fiber (g/L)	0	0	0	0	0	0	0
Other Carbs (g/L)	100	100	100	200	300	400	100
Fats (g/L)	0	0	0	0	0	0	0
kCal/L	375	375	375	750	1125	1500	375
Cornmeal (g/L)	33	33	33	33	33	33	100
Protein (g/L)	2.4	2.4	2.4	2.4	2.4	2.4	7.3
Fiber (g/L)	2.4	2.4	2.4	2.4	2.4	2.4	7.3
Other Carbs (g/L)	23.2	23.2	23.2	23.2	23.2	23.2	70.3
Fats (g/L)	0.6	0.6	0.6	0.6	0.6	0.6	18.2
kCal/L	110	110	110	110	110	110	333
Total							
Protein (µg/µl)	12.4	77.4	152.4	12.4	12.4	12.4	17.3
Fiber (µg/µl)	7.8	42.9	83.4	7.8	7.8	7.8	12.7
Other Carbs (µg/µl)	124.4	132.2	141.2	224.4	324.4	424.4	171.5
Fats (µg/µl)	1.8	9.6	18.6	1.8	1.8	1.8	19.4

Values that changed relative to 2Y10S3C (standard) medium are in red.

Table 7.3. Statistical reanalysis of individual measures from Con-Ex studies.

Figure	Statistical test	ExVial	ExAdj	ExVial+INT	ExAdj+INT
7.3A	One-way ANOVA: Blue 1 concentration	0.3674	0.3681	0.2255	0.3056
	Bonferroni's: 0.25 vs 0.5%	0.6216 (-7.7%)	0.6262 (-7.7%)	0.5707 (-8.1%)	0.5423 (-8.0%)
	Bonferroni's: 0.5 vs 1.0%	0.2778 (11.2%)	0.2771 (11.2%)	>0.9999 (4.6%)	0.9584 (6.5%)
	Bonferroni's: 1.0 vs 2.0%	>0.9999 (-6.0%)	>0.9999 (-5.9%)	0.4174 (-12.6%)	0.5550 (-10.8%)
7.4	One-way ANOVA: density	<0.0001	<0.0001	<0.0001	<0.0001
	Bonferroni's: 10 vs 20	0.5045 (-7.5%)	0.5059 (-7.5%)	0.6170 (-7.0%)	0.5800 (-7.2%)
	Bonferroni's: 20 vs 30	0.0003 (-29.9%)	0.0003 (-29.1%)	0.0007 (-28.4%)	0.0005 (-28.6%)
7.5	One-way ANOVA: time	<0.0001	<0.0001	<0.0001	<0.0001
	Bonferroni's: 0 vs 1 h	>0.9999 (n/a)	<0.9999 (n/a)	>0.9999 (n/a)	>0.9999 (n/a)
	Bonferroni's: 1 vs 4 h	0.7516 (3488%)	0.7555 (2787%)	0.0003 (886%)	0.0043 (955%)
	Bonferroni's: 4 vs 8 h	0.0015 (248%)	0.0015 (247%)	0.0135 (65.2%)	0.0063 (87.1%)
	Bonferroni's: 8 vs 12 h	0.2749 (36.7%)	0.2767 (36.6%)	0.5209 (21.3%)	0.4153 (24.7%)
	Bonferroni's: 12 vs 24 h	<0.0001 (88.0%)	<0.0001 (88.2%)	<0.0001 (57.3%)	<0.0001 (65.0%)
	Bonferroni's: 24 vs 48 h	<0.0001 (44.8%)	<0.0001 (44.7%)	<0.0001 (32.2%)	<0.0001 (35.8%)
7.7A	t test: r[A] vs LS	<0.0001 (99.9%)	<0.0001 (94.3%)	<0.0001 (92.3%)	<0.0001 (90.1%)
7.8A	t test: media dilution	<0.0001 (50.4%)	<0.0001 (42.4%)	<0.0001 (52.4%)	<0.0001 (44.5%)
7.8B	t test: yeast supplementation	<0.0001 (-65.8%)	<0.0001 (-58.7)	<0.0001 (-56.7%)	<0.0001 (-53.3%)
7.8C	t test: sucrose supplementation	0.0001 (-38.9%)	<0.0001 (-51.6%)	0.0003 (-32.2%)	<0.0001 (-45.4%)
7.8D	t test: cornmeal supplementation	0.7022 (-4.8%)	0.0411 (-26.4%)	0.2532 (-10.7%)	0.0121 (-27.0%)

Statistically significant p values (and percent changes) are indicated in red.

influences ExMedium and therefore ExAdj (Table 7.3, Fig. 7.8D, two-tailed t test). Importantly, the percent changes in ExVial, ExAdj, ExVial+INT and ExAdj+INT were highly correlated, comparable in size, and in the same direction in virtually all cases (Table 7.3). Our reanalysis suggests that INT and ExMedium do not contribute significantly to interpretations in many cases and therefore that measuring ExVial alone might be sufficient to detect major changes in Con-Ex in most studies.

Con-Ex power analyses

An important consideration regarding the utility of any experimental method is the number of replicates that must be performed to detect differences of varying magnitude (i.e. power). Using the average standard deviations for ExVial, ExAdj, ExVial+INT and ExAdj+INT derived from 23 experiments with control r[A] females, we found that all four measures had similar power to detect differences between groups (Fig. 7.13), consistent with the statistical reanalysis of these four measures (Table 7.3). Additionally, we found that 30%, 20% and 10% differences between two mean values can be detected at 80% power in Con-Ex studies with 4-6, 8-11 and 30-50 replicates, respectively (Fig. 7.13). Detection of differences of 20% or greater in Con-Ex studies therefore requires a reasonable number of replicates to be performed. The number of replicates required to detect differences in Con-Ex appears comparable to CAFE, but greater than when using radioactive methods [165].

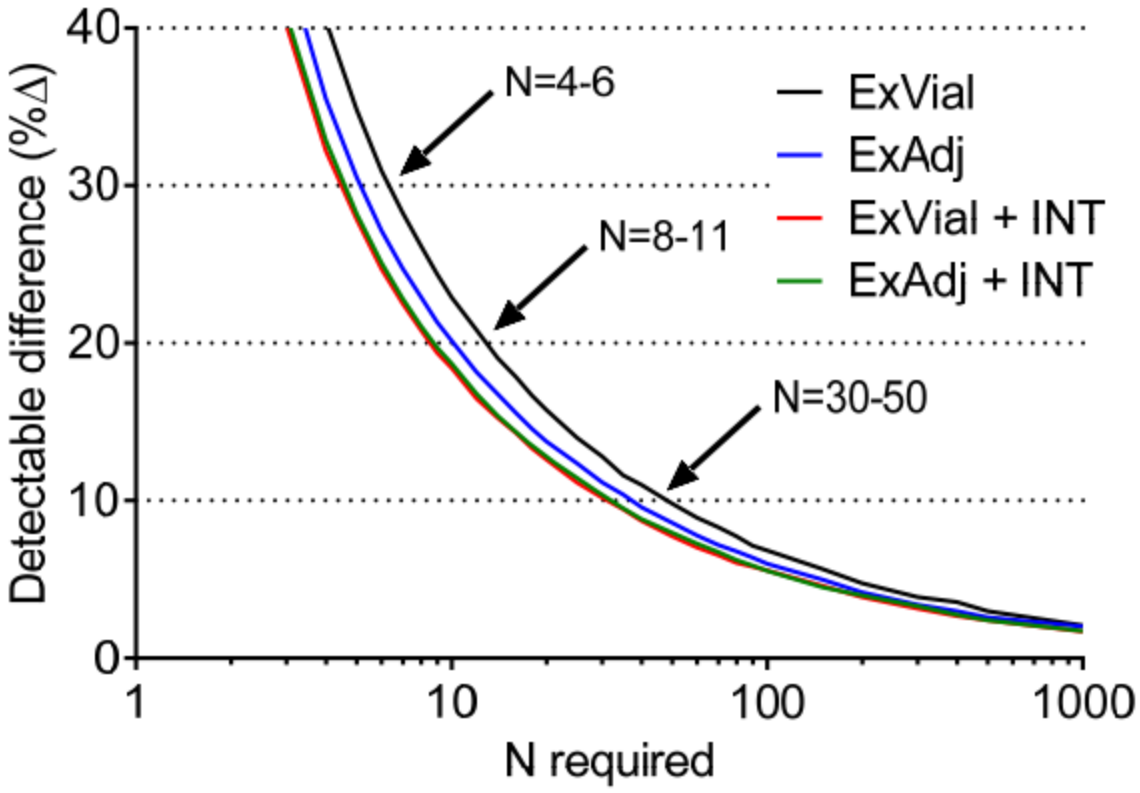

Figure 7.13. Power analysis of Con-Ex data. Data are the N required (number of replicates in each of two groups; X-axis) to detect the indicated % changes between the means of the two groups (Y-axis) using an unpaired two-tailed t test with 80% power when measuring ExVial, ExAdj, ExVial+INT and ExAdj+INT based on average standard deviations for the four measures of 0.058, 0.076, 0.057 and 0.081 µl/fly/d, respectively. The number of replicates required to detect 30%, 20% and 10% differences between means are indicated by arrows.

D. Discussion

Despite the growing interest in studies on diet in flies, it is often very challenging to determine the amount of dietary media consumed when flies are housed on solid, agar-based food [256]. We developed Con-Ex as a dye-based method for determining intake of solid media in *Drosophila*. Flies are provided continuous access to agar-based media for hours to days at a time in Con-Ex studies, mimicking routine fly housing conditions. Flies in Con-Ex studies consume food media labeled with dye and then excrete dyed waste throughout their environment within the vial for the duration of the experiment. Of the dyes that we tested in Con-Ex experiments, FD&C Blue No. 1 is the only dye that has a strong absorbance signal, has reasonably low background noise, and does not influence media consumption. Con-Ex studies with Blue 1 detect the predicted influences of feeding duration, strain, starvation, mating status and food composition on media ingestion. Additionally, the volume of Blue 1 consumed in liquid medium is comparable to, if not identical to, the volume of excreted Blue 1.

Furthermore, Con-Ex data generated in the Grotewiel and Pletcher laboratories were qualitatively similar. Our studies indicate that Con-Ex with Blue 1 as a food tracer is useful for assessing solid media consumption in adult flies. Identifying additional food dyes, particularly dyes that have distinct detection requirements (i.e. dyes with absorbances that do not interfere with the detection of Blue 1) would be valuable, for example, in studies aimed at measuring concurrent consumption-excretion of different media, experiments that require a specific absorbance spectrum due to interference by a transgenic marker, etc. Labeling food with fluorescent tracers like rhodamine B or

fluorescein [136, 262] might increase the sensitivity of or otherwise further broaden the utility of the Con-Ex method.

The accumulation of ExVial or ExVial+INT dye rises in a largely linear fashion out to ~24 h in Con-Ex studies, but thereafter it appears to begin to plateau as described previously in studies using a radioactive tracer [165]. Since flies are exposed to and consume the same food medium over several days in Con-Ex and radioactive labeling studies (as well as under standard housing conditions), non-linear food intake after ~24h on the medium might be the norm with routine fly husbandry. Additional studies are needed to determine whether the non-linear consumption at later feeding time-points is related to time-dependent loss of water from the medium, accumulation of waste on the medium, or other changes.

The dye used to label food media in Con-Ex studies as described here can be detected in 3 samples: INT, ExVial, and ExMedium. In studies that included all relevant measures, we calculated the total amount of excreted dye (ExAdj) by adjusting ExVial to account for the volume excreted on the food medium. Although ExAdj+INT is arguably the most comprehensive value to assess in Con-Ex studies because it likely reflects the total volume of media consumed, we found that assessing ExVial alone was typically sufficient. In the majority of studies, statistical analyses with or without ExMedium or INT led to the same interpretations and the magnitudes as well as the directions of change in the volumes consumed-excreted were comparable whether ExMedium or INT were considered. Thus, manipulations that cause large effects could likely be identified—at least in a preliminary fashion—by measuring ExVial alone.

Flies stand on or walk across the surface of the food medium in Con-Ex studies. This physical interaction with the food medium, which is required for consumption, raised the possibility that some of the dye we measured as excreted might instead have been transferred onto the vial wall by the flies' appendages, thereby potentially confounding the use of Con-Ex to measure consumption per se. Several observations lead us to believe that the vast majority of dye that is deposited in the vial during Con-Ex studies is from consumption and subsequent excretion of the food medium. In our time-course studies with Blue 1, we detect greater amounts of INT dye than ExVial dye at early time-points and there is a lag of at least 1 h before ExVial dye can be detected. This strongly suggests that flies can interact with the medium to consume it without transferring detectable amounts of dye from the food to the vial on their appendages and is wholly consistent with dye consumption preceding dye excretion. Additionally, in Con-Ex studies dye is clearly visible inside of flies and in excretion products on the walls of vials, and the total volume of media consumed is comparable to previously reported volumes determined using radioactive food labels [165]. The most parsimonious interpretation of our data is that the vast majority of the detectable dye in Con-Ex experiments is derived from consumed food media.

One of the most widely used approaches for measuring solid media consumption in flies is based on the internal accumulation of a radioactive tracer [165, 171, 260]. Consumption of media labeled with some radioactive tracers leads to the progressive accumulation of the tracer within flies for up to 3 days [165]. Although the utility of this method is well established, the possibility of inadvertently releasing radioactive flies in a laboratory could make this approach challenging to implement in some settings.

Additionally, the radioactive chemicals (leucine, ATP, CTP, etc. [165, 171, 260]) in this approach must be metabolized and then incorporated into long-lived molecules within the fly to be detected. This requirement for macromolecular incorporation is a potential confound for using accumulation of radioactive tracers given that changes in absorption of nutrients from the gut or metabolism of the tracers could occur in response to dietary manipulations.

FlyPAD is another method for assessing feeding behavior in *Drosophila* fed solid food media [259]. FlyPAD is a highly sophisticated approach that measures the interactions of individual flies with solid food medium over relatively short time periods of up to ~1 h. Major strengths of the FlyPAD method include that it assesses behavior in individual flies, it captures very detailed information regarding feeding behavior, and the number and duration of fly interactions with the food medium correlate with estimates of medium consumption [259]. In contrast to FlyPAD, Con-Ex requires equipment found in virtually any modern laboratory, was developed primarily to estimate consumption of solid medium, and assesses consumption of medium under routine housing conditions in groups of animals on media for hours to days. Although both FlyPAD and Con-Ex could certainly be adapted for uses beyond those described here, the two methods are quite different technically and are suitable for addressing distinct questions related to the biology of feeding in flies.

Of the previously described methods, Con-Ex is most similar to those based on the accumulation of radioactive isotopes. Both Con-Ex and the radioactivity accumulation methods label food with a tracer that does not impact consumption, report essentially linear rises in the volume of media consumed out to 24 h or more, can be

used for feeding durations lasting hours to days under conditions mimicking routine housing conditions, detect the effects of genetic background, starvation and mating status on consumption, and are able to measure the consequences of changing the nutritional composition of fly media [165]. Advantages of the radioactivity-based methods are that they are operationally straightforward and sensitive. Advantages of the Con-Ex method are that it uses a readily available dye tracer, does not rely on radioactivity, is inexpensive and circumvents potential confounds associated with metabolism of the food label. Our results using Con-Ex with Blue 1 indicate that it is a suitable, non-radioactive method for determining consumption of solid media in *Drosophila*.

The Con-Ex approach described here was designed to assess the effects of discrete treatments on feeding (e.g. starved versus fully fed flies). Since the amount of excreted Blue 1 corresponds to the amount of consumed Blue 1, it should be possible to use Con-Ex to estimate the total volume of medium flies consume when INT, ExVial and ExMedium are all determined. Importantly, though, Con-Ex has utility even when ExVial alone is determined. The utility and ease of assessing ExVial alone raises the possibility that the method could be adapted for larger scale approaches aimed at identifying genes, genetic pathways and neural circuits that regulate feeding behavior. The possibility that Con-Ex can be used as a large scale screening platform is a significant potential advantage not shared by other current methods for assessing consumption of solid food media in *Drosophila*.

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F. Author contributions

M.G., R.E.S., K.M.L., B.Y.C, J.C.J. and S.D.P. designed the experiments, R.E.S., B.C.S., K.M.L., B.Y.C., and J.C.J. performed the experiments, M.G., B.C.S., B.Y.C. and J.C.J. analyzed the data and generated the figures, M.G. wrote the manuscript and R.E.S., K.M.L., B.C.S., B.Y.C., J.C.J. and S.D.P. edited the manuscript.

G. Competing interests

The authors declare no competing interests.

7.2 – Protocols

P.1 - Basic Fly Handling and Husbandry

A. Standard Fly Lab Lingo:

1. Stock or strain: a culture of flies with a particular genotype. Balanced stocks have a special chromosome called a balancer that is marked with a dominant phenotype and suppresses recombination on the corresponding sister chromosome. Balanced stocks are often weak (i.e. grow poorly).

2. Seeding: putting adult flies into a new bottle or vial. Also called 'setting-up'.

3. Transfer: moving flies without anesthesia from one vial or bottle to another. One-to-one transfer means moving flies from one bottle/vial to one new bottle/vial. Two-to-one transfer means moving flies from 2 vials/bottles to 1 new vial/bottle. Also called 'flipping'.

4. Clearing: removing all of the adults from a bottle or vial. Can be done with or without anesthesia.

5. Anesthesia: CO_2 used to temporarily immobilize flies.

6. Brood: refers to the number of times a set of adults has been used to seed bottles. Using flies for 2 broods is common, with 3 broods being possible in some cases.

7. white plus (w^+): indicates eye color. white minus (w^-) flies have white eyes. w^+ flies have eyes that can vary from light peach to deep red.

8. Food: All of our fly food currently has antibiotics on it (ampicillin, tetracycline and chloramphenicol, ATC). Yeasted (Y) food vials and bottles have live yeast on added. Yeasted food should be used for seeding new vials and bottles for *growing* flies. Nonyeasted (NY) food has no yeast on it and should be used to *house* flies prior to behavioral studies and for *storing* virgin females and males prior to setting-up crosses.

B. Standard Fly Husbandry

1. Remove necessary number of yeasted bottles or vials from the cold room. Use bottles to grow lots of flies for behavioral, stress or other large experiments. Use vials for smaller numbers of flies in limited scale crosses or other small scale experiments.

2. Before putting in new flies, bottles and vials must be dried 2 hours to overnight in the environmental chamber so that all condensation on the walls evaporates. The food will pull away from the wall of the bottle or vial if they are over-dried. It is poor practice to use over-dried food.

3. Turn on the CO_2. Clean microscope, CO_2 pad and counter with ethanol. Clean before starting, between each genotype and after you are finished. Be sure the CO_2 is on before putting ethanol on the pad.

4. Open CO_2 to pipette, invert bottle or vial, insert pipette along cotton plug and tap bottle/vial gently. Flies will become anesthetized quickly and should fall onto the plug and/or the neck of the bottle/vial.

5. Clic off CO_2 to pipette, remove CO_2 pipette from vial/bottle. Hold inverted bottle/vial over CO_2 pad. Remove plug and gently shake/tap flies onto pad into a pile. Return plug to bottle/vial and set aside.

6. Place anesthetized flies in a row and sort flies according to needs. Short CO_2 times are important. For collecting flies that will be used in behavioral studies, goals are (1) all genotypes experience the same CO_2 exposure and (2) all flies are anesthetized for less than 5 minutes.

7. Set-up new bottles/vials by putting sorted flies from step 6 into dried bottles/vials. Anesthetized flies should be kept on the wall of the bottle/vial. If they fall into the food, many of them will stick there and die. Robust strains such as w[A], CS, etc. will do well with 10 females (♀, see below) per bottle or 3 females per vial. It is good practice to include a comparable number of males (♂, see below). Weaker stocks will need more females, up to as many as 50 per bottle and 15 per vial. When working with a stock that is new to you it is good practice to seed bottles or vials with a range of females (10-25/bottle for example) and then use an optimum number thereafter based on how the various bottles/vials grow.

8. Insert cotton plug, invert new bottle/vial and tap anesthetized flies onto the plug. Lay the bottle/vial on its side, label with genotype and date. First broods (i.e. bottles or vials in which the flies are new parents) are marked with a single slash.

9. Wait for flies to regain locomotor activity. Turn bottles/vials upright and place in environmental chamber to grow.

10. Beginning at around 4 days after seeding, check bottles/vials daily for larval activity. When larval activity is obvious, transfer adults to new bottles/vials (dried appropriately). Label second brood with genotype, date and two slashes.

11. Beginning at around 4 days after seeding the second brood, check bottles/vials daily for larval activity. Discard adults when larval activity is obvious. If necessary, a third brood is possible in some cases.

12. You should expect to see obvious larval activity 4 to 7 days after seeding and obvious pupae 5-10 days after seeding. New adults should begin emerging ~10 days after seeding. Some strains, especially balanced strains, can take up to 4 additional days to emerge. Perfectly seeded bottle/vials will have robust larval activity followed by large numbers of pupae that populate the bottom three-fourths of the wall. Pupae will not typically be in the food or on the plug in these bottles. Large numbers of healthy adults suitable for experiments will emerge from perfectly seeded bottles/vials.

13. Common Problems: If your bottles/vials are too dry or wet (as described below), the resulting adults should not be used for behavioral, stress or gene expression studies. The resulting adults are fine genotype-wise and reproduction-wise, though, and can be used to set-up new bottles/vials as necessary.

a. Food too dry after 4-7 days of new adults in bottle/vial: The food should not be so dry that it detaches from the wall of the bottle of vial and the pupae are in the food. In cases like this, the food was either over-dried, there were not enough females placed in the bottle/vial, or possibly both. If this occurs across several strains that have grown well in the past, it is likely due to over-drying. If it occurs with a subset of strains, it is more likely due to insufficient numbers of females being used for those specific strains. The appropriate fixes are to decrease drying time, add more females, or both.

When you transfer flies from the first to second brood or when clearing the second brood, note the quality of the culture and food. If the food in some bottle/vials is

detached from the wall after 7 days, go ahead and transfer/clear the adults and then add ddH_2O (NOT ETHANOL!) to the bottle/vial until the gap between the food and the wall is filled. In many cases this will help the larvae quite a lot and you still might get a decent yield of adults, although they might be delayed a few days due to lack of water.

b. Food too wet after 4-7 days of new adults in bottle/vial: The food should not be so wet that it runs down the wall of the bottle/vial when it is inverted and the pupae are on the plug. If this happens, the food was not dried sufficiently before adults were added, too many adults were added, or possibly both. If this occurs across several strains that have not had this problem in the past, it is likely due to under-drying the food. If it occurs with only a subset of strains, it is more likely due to too many females being added in those specific strains. The fixes are to increase the drying time for bottles/vials, decrease the number of females used, or both.

If you notice that your bottles are too wet when transferring from the first to second brood or when clearing the second brood, you can put a folded Kim wipe in the bottle/vial so that it touches both the food and the plug. This will not result in a miraculous drying of the bottle/vial, but it can convert a bottle/vial that is far too wet into one that can be managed with some care.

C. The Basics of Setting-Up Crosses

1. You will need males (♂, mated or unmated) and females (♀ with a ‘v’ on top, unmated or virgin) for your crosses. Grow bottles or vials as above for strains required to generate males and virgin females. For planning purposes, you can comfortably

collect 100 males and/or 50 virgin females from a robust bottle. Likewise, you can probably count on collecting 20 males and 10 virgin females from each well-seeded vial.

2. Around day 10 after seeding, begin to collect virgin females, identified by their light body pigmentation and female genitalia (see below). Typically, one would collect virgin females first thing in the morning, again around noon, and again last thing before leaving for the day.

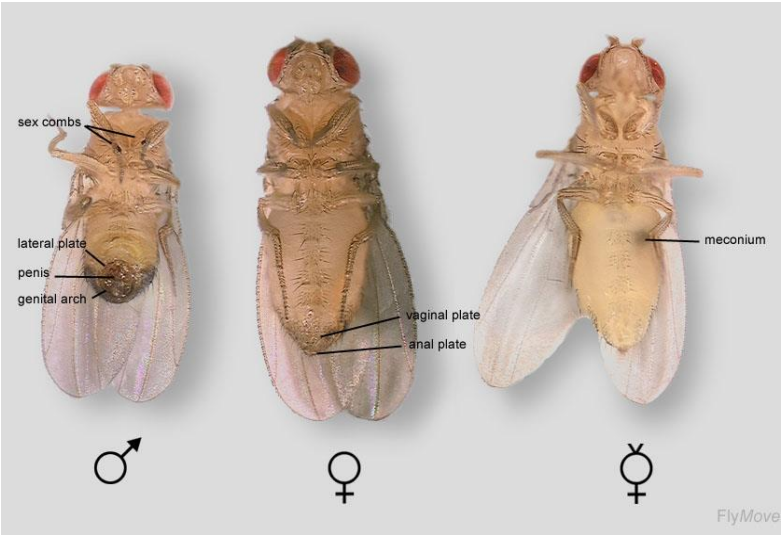
3. Keep virgin females in nonyeasted vials with no more than 25 females/vial. Label each vial with genotype, date and number collected. Keep collected females in environmental chamber until ready to use. One will often collect virgin females over several days or until a sufficient number of virgin females has been collected. Also, it is convenient to store virgin females in upside-down vials.

4. When sufficient numbers of virgin females have been collected (~10% more than you plan to use) or when it is obvious that you will be able to collect all the virgin females you will need, collect all males into nonyeasted vials needed for your crosses. Males are identified by their male genitalia (see below).

5. Set-out yeasted bottles or vials to warm and dry as described above. On the day of the cross, check all virgin female vials for larvae using the microscope. Any vials with larvae MUST be discarded because at least one of the females has mated. Use only virgin females from vials with no larvae.

6. To set-up a cross, anesthetize the males and check them, anesthetize the virgin females on the same plate and check them, and put appropriate numbers of males and females into yeasted bottles/vials as described in steps B7-B9 above. Handle them thereafter as described in B10-B12 above.

7. Make sure that you know what progeny to expect from your crosses before you set them up.



P.2 - Quarantine of New Fly Stocks

The following is an outline of steps which should be followed when new stocks are brought into the lab. It is important that stocks go through the entire quarantine procedure to prevent a mite infestation. All new stocks should be handled as if there are mites present. Quarantine stocks must be kept isolated from all other stocks.

1.New stocks must be brought directly to an isolated spot. Do not bring them near any current lab stock.

2.Transfer the adults to a fresh vial (P0) and keep the original vial for ~one week. Place vials in a water moat. Each stock needs its own individual moat.

3.When the P0 vial contains adequate larvae, second brood the adults to a backup vial. Save all vials for monitoring of mites later.

4.Shortly after the adults eclose from the P0 vial, transfer them to a fresh vial (F1) for ~one week and repeat step #3.

5.Keep the stock isolated for two more generations (F2 and F3).

6.When the adults eclose from the F3 vial, put them in a fresh vial and check vials from all previous generations for mites.

7.If ALL vials are clear of mites, the stock may be taken out of quarantine.

8.If mites are found at any time or suspected of being in a stock, restart the quarantine process until no mites are found.

Adapted from the recommendations of the

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P.3 - Ethanol Sedation Assay

A. Day before assay

1. Collect flies (reared for behavioral assays) in groups of 11 (single sex) under brief CO_2 (~5 minutes) following standard procedures for behavioral assays. Collect only those flies that look healthy, are relatively the same size, have normal wings, and appear dry. Flies should be transferred from the CO_2 plate into an Eppendorf tube using a funnel and then dumped from the Eppendorf tube into a non-yeasted vial.

2. Allow flies to recover overnight in upside-down non-yeasted food vials in the environmental chamber. It is possible to test a maximum of 24 vials of flies in a single experiment.

3. Dilute ethanol solution as necessary (85% is our standard concentration). ~250 ml of ethanol solution can be stored in a sealed 500ml bottle or other sealed container for a week without a problem. Make ethanol fresh <u>weekly</u>. Diluted ethanol is exothermic and should be <u>stored overnight at room temperature before use</u>.

B. Day of assay

1. For each vial of flies to be tested, you will need (a) a clean, empty food vial; i.e. testing vial, (b) a new Flug, (c) a silicone #4 plug and (d) 1.0 ml of ethanol solution (85% ethanol is our standard concentration).

2. Turn on humidifier and allow relative humidity in testing room to rise to 55-65%. Temperature should be 20-23°C. Record humidity and temperature on test log.

3. Have someone else in the lab assign a unique code to each group of vials for each genotype and—IMPORTANTLY—record the code for later. Place coded vials with flies in testing room to acclimate.

4. Label empty testing vials to match codes on fly vials from B.3.

5. Construct a testing log by entering the code for each vial into the Test Log E or Test Log EE sheet within the Excel Sedation file SA E EE 6 min SIGMOIDAL 2015.10.05. Use a random or cycling order. Add other pertinent information (% ethanol, sex, etc.) to the Test Log worksheet and print for use during testing.

6. Using the Test Log as a guide, arrange coded food vials with flies and empty testing vials into matching arrays with 4 vials in each row. The maximum possible number of vials that can be tested in a single experiment is 24 vials (i.e. 6 rows of 4 vials each).

7. Transfer flies from food vials into matched/labeled testing vials one at a time and immediately insert Flugs into testing vials until Flugs are a uniform distance below the vial tops. Use the Fluginator to push Flugs down into vials.

8. Time 0 assessment: Grasp each vial individually with thumb and forefinger, tap gently on the table three times to knock flies to the bottom of the vial, wait 30 seconds and then count the number of flies that are immobile. Typically, this is 0 or 1 at time 0. Record the number of immobile flies for each vial at time 0 in the printed Testing Log.

9. Hereafter, each row of four vials will be handled as a set at staggered one-minute intervals.

Start timer counting up at time 0 and immediately begin adding 1 ml of ethanol to the Flug in the vials for the first row/set of 4 vials. Add ethanol to the vials at 5 second intervals in the order they will be tested. Add ethanol to the Flugs in a circular motion so that all ethanol is absorbed as uniformly as possible. When ethanol has been added to all four testing vials in the set, insert a silicone #4 plug in each vial to seal it.

At times 1, 2, 3, 4 and 5 minutes on the timer, add 1 ml of ethanol to the second, third, fourth and fifth sets of 4 vials, respectively. Continue inserting #4 plugs after adding ethanol to each set of 4 vials.

10. At time 6 minutes, test the first set of 4 vials by grasping the first vial with thumb and forefinger and then tapping gently on the table three times to knock flies to the bottom of the vial. Tap the other 3 vials in the set the same way at 5 second intervals. 30 seconds after tapping the first vial, count and record the total number of flies that are sedated. Count and record the number of sedated flies in the other 3 vials at 5 second intervals. Flies are scored as sedated if they do not appear to have productive locomotion.

The specific schedule is:

Vial	Tap	Assess
1	6 min 0 s	6 min 30 s
2	6 min 5 s	6 min 35 s
3	6 min 10 s	6 min 40 s
4	6 min 15 s	6 min 45 s

At times 7, 8, 9, 10 and 11 minutes, test the second, third, fourth, fifth and sixth sets of vials, respectively, as done for the first set.

11. At time 12 minutes, test the first set of 4 vials again as described in B10 and continue testing the second, third, fourth, fifth and sixth sets of vials at 13, 14, 15, 16 and 17 minutes, respectively.

Continue testing flies as described in B10 and B11 until all flies are sedated (typically 60-90 min).

12. Record the total number of flies in each vial.

13. Clean-up is (a) turn off humidifier, (b) remove #4 plugs for washing and reuse, (c) discard Flugs/vials/flies, (d) remove any trash from and straighten up testing room and (e) turn off light in testing room.

14. Enter the total number of flies in each vial and the number of flies sedated at each time point in the Test Log within the Excel worksheet. Percent Active flies will be automatically calculated and graphed below the Test Log. Press ‘Ctrl + s’ to calculate ST50s for each vial and sort the data by group in the Sorted Data worksheet.

15. Note any flagged data in Sorted Data worksheet. Consider excluding data that looks qualitatively poor.

M Grotewiel, R Schmitt, K Lee: 7/2014, 3/2015, 7/2016

P.4 - Simple Locomotor Assay

A. Day before assay

1. Collect experimental flies (reared for behavioral assays) in groups of 11 (single sex) under brief CO_2 following standard procedures for behavioral assays. A maximum of 24 vials, 6 groups of 4, can be tested during the assay. You will need to collect 4 additional vials of controls flies to be the vortexed group.

2. Allow flies to recover overnight in non-yeasted food vials in the environmental chamber.

B. Day of assay – Flugged Vial Experiment

1. Turn on humidifier(s) and allow relative humidity in testing room to become/remain between 55-65%.

2. Prior to the experiment, print the locomotor test log sheet. For each vial of flies to be tested, you will need: a clean, empty testing vial and a new Flug

3. Transfer flies from all food vials into matched testing vials one at a time and immediately insert Flugs into testing vials until the bottom of the Flugs are just below the vial tops. For vials used for vortexing, use “The Fluginator” to force Flugs further down into vials until the tape is in contact with the vial.

4. Vortex the control vials for 4 minutes. Up to four vials will be vortexed at once. In order to prevent vial destruction during the vortex, use a couple of rubber bands on each vial and a few rubber bands to hold all four vials together. (See Below)

5. Have someone else in the lab assign a unique code to each vial for each genotype and—IMPORTANTLY—record the code for later*. Place coded vials with flies in testing room to acclimate.

6. Label empty testing vials to match codes on fly vials from B5

7. Using the Test Log as a guide, arrange coded food vials with flies and empty testing vials into matching arrays in the testing room. It is possible to test 6 sets of 4 vials simultaneously, so arrange 24 vials (maximum) in 6 sets or rows containing 4 vials each. Then transfer flies from food vials to the matching testing vials.

8. Time 0 assessment (# dead): For each vial individually: grasp with thumb and forefinger, tap the vial on the table three times to knock flies to the bottom of the vial, wait 30 seconds and then count the number of flies that are dead (no movement

whatsoever). Record this number of flies for each vial at time 0 in the printed Testing Log.

9. Hereafter, each row of four vials will be handled as a set at staggered one-minute intervals.

Start timer counting up at time 0 upon completion of recording the number of dead flies.

10. At time 6 minutes, test the first set of 4 vials by grasping each vial individually with thumb and forefinger, tapping on the table three times to knock flies to the bottom of the vial. Then wait 30 seconds to count and record the total number of flies that are on the bottom.

The specific schedule is:

Vial	Tap	Assess
1	6 min 0 s	6 min 35 s
2	6 min 5 s	6 min 40 s
3	6 min 10 s	6 min 45 s
4	6 min 15 s	6 min 50 s

At times 7, 8, 9, 10 and 11 minutes, test the second, third, fourth, fifth and sixth sets of vials, respectively, as done for the first set.

11. At time 12 minutes, test the first set of 4 vials again as described in B10 and continue testing the second, third, fourth, fifth and sixth sets of vials at 13, 14, 15, 16 and 17 minutes, respectively.

Continue testing flies as described in B10 and B11 for 60 minutes.

12. Record the total number of flies in each vial on the locomotor assay sheet.

13. Fill out the locomotor assay file. Percent Active flies will be automatically calculated. Compile the data as directed on the sheet to calculate aggregate percent active and fraction alive for each vial and sort the data by group in the Sorted Data worksheet. Clean-up is (a) turn off humidifier, (b) discard vials containing flies, (c) remove any trash from and straighten up testing room, and (d) turn off light in testing room.

I. Hines, 2015.10

M. Grotewiel, 2016.07

P.5 - Micro-Batch Food Protocol

1. Add 750mL distilled water to a large beaker and bring to boil on hot plate (turn it all the way up.) Use a large stir bar and the stir function around 4-5.

2. For complete food, measure out ingredients as follows:

	Complete	20% Sugar	30% Sugar	40% Sugar	10% Yeast	20% Yeast	30% Yeast	10% Cornmeal	Complete (no sugar Or cornmeal)	30% Yeast (no sugar Or cornmeal)
Sugar	100 g	200 g	300 g	400 g	100 g	100 g	100 g	100 g	0 g	0 g
Agar	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g
Yeast	20 g	20 g	20 g	20 g	100 g	200 g	300 g	20 g	20 g	300 g
Cornmeal	33 g	33 g	33 g	33 g	33 g	33 g	33 g	100 g	0 g	0 g

3. Alter any ingredients as needed.

4. Mix together the dry ingredients for each batch.

5. Once the water is boiling, turn it down to about 5 and VERY slowly sprinkle in the solid ingredients. If you go too fast, it will boil over. Consider wearing an autoclave glove for steam protection.

6. Bring volume up to 1 L with hot water. (Microwave water in beaker until boiling)

7. Cook on low boil for 20 minutes.

8. Turn off heat and periodically check the temperature of the food (roughly 15-20 mins)

9. When the food temperature is 70°C, add Tegosept solution:

    8.6 mL EtOH

    1.67 g Tegosept

10. Let the food cool to at least 65°C (not lower than 55°C), then pour using the vial despenser.

11. Let the food solidify/cool for an hour or more, then add antibiotic solution (100 µL per vial):

    12 mL total (1 tray):

    6 mL H_2O

    0.12 g Ampicillin

    0.024 g Tetracyline

    5.7 mL EtOH + 0.3 mL H_2O

    0.15 g Chloramphenicol

Brandon Shell 8/2016

P.6 - Standard Feeding Assay

Making the food (make the day before you begin the assay)

1. Determine number of Mocaps needed for feeding assay.
2. Each Mocap holds about 5 mL of food. Calculate amount of water needed to make food.
    a. Example: 32 Mocaps x 5 mL = 160 mL batch (up batch to 200 mL)
    b. It's easiest to make one big batch of food and then split it in two so there is one with dye and one without dye. *Note: Only split the batch if you have a way to keep both batches above 65°C. (2nd hot plate or water bath)
3. Measure out food ingredients. (Measurements in Table on back page)
4. Mix together dry ingredients for each batch.
5. Fill a flask with the appropriate amount of DI water. (Measurements in Table on back page)
6. Place flask on hot plate, add stir bar, and turn on stir function.
7. Turn the heat all the way up until the water begins to boil (roughly 10 minutes).
8. Once the water is boiling, turn the temperature down to about 100°C and VERY slowly sprinkle in the solid ingredients. If food is poured too fast, it will boil over. Wear autoclave gloves for steam and heat protection.
9. Cook food on low boil for 20 minutes.

10. While the food is cooking, make antibiotic solutions. (Measurements in Table on back)

11. Turn the heat completely off to let the food cool down (roughly 10 minutes) and periodically check the temperature with a thermometer.

12. When the food temperature is 70°C, pour the antibiotic solutions directly into the food.

13. After the antibiotic solutions are mixed in, split the food into two batches, one that will have no dye and one that will have dye added to it.

    a. Example: Split a 200 mL batch into two 100 mL batches. Then add 1 g of Blue 1 dye to one of the 100 mL batches.

14. Add 1% Blue #1 to one of the batches. (Measurements in Table)

15. Let the food cool to at least 65°C (not lower than 55°C), then pour into Mocaps using a small beaker for easier pouring.

16. Let the food solidify (about 10 minutes), then place inside a lidded container and keep at 4°C overnight.

Seeding Flies (day after making food)

17. Set food out and allow to return to room temperature.

18. Collect 15 flies/vial and place Mocap on top of vial.

19. Allow flies to consume food for 24 hours at 25°C and 65% relative humidity.

Next Day (day 2)

Preparing Samples

20. After 24 hours of feeding, record the number of dead flies.

21. Fill an ice bucket with ice.

22. Transfer flies from vials to 1.7 mL microcentrifuge tubes. Place each tube on ice after transfer. Do NOT discard vials.

23. When all flies have been transferred to tubes, put tubes in -20°C to euthanize.

Measuring Vial Excretion

24. Add 3 mL of sterile water to each vial.

25. Cover vial with parafilm. Make sure the parafilm is tight enough to be able to turn the vial upside down without any water escaping.

26. Vortex the vial for 15 seconds upright and 15 seconds upside-down. Check to make sure all the dye excreted on the sides of the vial has been absorbed into the water. If not, continue to vortex.

27. Pipette 1 mL of each sample into disposable cuvettes.

28. Spec at 630λ (for Blue 1 dye) and record each sample using sterile water as a blank.

Measuring Internal Fly Dye

29. Retrieve flies from -20°C and let cool back to room temperature.

30. Add 500µL of sterile water to each microcentrifuge tube.

31. Homogenize flies using drill and pestle for 30 seconds.

32. Add 1 mL of sterile water into each micrcentrifuge tube (for a total of 1.5 mL).

33. Vortex each tube for 15 seconds.

34. Spin all tubes in centrifuge for 10 minutes.

35. Pipette 1 mL of each sample into disposable cuvette

36. Spec at 630λ (for Blue 1 dye) and record each sample using sterile water as a blank.

Tegosept and Chloramphenicol Solvent = Ethanol (add both to the same ethanol)

Tetracycline and Ampicillin Solvent = Water (add both to the same water)

Example:

For a 100 mL batch, measure out 1 mL of Ethanol and add 0.200 g Tegosept and 0.0125 g Chloramphenicol to it. Then measure out 1 mL of Water and add 0.002 g Tetracycline and 0.010 g Ampicillin to it.

Component	50 mL batch	75 mL batch	100 mL batch	125 mL batch	150 mL batch	200 mL batch
Sugar	5 g	7.5 g	10 g	12.5 g	15 g	20 g
Yeast	1 g	1.5 g	2 g	2.5 g	3 g	4 g
Cornmeal	1.65 g	2.48 g	3.33 g	4.13 g	5.00 g	6.67 g
Agar	0.50 g	0.75 g	1.00 g	1.25 g	1.50 g	2.00 g
Antimicrobial	**50 mL batch**	**75 mL batch**	**100 mL batch**	**125 mL batch**	**150 mL batch**	**200 mL batch**
Tegosept	0.100 g in 0.5 mL	0.150 g in 0.75 mL	0.200 g in 1 mL	0.250 g in 1.25 mL	0.300 g in 1.5 mL	0.400 g in 2 mL
Chloramphenicol	0.0063 g in 0.5 mL	0.0094 g in 0.75 mL	0.0125 g in 1 mL	0.0156 g in 1.25 mL	0.0188 g in 1.5 mL	0.0250 g in 2 mL
Tetracycline	0.001 g in 0.5 mL	0.002 g in 0.75 mL	0.002 g in 1 mL	0.003 g in 1.25 mL	0.003 g in 1.5 mL	0.004 g in 2 mL
Ampicillin	0.005 g in 0.5 mL	0.008 g in 0.75 mL	0.010 g in 1 mL	0.013 g in 1.25 mL	0.015 g in 1.5 mL	0.020 g in 2 mL
1% Blue #1	0.50 g	0.75 g	1.00 g	1.25 g	1.50 g	2.00 g
Flask Size	250 mL	250 mL	250 mL	250 mL	500 mL	500 mL

Component	250 mL batch	300 mL batch	350 mL batch	400 mL batch	450 mL batch	500 mL batch
Sugar	25 g	30 g	35 g	40 g	45 g	50 g
Yeast	5 g	6 g	7 g	8 g	9 g	10 g
Cornmeal	8.25 g	9.91 g	11.66 g	13.32 g	14.86 g	16.65 g
Agar	2.50 g	3.00 g	3.50 g	4.00 g	4.50 g	5.00 g
Antimicrobial	**250 mL batch**	**300 mL batch**	**350 mL batch**	**400 mL batch**	**450 mL batch**	**500 mL batch**
Tegosept	0.500 g in 2.5 mL	0.600 g in 3 mL	0.700 g in 3.5 mL	0.800 g in 4 mL	0.900 g in 4.5 mL	1.000 g in 5 mL
Chloramphenicol	0.0310 g in 2.5 mL	0.0375 g in 3 mL	0.0438 g in 3.5 mL	0.0500 g in 4 mL	0.0563 g in 4.5 mL	0.0625 g in 5 mL
Tetracycline	0.005 g in 2.5 mL	0.006 g in 3 mL	0.007 g in 3.5 mL	0.008 g in 4 mL	0.009 g in 4.5 mL	0.010 g in 5 mL
Ampicillin	0.025 g in 2.5 mL	0.030 g in 3 mL	0.035 g in 3.5 mL	0.040 g in 4 mL	0.045 g in 4.5 mL	0.050 g in 5 mL
1% Blue #1	2.50 g	3.00 g	3.50 g	4.00 g	4.50 g	5.00 g
Flask Size	500 mL	1000 mL	1000 mL	1000 mL	1000 mL	1000 mL

P.7 - Single Fly Genomic DNA Preparation

1) The squishing buffer (SB) is 10 mM Tris-Cl pH 8.2, 1mM EDTA, 25 mM NaCl, and 200 ug/ml Proteinase K (diluted 1:100 in SB) from a frozen 20 mg/ml stock each day.
2) Place one fly, alive, in a 0.5 ml tube and freeze tube at -80 or -20°C for a few minutes to days to kill them.
3) Mash the fly for 5-10 seconds with a pipette tip containing 50 µl of SB without expelling any liquid (sufficient liquid escapes from the tip during mashing). Then expel any remaining SB from the tip.
4) Incubate at 25-37°C (room temp.) for 20-30 minutes.
5) Inactivate the Proteinase K by heating to 95°C for 1-2 minutes.
6) Spin down crud in centrifuge and store at 4°C for up to several months.
7) Use 3-5 µl of the DNA prep (supernatant) in a 50 µl PCR reaction.

adapted from Gloor et al. 1993

P.8 - Basic Polymerase Chain Reaction (PCR) Protocol

1. Dilute primers to 10 pmol/µl. The primers in the Primer Boxes in the -20°C freezer are at 1 nmol/µl (1000 pmol/µl), so those need to be diluted 1:100 in dd H2O.
2. Thaw in hand and/or store on ice:

   primers

   Taq Buffer (in Taq box; includes $MgCl_2$)

   2.5 mM dNTPs

   dd H_2O
3. Each PCR sample or tube should contain:

   1-5 µl template (e.g. 200 ng purified gDNA or 5 µl of squish prep)

   5 µl forward primer

   5 µl reverse primer

   5 µl Taq Buffer

   5 µl 2.5 mM dNTPs

   0.3 µl Taq polymerase

   X µl dd H_2O to 50 µl total

Making each individual sample separately is cumbersome, so make a Master Mix for n+1 samples according to the PCR Worksheet MASTER. The master mix contains all components that will be used in multiple samples. For example, if amplifying several different gDNA samples with the same primer pairs, the Master Mix should contain

everything except the gDNA templates. When making the Master mix, add all components except for Taq polymerase, vortex to mix, add the Taq polymerase, then vortex to mix.

4. Aliquot the Master Mix into labeled 0.5 ml thin-walled PCR tubes and add remaining component(s).

5. Close lids and pulse vortex. If necessary, gently tap tubes to bring liquid to bottom.

6. Place tubes in thermocycler, adjust thumb-wheel on lid until you start to feel a SMALL amount of pressure, set program to MSG1 (or other as appropriate), enable heated lid, press Proceed. A run takes ~3 hours, but can vary depending on cycling parameters.

P.9 - Fly Head/Body Prep

1. Bring the following equipment to the cold room: large plastic box, styrofoam box with sieve and tube holder in it, large metal forceps, a vortexer, and enough funnels, large orange-capped conical tubes, and labeled 1.7 snap-cap tubes for the number of preps you are doing.

2. Obtain liquid N_2 (in dewar) and dry ice (in styrofoam container) from 6th floor supply center. 3/4th full liquid N_2 and 1/2 full dry ice is sufficient for ~12 preps.

3. Store samples on dry ice before and after prep.

4. Fill conical tube 3/4 full (in styrofoam container) with liquid N_2.

5. Add flies to tube, screw on cap WITH HOLES (or it will violently explode), and vortex (stopping as little as possible) until the liquid N_2 is almost gone (~1 min).

6. Repeat—filling tube with flies 1/2 full and vortexing again.

7. Pour N_2 into sieve so it is sufficiently cold (otherwise the flies will stick and you'll get nothing). Dump flies into sieve and beat laterally with heavy forceps for several minutes.

8. Collect heads (middle layer) or bodies (top layer) using funnel and labeled 1.7 snap-cap tube.

9. Store at -80°C until use.

<u>Notes:</u>

1. Wear safety glasses, lab coat, 2 pairs of latex gloves and cryo gloves.

2. Between genotypes: take a break to clean and completely dry sieve (or it will freeze together and form an ice layer over the holes). Get a new funnel and conical tubes

J. Butler 4/2015

P.10 - RNA and cDNA Prep

Part A: Fly collection

Whole body fly collection

1. Collect 25 flies of desired age, genotype and gender in a 1.5mL snap cap tube. 1 tube = 1 n. Place tubes on ice immediately after flies enter tube.
2. Once done collecting, place flies in -80. Once flies are frozen and dead, you can proceed to Part B

Head Preps

1. Collect whole body flies of desired age, genotype and gender in a conical tube. 1 tube = 1 n. For head preps, about 250 flies should be in each tube (absolute minimum = 150 flies, but this is not recommended). Store flies on ice at all times. After each collection, place flies immediately back in the -80 freezer.
2. Once collected, bring the following equipment to the cold room
    - large plastic box
    - styrofoam box with sieve and tube holder in it
    - large metal forceps
    - a vortex
    - funnel(s)
    - large orange-capped conical tubes → ONLY USE CAPS WITH HOLES

- labeled 1.7 snap-cap tubes for the number of preps you are doing
- Cryogloves → WEAR AT ALL TIMES

3. Obtain liquid N_2 (in dewar) and dry ice (in styrofoam container) from 6th floor supply center. 3/4th full liquid N_2 and 1/2 full dry ice is sufficient for ~12 preps.
4. Store samples on dry ice before and after prep.
5. Fill conical tube 3/4 full (in styrofoam container) with liquid N_2.
6. Add flies to tube, screw on cap WITH HOLES (or it will violently explode), and vortex (stopping as little as possible) until the liquid N_2 is almost gone (~1 min).
7. Repeat—filling tube with flies 1/2 full with N_2 and vortexing again.
8. Pour N_2 into sieve so it is sufficiently cold (otherwise the flies will stick and you'll get nothing). Dump flies into sieve and beat laterally with heavy forceps for several min.
9. Using funnel, quickly collect heads (middle layer) or bodies (top layer) using funnel and labeled 1.7 snap-cap tube.

9. Between genotypes: take a break to clean and completely dry sieve (or it will freeze together and form an ice layer over the holes). Get a new funnel and conical tube.

10. Store at -80°C until use.

**Wear safety glasses, lab coat, 2 pairs of latex gloves and cryo gloves (plus warm clothes and long pants).

Part B: RNA extraction

** All water used is DEPC water

** Samples must be kept on ice at all times, unless otherwise stated

1. Wipe down bench and all pipettes, pipette boxes, ect with 100% ETOH. Place clean plastic pestles in 50mL conical tube, cover pestles with chloroform, and let them soak for 20 minutes. Transfer pestles to new clean empty 50mL conical tube, and allow to air dry for 20 minutes.

** all chloroform is stored under the hood, and any procedures involving chloroform should always be done under the hood

2. While under the fume hood, add 250µL Trizol (pink, stored in fridge) to each tube of flies. Homogenize for 1 minute with drill and pestle
3. Add 100µL of chloroform to each tube. Vortex for 15 seconds. Incubate for 3 minutes at room temperature
4. Centrifuge samples at maximum speed (14,000 x g) for 15 minutes in the cold room
5. Label new 1.5mL tubes appropriately. Remove roughly 200µL of the upper aqueous phase and place in new tube. If you accidently pipette any fly parts or other liquid, centrifuge that sample again (i.e. repeat step 4) and then attempt this step. Discard tubes with fly parts and the pink liquid.
6. Add 250µL isopropanol (labeled ISO in RNA reagents station) to each tube containing the upper aqueous sample. Invert the tube 10 times. Incubate samples for 10 minutes at room temperature. After, centrifuge samples at maximum speed (14,000 x g) for 10 minutes in cold room
7. A white pellet on the bottom of each tube should be visible. Remover liquid from the tube with a 200µL pipette. Add 500µL of 75% ETOH to each tube. Invert 10 times to wash. After, centrifuge samples at maximum speed (14,000 x g) for 5 minutes in the cold room

** 75% ETOH should be made with DEPC water. Large amounts are typically pre-made and stored at the RNA reagents station

8. While samples are in the centrifuge, re clean bench with ETOH. Clean a piece of glass plate (stored at RNA reagents station) with ETOH and then chloroform. Place the clean glass on a cleaned section of your lab bench.
9. Using 200µL and 20µL pipettes, remove as much liquid as possible surrounding the pellet. Dispose of all liquid.
10. Lay tubes with the lids open on the glass plate. Allow tubes to air dry until i) no liquid droplets remain and the pellet is clear or ii) 60 minutes has passed
11. Add 50µL of DEPC water to each pellet. Allow samples to sit at room temperature for 60 minutes. After, place samples in 4°C incubator overnight.

Part C: cDNA conversion

DNAse treatment (all reagents kept in DNAse treatment box in -20 freezer)

1. Re-suspend the pellet by pipetting up and down. Once re-suspended, vortex the sample
2. Measure RNA concentration. The reference is 100µL DEPC water. Samples are 98µL DEPC water, 2µL of the RNA sample. Absorbance is read at 260nm. Record absorbance's for each sample

** RNA can be stored in -80 until ready to proceed

3. Set heat blocks to 37°C (smaller holes) and 65°C (larger holes)
4. Using the RNA absorbance excel spreadsheet, calculate how much DEPC water and RNA sample is needed. For each sample, 6µg of RNA is added to a 0.5mL snap cap tube. The total volume is brought to 17µL by DEPC water. Vortex samples

5. Add 2µL DNAse buffer (10X DNAse 1 – buffer) and 1µL DNAse enzyme (R DNAse 1). Incubate samples at 37°C for 25 minutes
6. After incubation, switch heat block to 42°C (larger holes)
7. Pulse centrifuge samples. Add 2µL DNAse inactivation reagent. Incubate samples at room temperature for 2 minutes. While incubating, flick tube gentle to resuspend the inactivation reagent. Sample should be a cloudy white.
8. Centrifuge samples at 10,000 x g for 1.5 minutes
9. Use pipette to transfer 11µL of the clear upper phase to new 1.5mL snap cap tubes

Reverse transcription (all reagents kept in reverse transcription box in -20 freezer)

10. Add 1µL of oligo-dT to each sample of DNAse treated RNA
11. Incubate at 65°C for 15 minutes. While incubating, thaw 1st strand buffer, DTT and 10mM dNTP on ice
12. After incubation, put tubes on ice for 1 minute. Pulse centrifuge.
13. Keep all samples on ice throughout. Add the following to each tube:
    - 4µL of 5 X 1st strand buffer (5 X FS buffer)
    - 2µL of 0.1M DTT
    - 1µL of 10mM dNTP
    - 1µL of Superscript 2 enzyme (SSll) **must always stay on ice**
14. Incubate samples at 42°C for 50 minutes
15. Store cDNA samples at -20°C

<u>Notes</u>:

- When reagents are low, tell someone immediately!
- Use RNAse free reagents only

- All DNAse treatment reagents from Applied Biosystems (AM1906)
- Reverse transcriptase and buffers are from Invitrogen (18064014)
- Oligo-dT from Invitrogen (18418-012)

P.11 - Real-Time PCR: Primer Check

**Reserve your PCR machine time slot ahead of time

Note: RT-PCR is highly sensitive. Knobs on pipettes MUST be taped at all possible steps when pipetting replicate samples. Change tips after each volume is dispensed.

1. Dilute a single control cDNA to these four dilutions: 1:8, 1:16, 1:32, 1:64

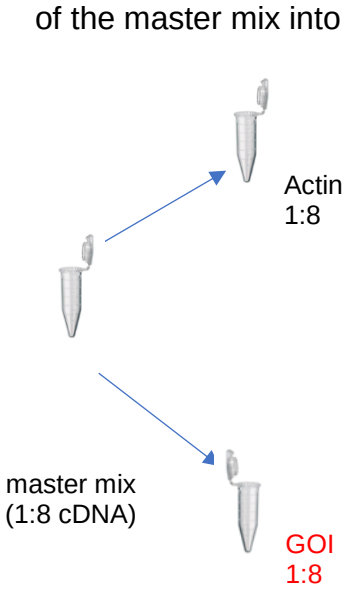
cDNA dilution	µL water	µL cDNA
1:8	168	24 (from sample)
1:16	100	100 (from 1:8 dilution)
1:32	100	100 (from 1:16 dilution)
1:64	100	100 (from 1:32 dilution)

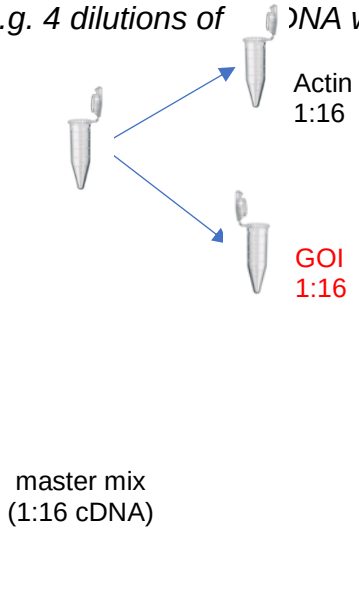
2. Dilute all primers to 3 pmol/µL (actin primers and primers for genes of interest (GOI))
    - All primers are stored at 1000 pmol/µL in the battle ship boxes in -20°C
    - Make the following dilutions:
        a. 5 µL of the 1000 pmol/µL primer + 45 µL water = 100 pmol/µL dilution
        b. 30 µL of the 100 pmol/µL primer + 270 µL water = 10 pmol/µL dilution
        c. 15 µL of the 10 pmol/µL primer + 35 µL water = 3 pmol/µL dilution

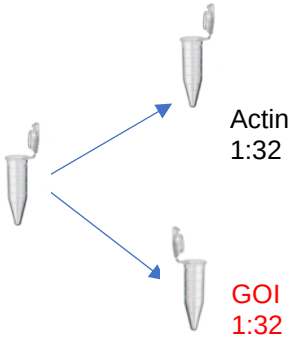
3. For each dilution of cDNA (four total from Step 1) make a master mix containing:

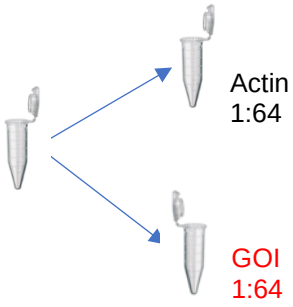
	1 primer pair	2 primer pairs	3 primer pairs	4 primer pairs	5 primer pairs	6 primer pairs	7 primer pairs	8 primer pairs
water	18 µL	36 µL	54 µL	72 µL	90 µL	108 µL	126 µL	144 µL
SYBR	30 µL	60 µL	90 µL	120 µL	150 µL	180 µL	210 µL	240 µL
cDNA	4 µL	8 µL	12 µL	16 µL	20 µL	24 µL	28 µL	32 µL

4. For each master mix, label a tube for each primer pair that will be run. Pipet **49.4 µL** of the master mix into each tube. *E.g. 4 dilutions of cDNA with 2 primer pairs.*

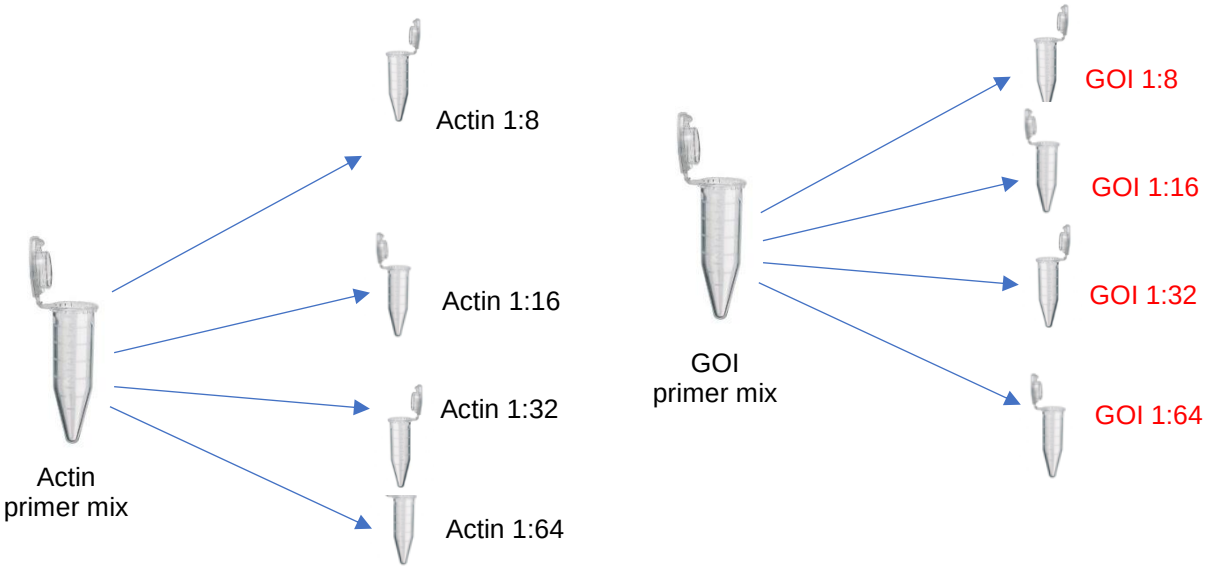








5. Mal master mix (1:32 cDNA) ‹ for each primer pair: 20 master mix (1:64 cDNA) mer + 20 µL reverse primer
6. Pipet **7.6 µL** of the primer mix (step 5) into the corresponding tubes containing the master mix (step 4) for each cDNA dilution



7. Vortex and spin down samples. Pipette 15 µL of each sample in triplicate into a 96 well real time plate. Which samples go into each well on the plate should be pre-determined ahead of time.
8. Put clear adhesive cover over the plate. Use the rubber tool to press the cover down. Be very careful to void bubbles and smudges. Use the razor blade to cut off the excess edges of the cover, and use the rubber tool again to make sure the edges are sealed to the plate.
9. Spin plate at 1000 rpm for 2 minutes using the centrifuge in Rita's lab
10. Run plate in real-time machine as described at end of protocol.

P. 12 – Real-Time PCR: Gene Expression

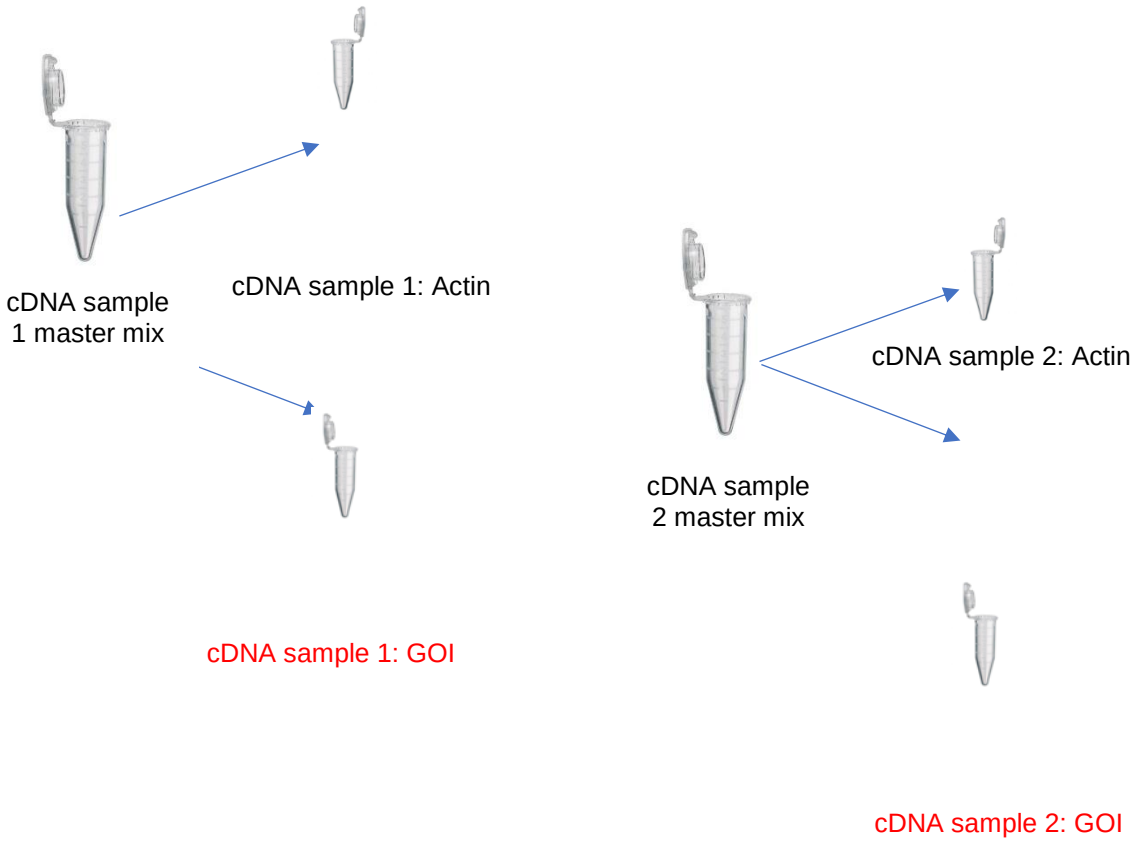
**Reserve your PCR machine time slot ahead of time

Note: RT-PCR is highly sensitive. Knobs on pipettes MUST be taped at all possible steps when pipetting replicate samples. Change tips after each volume is dispensed.

1. Dilute primers (e.g. Actin and gene of interest (GOI) primers) to 3 pmol/µL
    - All primers are stored at 1000 pmol/µL in the battle ship boxes in -20°C
    - Make the following dilutions:
        a. 5 µL of the 1000 pmol/µL primer + 45 µL water = 100 pmol/µL dilution
        b. 30 µL of the 100 pmol/µL primer + 270 µL water = 10 pmol/µL dilution
        c. 15uL of the 10 pmol/µL primer + 35µL water = 3 pmol/µL dilution
2. Dilute cDNA samples to a 1:10 dilution (or another dilution previously determined)
3. Make a master mix for each diluted cDNA sample using the table below. Pulse vortex and pulse centrifuge.

	1 primer pair	**2 primer pairs**	**3 primer pairs**	**4 primer pairs**	**5 primer pairs**	**6 primer pairs**	**7 primer pairs**	**8 primer pairs**
water	18 µL	36 µL	54 µL	72 µL	90 µL	108 µL	126 µL	144 µL
SYBR	30 µL	60 µL	90 µL	120 µL	150 µL	180 µL	210 µL	240 µL
cDNA	4 µL	8 µL	12 µL	16 µL	20 µL	24 µL	28 µL	32 µL

4. For each master mix, label one tube for each primer pair. Into each tube, pipet **49.4 µL** of the appropriate master mix. *E.g. 2 cDNA sample and 2 primer pairs (Actin and GOI).*

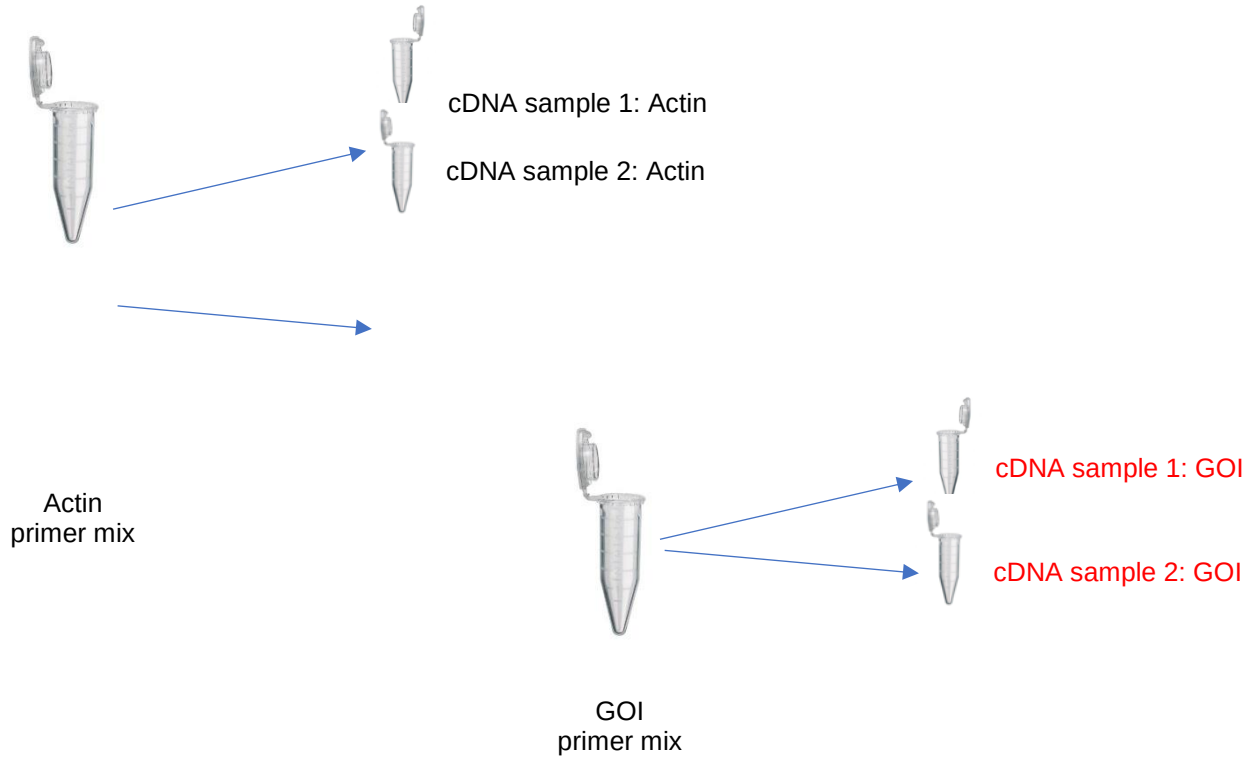


5. Make a primer mix for each primer pair: 50% forward primer, 50% reverse primer

Number of cDNA samples	uL of forward primer	uL of reverse primer
2	12	12
3	16	16
4	20	20
5	24	24

6	28	28
7	32	32
8	36	36
9	40	40
10	44	44

6. Pipet **7.6 µL** of the primer mix into the corresponding tubes containing the master mix for each cDNA sample from step 4. *E.g. 2 primers (Actin and GOI) and 2 different cDNA samples*.



7. Pulse vortex and pulse centrifuge samples
8. Aliquot **15µL** of each sample in triplicate into a 96 well real time plate. Which samples go into each well on the plate should be pre-determined ahead of time.

9. Put clear adhesive cover over the plate. Use the rubber tool to press the cover down. Be very careful to void bubbles and smudges. Use the razor blade to cut off the excess edges of the cover, and use the rubber tool again to make sure the edges are sealed to the plate
10. Spin plate at 1000 rpm for 2 minutes using the centrifuge in Rita's lab.
11. Run plate in real-time machine as described in final protocol.

REAL-TIME PCR: USING THE PCR MACHINE

Report any issues with the real time PCR machine to Dr. Grotewiel

1. Open the StepOne software
2. Use your name as the username
3. Select Advance Set Up
4. Experiment properties tab:
    - Enter experiment name
    - Select 96 wells
    - Select quantification comparative cT
    - Select SYBR green and check box to include melt curve
    - Select standard 2 hour run time
5. Plate set up tab:
    - Target = primers used
    - Samples = cDNA (include dilutions or sample number, as appropriate)
    - Click assign targets and samples
        - Assign wells but selecting with primer and cDNA sample are in each well
    - Select rox as the dye to use

- Select actin as the endogenous control

6. Run method tap:
    - Enter 15 as the reaction volume per each well
    - Use all other default settings on this page!
7. Click RUN
8. Click START RUN
9. Save file in the Grotewiel folder
10. Analyze data per standard lab protocols.

P.13 - Quantitative Measurement of β-Gal Activity in Flies

1) Homogenize (with drill/pestle in 1.5 ml snap-cap tubes) 10 lacZ-expressing flies of desired age and gender in 250 µl of extraction buffer (1X PBS with 1X protease inhibitor cocktail) for 25 seconds.
    - if using rotating spec, can only test 6 samples at a time (1 every 10 seconds for a minute)
2) Add 500 µl extraction buffer to each tube from step 1, mix by vortexing for 30 sec
3) Centrifuge extracts for 5 min at 14,000 rpm at room temperature
4) Transfer supernatants from step 3 into new labeled 1.5 ml snap-cap tubes
5) Set spectrophotometer at 562 nm.
6) Add 900 µL of 1 mM CPRG to 100 µl of water to a plastic cuvette for the blank. Set spec reference with the blank (water sample + CPRG).
7) Transfer 100 µl of each fly extract to an individual plastic cuvette. Add 900 µL of 1 mM CPRG to each of the fly extracts. Stagger the addition of CPRG to coincide with the order and timing of absorbance measurements.
8) Record absorbance of each cuvette every minute for 6 mins

<u>CPRG</u>

*CPRG is more sensitive than X-GAL and product measurement at 562 not interfered with by fly pigment

*CPRG solution is only good for 24 hours

Final volume of 1mM CPRG (mL)	CPRG added (g)
10	0.005
15	0.008
35	0.0205

Protease inhibitor cocktail

Stored in the -20

Sigma (P8340)

Vita

Rebecca Elaine Schmitt was born on May 9, 1993 in Green Bay, Wisconsin and is a United States Citizen. She went to Southwest High School (Green Bay, WI) and attended the University of Wisconsin – Platteville (Platteville, Wisconsin) from 2011 to 2014. She received her Bachelor of Science in Chemistry with a criminalistics emphasis. Rebecca came to Virginia Commonwealth University in 2014 and started in the Biomedical Science Doctoral Portal where she then entered the Department of Human and Molecular Genetics.