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The Role of Circadian Genes in Tolerance to Ethanol in *Drosophila melanogaster*

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The Role of Circadian Genes in Tolerance to Ethanol in *Drosophila melanogaster*

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To my loving, supporting, and amazing parents, Bertram and Toby Pohl

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The Role of Circadian genes in Tolerance to Ethanol in *Drosophila melanogaster*

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Alcoholism is a devastating inheritable disease that causes a large fiscal and societal impact worldwide. The fruit fly, *Drosophila melanogaster,* has proven to be a useful model system in identifying genetic factors underlying ethanol-associated behaviors. Many genes have been identified in the fly that are involved in the acquisition of tolerance, or the reduced response of an effect of a drug caused by previous exposure. In this thesis, I investigated whether circadian genes are involved in tolerance to ethanol in the fly. Circadian genes had previously been implicated in cocaine sensitization in flies, as well as some ethanol responses in mammals. I developed a novel assay using a bootstrapping paradigm to analyze tolerance to ethanol in the fly that allows for the investigation of multiple components of tolerance. I then used this assay to test whether circadian genes were necessary for the acquisition of tolerance in the fly.

Interestingly, only some circadian genes affect tolerance to ethanol. These results argues that circadian genes are involved in tolerance to ethanol, but because some mutants are arrhythmic but still acquire tolerance, that they are acting in a role outside of the circadian system. While in the course of this work, I intended to investigate if these mutations affected ethanol preference in a two-choice assay. Before employing this assay, however, we wished to determine if flies prefer ethanol for its pharmacological effect or for its value as a food. Weperformed experiments in which flies had a choice between food supplemented with ethanol and food supplemented with an isocaloric carbohydrate. When presented with the isocaloric alternative, flies no longer demonstrated preference for ethanol. Flies will even stop showing preference for ethanol when switched to a balanced assay after preference has already been attained. We conclude that the flies prefer ethanol not because of its effect as a drug, but as a food source.

Table of Contents....viii

List of Figures....xi

CHAPTER 1: GENERAL INTRODUCTION....1

Drosophila have become an important model for investigating ethanol-associated behaviors....1

The circadian system: an overview....3

Non-circadian roles of circadian genes....6

Circadian rhythms and drugs of abuse....7

Ethanol preference in Drosophila....10

Dissertation overview....12

CHAPTER 3: BOOTSTRAPPING ANALYSIS OF TOLERANCE TO ETHANOL IN DROSOPHILA MELANOGASTER....15

Introduction....15

Results....18

Quantification of knock-down during ethanol exposure....18

Quantification of movement during ethanol exposure....20

An ethanol array tests for rapid tolerance to ethanol at a range of concentrations....21

Bootstrapping as a tool to evaluate tolerance in a single experiment....22

Validation of the single vial method for detecting tolerance....25

Analyzing fly movement reveals more details on the effects of previous ethanol treatment....26

Indentification of mutants sensitive to ethanol....27

Discussion....28

Methods....44

Fly stocks....44

Ethanol Treatment....44

Monte Carlo Simulation 44
Availability of MATLAB scripts 45

CHAPTER 3: CIRCADIAN GENES DIFFERENTIALLY AFFECT TOLERANCE TO ETHANOL 46
Introduction 46
Results 49
Circadian mutations do not alter sensitivity to ethanol 49
Tolerance to ethanol sedation is eliminated by only some circadian mutations 51
Circadian mutations affect different components of tolerance 51
A functional clock is not necessary for the acquisition of ethanol tolerance. 54
An acute dose of ethanol does not shift the behavioral rhythmicity of the fly. 54
The effect of *per*^{01} on tolerance is dependent upon genetic background. 55
Discussion 55
Methods 68
Stocks and Media 68
Backcrossing 68
Tolerance and Sensitivity Assays 70
Statistical Analysis 71
Measurement of Circadian Activity 72

CHAPTER 4: ETHANOL PREFERENCE IN DROSOPHILA MELANOGASTER IS DRIVEN BY ITS CALORIC VALUE 74
Introduction 74

Results....78

D. melanogaster prefer food that contains ethanol when the non-ethanol choice contains fewer calories....78

Preference for ethanol is eliminated when the control food is balanced for calories....79

The peak ethanol concentration in the unbalanced CAFE

assay does not exceed 4 mM....82

The amount of ethanol consumed is insufficient to induce tolerance...84

Flies prefer the non-ethanol-containing food if it contains more calories....85

Switching to a calorically-balanced alternative after preference has developed eliminates ethanol preference....85

Discussion....87

Methods....99

Flies and Media....99

Calorie Determination....99

Preference
Assay....99

Proboscis Extension Response....100

Tolerance Assay....101

Gas Chromotography....102

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS....103

Summary and conclusions....103

Future directions....105

REFERENCES....107

VITA....121

List of Figures

Figure 1.1. The core components of the Drosophila circadian clock. 14

Figure 2.1. Schematic of measuring fly activity.....31

Figure 2.2. Comparison of hand count and computer count. 32

Figure 2.3. Curve fitting. 33

Figure 2.4. A behavioral array tests for tolerance at a range of concentrations. 34

Figure 2.5. Schematic of the moving block bootstrap for time series data.....35

Figure 2.6. The moving block method detects tolerance. 36

Figure 2.7. Comparison of moving block method to a Monte Carlo simulation. 37

Figure 2.8. Replicability of the moving block method. 38

Figure 2.9. "Random Graph" Bootstrapping. 39

Figure 2.10. The "Random Graph" method detects tolerance. 40

Figure 2.11. Bootstrapping the "Movement of Flies" data indicates only some parameters are affected by previous ethanol sedation. 41

Figure 2.12. Bootstrapping can be used to assess sensitivity to ethanol.. 42

Figure 3.1. Circadian mutants do not affect sensitivity to ethanol 58

Figure 3.2. Some circadian mutants disrupt tolerance to ethanol. 59

Figure 3.3: Wild type flies acquire tolerance to multiple components of tolerance 60

Figure 3.4: Flies carrying the *per*^{01} mutation fail to acquire tolerance to the sedative phase.....61

Figure 3.5: Flies carrying the *tim*^{01} mutation fail to acquire tolerance to any phase. 62

Figure 3.6: Flies carrying the *cyc*^{01} mutation do not acquire tolerance to some phases of tolerance.....63

Figure 3.7: Flies carrying the *Clk*^{JRK} mutation acquire tolerance. 64

Figure 3.8: Constant light does not eliminate ethanol tolerance in wild type flies. 65

Figure 3.9: An acute dose of ethanol does not disrupt circadian activity. ....und 66

Figure 3.10: The effect of *per*^{01} on tolerance is genetic backgrospecific 67

Figure 4.1. Drosophila show preference for liquid food supplemented with ethanol. 91

Figure 4.2. Balancing calories with sucrose eliminates preference for ethanol 92

Figure 4.3. Proboscis extension response to different calorie sources 93

Figure 4.4. Preference for ethanol food can be eliminated if the other solution has equal caloric content. 94

Figure 4.5. Consumption plots for isocaloric CAFE assays. 95

Figure 4.6. The amount of ethanol inside the fly is small and insufficient to induce tolerance to ethanol. 96

Figure 4.7. Drosophila prefer the non-ethanol solution if it contains more calories. 97

Figure 4.8. Switching to a balanced assay after preference has been established eliminates the preference for ethanol. 98

Chapter 1

General Introduction

Drosophila have become a useful model for the investigation of ethanol-associated behaviors

Alcoholism is a serious health concern worldwide. In the United States, almost 4% of the population meet the criteria for alcohol dependence, and alcohol related problems are estimated to cost over 184 billion dollars per year (Harwood, 2000; Grant et al., 2004). A large body of evidence points to alcoholism as having genetically heritable components. Adoption studies have shown that adoptees whose biological parents were alcoholics have an increased chance of becoming alcoholics when compared to other adoptees (Cadoret et al., 1980). A variety of twin studies show have shown a strong correlation of alcoholism between twins, and this correlation is stronger when the twins are monozygotic (Goldman et al., 2005). Based on these studies, researchers have estimated that the heritability of the disease is between 48% and 58% (Prescott and Kendler, 1999).

While great strides have been made to identify genes underlying alcohol addiction, this disease has proven to be genetically complex. There does not appear to be a single “alcoholism gene”, and the disorder is thought to arise from

a combination of a multitude of genes affecting various components, or endophenotypes, of alcoholism (Mayfield et al., 2008). Therefore, the identification of a single gene that underlies all facets of alcoholism is an unlikely expectation. A more reasonable expectation is that mutant analysis can identify regulatory genes that control one or more of the endophenotypes of alcoholism.

Tolerance, also called inducible resistance, is defined as a reduced response to an effect of a drug caused by prior drug exposure. Tolerance is an important endophenotype of addiction because following the acquisition of tolerance the individual will consume more of the drug in order to achieve the previous effect, thereby increasing the likelihood of addiction. Some changes that are thought to underly tolerance also cause withdrawal symptoms (Crabbe, 2001) While alcoholism is a complex phenomenon and difficult to study in model systems, the acquisition of tolerance can be readily measured. Despite its relevance to addiction, the molecular mechanisms that underlie the acquisition of tolerance have not been defined.

The fruit fly, *Drosophila melanogaster*, has been established as a model system for the investigation of ethanol induced behaviors (Moore et al., 1998; Dzitoyeva et al., 2003a; Atkinson, 2009). With its low-cost to maintain and short generation time, the fruit fly has been used for decades in genetic studies. Most mammalian genes are represented in Drosophila, and of 714 genes in humans designated as disease-causing, 548 are conserved in the Drosophila genome

(Reiter et al., 2001). Over the past decade, the fruit fly has been used to identify genes involved in both sensitivity to ethanol and the acquisition of tolerance. When given a dose of ethanol, flies demonstrate a biphasic response similar to mammals; they become hyperactive initially before eventually sedating. When the drug is removed, flies recover from the sedation. Numerous labs have shown that flies gain tolerance to a single dose of ethanol, in that they take longer to sedate and/or recover more quickly from a second dose of ethanol (Scholz et al., 2000; Cowmeadow et al., 2005). Using mutant analysis, genes affecting a variety of functions, such as octopaminergic and GABAergic signaling, the stress pathway, and a calcium-activated potassium channel have been identified as being involved in tolerance (reviewed in (Atkinson, 2009)).

The circadian system: an overview

Circadian rhythms, or rhythms that occur on a near 24-hour timescale, are present in a wide variety of species, from bacteria to mammals. This rhythm is innate, as animals that are allowed to “free-run” in constant darkness maintain these cycles. These rhythms are necessary for an animal to be able to predict daily changes in their environment (Sharma, 2003). Behaviors and physiological processes that are circadian-regulated in humans include sleep-wake patterns, as well as fluctuations in body temperature, respiratory rate, heart rate, and

metabolism (Bridges and Woods, 2001; Mortola, 2004; Refinetti, 2010; Huang et al., 2011). In Drosophila, circadian rhythms control physiological processes such as eclosion, locomotion, sleep, sensory perception, metabolism, and learning and memory (Konopka and Benzer, 1971; Krishnan et al., 1999; Hendricks et al., 2000; Lyons et al., 2008; Xu et al., 2011).

The first circadian gene was discovered in Drosophila (Konopka and Benzer, 1971). The identification of a single gene in which mutations could render flies behaviorally arrhythmic was one of the first major discoveries in the field of neurogenetics. Not only was a mutation identified that caused a complete lack of rhythmicity, but two other alleles were also identified in the same gene: one that caused the fly to have unusually long rhythms and one that caused the fly to have unusually short rhythms. This gene was named *period* (*per*). Many years later scientists discovered that three homologs of *per* exist in humans (*per1*, *per2*, *and per3*) and are necessary for circadian function (Maywood et al., 2007).

The core circadian clock has now been well characterized in Drosophila. The intracellular circadian clock consists of a network of interacting transcription factors that form a negative feedback loop ((Hardin, 2005) and illustrated in Figure 1.1). Clock (CLK) and cycle (CYC) are beta-helix-loop-helix transcription factors that recognize E-Box DNA regulatory elements that regulate gene expression. Clock-cycle heterodimers bind to E-Box elements in the

transcriptional control regions of *per* and *timeless* (*tim*), thereby activating their transcription. In Drosophila, peak mRNA levels of *per* and *tim* accumulate in the early evening, whereas peak protein levels do not accumulate until later in the night. The PER and TIM proteins form dimers that accumulate in the cytoplasm before entering the nucleus and disrupting the CLK-CYC complex. This disruption eventually leads to the degradation of PER, TIM, and CLK. The degradation of CLK-CYC causes a decrease in the transcription of *tim* and *per* mRNA, thereby reducing the presence of period and timeless proteins. The inhibition of CLK-CYC is relieved, bringing the cycle back to the beginning of the loop. This process takes approximately 24 hours, generating an innate clock. A null mutation in any of these genes renders the animal arrhythmic.

The molecular clock is well conserved in mammals (Maywood et al., 2007). Both Clock and the mammalian homolog of cycle, Bmal1, play the same role as they do in flies. Mammals, however, contain three *period* genes. Knocking out a single *period* gene is insufficient to produce arrhythmicity, but animals lacking all three are indeed arrhythmic. While mammals do have a conserved *timeless* gene, it appears to be involved in development and is not a core clock gene (Gotter et al., 2000). Instead, the dimer of *period* in the negative feedback loop is *cry.* Interestingly, in Drosophila, *cry* is the photoreceptor in circadian neurons necessary for entraining rhythms (Emery et al., 1998).

Both mammals and flies have peripheral and central pacemakers. All

pacemakers appear to be autonomous, in that they are capable of producing biological rhythms without any input (Lowrey and Takahashi, 2011). Indeed, pacemaking cells that have been isolated in culture still maintain rhythms (Michel et al., 1993). However, the central pacemaker neurons are necessary to synchronize the peripheral clocks. In mammals, these central pacemaker neurons are found in the SCN (suprachiasmatic nucleus) (Lowrey and Takahashi, 2011). In flies, a group of neurons called the lateral ventral neurons LN_vs play this role (Nitabach et al., 2006). The LN_vs contain a signaling peptide called pigment dispersing factor (pdf), which is released rhythmically in a 24 hour cycle and is necessary for the synchronization of peripheral clocks (Renn et al., 1999). Interestingly, mutations for the BK gene *slo* causes a buildup of pdf in the LN_vs and render the fly arrhythmic (Fernandez et al., 2007). BK channels are also involved in the mammalian clock, as BK mutants in mice are arrhythmic (Meredith et al., 2006).

Non-circadian roles of circadian genes

In Drosophila, a null mutation in any of the core clock genes renders the fly behaviorally and molecularly arrhythmic. One would expect, therefore, that all behavioral effects shoud be uniform across mutations that eliminate clock function. This assumption is, however, untrue. There are many documented

instances where an effect is not shared by all circadian genes. For instance, sleep-deprived *per* mutants show normal sleep rebound, whereas *tim* flies do not (Hendricks et al., 2000). In a courtship suppression learning and memory assay, *per* mutants demonstrate reduced learning, whereas *tim*, *cyc*, and *Clk* mutants do not (Sakai et al., 2004). Furthermore, *per* and *tim* mutants have longer copulation times than wild type flies, whereas *cyc* and *Clk* mutants do not affect this behavior (Beaver and Giebultowicz, 2004). Another example more relevant to this dissertation will be discussed in the next section. These results indicate that circadian genes have pleiotropic effects, and do not necessarily always work as a unit. These genes are involved in processes outside of regulating circadian rhythmicity.

Circadian rhythms and drugs of abuse

Substantial evidence implicates the regulation of circadian rhythms in drug related behaviors. Many alcoholics have a disrupted sleep/wake cycle. (Brower, 2001a). In rodent models, ethanol can can alter the circadian rhythms of the animal. For instance, chronic alcohol intake in rats can disrupt their circadian period (Rosenwasser et al., 2005a). In a slice preparation of the rat SCN, a single dose of ethanol modulates the firing pattern of the pacemaker (Prosser et al., 2008). These studies indicate that the drug is capable of altering mammalian

circadian rhythms.

The first link between circadian genes and drug responses was discovered in Drosophila. Studying sensitization to cocaine, Andretic et al., 1999 found that flies carrying null mutations for *per*, *cyc*, or *Clk* completely blocked sensitization. Interestingly, they found that mutants lacking functional *tim* were capable of sensitizing, even though these flies were also completely arrhythmic. These results demonstrate that the genes *per*, *cyc*, *and* Clk are necessary for this drug response while *tim* is not. However, because all of this mutations stop the circadian clock from running, their role in drug responivity must be independent and separate from their role in producing circadian rhymicity.

The link between cocaine responsivity and circadian genes was also later described in mammals. The negative feedback loop in mammals is similar to that of Drosophila, but one difference is that the mammalian genome has three separate genes that encode different forms of *period*. A knockout for *per1* eliminated cocaine sensitization in mice, whereas a knockout for *per2* caused an increase in sensitization, again suggesting a role for these genes outside of the clock (Abarca et al., 2002). Additionally, *per1* mutants exhibited a lack of place preference to cocaine when compared to wild type controls. The *period* gene is not the only circadian gene demonstrated to be involved in a cocaine response in mice, as *Clock* mutants show an increased place preference (McClung et al., 2005).

While most studies have looked at the involvement of circadian genes with regard to cocaine, some have also studied the effects of ethanol. Although the connection between circadian regulatory genes and the production of ethanol tolerance has not directly demonstrated, it has been shown that *per2* mutants in mice consume more ethanol than their wild type controls (Spanagel et al., 2005). Additionally, chronic exposure causes disregulation of *period* mRNA levels in a variety of brain regions in rats (Chen et al., 2004).

Further evidence from my lab supports the proposal that circadian system is important for the production of tolerance in Drosophila. We have shown that the gene *slo*, which encodes the BK calcium activated potassium channel, is both necessary for the acquisition of tolerance and that its induction produces a tolerance phenotype (Cowmeadow et al., 2005; Cowmeadow et al., 2006). Interestingly, *slo* has been shown to be involved in the circadian clock. Mutants for *slo* do not show circadian rhythmicity, and *slo* message levels cycle in a circadian fashion (Ceriani et al., 2002; Fernandez et al., 2007). Additionally, the transcription factor CREB, which is necessary for slo-induced benzyl alcohol tolerance, is also necessary for circadian rhythms and its activity cycles in a circadian manner (Belvin et al., 1999; Wang et al., 2007; Wang et al., 2009). These data point to an interaction between *slo,* which has been shown to be involved in the ethanol response, and the circadian system.

Ethanol preference in Drosophila

Ethanol preference studies in rodents have provided insight into the genetics associated with ethanol consumption. Most of these studies use a two-bottle choice assay in which the animal has a choice between a bottle that contains water and a bottle that contains water with ethanol. Using these assays, a variety of genes have been found to be associated with ethanol preference, including genes involved in the immune response (Blednov et al., 2011), the stress pathway (Olive et al., 2003), and ion channels (Blednov et al., 2003; Kamens et al., 2010). A Drosophila model looking at ethanol preference would be valuable to further study the genetics of this behavior. However, before undertaking this endeavor, we contend that it is necessary to understand if the animal is drinking ethanol for its pharmacological effect, or if it is consuming the drug for other reasons.

Drosophila melanogaster naturally consume ethanol in the wild. Their major food supply is rotting fruits, which can contain up to 7% ethanol (McKenzie and McKechnie, 1979). Indeed, *D. melanongaster* have been found in wineries, where ethanol levels are much higher (Gibson et al., 1981). Therefore it should

not be surprising that they have adaptive mechanisms to thrive in high ethanol environmments. *D. melanogaster* metabolize ethanol rapidly, and can survive with much larger internal ethanol concentrations than rodents or humans (Cowmeadow et al., 2005). Additionally, *D. melanogaster* can use ethanol as a food. Flies that are fed a diet of pure ethanol can survive for longer than flies receiving no sustenance (Libion-Mannaert, 1976; Pecsenye et al., 1994).

There is a long history investigating ethanol preference in Drosophila. Most of these studies are ecological in nature, in which investigators were interested in whether flies that metabolize ethanol better are more attracted to the drug. Both larval and adult flies have been shown to demonstrate preference for ethanol. Indeed, larvae will spend more time on the half of an agar plate that contains ethanol (Gelfand and McDonald, 1980; Parsons, 1980; Depiereux et al., 1985). Adults will extend their proboscis on the portion of a food plate (Cadieu et al., 1999). However, the conclusions from these studies were that they preferred ethanol for its value as a food.

Ethanol preference has recently been revisted in Drosophila as a model for investigating pharmacological preference to ethanol. Devineni and Heberlein, 2009 used the CAFE assay to investigate ethanol preference. This assay places flies in a vial, and their sole food and water source comes from capillary pipets that protrude into the vial (Ja et al., 2007). Flies have a choice between drinking

pipets that contain just liquid food and food supplemented with ethanol. When tested, flies showed a strong preference for ethanol that increased over time. The authors furthermore performed an experiment that they claimed demonstrated preference could not be due to the caloric effects of ethanol. They changed the caloric ratio between the ethanol-containing pipet and the ethanol-free pipet by varying the concentration of the food solution. They showed that no matter how much they varied the caloric ratio, preference for ethanol remained constant. However, the lowest ratio they tested was 2:1 (the ethanol contaning pipet contained twice as many calories as the control pipet). Therefore, it is possible that the flies can sense this caloric difference and prefer the ethanol-containing pipet.

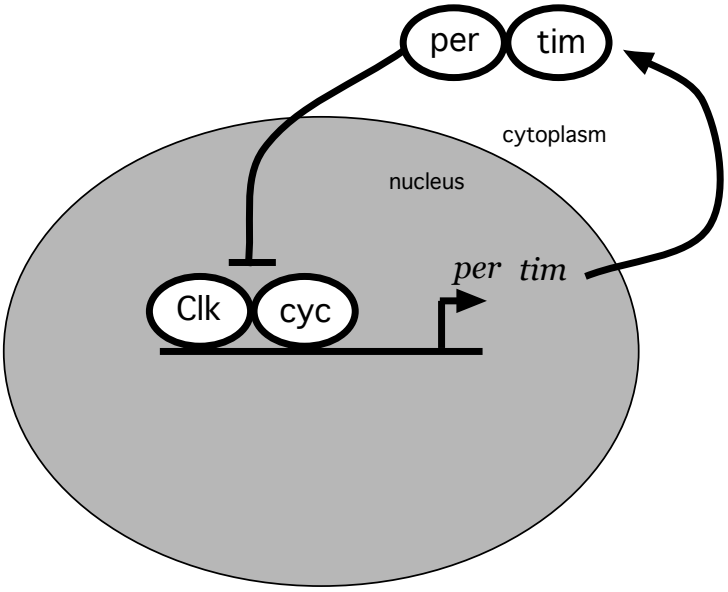
Dissertation Overview

The goal of this dissertation is to investigate the role of circadian genes on ethanol-associated behaviors in *Drosophila melanogaster*. Chapter 2 describes the method I use to analyze tolerance to ethanol. This method uses a curve-fitting and bootstrapping procedure to identify multiple components of tolerance. This method can also successfully identify mutants that are sensitive to ethanol.

Chapter 3 investigates the role of circadian genes in tolerance. Each of the core circadian genes are tested, and only some are necessary for tolerance to ethanol. Disruption of circadian rhythmicity alone is insufficient to eliminate ethanol tolerance, and an acute dose of ethanol does not shift the behavioral rhythm of the fly.

During the course of my dissertation, I had planned on testing circadian mutants for their preference for ethanol. However, I first conducted experiments to confirm that Drosophila consume ethanol for the pharmacological effects or for its nutritonal value. Chapter 4 contains this analysis, in which I test preference between food solutions that contain ethanol and food solutions with a calorie matched control. Preference for ethanol is eliminated when the control solution is isocaloric, and we therefore conclude that flies prefer ethanol not for its effect as a drug, but for its value as a food source. General conclusions are presented in Chapter 5.

Figure 1.1: The core components of the Drosophila circadian clock. Clk/cyc heterodomers bind to the promoter of *per* and *tim* and initiate their transcription. These transcripts are translated in the cytoplasm where they accumulate as proteins. These proteins then dimerize and enter the nucleus, where they inhibit their own transcription by inhibiting Clk and cyc. This causes a decrease in the amount of *per* and *tim*. Eventually, this will release the suppression of Clk and cyc, causing them to initiate transcription again. This process takes approximately 24 hours.



Chapter 2

Bootstrapping Analysis of Tolerance to Ethanol in *Drosophila melanogaster*

INTRODUCTION

Alcoholism (alcohol dependence) continues to be a devastating disease that affects millions of people worldwide. This is a complex disease whose diagnosis is based on the compulsive use of alcohol despite an accumulation of social problems that compromise the quality of one's life. Typically the diagnosis also includes alcoholism endophenotypes such as the incremental increase in consumption, alcohol tolerance, and withdrawal symptoms (American Psychiatric Association, 2000). While the totality of the disease is not captured in any model organism, the physiologically-based endophenotypes do lend themselves to study in model systems. Tolerance, or the reduced effect of a drug due to previous exposure, is an important component of addiction as the user consumes more of the drug in order to attain the previous effect, thereby speeding the user to addiction. Tolerance is an especially convenient endophenotype because it can reproducibly be assayed in many model systems (Wolf and Heberlein, 2003; Crabbe et al., 2006; Atkinson, 2009).

The fruit fly *Drosophila melanogaster* has been a useful model for identifying genes associated with the development of tolerance to ethanol

(Atkinson, 2009). A single dose of ethanol is capable of inducing rapid tolerance to ethanol sedation (Scholz et al., 2000; Cowmeadow et al., 2005). Rapid tolerance is the tolerance that exists after drug clearance that was produced in response to a single dose of the drug. Using the powerful genetic tools available in Drosophila, a diverse range of genes have been discovered that are necessary for the acquisition of tolerance. These include genes that encode for ion channels, neurotransmitter biosynthetic enzymes, and post-synaptic density scaffolding proteins. (Scholz et al., 2000; Dzitoyeva et al., 2003b; Cowmeadow et al., 2005; Urizar et al., 2007). Many of these genes have been shown to be involved in similar ethanol phenotypes in mammalian systems. A higher throughput method for detecting tolerance should facilitate future mutant analysis.

In this paper, we use a bootstrapping technique to further understand and simplify the detection of tolerance in Drosophila. Bootstrapping is a widely used statistical technique that allows for the determination of both an estimate of any parameter and the variability associated with that estimate (Efron, 1982). Unlike standard parametric statistics, bootstrapping does not assume that the data are Gaussian. Bootstrapping randomly samples, with replacement from independent observations in order to perform theoretical replicates of the experiment. As computing power has increased, one can perform many iterations of this resampling to generate accurate parameter estimates with error measurements.

To our knowledge, bootstrapping has never been applied to analyze Drosophila behavior.

As in mammals, low concentrations of ethanol in flies stimulate an increase in locomotion while higher concentrations cause a decrease in locomotion, eventually leading to a loss of postural control (Moore et al., 1998). Using a video-tracking system, it was previously demonstrated that flies receiving their second dose of ethanol have a prolonged hyperactive phase and take longer to sedate (Scholz et al., 2000). Teasing apart the hyperactive phase from the sedative phase may be important in understanding the role a gene plays in tolerance. In this work, we show how we were able to use a combination of time-lapse photography, automated image analysis, and bootstrapped error estimation in order to quantify five facets of fly behavior that are potentially useful indicators of tolerance. These are: 1) the time in which 50% of the flies are fully sedated or "knocked down" (K_{50}); 2) the time that it takes for flies to become maximally hyperactive (T_{max}); 3) the rate in which flies become hyperactive (R_{+}); 4) the rate in which the flies sedate (R-); and 5) the maximum amount of excitation (E_{max}). We show the degree to which these parameters, particularly K_{50}, distinguish between groups expected to have differing degrees of physiological tolerance.

RESULTS

Quantification of knock-down during ethanol exposure

Tolerance is defined as the reduced response to an effect of a drug due to previous drug exposure. In this study, a stock is said to be capable of acquiring ethanol tolerance if a single ethanol exposure reduces the sensitivity of the flies to a second ethanol exposure delivered twenty-four hours later. Ethanol is delivered by placing the mouth of a vial containing flies over an aliquot of ethanol that, through evaporation, generates a sedating dose of ethanol vapor (Figure 2.1A).

We developed two methods to characterize the response of flies exposed to gradually increasing ethanol vapor (both implemented via software written in MATLAB (The Mathworks, Natick, MA)). During the ethanol sedation, the vials are photographed once every 20 seconds. This time interval was chosen to allow time for flies to move between photographic frames. The first method identifies the number of flies on the vial walls at each time point. Non-sedated wild type flies are negatively geotactic, and will tend to cling to the walls of the vial. To count the number of flies at each time point, the program first isolates the flies by subtracting each frame from an "average" frame – the pixelwise mean of the last 150 frames. Movement of the flies over time ensures that averaging acts to fade

the image of the flies while reinforcing static features such as the vial and the background. We noticed that after the subtraction there was some resulting noise due to fluctuations in the lighting. To remove the noise, we used a Wiener filter, followed by a low-pass filter. The resulting image contains only the flies on the vial at the current time point because all of the common features in the image (i.e. the background) are removed by the subtraction. A subtraction at a random time point is shown in Figure 2.1B. The program then counts the number of flies using a particle counting algorithm. This task has been simplified because the flies are the only high contrast objects remaining in the processed images. Any object that covers at least 10 pixels is counted as a fly. Because flies are sometimes close enough together to form a single object, we counted any object larger than 25 pixels to be two flies. The result is a count of the number of flies vs. time, which we call a "knock-down curve". The accuracy of this method was demonstrated by comparing the output to a hand-count (Figure 2.2A). While the hand-count was strongly correlated with the computer generated count (r=0.98), small inaccuracies in the computer-generated count could arise from rare errors in fly detection or when one fly occludes another. The knock-down curve has an inverted sigmoidal shape that is well fit by an inverse cumulative normal function (Figure 2.3A). This fit has two parameters: μ (mean) and σ (variance). The μ parameter is the time when half of the flies are knocked down (K_{50}).

Quantification of movement during ethanol exposure

The second method we employ measures the activity level of the flies by quantifying their movement over the duration of the ethanol treatment. This method identifies how many flies have moved between each subsequent frame. In this method, we again use subtraction analysis, but instead of subtracting each frame from an average frame, each frame is subtracted from the subsequent frame. All the other filtering remains the same as previously described. Flies that have not moved are, along with the background, subtracted out and only the flies that have moved remain. A depiction of an example subtraction is shown in Figure 2.1C. The program then uses the particle counter to determine the number of flies that have moved between frames. Because each fly that moves generates two flies in a difference image (i.e. its initial and subsequent position), we divide the count by two. We again only counted an object as a fly if it contained ten or more pixels. Therefore, flies that did not move more than their body length were not counted. As above, the accuracy of the software was verified by comparison with hand counts (Figure 2.2B). The hand count and computer count exhibited a high correlation (r=0.93), indicating the effectiveness of our software.

As has been previously described (Wolf et al., 2002), the movement curves typically show a small startle response in the first 2-3 minutes, followed by a period of lesser activity before excitation, a maximal peak of excitation, and

finally a sedative phase during which the flies fall to the bottom of the vail or stop moving altogether (Wolf et al., 2002). The curves representing movement were more complicated than the curves representing the "awake" flies and contains multiple peaks and troughs. Therefore, we used a piecewise fit to analyze these curves. The first 3 minutes were skipped to eliminate the startle response and the remaining portion of the curve was divided into two segments and each segment was fit with a smooth curve. The remaining two segments were the hyperactive phase which exhibits a monotonic rise and can be fit with a straight line, and the sedative phase that is best fit with an inverse cumulative Gaussian (Figure 2.3B). Importantly, the break between these two segments was also a free parameter in the fitting (as opposed to being set *a priori*). Therefore, our fit has four free parameters: 1) T_{max}: the time when the two segments meet and is therefore the time when flies are maximally active. 2) E_{max}: the height of the peak and therefore indicates the maximal activity of the flies. 3) R_{+}: the slope of the rising phase and therefore corresponds to the rate of hyperactivity. 4) R-: the sigma of the declining phase and therefore corresponds to the rate of sedation of the flies.

An ethanol array tests for rapid tolerance to ethanol at a range of concentrations

We used a simple assay to determine the dose response relationship between ethanol and functional ethanol tolerance. In this assay, flies are placed into a vial

with a cotton plug that contains 1 mL of either 75%, 50%, 25%, 12.5%, or 0% ethanol. The flies are exposed to the vapor for one hour, after which they are removed and transferred to fresh food vials to recover. Twenty-four hours later they are tested for the acquisition of tolerance. Flies from every first-day treatment are randomly given either 75%, 50%, or 25% ethanol so that one vial from each first-day treatment receives each second-day treatment (Figure 2.4). In this manner, we are able to assess tolerance induced by increasing amounts of ethanol. For each second day concentration, we used our analysis to examine the effect of previous ethanol treatment on their response to ethanol by using both the flies counted and flies moving metrics.

Bootstrapping as a tool to evaluate tolerance in a single experiment

In our previously published ethanol studies, we determined that flies are capable of acquiring tolerance using a large number of replicate vials and testing for a statistically significant separation in the behavioral response between animals receiving their first dose and animals receiving their second dose of ethanol (Cowmeadow et al., 2005). However, the need for replicates diminishes the forte of Drosophila–high throughput mutant analysis. A single vial assay would have great utility when testing large numbers of mutant animals. This utility

underlies our interest in being able to statistically test for the acquisition of tolerance in a single vial of 10 flies.

In a single experiment, we have only one observation (one fly count) at each time point. However, each vial contains ten flies, each of which are independently being affected by the ethanol treatment. The data at adjacent time points are presumably subject to independent and identically distributed noise that arises from factors intrinsic to the flies and the environment. Each time point is individually affected by noise from differences in behavior (e.g. flies that move to the bottom of the vial and are not scored) and from noise in the fly counting software. As is common in time series analysis, we removed some of this random component by smoothing the data with a moving average (in which the original n data points are replaced by the average of b points in a neighborhood or "window", resulting in b - k + 1 new data points that form a much smoother curve, see Figure 2.5). As long as the sliding window is small relative to the important aspects of the curve (e.g., the length of the monotonically decreasing segment), the result has much of the noise removed yet still has the important features of the phenomenon under study. We chose to use a sliding window of ten time points.

The resulting smooth curve is easily fit (as described above) to yield the time when 50% of the flies were knocked down (K_{50}). An increase in K_{50} due to previous ethanol exposure indicates the acquisition of tolerance. In order to

estimate the error associated with each K_{50}, we used a bootstrapping method commonly used in time series analysis (block bootstrapping, (Davison, 2003)) that is a very natural extension of the sliding window method used to smooth the original data. All curve-fitting and bootstrapping analysis was performed in Matlab. For each of 1000 bootstrap replicates, we repeat the same moving average to smooth the data but, at each position of the sliding window, we resample from the original data (10 points in our case) before computing the average. The fits were done exactly as for the original data, yielding 1000 estimates of K_{50} containing noise intrinsic to the data. From this sampling distribution we can compare knockdown times from vials which had previously received different amounts of ethanol on the first day. The resulting boxplots are shown for second day treatments of 75% ethanol (Figure 2.6A), 50% ethanol (Figure 2.6B), and 25% ethanol (Figure 2.6C). We used the "percentile method" to determine statistical significance (Efron, 1982). This method compares the values contained within the 2.5 and 97.5 percentiles of the bootstrap distributions of any two treatments. If these values do not overlap, the distributions are said to be significantly different. For each of these second day treatments, our bootstrapping analysis clearly shows that K_{50} is increased in flies that previously received the larger doses of ethanol.

Despite moving block bootstrapping being a common technique in the bootstrapping/time-series literature, we wished to verify that our bootstrapped

estimates of the experimental error associated with the K_{50} values were reasonable. To do so, we performed a Monte Carlo experiment to show that the distribution of K_{50} values obtained by the block bootstrapping method were equivalent to those that would be obtained from a large number of actual experimental replications assuming 1) independent and identically distributed Gaussian noise at each time point equivalent to the magnitude of measurement noise around the fit in our original samples and 2) the same data processing (clipping the fly counts at the maximum number of flies at the upper end and zero at the lower end, smoothing via the moving average, etc.). Additional details of this simulation are in the Methods. The resulting K_{50} values agreed exquisitely (Figure 2.7 and see Methods for details of this simulation). This was found to be highly replicable as four repetitions of the simulation yielded almost identical results (Figure 2.8).

Validation of the single vial method for detecting tolerance.

The only assumption in bootstrapping is that the data being bootstrapped is representative of the population. Small sample sizes, as in any statistical technique, can be misleading. To validate the single vial bootstrapping method we compared it to the outcome from the analysis of multiple vials (increased sample size). We performed bootstrapping analysis of data from three experiments that were conducted on consecutive days exactly as described for

the single vial method. Each individual single vial experiment produced a trend, as seen before, in which larger doses of ethanol on the first day cause an increase in K_{50} (summarized in Table 2.1). Each replicate positively detected tolerance (K_{50}/concentration curve with a positive slope).

We then employed a second bootstrapping technique that we called "Random Graph Bootstrapping" (Figure 2.9) to determine the K_{50} when calculated from three replicates. We used our software to find the number of flies that are on the vial at every time point in each vial. Then, at each time point, a data point was randomly selected from one of the three experiments. A cumulative normal was fit to the resulting curve, and K_{50} was extrapolated. This was repeated 1000 times, generating 1000 independent K_{50} values. As seen when looking at individual experiments, K_{50} was increased by prior ethanol treatments(Figure 2.10 A-C). The similarity in the Figure 2.6 and Figure 2.10 further validate the single vial bootstrapping method as for the detection of tolerance.

Analyzing fly movement reveals more details on the effects of previous ethanol treatment.

When flies are exposed to large doses of ethanol, they proceed through two drug-induced phases (Wolf et al., 2002). They initially become hyperactive before sedating. The above analysis demonstrated that increasing doses of

ethanol on the first day increases the duration of time that flies resist sedation during a subsequent ethanol exposure. However, we wanted to test if prior drug treatment affects the hyperactive phase, the sedative phase, or both. To do this, we analyzed the number of flies that moved within each 20 second frame.

To determine the effect of the various first-day ethanol treatments on the 25% second-day ethanol treatment, we generated 1000 bootstrap replicates from the movement data across the three experiments using the "Random Graph" method. For each replicate, we fitted the piecewise half-linear, half-Gaussian fit to the resulting data. From this fit, we were able to analyze four parameters (T_{max}, R_{+}, R-, and E_{max}). Boxplots of all four parameters are shown in Figure 2.11. T_{max} is right shifted as the dose of ethanol increases (Figure 2.11A), indicating that the transition to the sedative state is delayed. R_{+} decreases with larger ethanol doses (Figure 2.11B). R- is unchanged (Figure 2.11C), while E_{max} (Figure 2.11D) is lower in larger ethanol doses.

Indentification of mutants sensitive to ethanol

Mutant analysis has been a fruitful approach in identifying genetic regulators of ethanol sensitivity (Guarnieri and Heberlein, 2003). We can use this same analysis we have used to determine if flies are capable of acquiring tolerance to determine if mutants are sensitive or resistant to ethanol. In this example, we

tested 4 mutant stocks (JC03, JC05, JC08, and JC12) that have previously been identified as sensitive to the anesthetic halothane (Campbell et al., 2009). Ethanol and volatile anesthetics share many of the same molecular targets (Mihic et al., 1997) and show cross tolerance in humans (Fassoulaki et al., 1993). We therefore reasoned that many of these halothane-sensitive lines would show altered sensitivity to ethanol. We used the same treatment protocol, but took pictures of them receiving an initial dose of 35% ethanol. We tested six vials for each genotype, and compared them to their appropriate genetic background control (ry^{506}). Using the "Random Graph" technique described above to generate bootstrap replicates across experiments, we compared the K_{50} of each mutation. JC03, JC05, JC08 were all more sensitive to ethanol compared to the ry^{506} control, while JC12 was resistant (Figure 2.12).

DISCUSSION

In this paper we show that two different paradigms, both employing the statistical method of bootstrapping, are capable of detecting the acquisition of ethanol tolerance in Drosophila. Importantly, these methods can capture the uncertainty associated with several intuitive measures of tolerance. One method uses variability associated within a single vial of ten flies to simulate replicated experiments. Bootstrapping, or any other statistical technique, does not substitute for more data or replication. To further validate our technique, we

performed two additional identical single vial experiments, each of which produced similar results after bootstrapping (Table 1). Additionally, we used a second bootstrapping technique that incorporates data from multiple vials collected on different days. Both single vial and multiple vial methods detect the acquisition of tolerance with high fidelity. The multiple vial method, which samples from a larger pool of data, might be more appropriate for other behavioral assays or features of tolerance not envisioned here. Both methods clearly show that large doses of ethanol twenty-four hours prior to a second dose cause flies to take longer to sedate when measured by how many flies are climbing on the side of the vial.

We used our bootstrapping technique to look at the effect that previous doses of ethanol had on both the hyperactive and sedative effects of ethanol. We find that tolerant flies take longer to reach maximal excitation (T_{max}) and that their maximum amount of excitability is lowered (E_{max}). This is in contrast to previous work that indicated that flies receiving their second dose sensitize to the hyperactive effects of ethanol (Scholz et al., 2000). The reason for this difference may be due the difference in drug delivery as the previous work delivered a constant air-stream of ethanol to the flies. Another possible reason could be that we measured tolerance 24 hours after initial ethanol exposure, whereas the cited work measured after only 4 hours. The differences may prove interesting to study.

There are certainly other statistical methods that could be used to analyze this type of data. The most obvious would be to select a time point, and then perform a Student's *t*-test between control and experimental groups. The problems with this approach are that it only uses a single time point instead of the entire distribution, that it implicitly assumes that the data has a Gaussian distribution, and that it contains post-hoc bias as the investigator would have to pick the time point to test. A second approach would be to perform a Student's *t*-test at all the time points. This also ignores the actual patterns in the data, and also assumes a Gaussian distribution. Neither of these would have allowed us to statistically compare differences in the time to an event (e.g. T_{max}). By bootstrapping, we are able to not only identify various parameters involved in tolerance, but to assign error estimates to them.

The single vial method probably will have greatest utility in genetic mutant screens since it permits substantially higher throughput on the first pass of analysis. Secondary analysis of mutant might be performed with the multiple vial method which should allow extraction of fine difference in behavioral parameters. Finally, we believe that these bootstrapping techniques will be applicable for statistically analyzing responses to other drugs and other behavioral responses.

Figure 2.1: Schematic of measuring fly activity. A) 10 flies are placed in a standard fly vial with a permeable platform to prevent them from escaping. 1 mL of ethanol is pipetted onto a cotton plug which is placed into the vial. Pictures are taken every 20 seconds until all animals are sedated. B)The number of flies climbing on the wall of the vial is scored. At each time point, a filtered image of the vial is subtracted from an average vial of the last 150 images. The resulting image shows the flies that are on the vial, which is then counted by the program. C) The number of flies that have moved between two subsequent frames is scored. The image depicting the number of flies on the vial is subtracted from the subsequent image depicting the number of flies on the vial. The resulting image shows the number of flies that have moved. As a fly that moves will be scored twice, the resulting count is divided by two.

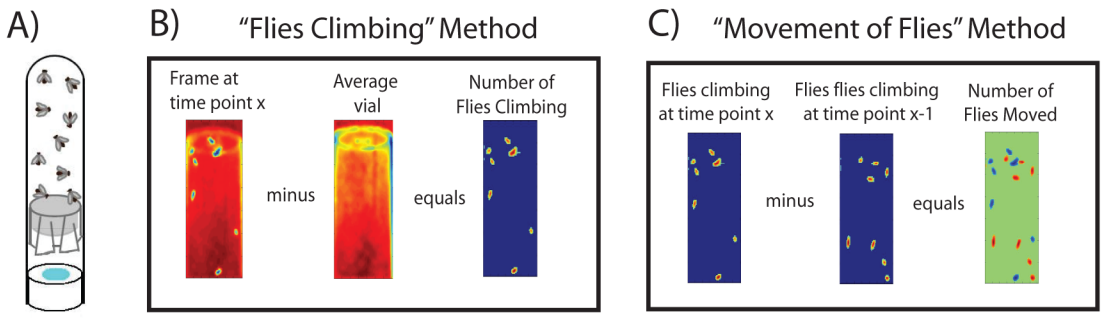


Figure 2.2: Comparison of hand count and computer count: A) A comparison between the computer generated count of the number of flies climbing on the vial and a hand count. One out of every ten frames were scored for a vial of 10 flies receiving 25% ethanol. The inset depicts the computer count plotted against the hand count. The dashed gray line indicates a theoretically perfect correlation. B) A comparison between the "the computer-generated count of the number of flies moving in the vial and a hand count. One out of every ten frames were scored for the same vial as analyzed in the previous panel. The inset depicts the computer count plotted against the hand count. The dashed gray line indicates a theoretically perfect correlation.

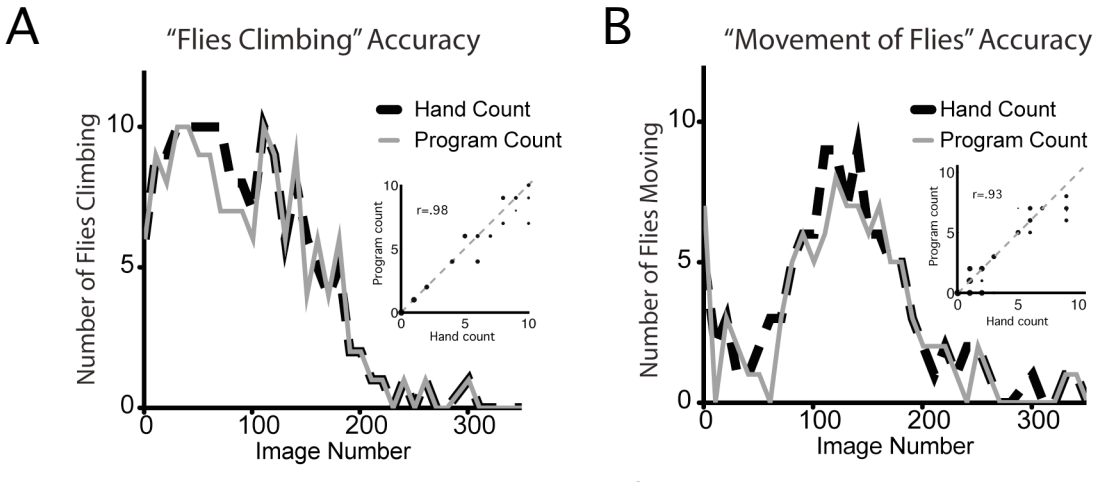


Figure 2.3: Curve fitting A) A cumulative normal fits the flies climbing data. In a representative vial receiving 25% ethanol, the raw data (red circles), smoothed data in which every 10 time points are averaged (blue triangles), and an inverse cumulative normal fit (black line) are shown. B) A piecewise curve with a linear hyperactive phase and a Gaussian sedative phase fits the movement of flies data. For the same vial as the pervious panel, the raw data (red circles), smoothed data in which every 10 time points are averaged (blue triangles), and an inverse cumulative normal fit (black line) is shown.

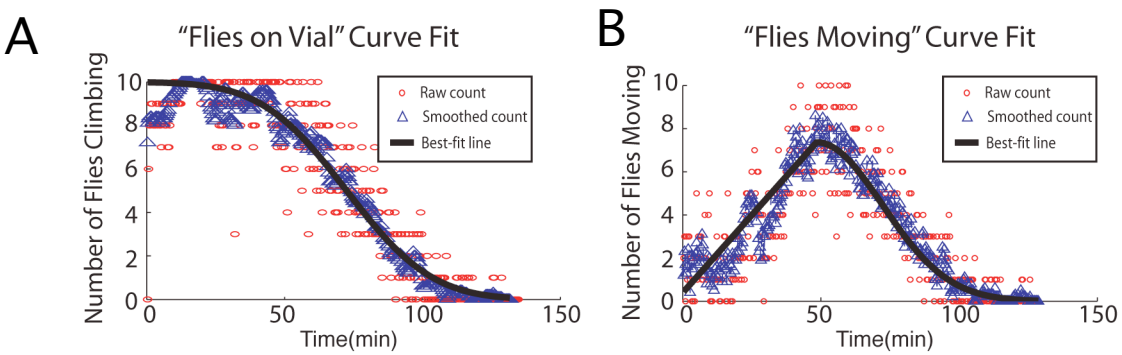


Figure 2.4: A behavioral array tests for tolerance at a range of concentrations. On the first day of treatment, flies receive either 75%, 50%, 25%, 12.5%, or 0% ethanol. Twenty-four hours later, one vial from each of the previous day's treatments receives 75%, 50%, and 25% ethanol. Photos are taken every twenty seconds, and comparisons within each second day treatment are made to identify flies that have acquired tolerance. Fitted curves for the number of flies climbing and the number of flies moving are shown for every treatment group.

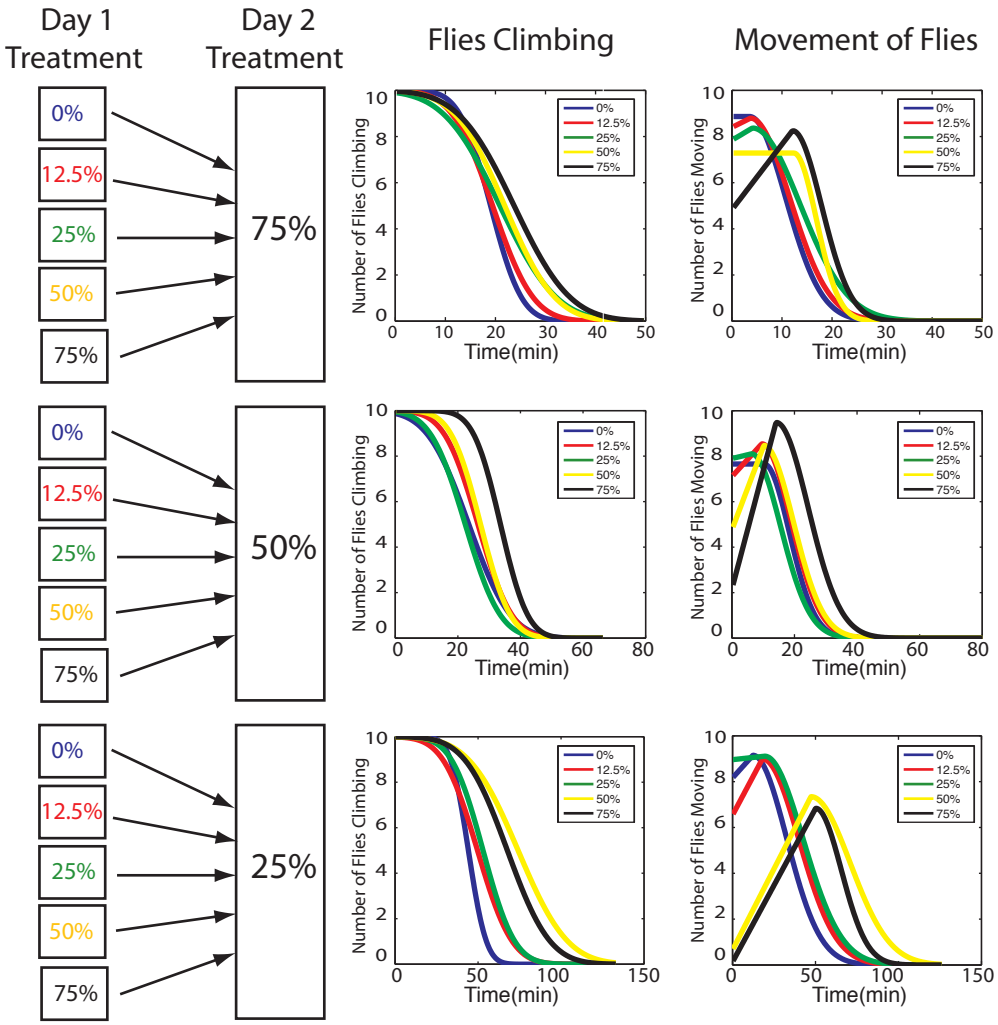


Figure 2.5: A schematic of the moving block bootstrap for time series data. As with any moving average, a sliding window (or "block") of some size **b** (black oval in upper panel) is moved across the original data (blue). Only four blocks are shown here for clarity; in reality, a block was defined around each data point except those within **b**/2 samples from the beginning and end. Within each block, the original data were resampled with replacement and then averaged, and this average replaced the central data point of the block (red points and curve). Once a smoothed bootstrap replicate of the data set was generated (middle panel), it was fit with an inverse cumulative normal (dashed black line) using the method of least-squares. From this curve, the K_{50} value was calculated (blue point on the x-axis; this corresponds to the mean of the cumulative normal). This entire process was repeated a large number of times – 1000 in our case. The resulting distribution (lower panel) is the bootstrap estimate of the sampling distribution of K_{50} values, from which standard errors and 95% confidence intervals can be easily calculated. The latter is simply the interval on the x axis that excludes the 50 most extreme upper and lower values corresponding to the 2.5% upper and lower tails (double headed arrow and dashed lines).

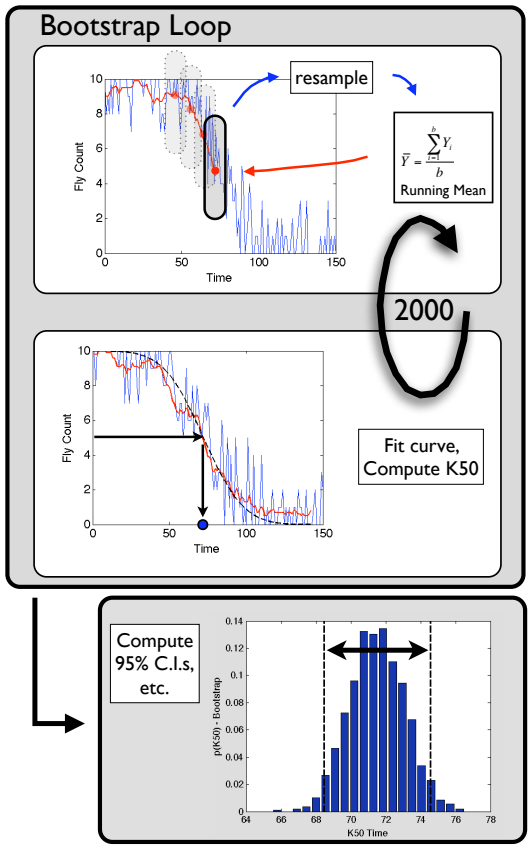


Figure 2.6: The moving block method detects tolerance. Boxplots of the time in which 50% of the flies (K_{50}) are shown. K_{50} was determined by using the moving block method to resample the data 1000 times for each vial. In each resampling, an inverse cumulative normal was fit to the flies climbing curve, and K_{50} was scored. Shown are boxplots for second day concentrations of A) 75% EtOH, B) 50% EtOH, and C) 25% EtOH. A trend at all second day concentrations demonstrates that flies with a higher first day dose of EtOH take longer to sedate on the second day. For these and all subsequent boxplots, the red line indicates the median, the box indicates upper and lower quartile values, the whiskers are the coverage of the data within 1.5 of the interquartile regions (corresponding to approximately 99.7% of the data), and the red crosses indicate outliers. Asterisks indicate a significantly higher K_{50} than the untreated vial as determined by the percentile method.

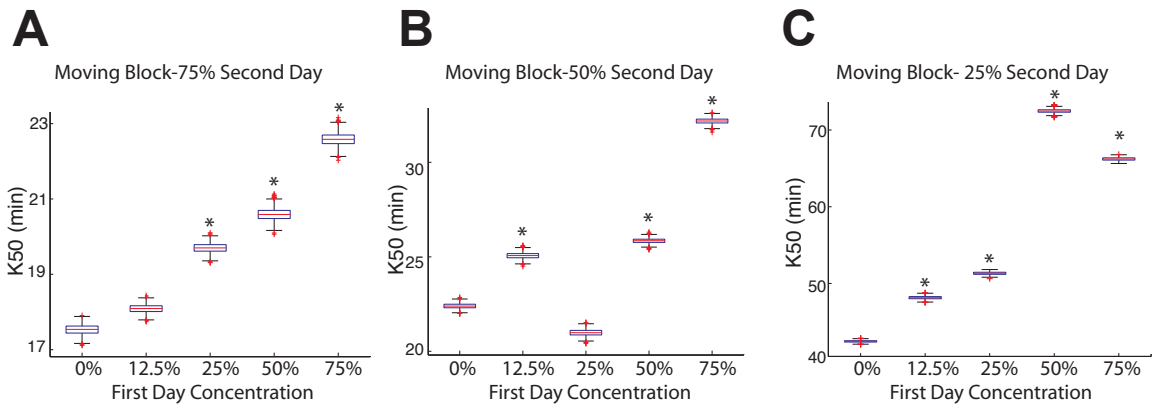


Figure 2.7: Comparison of moving block method to a Monte Carlo simulation. This figure illustrates the method we used to verify our blocked bootstrapping method. The upper panel shows the simulated-data generator, which consisted of an inverse cumulative normal (representing the "ideal" population behavior) and Gaussian noise with an amplitude equal to that in our actual data (i.e. the average standard deviation of the residuals obtained when we fit our experimental data). As shown on the left side of the figure, we generated one "original" data set and then ran it through the blocked bootstrapping procedure described previously to estimate the sampling distribution of K_{50}s (lower graph, top panel). The right side of the figure illustrates the computation of the "true" sampling distribution of K_{50}s from this simulated world. In each of 2000 experiments, a new data set was collected by getting a new draw of (pseudo)random Gaussian noise (upper right) and adding it to the ideal behavior (upper left). Each of these data sets (blue curves in the middle panel), were clipped and smoothed (red curves), fit (black curves), and a K_{50} estimate was read out from each fit (arrows). The resulting estimate of the K_{50} sampling distribution is shown in the bottom graph of the lower panel – this is the estimate for this simulated world, because it was obtained by doing a large number of experimental replications within the simulated world. Clearly, the shapes of the two distributions are very similar (and roughly Gaussian). Most importantly, the widths of the distributions are nearly identical, as indicated by the excellent agreement between the 95% confidence intervals, shown by the dashed lines.

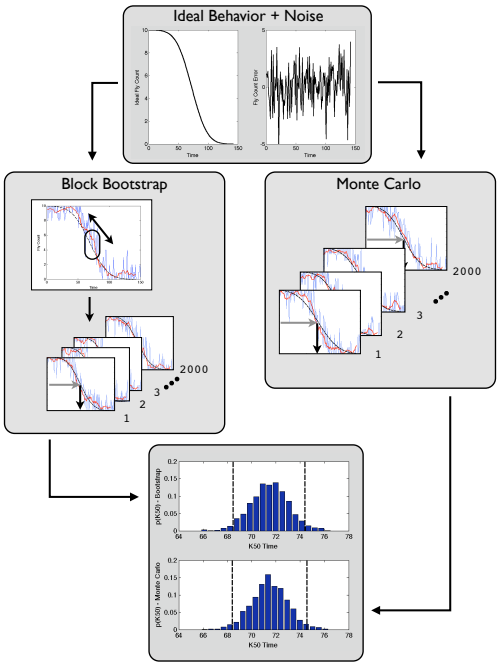


Figure 2.8: Replicability of the moving block method: Each of the four rows shows the results from an entire bootstrap/Monte Carlo comparison as described in the previous figure. The top row shows the same results from the previous figure, and the remaining rows show three successive repeats of the entire comparison. In the left column, the upper and lower graphs in each row show the distributions obtained by bootstrapping and Monte Carlo simulation, respectively. All axes are scaled identically, so a glance down the column confirms that all the distributions and 95% confidence intervals (dashed vertical lines) are virtually identical both within and across successive analyses.

The right column shows quantile-quantile plots of the bootstrapped distribution against the corresponding Monte Carlo distribution. The dashed gray line shows where the data would fall if the two distributions were identical. Clearly, the agreement is excellent except occasionally in the extreme tails (well beyond 95%).

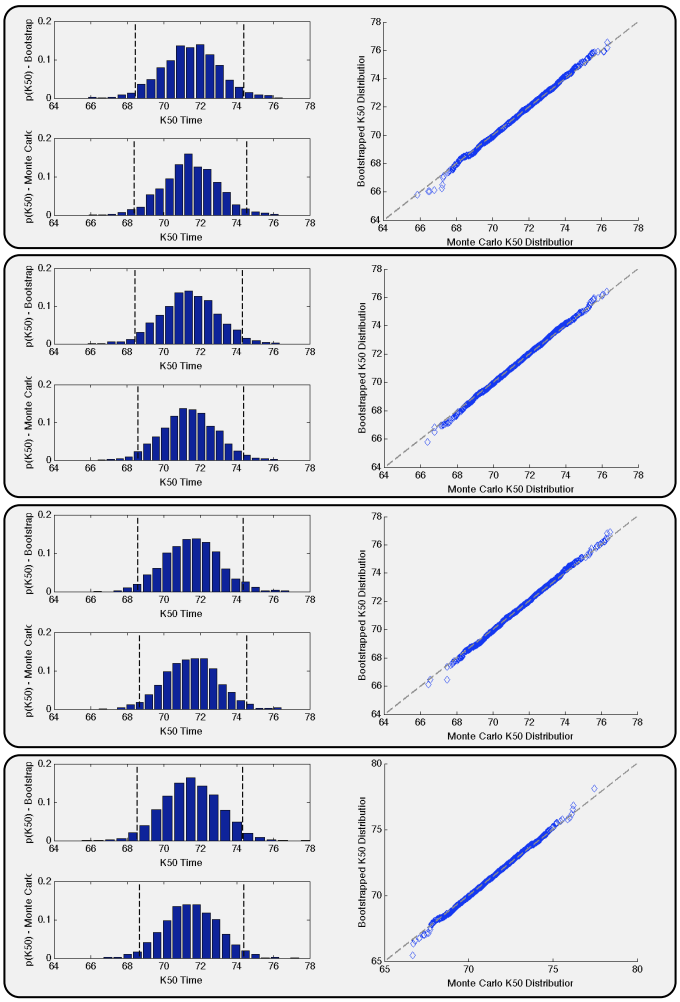


Figure 2.9: "Random Graph" Bootstrapping. In this example, data were obtained from three identically performed experiments. Data is resampled by randomly selecting a point from one of the three graphs at each time point. This is repeated 1000 times to generate 1000 randomly sampled graphs.

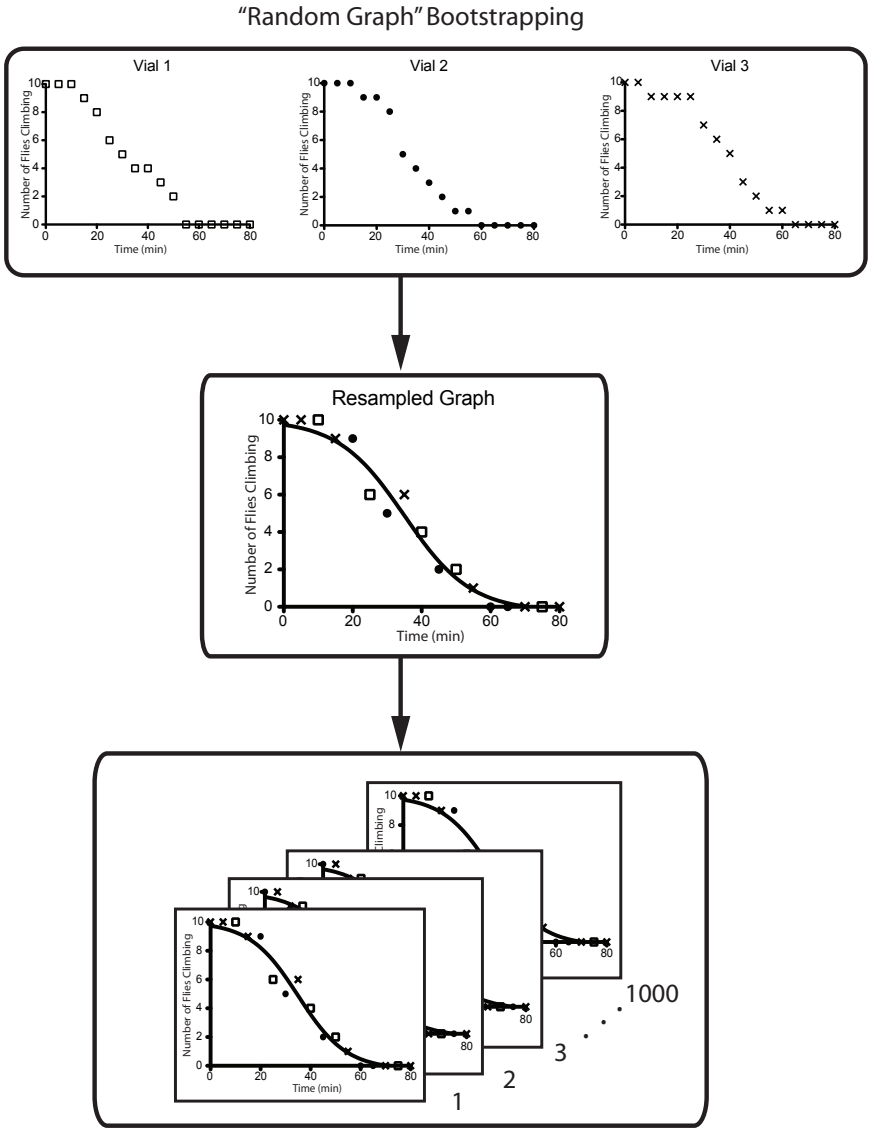


Figure 2.10: The "Random Graph" method detects tolerance. Boxplots of the time in which 50% of the flies (K_{50}) are shown. K_{50} was determined by using the "Random Graph" method to resample the data 1000 times for each concentration. For each resampling, K_{50} was determined by randomly selecting from three identically performed experiments at each time point. The resulting resampled graph was fit to a cumulative normal using the flies climbing method and K_{50} was extrapolated. Shown are boxplots for second day concentrations of A) 75% EtOH B) 50% EtOH, and C) 25% EtOH. A trend at all second day concentrations demonstrates that flies with a higher first day dose of EtOH take longer to sedate on the second day. Asterisks indicate a significantly higher K_{50} than the untreated vial as determined by the percentile method.

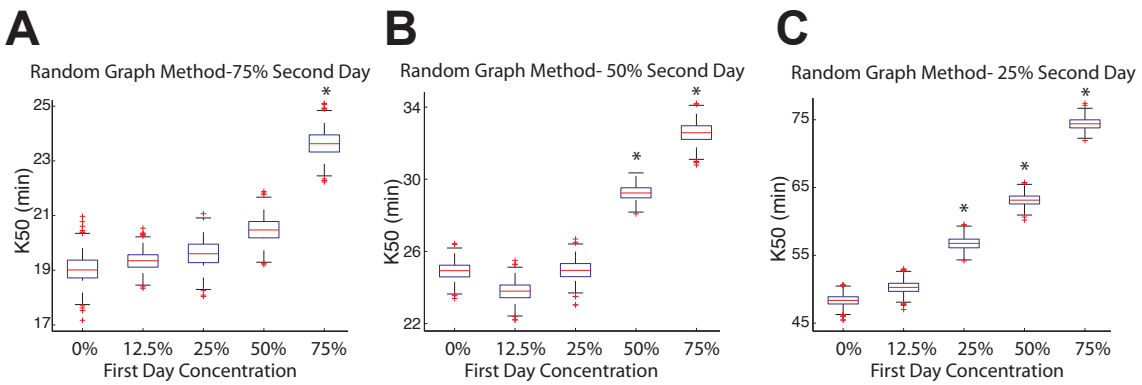


Figure 2.11: Bootstrapping the "Movement of Flies" data indicates only some parameters are affected by previous ethanol sedation. Boxplots generated from the "Random Graph" bootstrapping method display parameters from 1000 resampled curves (taken from the same three days as Figure 6) fitted using the "Movement of Flies" method. All data is from flies that received 25% EtOH on the second day, and comparisons are made between flies that received different first day treatments. A) The time at which the flies are maximally hyperactive (T_{max}). T_{max} increases as ethanol treatment increases. B) The rate in which flies become hyperactive (R_{+}). A trend indicates that the rate is decreased with higher previous day treatments. C) The rate in which flies sedate (R_{-}). D) The maximum amount of activation (E_{max}). E_{max} decreases with bigger first day treatments. Asterisks indicate a significant difference from the untreated vials as determined by the percentile method.

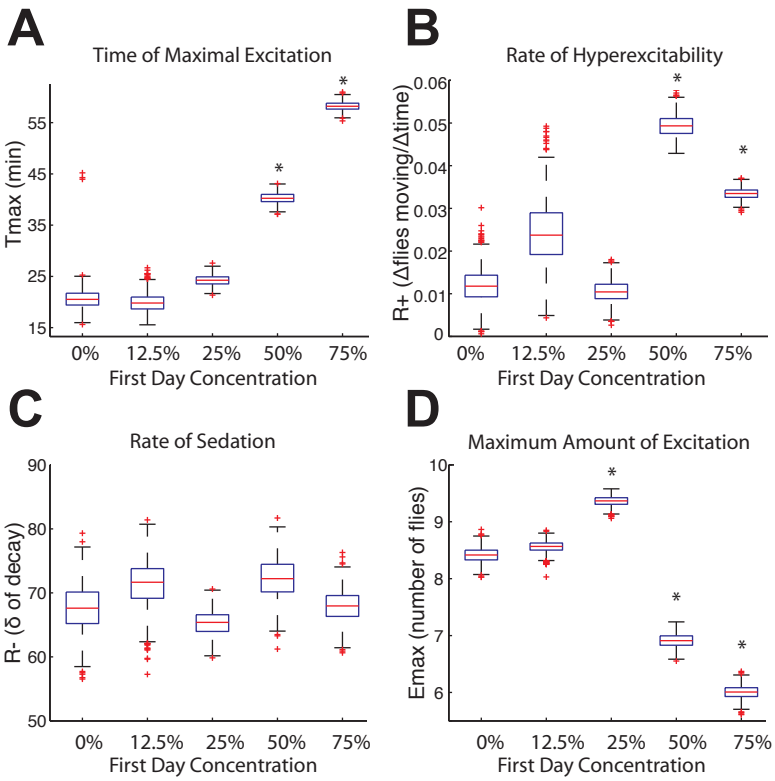


Figure 2.12: Bootstrapping can be used to assess sensitivity to ethanol. A boxplot displays the time when 50% of the flies are knocked down (K_{50}) for the wildtype line *ry*^{506} and four halothane sensitive mutants (JC03, JC05, CJ08, and JC12). The "Random Graph" method was used to randomly select a value at each time point from six vials of each genotype. Asterisks indicate a significant difference from *ry*^{506} as determined by the percentile method.

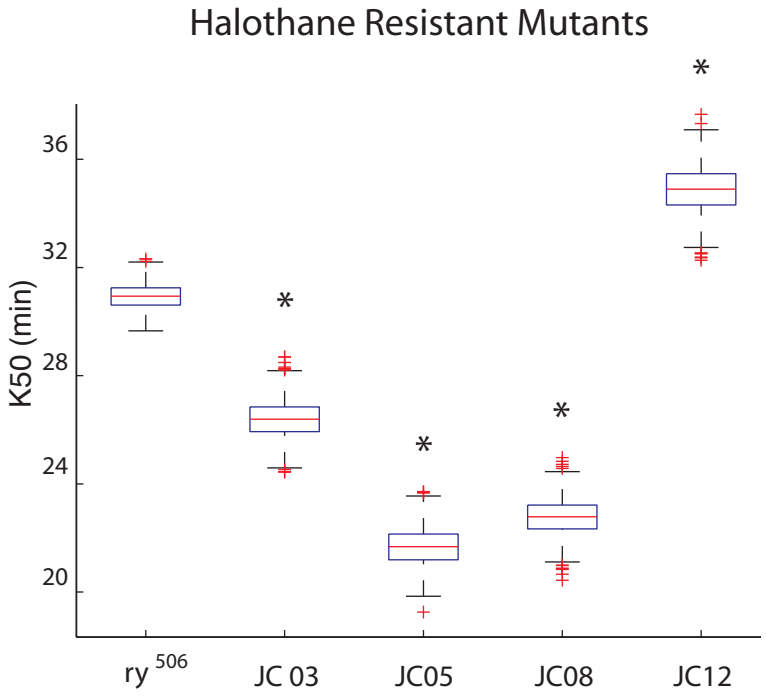


Table 2.1: Over three identical experiments, tolerance develops at all second day treatments. The moving block bootstrapping method was employed on three identically performed experiments. For each second day treatment, we assessed the point at which 50% of the flies were knocked down (K_{50}) for 1000 bootstrapped curves for flies that had previously received either 0%, 12.5%, 25%, 50%, or 75% EtOH first day treatment. The slope and correlation coefficient (r) are displayed here and correspond to the relationship between the previous day treatment and the K_{50} of the three experiments. Experiment 1 shows the slope and correlation for the experiment shown in Figure 4. Each experiment shows a positive slope, indicating an increase in sedation time as first day dose increases.

	Experiment 1		**Experiment 2**		**Experiment 3**	
Second Day Treatment	**slope**	**r**	**slope**	**r**	**slope**	**r**
75% EtOH	1.26	0.98	0.47	0.46	1.61	0.84
50% EtOH	2.04	0.74	2.77	0.84	1.63	0.78
25% EtOH	7.19	0.89	4.82	0.85	8.02	0.89

METHODS

Fly Stocks: All flies were reared on standard corn/molasses medium and kept on a 12:12 light-dark cycle. For all assays, female flies aged 4-6 days old at the time of initial treatment were used. For all tolerance assays, the Canton-S (CS) wildtype strain was used. For the sensitivity assays ry^{506},JC03, JC05, JC08, and JC12 were kindly provided by Howard Nash (Campbell et al., 2009).

Ethanol Treatment: Ten flies are placed into a standard fly vial with an air-permeable platform inserted to prevent them from escaping. We then pipet 1 mL of either 75%, 50%, 25%, 12.5%, or 0% ethanol onto a cotton plug and insert it into the vial with the flies, allowing ethanol vapor to gradually equilibrate into the chamber. The flies are exposed to the vapor for one hour, before being removed and placed into fresh food vials to recover. Twenty-four hours later, flies are given a second dose of ethanol and a camera takes pictures every 20 seconds until all flies are sedated. For the sensitivity assay, all flies receive 35% ethanol. Pictures are taken every 20 seconds until all the flies are knocked down.

Monte Carlo Simulation: A simulated knock-down curve (with parameters similar to a real knock-down curve) was used to compare the moving block analysis with a Monte Carlo simulation. For each repetition, a noise factor of 1.7

(approximating the noise from our curves) was added to simulate real-world noise. For 2000 repetitions, a curve was fit both to the moving block analysis and the Monte Carlo simulation, and a K_{50} was generated. The resulting distributions of K_{50}s were compared. This analysis was performed in Matlab.

Availability of MATLAB scripts: The scripts used in this analysis are available at:

http://w3.biosci.utexas.edu/atkinson/software/Home.html.

Chapter 3: Circadian genes differentially affect tolerance to ethanol

INTRODUCTION

Alcoholism is a devastating disease worldwide. While environmental and societal factors play a role in succumbing to this disease, a large body of evidence indicates that alcoholism is genetically heritable (Prescott and Kendler, 1999). While this disease is too complex to model completely in animal systems, the endophenotypes of alcoholism can be defined and studied. One such endophenotype is tolerance: a decrease in the effect of a drug due to previous exposure. Tolerance is an important endophenotype as the reduced response can trigger increased drug consumption in order to reach the desired effect, thereby speeding the user down the path to addiction.

The fruit fly, *Drosophila melanogaster*, has proven to be a useful tool for identifying the genetic components underlying ethanol tolerance (Atkinson, 2009). A diverse set of genes have been shown to affect tolerance in the fly, including genes involved in neurotransmitter synthesis, (Scholz et al., 2000) synaptic structure (Urizar et al., 2007), and ion channel activity (Cowmeadow et al., 2005). Importantly, many of these genes have also been shown to have similar effects in mammals as well, demonstrating that the genes involved in this process are conserved from the fly to higher organisms.

The circadian clock, prevalent in most animals, allows the organism to anticipate circadian changes in the environment, even in the absence of environmental cues. It is well known that ethanol has a disruptive effect on the circadian sleep/wake cycle in humans (Brower, 2001a) and estimates of the number of alcoholics who are also insomniacs range from 36-72% ((Brower, 2001b)). Furthermore, circumstantial evidence indicates that the circadian clock has a role in generating some endophenotypes of alcoholism. In rat behavioral assays, chronic alcohol intake can disrupt the circadian period (Rosenwasser et al., 2005b) and in slices of the rat central pacemaker, a single dose of ethanol changes the firing pattern of the circadian pacemaker. (Prosser et al., 2008).

In Drosophila, the genes that regulate the clock are well characterized, and most have homologous mammalian counterparts (see Figure 1.1). In a negative feedback loop, the CLK and CYC proteins stimulate the transcription of the *period (per)* and *timeless (tim) genes*. The PER and TIM proteins build up in the cytoplasm, where they dimerize and eventually enter the nucleus. These PER-TIM dimers disrupt the CLK-CYC dimers, thereby inhibiting the transcription of the *per* and *tim* genes. This inhibition causes PER and TIM levels to fall and eventually they are not sufficiently abundant to inhibit the activity of CLK and CYC. The CLK and CYC transcription factors can then once again stimulate the transcription of the *per* and *tim* genes. This cycle takes approximately 24 hours, thereby establishing the periodicity of the circadian clock in the fly.

The first study to show that circadian genes play a role in a drug response was conducted in Drosophila. Andretic et al. (1999) found that flies carrying a loss of function mutation in *per*, *cyc*, or *Clk* failed to show normal sensitization in response to multiple applications of cocaine. Interestingly, a loss of function mutant for *tim*, did not have an effect on cocaine sensitization. The fact that mutations that eliminate circadian rhythmicity have different effects on a drug response suggested that the circadian genes affect drug responses through a mechanism that is independent of their role in maintaining rhythms.

The link between drug responsivity and circadian genes was also later described in mammals. While some have looked at cocaine responses (Abarca et al., 2002; McClung et al., 2005), others have identified a link between circadian genes and ethanol. Consumption of ethanol is increased in *per2* mutant mice (Spanagel et al., 2005). Additionally, chronic ethanol exposure causes dysregulation of *per* levels in a variety of brain regions (Chen et al., 2004).

More recently, work from Drosophila has implicated circadian-related genes in the production of ethanol tolerance. These genes are not part of the master circadian clock but are circadian genes in that they are probably transmitting the rhythm to other genes or cells (Belvin et al., 1999; Ceriani et al., 2002). In both flies and mammals, mutations in *slo*, which encodes the BK-type Ca^{2+}-activated K^+ channels, cause animals to be behaviorally arrhythmic (Ceriani et al., 2002; Meredith et al., 2006). Mutations in *slo* also eliminate the capacity to

acquire tolerance to ethanol in at least one behavioral paradigm (Cowmeadow et al., 2005). Additionally, flies carrying mutations in the CREB transcription factor also lack circadian rhythms and fail to acquire tolerance to the organic solvent benzyl alcohol (Belvin et al., 1999; Wang et al., 2007).

In this paper, we show mutations in the circadian genes *per*, *tim*, *cyc* and *Clk* differentially affect tolerance to ethanol in Drosophila even though all of these mutations eliminate circadian rhythmicity. While some mutations completely ablate tolerance, others alter it substantially without eliminating it. These results further reinforce the importance of circadian genes in the production of tolerance, but also indicate that these genes are acting in a role independent of their role in maintaining circadian rhythmicity.

RESULTS

Circadian mutations do not alter sensitivity to ethanol

We tested mutants from each of the core circadian genes, each of which render the fly behaviorally arrhythmic. These mutations are: per^{01}, tim^{01}, cyc^{01}, and Clk^{JRK}. To eliminate any differences due to genetic background, each of these mutations were back-crossed into our wild type Canton-S (CS) strain for six generations.

Sensitivity to ethanol was tested for each of these mutants by placing ten age-matched female flies into vials with a kimwipe platform that prevented them from escaping. One mL of 35% ethanol was placed onto a cotton plug and inserted into the bottom of the vial. Pictures of the flies were taken every 20 seconds until all the flies were sedated. Software-generated counts of the number of flies climbing the walls of the vial were made at each time point. A curve-fitting and bootstrapping method determines the time when which half of the flies are sedated (K_{50}) (Pohl et al., in review). Because day-to-day variability affects how quickly the flies knock out, the comparison of mutant and wild type ethanol responses were only made on data collected from the same experiment. As demonstrated in Figure 3.1, none of the mutations knock down signifcantly different from wild type, indicating that these mutations do not affect the sensitivity to the drug. We must point out that for *Clk*^{JRK}, replication experiments did not always show a complete lack of sensitivity change. In one experiment, *Clk*^{JRK} showed sensitization, and in another, it showed resistance.

Tolerance to ethanol sedation is eliminated by only some circadian mutations

We used a similar method to test for tolerance to ethanol. Flies are divided into two groups and one group receives a 75% dose of ethanol for one hour while the other group is mock treated. Twenty-four hours later, both groups receive 35% ethanol until sedated. Using the same curve-fitting and bootstrapping method, we determine the time when half of the flies are knocked down (K_{50}). A population is said to be tolerant if the K_{50} of the group receiving its second sedation is significantly higher than the group receiving the first sedation. Wild type CS flies demonstrate tolerance to this dose of ethanol (Figure 3.2A, 3.2F). However, *per*^{01}(Figure 3.2B, 3.2G) and *tim*^{01} (Figure 3.2C, 3.2H) mutants fail to acquire tolerance. The other circadian mutants tested, *cyc*^{01}(Figure 3.2D, 3.2I) and *Clk*^{JRK} (Figure 3.2E, 3.2J), acquire tolerance.

Circadian mutations affect different components of tolerance

When flies first receive ethanol, they first exhibit a hyperactive phase before they enter the sedation phase (Moore et al., 1998). The analysis of tolerance described above does not distinguish whether a mutation affects overall tolerance because it alters the hyperactive phase or because it alters the

sedation phase. We have observed that the wild type acquires tolerance to both the hyperactive and sedative phases of ethanol intoxication (Pohl et al., in review). We decided to investigate whether the circadian mutations differentially affected these sub-phases of tolerance. Using the same tolerance paradigm described above, we measured the movement of the flies. We next employ a curve-fitting method in which a linear line fits the hyperactive phase and a normal distribution fits the sedative phase. This fit allows us to determine four parameters: 1) T_{max}: the time of the height of the peak, which signifies the time when the flies switch from hyperactivity to sedation; 2) R_{+}: the slope of the hyperactive phase; 3) R-: the decay of the sedative phase; and 4) E_{max}: the maximal amount of excitation.

Ethanol tolerance can be detected in some of these parameters. During their second sedation wild type animals reach maximal activity (T_{max}) after animals that are receiving their first sedation (Figure 3.3A,B). Additionally, animals receiving their second sedation had a reduced rate of excitation (R_{+}) (Figure 3.3C). However, the rate of sedation (R-) was unaffected by a prior ethanol sedation. Finally, during the second exposure to ethanol vapor, animals show a reduction in the maximal amount of excitation (E_{max}) in comparison to animals receiving their first sedation (Figure 3.3D). While this analysis looks at movement of the flies, and not the number of flies awake, we can still be confident that the decline in motion corresponds to the sedation phase as T_{max}

matches up well with the start of the decline in the number of flies on the vial (Compare Figures 2A and 3A).

We found that circadian mutations differentially altered tolerance. In the case of the *per*^{01} mutation, ethanol only showed a significant change in the R_+ parameter (Figure 3.4). However, the other parameters were unaffected. This suggests that the mutation may not be affecting tolerance to the hyperactive effects, but to the sedative effects. The *tim*^{01} mutants do not show tolerance in any of the four quantitative parameters (Figure 3.5). This indicates a complete absence of the tolerance response for this mutation.

The *cyc*^{01} mutants exhibit a far different profile from *per*^{01} and *tim*^{01} (Figure 3.6). As in wild type, *cyc*^{01} mutant flies receiving their second sedation take longer to transition from the hyperactive phase to the sedative phase, indicated by an increase in T_{max} (Figure 3.6B). However, unlike wild type, the slope of the hyperactive phase (R_+) is not significantly different (Figure 3.6C) Both R- and E_{max} are not significantly different (Figure 3.6D-E).

The *Clk*^{JRK} mutants are the most similar to wild type (Figure 3.7). T_{max}, R_+, and E_{max} are significantly different (Figure 3.7B,C,E) and R- is not significantly different (Figure 3.7D). This indicates that the *Clk* gene may not be involved in tolerance to ethanol.

A functional clock is not necessary for the acquisition of ethanol tolerance

As some circadian mutants are capable of acquiring tolerance even though the animal is arrhythmic, we hypothesized that a functional clock is not necessary for tolerance. To test this, we placed wild type CS flies in constant light for 5 days, which has been shown to make flies arrhythmic (Price et al., 1995). Flies were grown in a 12:12 light cycle, but once 1-2 day old flies were collected, they were placed in constant light for five days. They were then treated with either 75% or 0% ethanol for one hour as previously performed. These flies remained in constant light for the twenty-four hour period before their second sedation. A separate group was kept in 12:12 light-dark. As shown in Figure 3.8, constant light did not completely block the acquisition of tolerance.

An acute dose of ethanol does not shift the behavioral rhythmicity of the fly

As circadian genes interfere with tolerance to ethanol, we decided to test if an acute dose of ethanol is capable of altering behavioral rhythmicity of the fly. To do this, we placed flies in constant darkness so that their free-running rhythms could be assessed. At subjective mid-day for the fly, they were treated with an identical 75% EtOH dose for one hour as has been previously described. This treatment was performed in dim-red light, which does not alter the circadian rhythm of the fly. Immediately after sedation, flies were individually placed in a

Trikinetics Activity Monitor, where their rhythms were monitored for 7 days (Figure 3.9A). This dose of ethanol did not significantly alter the strength of the circadian rhythm (Figure 3.9B) or the period length (Figure 3.9C). Total locomotor activity of the fly was also unaffected (Figure 3.9D).

The effect of *per*^{01} on tolerance is dependent upon genetic background

We wished to test if the *per*^{01} mutation caused the elimination of tolerance in other genetic backgrounds. We therefore obtained this mutation in a +;+;*ry*^{506} background from Dr. Joel Levine. We tested both the +;+;*ry*^{506} line and the *per*^{01};+;*ry*^{506} line for tolerance by determining the K50 of each. Both of these lines showed significant tolerance (Figure 3.10). These results indicate that the effect of the *per*^{01} in the CS background is specific to that genetic background.

DISCUSSION

In this paper, we show that circadian genes are involved in tolerance to ethanol in the fruit fly, but that they are acting in a manner independent of the regulation of circadian rhythmicity. Mutations for *tim* and *per* block tolerance to the sedative effects of ethanol, whereas mutations for the genes that encode for their transcription factors, *cyc* and *Clk* do not. Additionally, mutations for *tim* and

cyc, disrupt tolerance to the hyperactive phase, whereas *Clk* and *per* do not. Subjecting wild type flies to constant light, which knocks out rhythmicity, does not eliminate tolerance. These results indicate that some of the circadian genes of the fly are necessary for tolerance, but that a functional clock is not necessary.

Our results share several similarities to the cocaine sensitization story in Drosophila. The circadian mutations per^{01}, cyc^{01}, and Clk^{JRK} disrupt cocaine sensitization, whereas tim^{01} does not (Andretic et al., 1999). While the genes that are involved in ethanol tolerance are not identical, the pattern that these genes act in a pleiotropic role to produce drug phenotypes is consistent. Both these cases argue that these genes act in a role independent of circadian rhythmicity to promote drug responses.

We do not find that a single tolerance-inducing dose of ethanol is sufficient to alter the behavioral rhythmicity of the fly. Both acute and chronic doses of ethanol are sufficient to shift circadian rhythms in mammals (Rosenwasser et al., 2005a; Brager et al., 2011). It is possible that a chronic dose could have an effect on the behavioral rhythmicity of the fly. More work is necessary to determine if ethanol can shift the clock in the fly.

The per^{01} mutation only blocks tolerance to sedation when it is in a Canton-S background. We found robust tolerance when this mutation was in the ry^{506} background. These results indicate that per^{01} only affects tolerance in specific genetic backgrounds. It is likely that per^{01} attains modifiers that allow the

flies to acquire tolerance. Cantonized *per*^{01} is a weak stock compared to the *ry*^{506} background, so it possible that these modifiers would be highly selected for.

In conclusion we show that specific circadian genes disrupt the capacity for the acquisition of ethanol tolerance in Drosophila. However, a functioning clock is not necessary. These results add to the growing literature documenting the importance of circadian genes on drug responses.

Figure 3.1: Circadian mutants do not affect sensitivity to ethanol

Knockdown curves of flies receiving their first dose are shown comparing wild type Canton-S (CS) with A) *per*^{01}, B) *tim*^{01}, C) *cyc*^{01}, and D) *Clk*^{JRK}. Boxplots depicting the time when 50% of the flies are knocked down (K_{50}) is compared between CS and the mutants E) *per*^{01}, F) *tim*^{01}, G) *cyc*^{01}, and H) *Clk*^{JRK}. For these and all subsequent boxplots, the red line indicates the median, the box indicates upper and lower quartile values, the whiskers are the coverage of the data within 1.5 of the interquartile regions (corresponding to approximately 99.7% of the data) None of the curves are statistically different (bootstrapping and the percentile method). n=4 vials for *per*^{01} test, n=6 for all others.

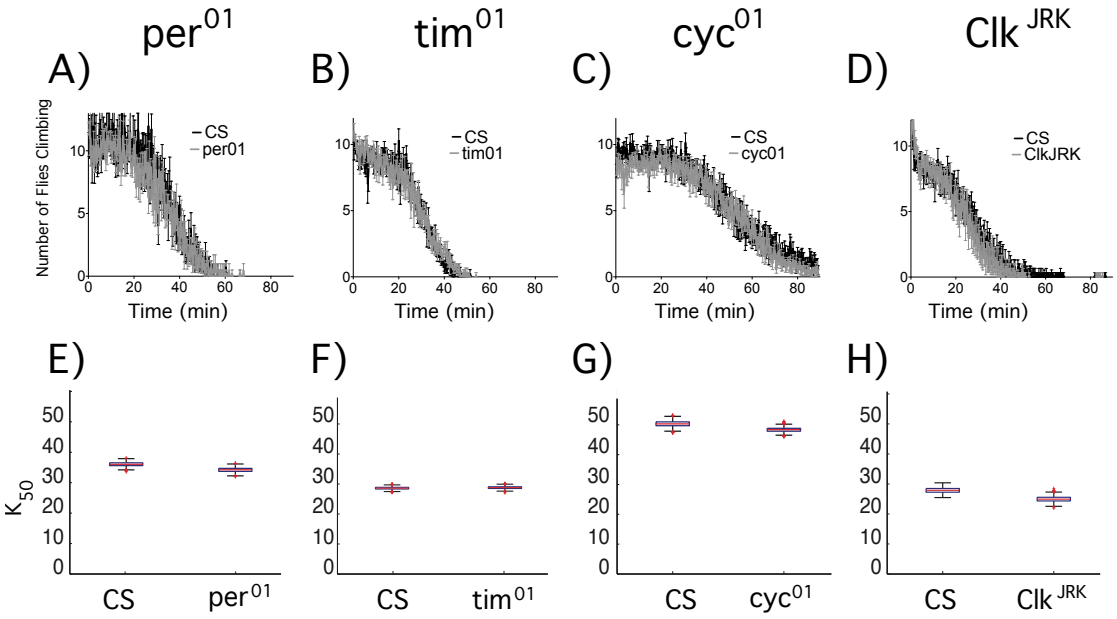


Figure 3.2: Some circadian mutants disrupt tolerance to ethanol

Knockdown curves are shown comparing flies recieving their first sedation to flies receiving their second sedation for A) CS, B) *per*^{01}, C) *tim*^{01}, D) *cyc*^{01}, and E) *Clk*^{JRK}. The corresponding boxplot for K_{50} is shown for F) CS, G) *per*^{01}, H) *tim*^{01}, I) *cyc*^{01}, and J) *Clk*^{JRK}. Significance was determined using bootstrapping and the percentile method. n=4 vials for *per*^{01} test, n=6 for all others.

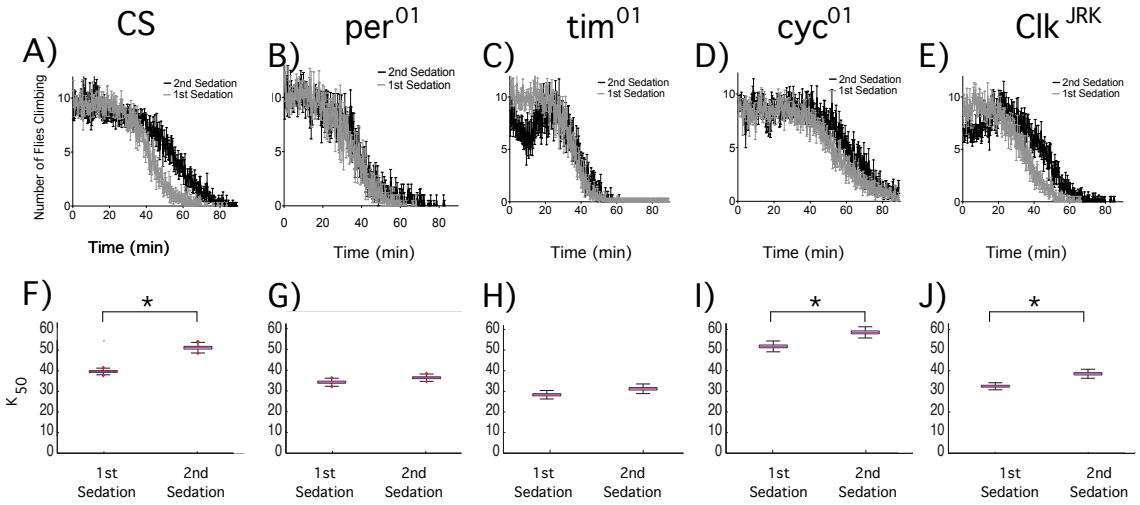


Figure 3.3: Wild type flies acquire tolerance to multiple components of tolerance

A) A graph comparing the number of flies moving for wild type CS flies receiving their first or second dose of ethanol. Boxplots depicting B) T_{max} (the time of maximal excitation) C) R_{+} (the rate of hyperactivity) D) R_{-} (the rate of decay) and E) E_{max} (The maximal amount of activity) are shown. Significance was determined using bootstrapping and the percentile method. n=6 vials.

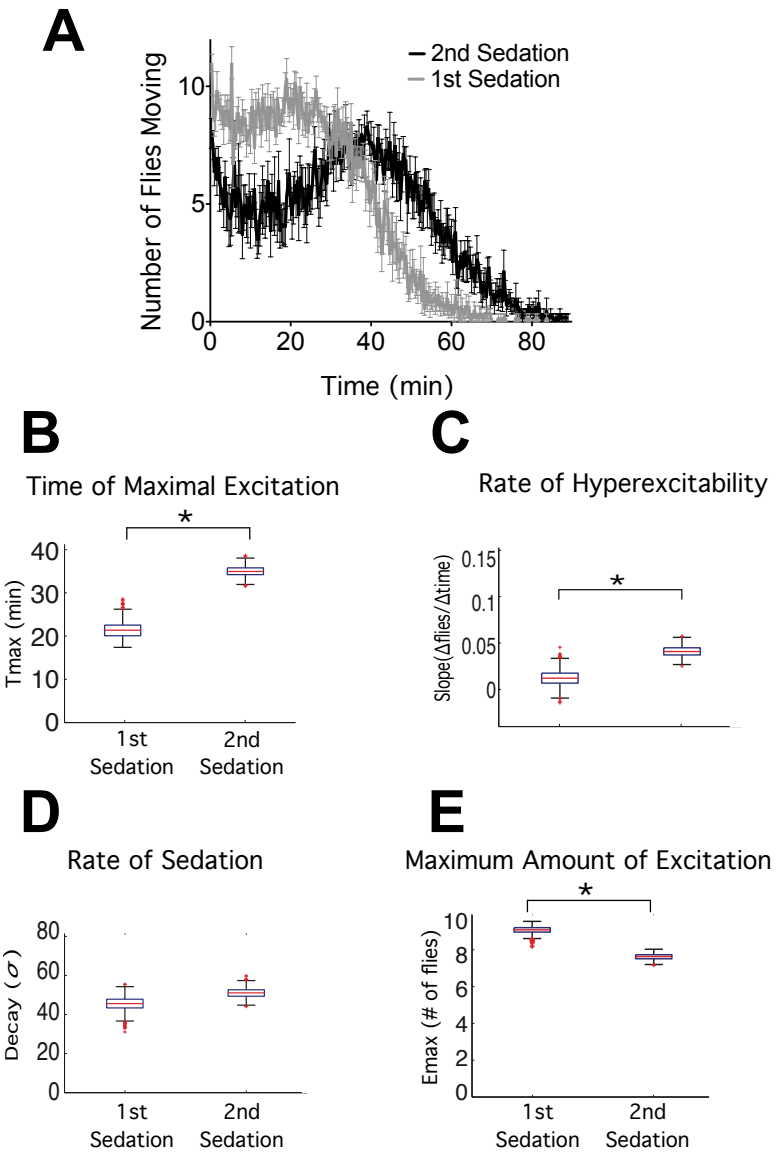


Figure 3.4: Flies carrying the *per*^{01} mutation fail to acquire tolerance to the sedative phase.

A) A graph comparing the number of flies moving for *per*^{01} flies receiving their first or second dose of ethanol. Boxplots depicting B) T_{max} (the time of maximal excitation) C) R_{+} (the rate of hyperactivity) D) R_{-} (the rate of decay) and E) E_{max} (The maximal amount of activity) are shown. Significance was determined using bootstrapping and the percentile method. n=4 vials.

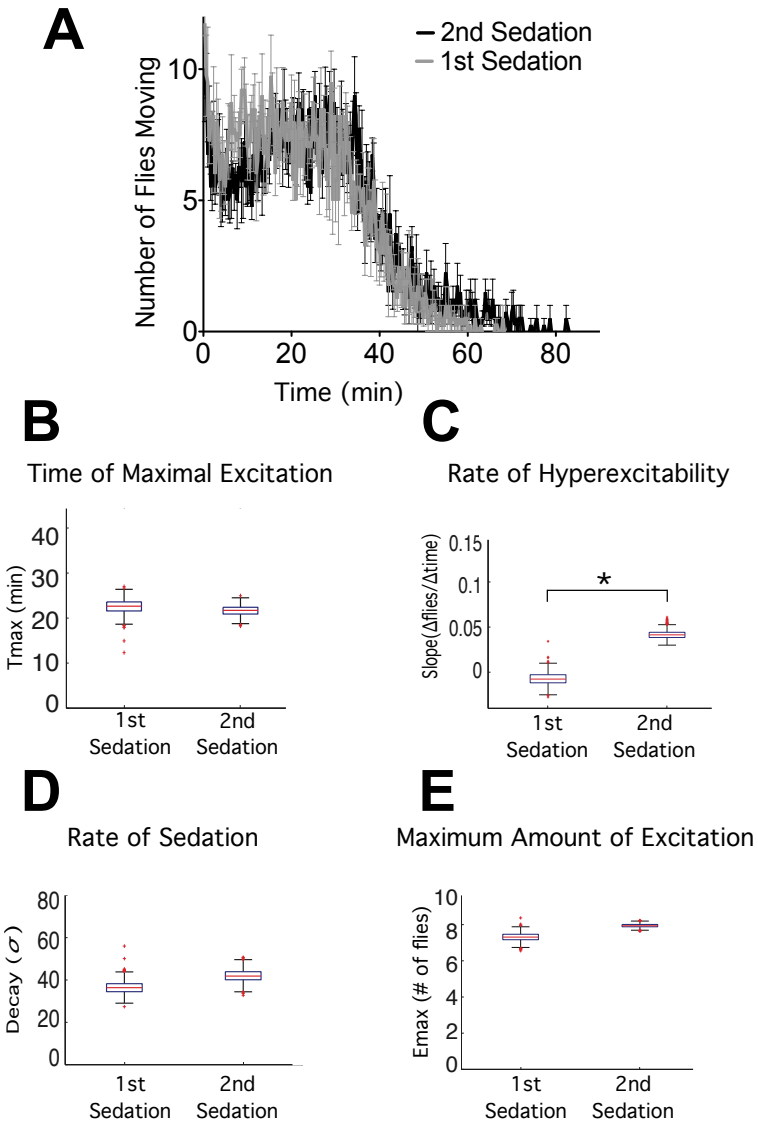


Figure 3.5: Flies carrying the *tim*^{01} mutation fail to acquire tolerance to any phase.

A) A graph comparing the number of flies moving for *tim*^{01} flies receiving their first or second dose of ethanol. Boxplots depicting B) T_{max} (the time of maximal excitation) C) R_{+} (the rate of hyperactivity) D) R_{-} (the rate of decay) and E) E_{max} (The maximal amount of activity) are shown. Significance was determined using bootstrapping and the percentile method. n=6 vials.

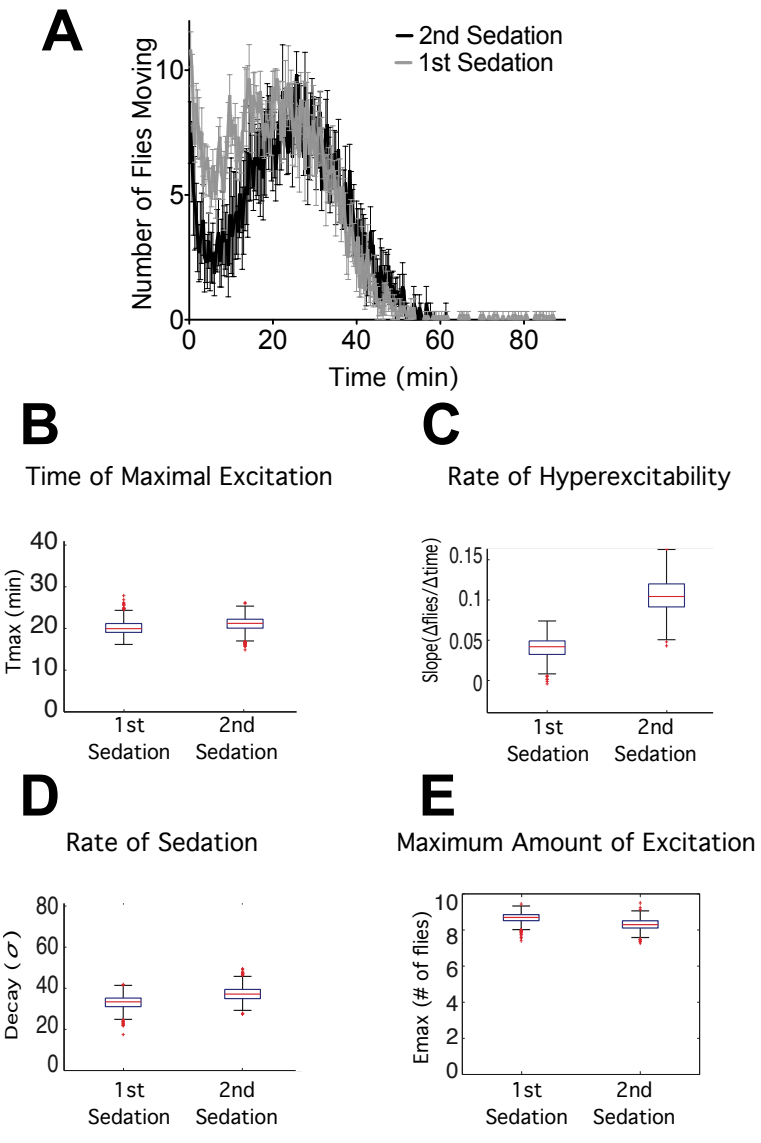


Figure 3.6: Flies carrying the *cyc*^{01} mutation do not acquire tolerance to some phases of tolerance.

A) A graph comparing the number of flies moving for *cyc*^{01} flies receiving their first or second dose of ethanol. Boxplots depicting B) T_{max} (the time of maximal excitation) C) R_{+} (the rate of hyperactivity) D) R_{-} (the rate of decay) and E) E_{max} (The maximal amount of activity) are shown. Significance was determined using bootstrapping and the percentile method. n=6 vials.

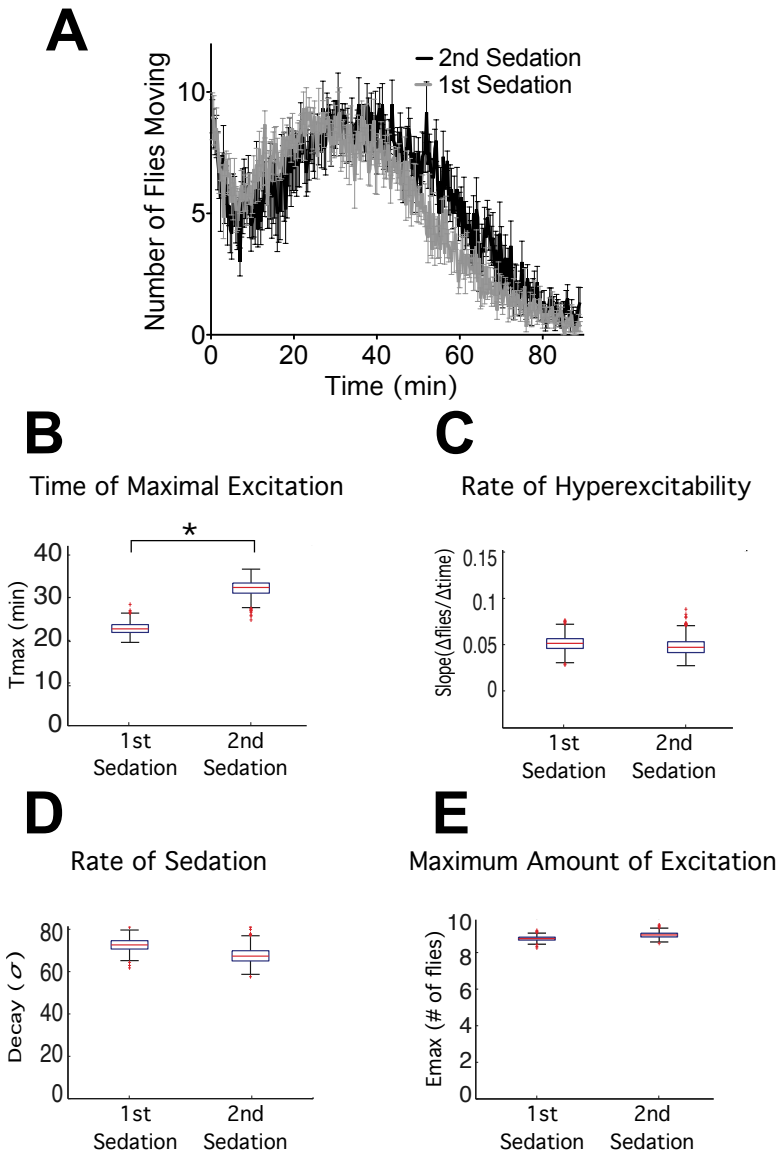


Figure 3.7: Flies carrying the *Clk*^{JRK} mutation acquire tolerance.

A) A graph comparing the number of flies moving for *Clk*^{JRK} flies receiving their first or second dose of ethanol. Boxplots depicting B) T_{max} (the time of maximal excitation) C) R_{+} (the rate of hyperactivity) D) R_{-} (the rate of decay) and E) E_{max} (The maximal amount of activity) are shown. Significance was determined using bootstrapping and the percentile method. n=6 vials.

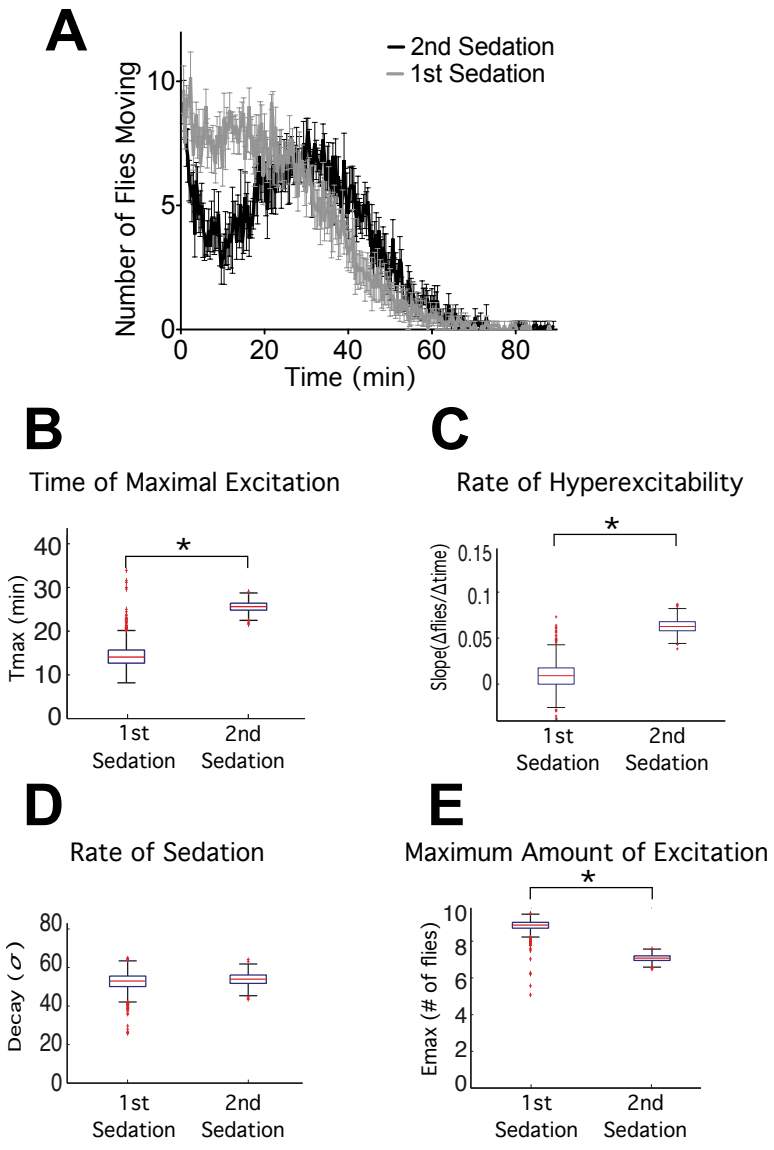


Figure 3.8: Constant light does not eliminate ethanol tolerance in wild type flies.

Knockdown curves are shown comparing flies recieving their first sedation to flies receiving their second sedation for A) flies maintained in a 12:12 light:dark cycle and C) flies maintained in constant light. Corresponding boxplots depicting K_{50} comparing flies receiving their first and second treatment are shown for B) Light:Dark flies and D) Constant light flies. Significance was determined using bootstrapping and the percentile method. n=6 vials.

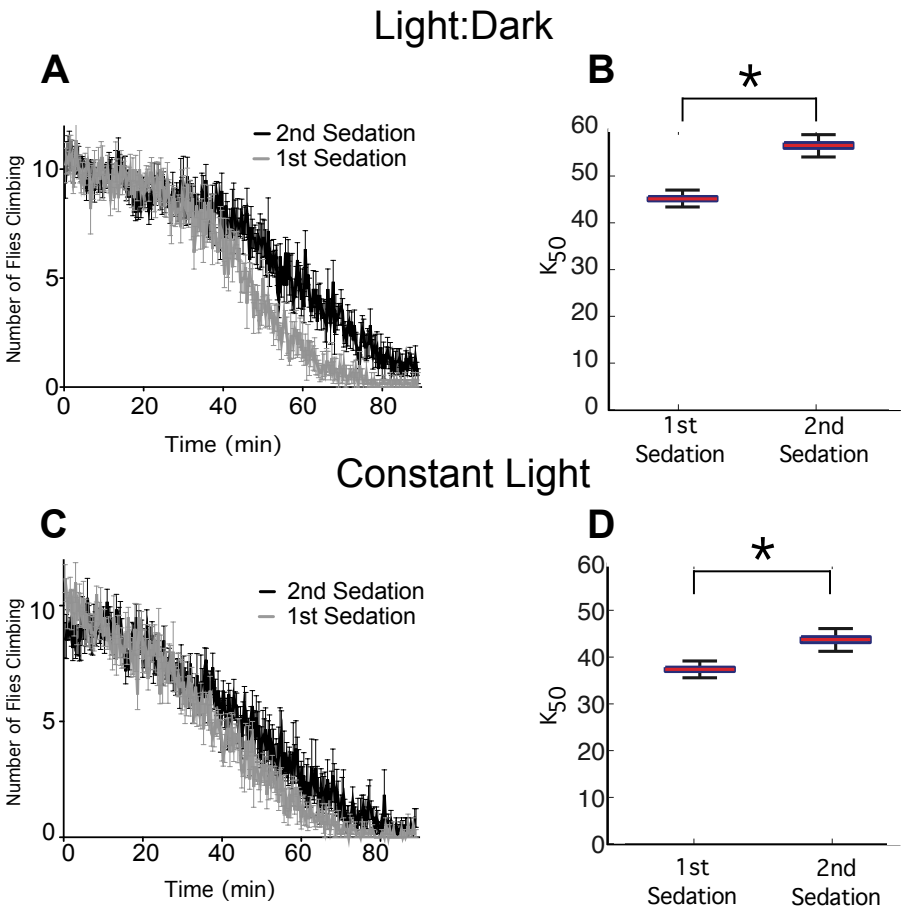


Figure 3.9: An acute dose of ethanol does not disrupt circadian activity

A) Average movement for flies that were maintained in constant darkness and then received (in dim red light) either 75% ethanol or water for one hour and were then placed in activity monitors in constant darkness. Bar plots indicate the B) rhythmic index, C) period length, and D) total number of times the flies moved. None of these values were significantly different (Student's *t*-test).

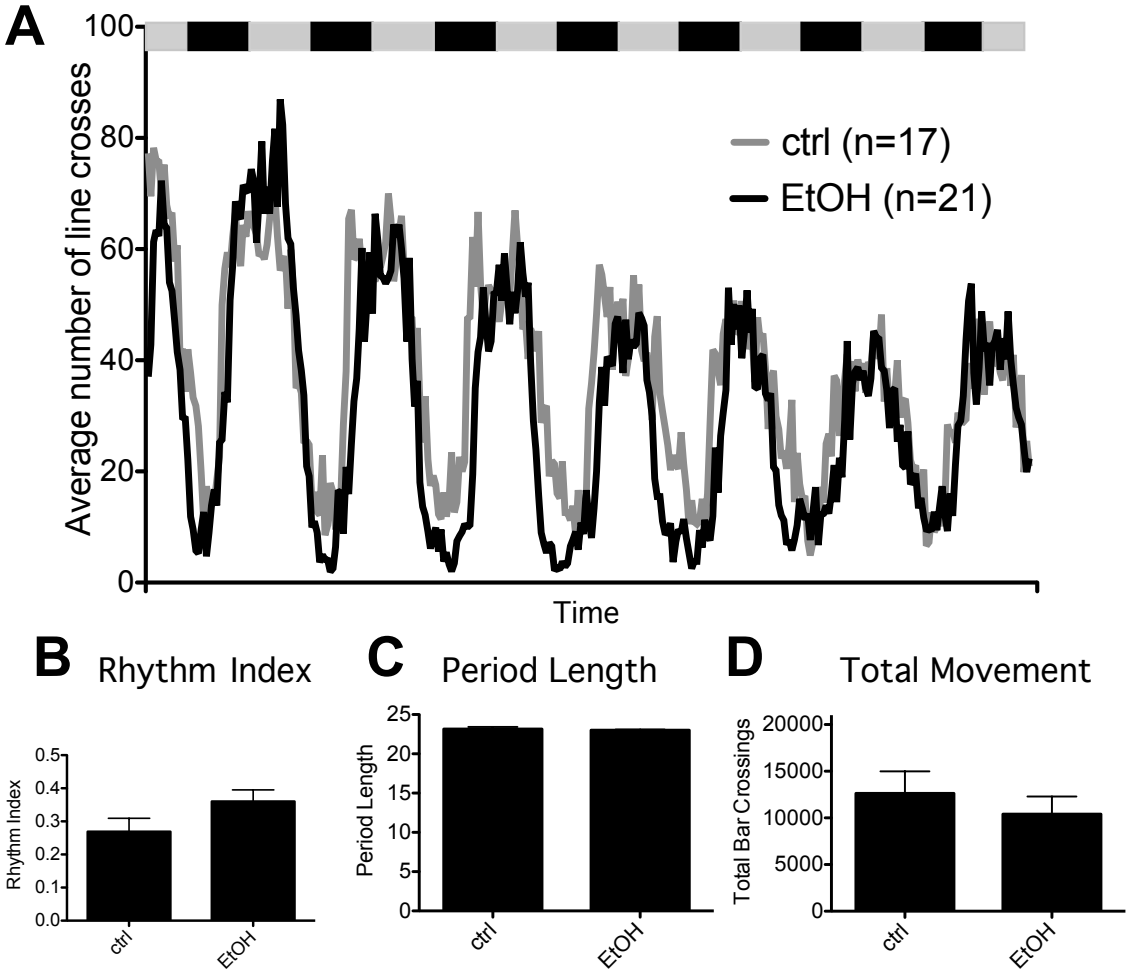
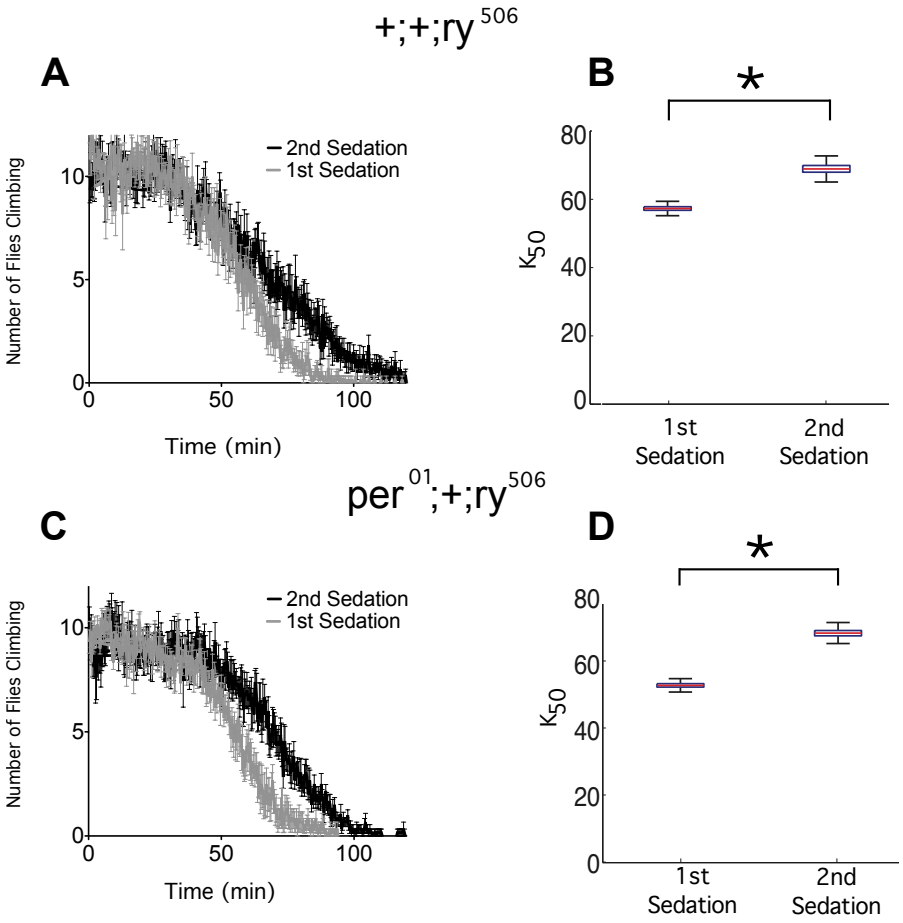


Figure 3.10: The effect of *per*^{01} on tolerance is genetic background specific

Knockdown curves are shown comparing flies recieving their first sedation to flies receiving their second sedation for A) +;+*ry*^{506} flies and C) *per*^{01};+*ry*^{506} flies. Corresponding boxplots depicting K_{50} comparing flies receiving their first and second treatment are shown in B and D. Significance was determined using bootstrapping and the percentile method. n=6 vials.



METHODS

Stocks and Media

All flies were raised in 12:12 light conditions on standard corn/yeast medium. The wild type strain used in this study in Canton-S (CS). The non-backcrossed mutants *per*^{01}, *tim*^{01}, *cyc*^{01}, and *Clk*^{JRK} were kind gifts from Dr. Paul Hardin. The +;+;*ry*^{506} and *per*^{01};+;*ry*^{506} lines were gifts from Dr. Joel Levine.

Backcrossing

All mutants were crossed into the CS background. Mutants were initially crossed to CS. The resulting heterozygote females were then crossed to CS males. The resulting female offspring, in single-pair crosses, were then crossed again to CS males. After eggs had been produced, the female was sacrificed and DNA was extracted using standard single-fly DNA extraction techniques. PCR was performed to identify the presence of the mutation. This cross was repeated until the mutation had been crossed into the CS background for 6 generations. Homozygous mutants were detected by PCR and kept as stable stocks.

The primers used for the detection of mutants were:

perwt-18U (for detection of the wild type *per* allele):

5'-CATACCGCTTCCTCATCC-3'

per01-18U (for detection of the *per*^{01} allele):

5'-CATACCGCTTCCTCATCT-3'

perL1 (common *per* lower primer):

5'-GGTCCTGGAAGGTGAAATGA-3'

timWT-20U (for detection of the wild type *tim* allele):

5'-GTCCGATCTGAGGTGCCTTT-3'

tim01-20U (for detection of the *tim*^{01} allele):

5'-TGCAGATTAGCGAAATCAGT-3'

timL1 (common *tim* lower primer):

5'-CAGCGTGGCAAACCTGTAAT-3'

cycwt-18U (for detection of the wild type *cyc* allele):

5'-TCGACGTCCTGCATCCGA-3'

cyc01-18 (for detection of the *cyc*^{01} allele):

5'-TCGACGTCCTGCATCCGT-3'

cycL1 (common *cyc* lower primer):

5'-CTCTGTGGAACGTCGGTCTT-3'

Clkwt-18U (for detection of the wild type *Clk* allele):

5'-ATCTGCACACGCAGCACC-3'

ClkJRK-18U (for detection of the *Clk*^{JRK} allele):

5'-ATCTGCACACGCAGCACT-3'

ClkL1 (common *Clk* lower primer):

5'-CATCGATATCCTCACGCAGT-3'

Tolerance and Sensitivity Assays

Tolerance assays are conducted in a manner similar to Pohl et al. Four to six day old females are loaded into vials with 10 flies in each vial. A platform with an air-permeable Kimwipe prevents the flies from being able to get to the bottom of the vial. In half the vials, 1 mL of 75% ethanol is pipetted onto one third of a Flug (Genesee Scientific, San Diego, CA) and placed onto the bottom of the vial. Flies are exposed to this ethanol vapor for one hour before being removed and placed into fresh food vials to recover. The other half of the vials receive a 0% ethanol control. Twenty-four hours after the first treatment, both groups are subjected to 35% ethanol until they are completely sedated. Vials are interdigitated so that each alternating vial contains flies from a different group. A camera takes pictures every 20 seconds. For sensitivity assays, interdigitated vials of mutant and wild type flies that have never previously been exposed to ethanol are sedated with 35% ethanol. As cantonized *per*^{01} is a weak stock and it was difficult to attain sufficient flies for an experiment, tolerance and sensitivity were tested using the

same flies for this mutant. All experiments are conducted between the hours of ZT5 to ZT9.

Statistical Analysis

Statistical analysis was performed as described in Pohl et al. using Matlab. Briefly, the number of flies climbing on the vial and the number of flies moving during each 20 second frame was counted using an automated particle counter. The time when 50% of the flies are knocked down (K_{50}) was determined by fitting an inverse cumulative normal function to the "flies climbing" curve. Bootstrapping analysis resampled this distribution 1000 times by randomly selecting a point at each time from one of the vials. Significance was determined using the "Percentile Method": if the values contained within the 2.5 and 97.5 percentiles of the K50 bootstrap distributions of any two treatments do not overlap, they are said to be statistically different. In the case where flies in the first few minutes spent time on the platform where they could not be scored by the camera (i.e. *tim*^{01} tolerance test), the first 15 minutes were set to having 10 flies after confirmation by looking at the images in question.

To evaluate the more detailed parameters (Tmax, Emax, R+, and R-), a piecewise function was fit to the "flies moving curve". The startle response, which was determined to be the time when the lowest amount of movement prior to

sedation was seen in each experiment, was eliminated to simplify the analysis (The amount of time eliminated was later added back to Tmax to accurately represent the time of the peak). The piecewise function contains a linear component for the hyperactive phase and a Gaussian component for the sedative phase. Bootstrapping was performed by resampling 1000 times from a random vial at each time point. Each of the four parameters were compared using the "Percentile Method."

Measurement of Circadian Activity

Circadian activity was measured using standard procedures (Rosato and Kyriacou, 2006). Individual flies were loaded into 5mm X 65mm glass tubes. One end of the tube contained a food solution composed of 5% sucrose and 2% agar which was sealed with wax. After the fly was loaded into the tube, a piece of cotton was inserted to prevent it from escaping. These tubes were placed into DAM2 Drosophila Activity Monitors (Trikinetics Inc., Waltham, MA) which records a signal when a fly crosses an infrared beam. They were then placed in an incubator which maintained constant darkness. Activity was recorded every 30 minutes for seven days. Any fly that did not move at least once in any 24-hour period was discarded. Analysis of rhythmic index, period length, and total movement was performed in Matlab using software attained from Dr. Joel Levine

(Levine et al., 2002). One untreated fly moved over seven standard deviations over the mean value and was discarded as an outlier.

Chapter 4

Ethanol preference in *Drosophila melanogaster* is driven by its caloric value

INTRODUCTION

Alcoholism is a devastating disease that has both large financial and social costs. The manifestation of this disease is complex and both genetic and environmental factors are believed to play important roles (Mayfield et al., 2008; van der Zwaluw and Engels, 2009). The fruit fly, *Drosophila melanogaster*, has been a useful model system for identifying genes associated with rudimentary facets of alcohol-associated behavior (Atkinson, 2009). As in mammals, low doses of ethanol cause an excitatory effect while higher doses cause sedation in flies (Moore et al., 1998). Additionally, flies are capable of acquiring both rapid and chronic tolerance to the drug (Berger et al., 2004). A variety of Drosophila genes, most of which are conserved in mammals, have been shown to be involved in both locomotor sensitivity and tolerance to ethanol vapor (Moore et al., 1998; Bainton et al., 2000; Scholz et al., 2000; Dzitoyeva et al., 2003b; Cowmeadow et al., 2005; Rothenfluh et al., 2006; Corl et al., 2009).

Over 50 years of research documents the ecological relationship between *D. melanogaster* and ethanol. In natural habitats, these flies feed on rotting fruits that can contain over 5% ethanol. They have also been found to breed inside

wineries, where the concentration of ethanol is even higher (McKenzie and McKechnie, 1979; Gibson et al., 1981). One would expect that an animal whose food regularly undergoes ethanol fermentation to be attracted to the odor of ethanol because it is the odor of its food. Furthermore, if the food were to regularly contain potentially debilitating amounts of ethanol, then one would expect the flies to be resistant to the pharmacological effects of ethanol (either via innate resistance or rapid-inducible resistance). Perhaps the most tantalizing support for these suppositions are the comparative studies of the distribution of *D. melanogaster* and *D. simulans* in wineries. These species are thought to have diverged from a common ancestor only 3.4 million years ago and in the laboratory can productively interbreed (Capy and Gibert, 2004). Both species are found in the grounds and superstructures of wineries, with *D. simulans* outcompeting *D. melanogaster* in the non-alcohol-rich regions. However, *D. melanogaster* occupies all of the high alcohol content niches, such as the wine cellars, to the exclusion of its sister species. In all developmental stages, *D. melanogaster* proves to be more resistant to ethanol toxicity than *D. simulans*. Additionally, *D. melanogaster* females lay eggs in ethanol-containing mash where larvae thrive. It seems that once fermentation begins, the high alcohol content niche belongs to *D. melanogaster*, both as a food source and a nursery. These differences in distribution are believed to be a product of the capacity to

catabolize and tolerate ethanol (McKenzie and Parsons, 1972; McKenzie and Parsons, 1974; McKenzie and McKechnie, 1979).

D. melanogaster shows an obvious preference for ethanol-containing food over non-ethanol-containing food. Many investigators have concluded that *D. melanogaster* are attracted to ethanol as a food source. *D. melanogaster* larvae will spend more time on the ethanol-containing half of an agar plate compared to the half without ethanol (Gelfand and McDonald, 1980; Parsons, 1980; Depiereux et al., 1985) and adult flies will extend their proboscis more on the ethanol-containing half of a food plate compared to the half without ethanol (Cadieu et al., 1999). *D. melanogaster* are capable of breaking down ethanol and using its calories. *D. melanogaster* that consume only ethanol as a calorie source live significantly longer than flies that receive no calories (Libion-Mannaert, 1976; Pecsenye et al., 1994).

Recently, the role of ethanol in the life of *D. melanogaster* has been revisited in the context of alcoholism. The question addressed by Devineni and Heberlein (2009) was whether flies show an interest in ethanol because of its neuropharmacological effects and whether these effects could precipitate increased consumption. These experiments used the CAFE (CApillary FEeder) assay in which the flies choose between pipets that contain food alone and pipets that contain food supplemented with ethanol (Ja et al., 2007). Devineni and Heberlein (2009) showed that flies prefer ethanol, and that preference is

maintained when the caloric ratio between the ethanol-containing and non-ethanol-containing solutions is varied. However, in these experiments, the food containing ethanol had at least twice the calories as the control food. This leaves open the possibility that the flies can detect this caloric difference and prefer the ethanol-containing food for the calories that it provides. Recently it has been shown that, independent of taste, flies sense and value food for its caloric content (Dus et al., 2011). Furthermore, high calorie tasteless food can be successfully used as an unconditional stimulus in learning assays (Burke and Waddell, 2011; Fujita and Tanimura, 2011). Therefore, when evaluating preference one needs to account for the caloric content of the solution.

Here we further investigate whether flies prefer food containing ethanol because it is more caloric or because of its pharmacological effects. Distinguishing between the nutritive and pharmacological drive to consume ethanol is a maddeningly difficult question to answer because it requires determining the motivation of a fly. Indeed, this issue that has not been completely resolved in rodents where the pharmacological effects of ethanol have been much longer studied (Forsander, 1998). In this paper, we show that when we use a variety of carbohydrates to balance the calories between the ethanol and non-ethanol-containing pipets that the preference for ethanol is eliminated. This occurs even if the carbohydrates used to balance calories are not gustatorily attractive to the fly. We conclude that the preference of

D. melanogaster for ethanol is primarily due to its nutritive value rather than its pharmacological effects.

RESULTS

D. melanogaster prefer food that contains ethanol when the non-ethanol choice contains fewer calories.

We used the CAFE (CApillary FEeder) assay to verify that flies prefer food that contains ethanol (Ja et al., 2007; Devineni and Heberlein, 2009). Eight flies were placed in vials with access to four capillary pipets. Two pipets were loaded with control food (5% sucrose and 5% yeast extract). Two other pipets contained the control food supplemented with ethanol. The volume of liquid consumed from each pipet was scored daily for five days and each day the pipet was replaced with a new pipet with fresh food. For each day, a preference index was determined by the following formula: (Amount of ethanol food consumed - Amount of control food consumed) / Total food consumed.

As previously demonstrated, flies prefer food that contains a high concentration of ethanol (Figure 4.1 and (Devineni and Heberlein, 2009)). Flies show no preference for food supplemented with 1% ethanol (Figure 1A, 1B). However, flies show a strong preference for food supplemented with 5%, 10%, or 15%

ethanol when compared to ethanol-free food (Figure 4.1C-4.11J). As reported by Devineni and Heberlein (2009), the ethanol preference in flies is highly variable during the first day of the assay. Therefore, we calculate the total amount of the ethanol food consumed over the final four days and compared it to the amount of control food consumed. Only at 1% ethanol was the difference not statistically significant (Figure 4.1I). Preference indices for each condition are shown in Figure 4.1J.

Preference for ethanol is eliminated when the control food is balanced for calories.

Ethanol is rich in calories, and can be used as a food by *D. melanogaster* (Libion-Mannaert, 1976; Pecsenye et al., 1994). Indeed, ethanol is estimated to contain 7 kcal/g, compared to 4 kcal/g for sucrose and 1.58 kcal/g for yeast extract (Ja et al., 2007; Devineni and Heberlein, 2009). In our 5%, 10%, and 15% ethanol assays, the ethanol pipet contained 1.99, 2.98, and 3.97 times the number of calories than the control pipet, respectively. This led us to hypothesize that the flies were choosing the ethanol-containing food because it is richer in nutrients. To test this hypothesis, we added sufficient carbohydrate to the control pipet to equal the calories provided by ethanol in the ethanol-containing pipet. We first used sucrose to balance calories. In a balanced assay with 10% ethanol, flies not only failed to show a preference for ethanol, but instead showed a preference for

sucrose (Figure 4.2A, 4.2B). This was also the outcome when ethanol was lowered to 5% and the amount of sucrose added to the control pipet matched the calories provided by 5% ethanol (Figure 4.2C, 4.2D). Cumulative plots from days 2-5 show a significant preference for the balanced sucrose solution (Figure 4.2E, 4.2F). These data indicate that the presence of an isocaloric sucrose mix is sufficient to eliminate preference for ethanol.

The fact that the flies no longer preferred ethanol in the balanced sucrose assay could be due to an overwhelmingly strong attraction for the taste of sucrose. To find calorie sources that may be less gustatorily attractive, we tested a palette of carbohydrates for gustatory attractiveness using the Proboscis Extension Response (PER) assay. In this assay, a solution is applied to the proboscis of the fly, and if it is gustatorily attractive, the fly will extend its proboscis. The PER assay has been used by many investigators to determine the attractiveness of carbohydrates and other substances (Tompkins et al., 1979; Dahanukar et al., 2007; Gordesky-Gold et al., 2008). The carbohydrates were tested to identify those that were more palatable than ethanol, less palatable than ethanol, or equally palatable to ethanol. All of the carbohydrates were tested at a concentration calculated to have the same caloric content as 10% ethanol. We tested sucrose, glucose, mannose, ribose and two different types of maltodextrins (M100 and M180). Mannose and ribose have been previously shown not to activate the sugar receptor of the fly (Dahanukar et al., 2007). When tested in the PER assay, only

sucrose and glucose showed a response higher than the water control (Figure 4.3A). These results indicate that the maltodextrins, ribose, and mannose are not gustatorily attractive to the fly.

To determine whether any of these carbohydrates were gustatorily aversive to the fly, we asked whether the addition of the other carbohydrates to the standard food solution (5% yeast extract, 5% sucrose) would reduce the number of responses in the PER assay. All of the carbohydrates were tested at a concentration calculated to have the same caloric content as 10% ethanol. When these carbohydrates are added to the food solution of 5% sucrose and 5% yeast, only ribose supplementation reduced the attractiveness of the standard food solution indicating that only ribose is aversive (Figure 4.3B).

The six carbohydrates characterized in the PER assay were used in the CAFE assay to supplement the control pipet to a level calculated to counterbalance the calories provided by the ethanol in the ethanol-supplemented pipet (Figure 4.4 for summary data and Figure 4.5 for daily consumption and preference index plots). When calorie balancing was performed with glucose, ethanol preference was eliminated. Furthermore, despite not being gustatorily attractive, ethanol preference was also eliminated when the control solution was balanced with either M100 or M180 maltodextrin. However, preference for ethanol persisted when calorie balancing was performed with ribose. Ribose was shown to be strongly aversive in the PER assay and therefore, in this instance the fly may

choose the isocaloric ethanol solution merely as a way to avoid ribose. These data support a model in which the fly chooses ethanol-containing food only if another isocaloric (and palatable) calorie source is not available. However, we did encounter one carbohydrate that belied this model. When mannose was used to balance the calories in the ethanol pipet, we observed a preference for ethanol despite the fact that the PER assay shows that the taste of mannose is neither gustatorily aversive nor attractive. Nevertheless, the model in which ethanol is sought for its caloric content should not be discounted since four out of five of the non-aversive carbohydrates that were used to balance the calories in the ethanol pipet produced results that are consistent with this long-standing ecologically-based model.

The peak ethanol concentration in the unbalanced ethanol CAFE assay does not exceed 4mM.

Previously, Devineni and Heberlein (2009) found that flies with the option of drinking 15% ethanol food had about 1 mM more ethanol in them than control flies that had no access to ethanol. However, the authors point out that the sample consisted of multiple flies, that although collected at a single moment in time, was likely to be a mixture of flies that had recently consumed ethanol and flies that had consumed ethanol a long time ago and may have already metabolized it. To determine the maximum possible ethanol concentration that accumulates when

flies are showing strong ethanol preference, we determined the amount of ethanol inside single flies immediately after ethanol consumption in an unbalanced CAFE assay. One set of pipets contained standard food and one set of pipets contained standard food supplemented with 10% ethanol. All samples were collected on the third day of the assay at which point the flies were showing strong ethanol preference (Figure 4.1). We assayed single flies immediately after they drank from the ethanol pipet. The ethanol concentration maxima is likely to occur immediately after the sip is taken because the inter-sip interval is very long (~120 minutes as reported in (Ja et al., 2007; Devineni and Heberlein, 2009)) and that the rate of ethanol catabolism is very large (Middleton and Kacser, 1983; Moore et al., 1998; Cowmeadow et al., 2005). Thus, there is little opportunity for ethanol to accumulate between sips.

The ethanol content of each fly was measured by gas chromatography (Figure 4.6A). Water content of the flies was determined as described (Cowmeadow et al., 2005). The minimum concentration of ethanol that can be detected by this method is 0.5 mM. Flies drinking non-ethanol food contained no detectable ethanol above background (<0.5 mM, n=4). The highest observed concentration of ethanol in a fly immediately after drinking was 3.08 mM. Figure 4.6B shows the distribution of ethanol content.

The amount of ethanol consumed is insufficient to induce tolerance.

One criterion for addiction in humans is that the user becomes tolerant to the drug (American Psychiatric Association, 2000). We investigated whether the amount of ethanol consumed in the CAFE assay was sufficient to induce ethanol tolerance. In pharmacology, tolerance is defined as the reduced response to an effect of a drug caused by previous drug exposure. Adult flies have been shown to acquire functional behavioral tolerance when they are forced to breath ethanol vapor (Scholz et al., 2000; Cowmeadow et al., 2005). Flies that have received a previous vapor treatment take longer to sedate than flies receiving their first dose. To test for tolerance, we used a knockdown assay in which flies are placed into vials and 1 mL of a 35% ethanol solution is applied to a cotton plug and placed at the bottom of the vial. The amount of time it takes for 50% of the flies to sedate is determined from this assay. We compared flies after Day 5 of the 10% unbalanced CAFE assay to two control groups of flies. In the first control group, all four pipets contained only the base liquid food (5% sucrose, 5% yeast extract). We were concerned that this group might sedate more quickly than the ethanol group solely because they consumed fewer calories. Therefore, we included a second control group in which two pipets also included sufficient sucrose to equal the calories provided by the 10% ethanol solution in the experimental group. As shown in Figure 4.6C-D, all three groups knock down at the same rate, indicating the ethanol consumed was not sufficient to induce tolerance.

Flies prefer the non-ethanol-containing food if it contains more calories.

We have shown that in an unbalanced assay in which flies have a choice between food supplemented with ethanol and unsupplemented food that flies prefer the ethanol food. We have shown that this preference can be eliminated if we calorically balance the control pipet by adding non-sweet M100 maltodextrin. Given the hypothesis that flies are choosing the pipets based on calories, one would expect that the flies would prefer the non-ethanol-containing pipet if it contained more calories than the ethanol pipet. To test this hypothesis, we tested preference between a pipet supplemented with 10% EtOH and a pipet supplemented with enough M100 to provide 1.5-fold more calories than the ethanol. Like the isocaloric M100, this concentration of maltodextrin was neither gustatorily attractive nor aversive in the PER assay (Figure 4.7A). When tested in the CAFE assay, flies showed a significant preference for the M100 solution (Figure 4.7B-D).

Switching to a calorically-balanced alternative after preference has developed eliminates ethanol preference.

Flies consume more ethanol in the calorically-unbalanced but not in the calorically-balanced assay. One reason that the flies do not show ethanol prefer-

ence in the calorically-balanced assay might be that they do not consume sufficient ethanol to experience the pharmacological or intoxicating effects due to the presence of a gustatorily attractive and high calorie ethanol-free pipet. Indeed, Shtonda and Avery (Shtonda and Avery, 2006) have shown that *C. elegans* are not interested in other food if presented with a high quality food. To address this issue, we performed assays which began in the unbalanced state but were switched to a balanced state once clear preference had been established (3 days). For the first three days, the flies had the standard choice between food supplemented with 10% EtOH and non-ethanol containing food. Then, for the fourth and fifth days, the non-ethanol containing food was supplemented with M100 maltodextrin, chosen because of its lack of gustatory attractiveness, at a level sufficient to balance the calories from ethanol. Flies demonstrated preference for ethanol on days 2 and 3, but immediately following the switch to a balanced assay, preference was eliminated (Figure 4.8A-D). To test if this effect was strain or sex dependent, we repeated the switch assay with male flies of the w;Berlin strain (kind gift from U. Heberlein) and found a similar effect to the Canton-S strain (Figure 4.8E-H). These results indicate that even when given sufficient time to develop preference for ethanol, flies do not prefer ethanol if presented with an isocaloric alternative.

DISCUSSION

In this paper, we show that preference for ethanol in *D. melanogaster* is not due to the pharmacological effects of ethanol. While flies show preference for ethanol over control food that contains fewer calories, this preference is lost once the control food is adjusted to be isocaloric with the ethanol food. This holds true even for food supplemented with maltodextrins, which are not gustatorily attractive to the fly. Flies will even shift their preference toward the maltodextrin solution if it contains more calories. In this study, we use values for calories based on FAO (1998) estimates. It is possible that the number of calories available to Drosophila from its foodstuffs are different from the FAO estimates. An accurate measurement of effective number of calories provided by each food ingredient would be invaluable for studies of this kind. Because we could not be certain of the actual number of calories available to the fly, we balanced calories using a variety of carbohydrates. Four of the five non-repulsive carbohydrates that were tested abolished ethanol preference, which is consistent with the idea that the flies would reject ethanol as long as the calorically balanced alternative was not gustatorily repugnant. This is consistent with the interpretation that it is calories and not pharmacological effects that are motivating flies to drink ethanol.

One might argue that the flies need time to become accustomed to ethanol and that this will never occur in the presence of a calorically-balanced alternative. A related counter argument is that flies can develop pharmacological

attraction to ethanol but not if an alternative is simultaneously presented. Both of these caveats were discredited by our food switching experiment in which we allowed obvious ethanol preference to develop under non-calorically balanced conditions and then switched to a calorically balanced alternative food. Once offered another choice with equal caloric value, flies immediately lost their preference for ethanol and showed strong preference for the non-alcoholic food. If flies seek ethanol for pharmacological reasons, then one would assume that they would continue prefer it.

Furthermore, if flies seek and consume ethanol for its pharmacological effects then one would expect that the consumed ethanol would be sufficient to trigger other pharmacological responses. However, functional tolerance did not develop in these animals. Indeed the maximum amount of ethanol to accumulate inside the fly was quite low (less than 4 mM or .02 BAC). In humans, this amount of ethanol, while slightly compromising complex behavioral tasks, is insufficient produce euphoria (King et al., 2002), and therefore may be insufficient to trigger events associated with addictive behavior.

The role of calories in ethanol intake in rodents has been a contentious issue (Gill et al., 1996; Forsander, 1998). Early work showed that rats will eat considerably less food when ethanol is present and will drink more ethanol on a calorically restricted diet, suggesting that the calories provided by ethanol are not without consequence (Richter, 1926; Westerfieid and Lawrow, 1953). Furthermore, it

was more recently demonstrated that blocking the leptin pathway, which is involved in feeding and appetite, reduces ethanol consumption in mice (Forsander, 1998; Blednov et al., 2004). For rodents, it is probably reasonable to discount the possibility that this animal seeks ethanol for its caloric value because this would be such an unnatural situation. Rodents do not consume ethanol in the wild. However, wild *Drosophila melanogaster* do consume ethanol. The evolutionary history of *D. melanogaster* supports the interpretation that this species uses ethanol as a food source. In the wild, ethanol is a common component of high quality *D. melanogaster* food and high value egg-laying sites. While ethanol-rich foods are avoided by competitor Drosophila species–apparently because ethanol reduces the fecundity of these competitors–*D. melanogaster* is better able to metabolically handle high concentrations of ethanol than its close relatives (McKenzie and Parsons, 1972). This makes the ethanol-rich niche a protected area for *D. melanogaster* feeding and reproduction.

Calories are most likely an important factor in determining food selection by the fly. Indeed, flies have a putative taste-independent metabolic sensor that can be used to assess the caloric content of a food source (Dus et al., 2011). Additionally, flies will learn to be attracted to odors that are associated with tasteless calorie-rich foods (Burke and Waddell, 2011; Fujita and Tanimura, 2011). Our data indicates that flies are able to assess the caloric difference between the ethanol and non-ethanol pipets and prefer the one with a higher nutritional value.

Our data do not exclude the possibility that conditions exist that will cause flies to prefer ethanol for its pharmacological effects. However, preference does not seem to exist in the CAFE assay under conditions in which the alternative food is calorically balanced, perhaps because the ethanol content in the fly is too low (<4 mM). Devineni and Heberlein (2009) have generated higher ethanol levels in CAFE assays after flies have been starved for 20 hours. In this instance, ethanol preference is still more likely to be calorically based because the flies were extremely hungry and because the alternative food provided far fewer calories (unbalanced CAFE assay). Exposing flies to ethanol vapor or direct injection of ethanol is a standard way of generating high ethanol hemolymph levels (Moore et al., 1998; Manev et al., 2003; Cowmeadow et al., 2005; Devineni and Heberlein, 2009) and thus this approach may help generate pharmacological-based preference that can later be studied in the CAFE assay. In conclusion, our data strongly indicate that *D. melanogaster* do not prefer ethanol for its pharmacological effects in the confines of the CAFE assay.

Figure 4.1: Drosophila show preference for liquid food supplemented with ethanol. Food consumption plots are shown for five-day preference assays in which flies had a choice between liquid food (5% sucrose, 5% yeast) and liquid food to which we added either (**A**) 1% ethanol , (**C**) 5% ethanol, (**E**) 10% ethanol, or (**G**) 15% ethanol. Panels **B**, **D**, **F**, and **H** are preference index plots for the data presented in panels **A**,**C**,**E** and **G**, respectively. Panel **I** shows the cumulative consumption for both the ethanol-containing and ethanol-free food (control) for each concentration of ethanol tested during days 2 to 5. Panel **J** shows the cumulative preference indices calculated from the cumulative data. Significance was determined using Student's *t* test (*= $p<0.05$). n=16 vials for experiments.

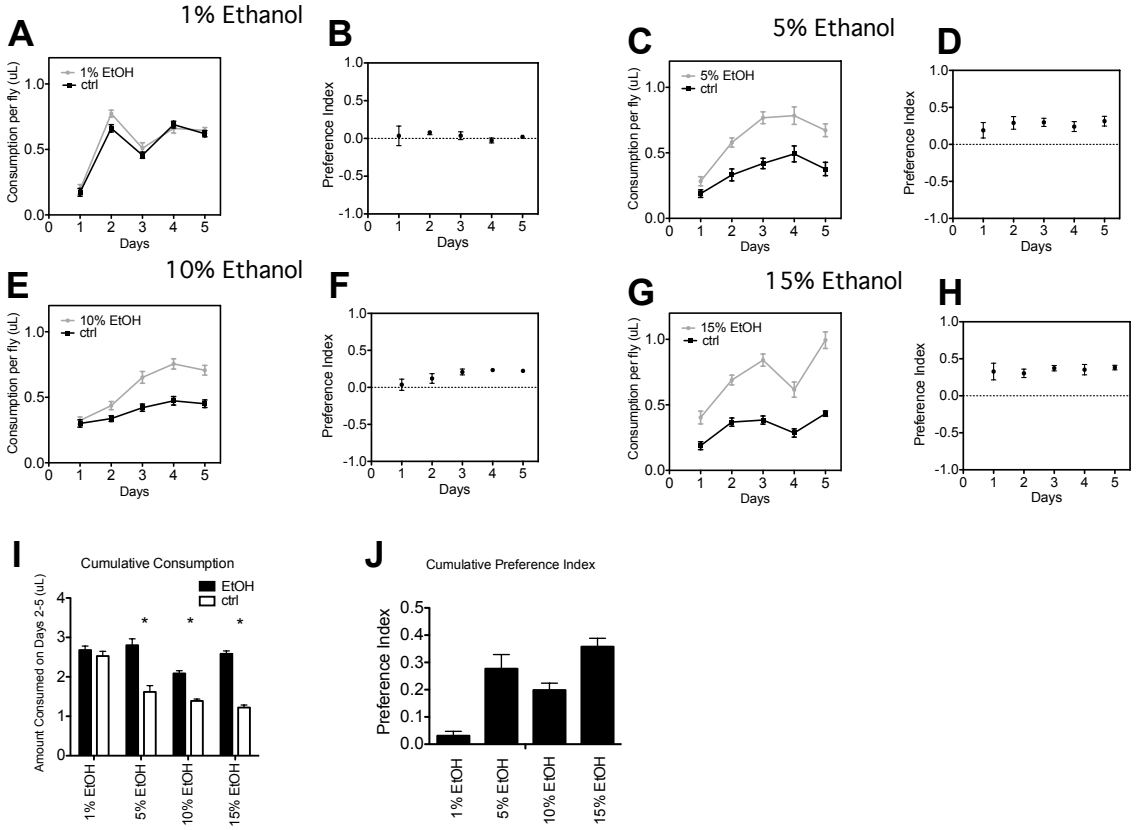


Figure 4.2: Balancing calories with sucrose eliminates preference for ethanol. Food consumption plots are shown for five-day preference assays in which flies had a choice between two pipets that contained liquid food (5% sucrose, 5% yeast) and ethanol and two pipets that contained liquid food supplemented with sufficient sucrose to counter-balance the calories contributed by ethanol in the other pipets. In panel **A**, 5% ethanol was tested while in panel **C**, 10% ethanol was tested. Panels **B** and **D** are preference index plots for the data in A and C, respectively. Panel **E** shows the cumulative consumption from days 2-5 for both the ethanol and non-ethanol-containing food. Panel F shows the preference index calculated from the cumulative data. Significance was determined using Student's *t* test (*= $p<0.05$ for preference for ethanol, #=$p<0.05$ for preference for control food). n=16 vials for all experiments.

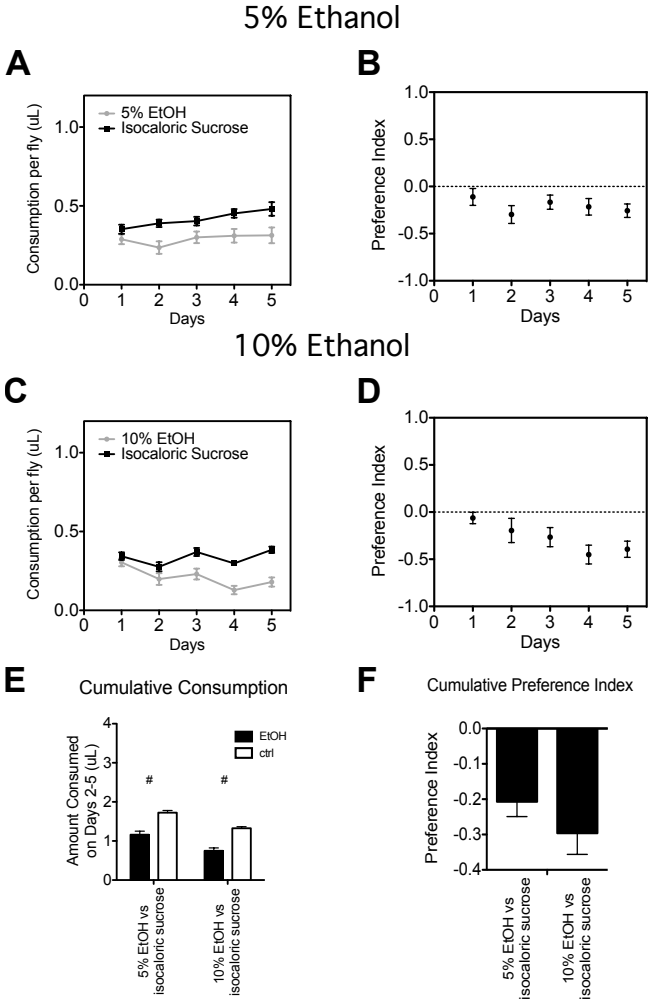


Figure 4.3: Proboscis extension response to different calorie sources.

A) Proboscis extension response to the substances in water. Flies were given 5 trials with each carbohydrate solution and the number of proboscis extensions counted. Shown are the mean number of extensions to each carbohydrate solution. Water was used as a negative control and 4% sucrose as a positive control. Significance was determined using a One-way ANOVA with a Dunnett's correction to compare to the water control (*=p<0.05). **B**) Proboscis extension response to the same carbohydrate series dissolved in liquid food (5% sucrose, 5% yeast). Water was used as a negative control and liquid food as a positive control. Significance was determined using a One-way ANOVA with a Dunnett's correction to compare to the liquid food control (*=p<0.05). The number of flies tested for each solution is indicated by the number in the bottom of each bar.

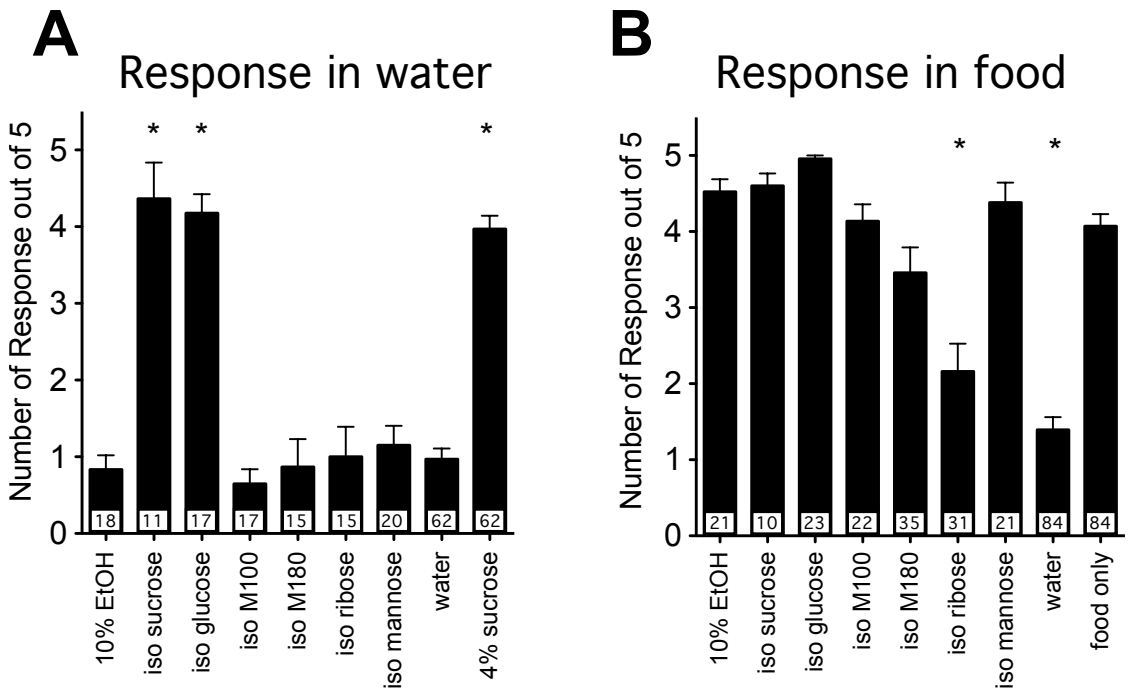


Figure 4.4: Preference for ethanol food can be eliminated if the other solution has equal caloric content. A) Cumulative consumption from days 2-5 of CAFE assays in which flies had a choice between food supplemented either with ethanol (EtOH) or an isocaloric carbohydrate alternative (ctrl). Significance was determined using Student's *t* test (*= p<0.05). n=16 vials for all experiments. **B)** The corresponding preference indices from Panel A.

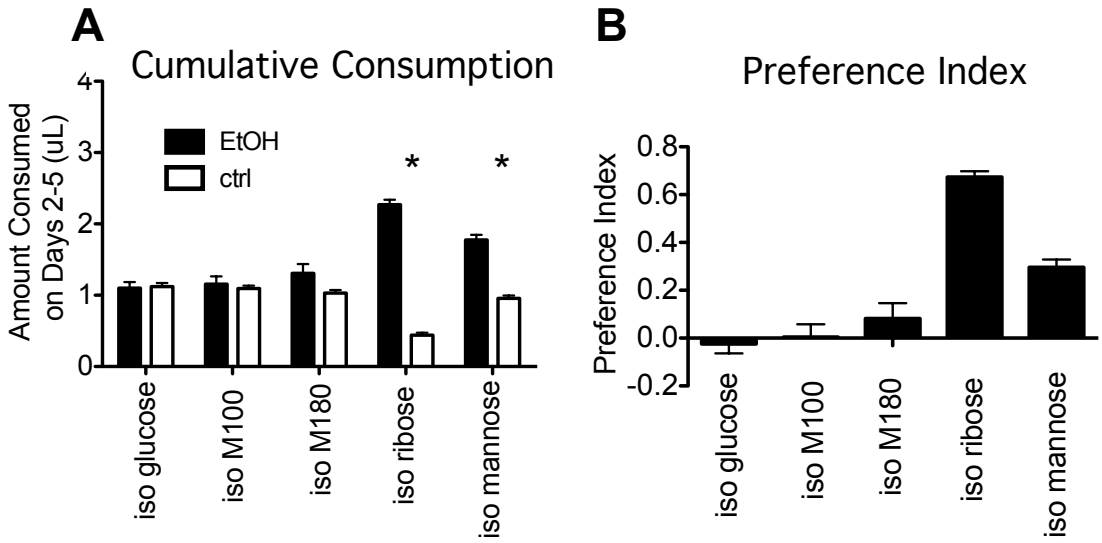


Figure 4.5: Consumption plots for isocaloric CAFE assays.

Consumption plots are shown for five-day preference assays in which flies had a choice between two pipets that contained liquid food (5% sucrose, 5% yeast) and ethanol and two pipets that contained liquid food supplemented with an isocaloric amount of (**A**) glucose, (**C**) M100 maltodextrin, (**E**) M180 maltodextrin, (**G**) ribose, or (**I**) mannose. Panels **B**,**D**,**F**, **H**, and **I** are preference index plots for the data presented in panels A,C,E, G, and I, respectively.

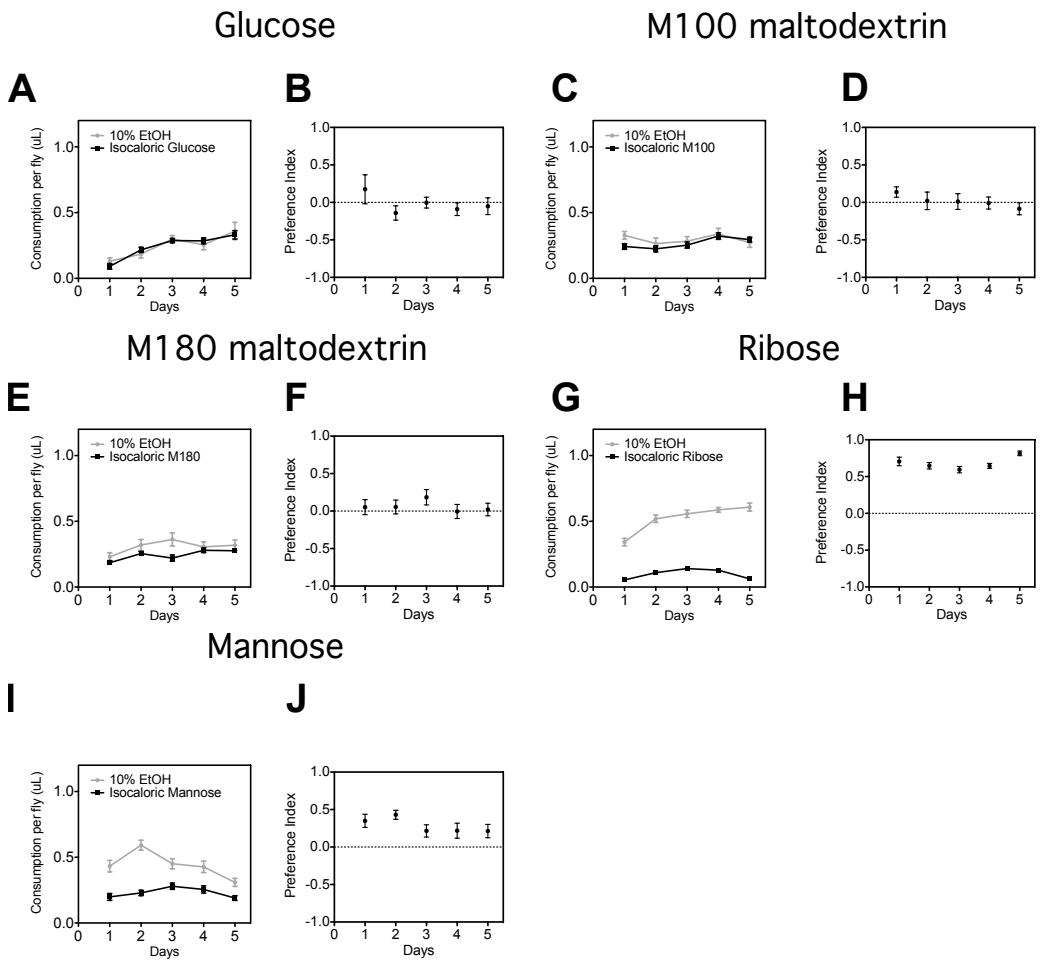


Figure 4.6: The amount of ethanol inside the fly is small and insufficient to induce tolerance to ethanol. A) Chromatograph tracings at the elution time of ethanol from a standard curve of ethanol dilutions and representative extracts from flies. The standard curve consists of two-fold ethanol dilutions into toluene. The labels on the x-axis pertain to the molarity of an amount equivalent to the water weight of one fly. Individual flies were prepared by crushing them in 7 uL of toluene immediately after they were observed to drink from a pipet. Tracings from a representative fly that only got control food (ctrl fly) and two representative flies that were in a 10% ethanol assay (EtOH fly) are shown. **B**) A histogram showing the ethanol content observed in flies that only received control food (ctrl) and flies that were immediately assayed after drinking from an ethanol pipet. **C**) Knock-down curves for flies receiving a sedating dose of ethanol vapor. Prior to the test, the flies had consumed liquid food for five days supplemented with 10% ethanol (EtOH), nothing (ctrl), or sucrose to equal the calories predicted for 10% ethanol (isocaloric sucrose). n= 5 vials of 10 flies. **D**) The time required for 50% of the flies to be sedated by the ethanol vapor is shown for flies that had consumed the liquid food described in for panel C for 5 days. None of the groups are significant-ly different from each other (Student's *t* test *= p<0.05). n=16 vials for all experiments.

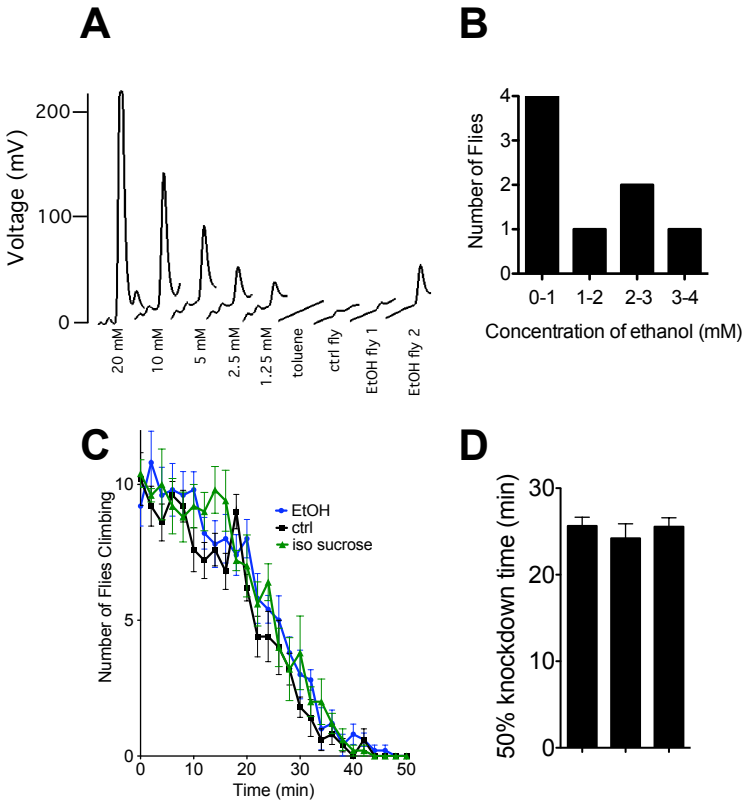


Figure 4.7: Drosophila prefer the non-ethanol solution if it contains more calories. A) Proboscis extension response data is shown for M100 maltodextrin at a concentration sufficient to provide 1.5 times the amount of calories of 10% EtOH (1.5X M100). This concentration was tested both in water and in food and compared to the appropriate control. The response to the 1.5X M100 solution was not statistically different from the controls (Student's *t* test). n=13 flies. **B**) Food consumption plots are shown for five-day preference assays in which flies had a choice between two pipets that contained liquid food supplemented with 10% ethanol and two pipets that contained liquid food supplemented with 1.5X M100. **C**) Preference indices based on the data from Panel B. **D**) Cumulative consumption from days 2-5 for both the ethanol and non-ethanol-containing food. Significance was determined using Student's *t* test ($p<0.05$).

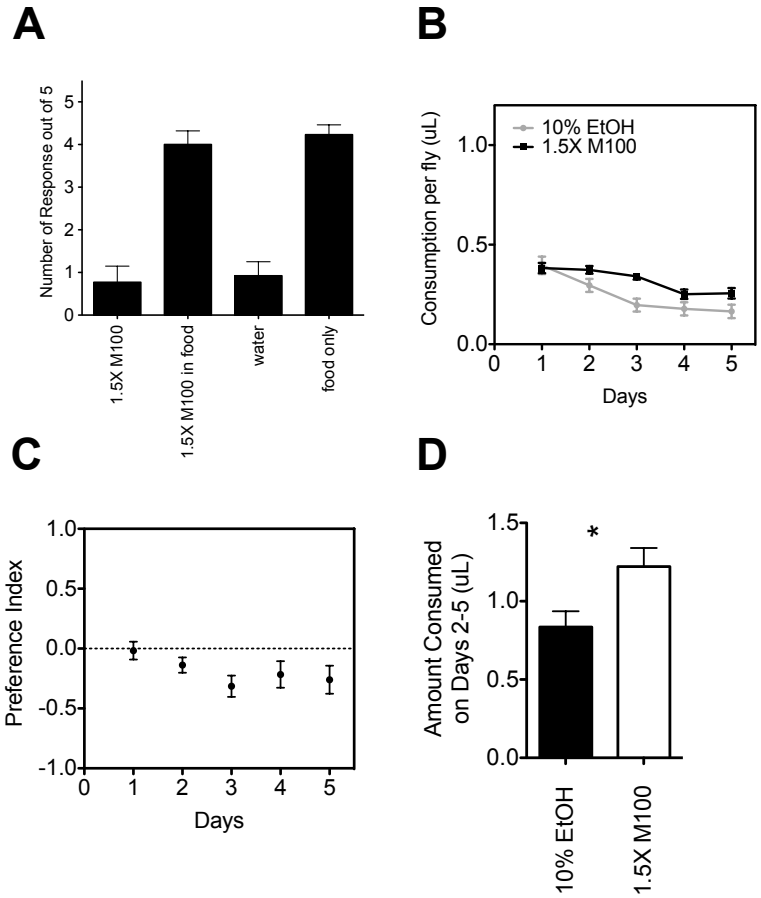
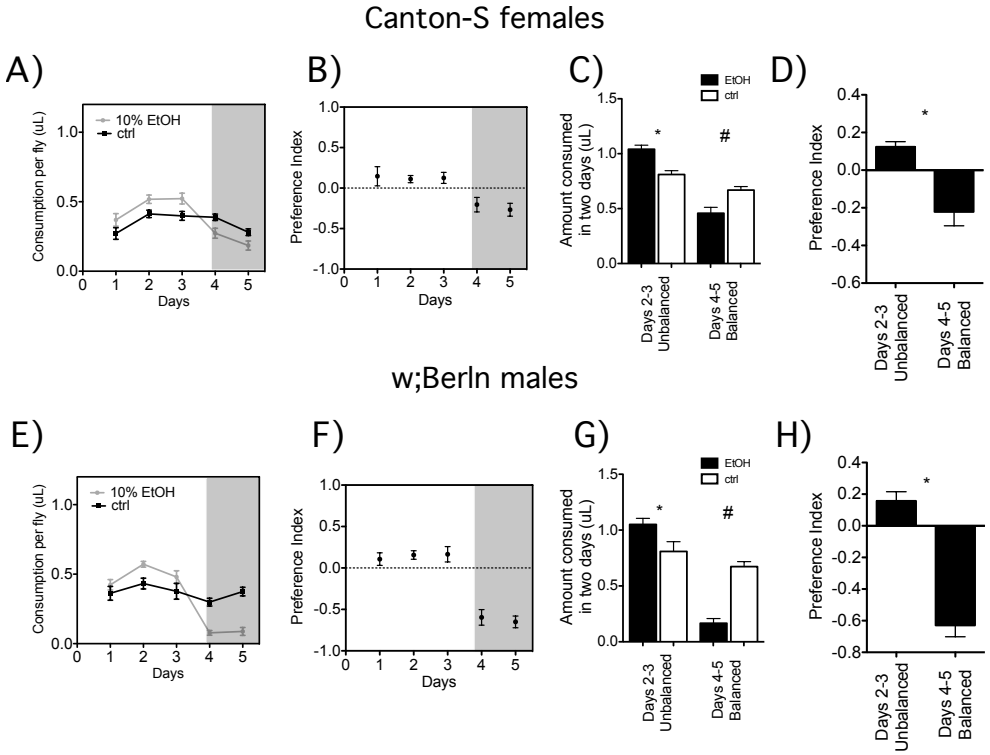


Figure 4.8: Switching to a balanced assay after preference has been established eliminates the preference for ethanol. A and **E**) Food consumption plots for five-day preference assays in which flies had a choice between pipets containing liquid food (ctrl) or liquid food supplemented with 10% ethanol (EtOH). On days 1-3, the control food was 5% sucrose, 5% yeast. On days 4 and 5, the control food is supplemented with enough M100 maltodextrin to balance the calories contributed by ethanol in the 10% EtOH pipets. **B** and **F**) Preference index plots for the data presented in panels A and E respectively. **C** and **G**) Cumulative consumption of control or ethanol-containing food before and after the flies were switched to a balanced assay. For days 2-3, the calories in the control pipet did not counterbalance the calories from ethanol in the EtOH pipet (Days 2-3 unbalanced). During days 4-5 (Days 4-5 balanced), the calories from ethanol in the EtOH pipet was counterbalanced in the control pipet with M100 maltodextrin. Significance was determined using Student's *t* test (*= p<0.05 for preference for ethanol food, #=p<0.05 for preference for control food). **D** and **H**) Preference indices calculated from the cumulative data in C and G respectively. Significance was determined using Student's *t* test (*= p<0.05)**.** Panels A-D use females from the Canton-S strain. Panels E-H use males from the w;Berlin strain. n=16 vials for all experiments.



METHODS

Flies and Media

Flies for all experiments, unless otherwise stated, were female 1-3 day old flies of the Canton-S strain. For the M100 switch assay, we additionally used 1-3 day old male flies of the w;Berlin strain which were kindly provided to us by Ulrike Heberlein. Flies were raised on standard cornmeal/molasses/agar medium that had no detectable amount of ethanol.

Calorie Determination

We calculated caloric content using the FAO (Food and Agricultural Organization of the United Nations) values of 4 kcal/g for disaccharides (sucrose), 3.75 kcal/g for monosaccharides (glucose, mannose, ribose), and 7 kcal/g for ethanol (FAO, 1998). The caloric content for maltodextrin is assumed to be 3.76 kcal/g based on information from the manufacturer (Grain Processing Corporation, Muscatine, IA).

Preference Assay

We used an assay adapted from Devineni and Heberlein (2009). Groups of eight flies were placed into standard plastic fly vials (Genesee Scientific, San Diego,

CA) in which holes were made to allow air to circulate. Filter paper was placed around the vial to prevent visual stimuli for the flies. One-third of a Flug (Genesee Scientific, San Diego, CA) was placed at the bottom of the vial to minimize the collection of moisture. Four holes were made in another one-third of a Flug which was placed at the top of the vial. 200 uL yellow pipet tips were cut to the appropriate length to hold a capillary tube and placed in each of the holes of the top Flug. Four uL of food was filled by capillary action into 1-5 uL capillary tubes (Catalog number 21-180-11, Drummond Scientific Company, Broomall, PA) and placed into the yellow tips so that the flies have access to them. Unless otherwise stated, two pipets (facing opposite of each other) contain ethanol-containing food and two contain non-ethanol-containing food. All vials were placed in a 5 gallon aquarium, which contained wet sponges on the bottom and wet filter paper on the sides to increase humidity. The top of the aquarium was sealed with plastic wrap and the aquarium was kept in a 12:12 light:dark cycle. Pipets were scored and replaced daily for five days.

Proboscis Extension Response

We used a method adapted from (2007). Individual flies were starved with only access to a water-soaked Kimwipe for 24-48 hours. The flies are then individually immobilized by placing them into yellow tips in which the end was cut so that just the head of the fly protrudes. Wicks made from thin strips of Kimwipes are dipped

into the food and applied to the labellum of the fly. To minimize sensitization from the sweet sugars, we only tested two carbohydrates per day plus a positive (4% sucrose) and negative control (water) at the beginning and end of the experiment. For each food source, the labellum was touched five times in one minute intervals, and the number of responses noted. We waited five minutes when switching to a new food source. Any fly that responded less than three times to the initial 4% sucrose or more than two times to the initial water was discarded.

Tolerance Assay

Flies were tested for tolerance immediately after being removed from the preference assay. For each condition tested, five groups of ten flies were placed into empty vials with an air permeable platform to prevent them from escaping. One mL of 35% ethanol was pipetted onto a cotton plug and placed on the bottom of each vial, allowing ethanol to gradually equilibrate into the chamber. Naturally flies will stay on the sides of the vial as they are negatively geotactic, but they lose the ability to hold onto the vial as ethanol concentration rises and will eventually pass out. The number of flies that cling to the side of the vial was digitally counted every 20 seconds until all flies were knocked down. For analysis, the data was smoothed into 1 minute bins.The time when 50% of the flies were

knocked down was extrapolated for each vial by fitting a log-inhibitor curve to each knockdown curve using Graphpad Prism (San Diego, CA).

Gas Chromatography

Single flies were placed in the 10% ethanol preference assay. On the third day, we observed the vials. Immediately after a fly was observed to extend its proboscis onto the tip of a pipet tip, we immediately transferred it into a vial with 7 uL of toluene. The fly was then crushed with a 200 uL pipet tip. Three microliters of the extract was manually injected into an SRI-310C Gas Chromatograph (SRI Instruments, Torrance, CA). The temperature protocol is: 50°C for 1 minute, ramp for 10 minutes to 150°C, and hold for 10 minutes. An ethanol peak is observed at approximately 2.2 minutes and toluene at approximately 10 minutes. All data was analyzed using PeakSimple (SRI Instruments, Torrance, CA). The area of the ethanol peak was determined using the integration tool with a threshold area size of 100. Determination of the ethanol content of the fly was determined by comparison to a known standard curve of ethanol. Water content of the female was ascertained by taking the difference in weight immediately after being removed from the CAFE assay and after desiccation at 65°C for 24 hours and was 0.86 uL per fly. This value is close to that reported by (Telonis-Scott and Hoffmann, 2003; Folk and Bradley, 2004; Cowmeadow et al., 2005)

Chapter 5: Conclusions and future directions

SUMMARY AND CONCLUSIONS

Drosophila have become a useful model for investigating the genetics of ethanol-associated behaviors. Our lab and others have previously identified mutations that are involved in sensitivity and tolerance to ethanol, many of which are conserved in mammals (Wolf and Heberlein, 2003; Atkinson, 2009). While circadian mutations ave previously been investigated for their role in cocaine sensitization in fruit flies, their role in ethanol responses has not been studied.

In this dissertation, I used a novel curve-fitting and bootstrapping assay to test for tolerance to ethanol. This assay has the advantage in that one can analyze many parameters in the tolerance response. Not only can one statistically test for the time when half the flies are knocked down, but one can test the time when flies transition from the hyperactive phase to the sedative phase, the maximal excitation, and the rates of excitation and sedation. I was able to use this assay in order to test circadian mutations.

I found that some circadian genes are necessary for tolerance to ethanol. Interestingly, this is not universal amongst circadian genes. *Clk*^{JRK} shows normal tolerance to ethanol, whereas *cyc*^{01} only shows tolerance to the hyperactive effects of ethanol. This is contrast to *per*^{01} and *tim*^{01}, which show tolerance to sedation. These differential effects of circadian mutations are both surprising and

unsurprising. While all of these genes are components of the core circadian clock, and a null mutation in any of them will render the fly behaviorally arrhythmic, these genes also have independent roles. There are other examples in the Drosophila literature that show differential effects of these genes (Andretic et al., 1999; Hendricks et al., 2000; Beaver and Giebultowicz, 2004; Sakai et al., 2004). Ethanol tolerance can now be added to this list.

Recently, there has been much excitement in the Drosophila field about the development of a two-choice assay to assess preference for ethanol (Devineni and Heberlein, 2009). A two-choice assay in such a genetically tractable organism as Drosophila would be invaluable to identify genes that are necessary for ethanol preference. We wished to test circadian mutants for ethanol preference. However, we wanted to be first investigate whether flies choose ethanol as a food source or for the pharmacological effects of the drug. Although Devineni and Heberlein, 2009 tested this by varying the ratio of calories provided by the food compared to ethanol, the lowest caloric ratio that they tested was approximately 2:1, in which the ethanol containing pipet contained twice the calories as the control pipet. We therefore decided to further test whether calories played a role in preference. We found that if the non-ethanol-containing pipet contained sufficient calories to balance the calories from ethanol, preference was eliminated. Flies would even prefer the control pipet if it contained more calories than the ethanol pipet. This was not due to the

possibility that the flies never got enough ethanol to become "addicted", as flies that show preference for ethanol lose it immediately after the pipet is balanced. We can therefore conclude that flies prefer ethanol not for its pharmacological effect, but for its value as a food source.

FUTURE DIRECTIONS

In this dissertation, circadian genes are shown to affect the acquisition of tolerance to ethanol in Drosophila. Previous research from our lab has implicated the BK channel *slo* as also being necessary for tolerance to ethanol. (Cowmeadow et al., 2005). This channel is also necessary for behavioral rhythmicity (Ceriani et al., 2002). Additionally, another gene necessary for circadian rhythmicity, CREB2, has also been shown to affect tolerance to the anesthetic benzyl alcohol (Belvin et al., 1999; Wang et al., 2009). These genes all must either interact directly or indirectly in the regulation of rhythmicity. It is possible that these interactive mechanisms are conserved for the development of tolerance to ethanol. More work would be needed to investigate the relationship between these genes in tolerance.

While we conclude that Drosophila prefer ethanol in the CAFE assay due to calories, it is possible that flies could prefer ethanol for its pharmacological effect under different conditions. Indeed, there is already an example demonstrating that flies most likely are attracted to the pharmacological effects of

ethanol. Flies will cross an electric barrier to reach an odor associated with ethanol vapor treatment (Kaun et al., 2011). It is possible that manipulating the assay, perhaps by providing flies with a higher ethanol content, will change their behavior so that they will prefer ethanol, even if a calorically balanced alternative is presented.

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Vita

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