Abstract

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Drug response is a polygenic trait that varies as a result of many factors, including the rate of drug absorption, metabolism and secretion. It is an important trait that can result in physiological and behavioral changes and can affect both health and survival. Associations between drug response and genes have been suggested but no clear picture of the relationship between genetic and pharmacological variation has yet emerged. Dissection of the genetic architecture of drug resistance is further complicated in that it involves the activity of multiple genes, which can interact with each other and the environment. My research uses *Drosophila melanogaster* to study resistance to the behaviorally active substances nicotine and caffeine. Both of these substances can exhibit adverse health effects at high doses or after chronic use by humans and are lethal when added to the diet of *Drosophila*.

For this study, several approaches were used to study drug response, including an analysis of quantitative genetic variation for drug resistance in natural populations, a P-element mutagenesis screen and association tests with candidate genes. These were used to assess drug resistance by measuring survival time on diets containing either nicotine or caffeine, and revealed that abundant genetic variation exists for drug resistance in *Drosophila*. This variation involves a complex genetic architecture and the interaction of many genes. Nevertheless, a classical forward genetic mutagenesis screen identified individual genes involved in drug resistance. These genes were not those typically studied for drug resistance, such as those in neurotransmitter systems and drug metabolism, but were involved in the development of the CNS and neuronal differentiation. Furthermore, an association study between nicotine and caffeine resistance and single nucleotide polymorphisms in three serotonin receptor genes, 5-HT1A, 5-HT1B and 5-HT2 detected significant associations between a SNP and nicotine resistance in the 5-HT1A gene. This suggests that although drug resistance is a complicated trait involving the interaction of many genes and environmental effects, mutations in individual genes and naturally occurring polymorphisms affecting survival time upon chronic exposure to nicotine and caffeine can be detected in *Drosophila*.

Pharmacogenetic analysis of nicotine and caffeine resistance in *Drosophila melanogaster*

By

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GENETICS

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Chair of Advisory Committee

Biography

I was born in August of 1978 in Miami, Florida. Time passed, I grew up, did many things, went many places, etcetera. Along the way, I developed an interest in several subjects but at some point chose to focus on science. I continued to do so while attending the University of Miami, which was nevertheless quite an enjoyable experience. After graduation, I decided to continue my studies by pursuing a doctoral degree in genetics. For that reason I moved to Raleigh, North Carolina in 1999. After brief deliberation I joined the lab of Dr. Greg Gibson to study the genetic architecture of drug resistance. Five, at times long, yet rewarding years later, I graduated and...

"Is not life a hundred times too short for us to bore ourselves?"
Friedrich Nietzsche

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Summary

How an organism responds to chemicals in its environment is important because it can influence an individual's health and survival. Organisms can respond to these substances through physiological and behavioral changes that are highly variable, with variation both between and within species. Although studies in both humans and model organisms have helped to elucidate the effects of various drugs and the changes they can elicit, questions remain as to how an organism receives, integrates and responds to a particular chemical (Mori, 1999). The genetics of drug response is therefore complex and shows significant variation between individuals. This variation likely has a polygenic basis resulting from variation in factors such as the rate of drug absorption and metabolism (Evans and Relling, 1999) as well as the activity of the drug on its target. Dissecting the genetic variation for drug and chemical response can also be complicated by the influence of environmental factors and previous experiences (Bernhard and van der Kooy, 2000; Sawin *et al.,* 2000).

Nevertheless, many studies have already been successful in identifying genetic loci affecting drug response and other complex behaviors. In fact, linkages have been made between several genes and behaviors such as depression, alcoholism or drug response in mice (Barrantes *et al.,* 1995; Pietila and Ahtee 2000), humans (Connings *et al.,* 2999; McLeod and Evans, 2001), nematode worms (Wagoner *et al.,* 1998), and fruit flies (Hirsh, 1998; Heberlein, 2000; Bainton *et al.,* 2000). Many aspects of the relationship between genetic and pharmacological variation affecting drug resistance are still unclear however, even though many of the biochemical pathways through which drugs are absorbed, metabolized and function have been characterized. Recent advancements in genomic information and technologies and the potential of

pharmacogenetics to improve human health have raised interest in the genetic basis of pharmacological variation for drug response. This task is well suited for the use of model organisms such as *Drosophila melanogaster,* with its relatively simple and well characterized nervous system, complete genome sequence and the availability of vast genetic resources.

This introduction is divided into four sections. The first section will give an outline of the project and its aims. The second section will give an overview of organism-drug interactions, focusing on behavioral responses and pharmacogenetics and the role of monoamine neurotransmitters on these. The third section will detail the behavioral and physiological responses to caffeine and nicotine, the two primary drugs studied in this project, and the current theories on the biochemistry of these responses. The last section will focus on the use of *Drosophila melanogaster* as a model system for drug response. This will include a brief overview of the neurotransmitter systems present in *Drosophila* and previous research on drug response and resistance.

1.1 Project Overview and Aims

The discovery of the genetic components affecting variation in phenotypic traits is of interest for both basic and practical reasons. The identification of loci or specific nucleotides within loci that contribute to phenotypic differences could lead to advances in agriculture and medicine as well as other areas. A variety of methods have been used for the last hundred years to accomplish this, from studies of Mendelian inheritance and mutagenesis screens to quantitative genetic analysis of polygenic traits to recent studies into association tests between nucleotide variation in candidate loci and traits of interest. These approaches all offer unique advantages as well as weaknesses in dissecting the genetic variation affecting phenotypic traits. For behavioral and pharmacological traits, such as drug response, this can be complicated by the polygenic nature of the traits and relatively large influence of environmental factors. Therefore, to identify the basis of genetic variation affecting these traits, animal models that are amenable to genetic manipulation and can be grown in controlled environments will be of great importance.

The overall plan of my thesis was to use *Drosophila melanogaster* to dissect the genetic architecture of drug resistance and to identify loci and nucleotide variants affecting this trait. This project focused primarily on genetic variation for resistance and sensitivity to nicotine and caffeine, with some work also on the role of the neurotransmitters dopamine, serotonin and octopamine. In humans, these neurotransmitters affect synaptic transmission in the nervous system and have been implicated in several facets of behavior, including drug response. Detailed analysis of this multifactorial trait presents many challenges, but *Drosophila melanogaster*, with its comparatively simple,

well-characterized nervous system, amenability to genetic manipulation and availability of genetic resources such as a complete genome sequence, offers the potential to answer many questions about the physiological and behavioral effects of drug response. Several approaches were used to study drug response, including a mutagenesis screen to identify genes affecting drug response and association tests with three serotonin receptor genes. Furthermore, the genetic architecture of drug resistance and the extent of natural variation present for drug resistance were also analyzed. The specific aims of this project were therefore to:

1. Examine the genetic architecture of natural variation for response to the neurotransmitters dopamine, octopamine and tyramine as well as nicotine and caffeine.

2. Identify single insertion mutations affecting survival time upon chronic exposure to nicotine and caffeine.

3. Test for associations between naturally occurring single nucleotide polymorphisms in three candidate genes and nicotine and caffeine resistance.

1.2 Organism-Drug Interactions

(A) Behavioral Response

Understanding the sources of variation in behavioral responses to environmental stimuli has long been a goal of genetic and biochemical research. The genetic architecture of behavioral traits however can be very difficult to dissect. This is because of the multifactorial nature of these traits, as well as the large environmental and genotype-environment interactions. The quantitative nature of behavioral and pharmacological traits can make isolating single loci involved in these traits difficult. It has in fact been suggested that the phenotypic contributions of individual genes are too small to detect in classical mutagenesis screens. Furthermore, analysis of mutations in neurotransmitter receptor and transporter genes has suggested that their phenotypic effects on behavior can be subtle (Yoshihara *et al.,* 2001).

Classical genetic screens have, however, identified genes involved in several behaviors in humans, mice, nematodes, and fruit flies, as well as others. Even mutations with very specific effects on behavior, such as the disruption of a single step in the learning process, have been isolated (Goodwin *et al.*, 1997). Many of these genes involve the transport, reception, production and metabolism of monoamines. Biogenic monoamines are a specific class of neurotransmitters that include dopamine, octopamine and serotonin and are involved in synaptic transmission. These neurotransmitters can exert their effects both presynaptically and postsynaptically (Pereda *et al.,* 1994) and are required for a variety of processes (Bainton *et al.*, 2000). Most of the receptors for these neurotransmitters are seven-helix receptors that are coupled to G-proteins, which regulate secondary messenger systems that can have a variety of effects within a cell.

The role of monoamines is highly conserved in most animals (Walker *et al.*, 1996) and they are believed to modify and regulate several personality traits and environmental responses, as well as having physiological effects. In humans, mutations in dopamine and serotonin receptors and transporters have been associated with impulsive, compulsive and addictive behaviors, including those that arise in response to drug use, among others (Comings *et al.,* 1999). Dopamine and serotonin have also been linked to locomotor, spatial and incentive learning, as well as to the modulation of an organism's response to environmental stimuli following experience (Sawin *et al.,* 2000; Saeki *et al.,* 2001). Therefore, both dopaminergic and serotonergic receptors are involved in the synaptic and neuronal adaptations that result in behavioral plasticity.

Drugs of abuse can alter multiple brain pathways, but similarities have been identified. These similarities center on the dopaminergic and serotonergic neurons. The destruction or inhibition of these systems is known to prevent some of the effects of these drugs (Koob *et al.,* 1998) and chronic drug use can disrupt the baseline levels of these neurotransmitters and their secondary messenger systems (Gawin, 1991). In *Drosophila*, a dopamine transporter moderates cocaine response (Porzgen *et al.,* 2001) and activation of serotonin receptors by LSD may produce changes in perception and behavior (Nichols *et al.,* 2002). The effects of drug withdrawal are also thought to result from the unmasking of compensatory adjustments to dopamine and serotonin pathways during drug use (Seth *et al.,* 2002). These findings have led to the theory that drugs of abuse share a common biochemical mechanism, with dopamine and serotonin having central roles (Betz *et al.,* 2000).

(B) Pharmacogenetics

Pharmacogenetics is an emerging field that examines the genetic basis of variation in drug response and toxicity. Pharmacogenetics has received substantial interest because it promises improvements in drug design and development and offers the possibility of shifting from the traditional trial-and-error process of drug discovery to a methodical approach where drugs are designed to act on a specific molecular target (Shi *et al.,* 2001). Research could also lead to the discovery of novel targets for therapy and for diagnostic tests based on the identification of disease susceptibly factors (McLeod and Evans, 2001). The ultimate goal of pharmacogenetics would be to allow physicians to select the drug and dosage with the greatest potential benefit and least side effects in individuals based on their genotype (Johnson and Evans, 2002). Research in pharmacogenetics has surged in recent years with advances in high-throughput sequencing and SNP genotyping methods that allow for the identification of multiple candidate genes much faster and cheaper than was previously possible. Developments in DNA microarrays and proteomics have also helped to clarify the biological and pharmacological pathways through which drugs act on and affect organisms.

The most abundant types of sequence variation in the human genome are single nucleotide polymorphisms, single base pair sites in DNA that vary among individuals in a population, and are estimated to occur at a frequency of 1 per 1000 bases (Brooks, 1999). Given the large number of genes involved in drug response and disease, and the possibility of several SNPs in each gene, it is unlikely that any single polymorphism in one gene would be responsible for a large amount of the variation in these traits. Human studies have however shown association in single nucleotide polymorphisms with addictive and compulsive behaviors (Comings *et al.,* 1999), bipolar disorder (Ranade *et al.,*

2003), depression (Frisch, 1999; Choi *et al.,* 2004), and schizophrenia (O'Donovan and Owen, 1999; Malhotra et al., 1998; Jonsson *et al.,* 2001) as well as others. These associations however, have not consistently held up, and there is not great confidence in the ability to discriminate between false positives and true polymorphisms.

Pharmacogenetic studies into the genetic basis of drug response and toxicity have focused primarily on genetic polymorphisms in drug metabolizing and elimination enzymes. These studies have located polymorphisms in the majority of genes for drug metabolizing enzymes (Evans and Relling, 1999). One example is cytochrome P-450, a liver enzyme that metabolizes at least 40 drugs and contains multiple pharmacologically significant variants (Rettie *et al.,* 1994; Daly *et al.,* 1996). Specific allelic variants have been identified in isozymes of cytochrome P-450 with distinct metabolism rates and clinical phenotypes (Rettie *et al.,* 1994). Several drugs of abuse, including nicotine, amphetamines and codeine are broken down by cytochrome P-450. Variation in cytochrome P-450 has also been suggested to affect resistance to the toxic effects of these drugs and the risk of drug dependence (Howard et al., 2002). Recent studies have also implicated receptor and transporter polymorphisms in the modulation of drug response and resistance (Evans and Relling, 1999). As with associations between SNPs and behavioral response, these results are still preliminary and not always consistent. Many challenges still remain in identifying genetic variants affecting drug resistance, but it is apparent that there is ample genetic variation for drug response and toxicity.

1.2 Caffeine and Nicotine

Two pharmacological substances of particular importance to human health are nicotine and caffeine. Both of these drugs are central nervous system stimulants and are the most prevalently used behaviorally active substances in the developed world. Caffeine is the more widely used of the two, with an estimated ninety percent of Americans consuming it daily (Betz *et al.,* 2000). Although it possesses many of the characteristics of regulated drugs, such as withdrawal, tolerance and dependence, the use of caffeine is not restricted or heavily regulated. This is in large part because of its low toxicity and the absence of any characterized substantial detrimental effects from caffeine use. For this reason, in the United States, as well as in most countries, there are only a few restrictions on the content of caffeine in consumer products.

The second most often used behaviorally active substance by humans is nicotine. It shares many of the same characteristics that caffeine does with illicit drugs, such as tolerance, withdrawal and addiction. Unlike caffeine, however, nicotine is regulated because of the deleterious effects of tobacco smoking on human health. Although anti-smoking campaigns and regulations have often highlighted the negative effects of other substances in cigarette smoke and focused only on the addictive properties of nicotine, nicotine itself is also extremely toxic. In fact, nicotine is often used commercially as an insecticide, and is a risk factor for cardiovascular and lung diseases, as well as cancer. The extensive use of these two substances and their potential consequences on human health make dissecting their physiological, behavioral and biochemical properties an important area of study.

(A) Caffeine

Caffeine, 1,3,7-trimethylxanthine, is an alkaloid of the methylxanthine family that occurs naturally in the leaves, seeds or fruit of more than fifty plant species, the most well known being coffee, tea and cocoa. In its pure state, caffeine is an intensely bitter white powder. It is usually consumed in beverages such as coffee and tea, as well as in many carbonated drinks as summarized in Table 1.1 (Barone and Roberts, 1996; Stavric *et al.,* 1988). Caffeine is also a component in pharmacological preparations and medications including diet aids and cold/flu remedies. Although it varies by individual, the behavioral effects of caffeine are experienced by the consumption of 50 to 300 mg (Stavric *et al.,* 1988), with the average daily caffeine consumption for Americans ranging from 250 to 600 mg (Barone and Roberts, 1996).

Following consumption, caffeine is rapidly absorbed into the blood stream and travels to various tissues throughout the body, including the brain. Caffeine reaches its peak level in blood plasma within thirty to seventy-five minutes after ingestion, but does not accumulate in the body and is easily metabolized (Mandel, 2002). In the United States, the use of caffeine is not restricted and its content in consumer products is not highly regulated. In fact, certain beverages, such as soda, can only contain 6 mg per liquid ounce and energy pills, such as Vivarin, can only contain 200 mg each. This is far below a toxic dose of caffeine, for which the LD50 (the dosage that would be lethal to 50% of the population) is estimated at 10 grams for oral consumption (Dews *et al.,* 2002). This lethal dosage to humans varies between individuals in response to several factors, the most important being weight, but ingestion of 150 mg of caffeine per kg of bodyweight is considered the LD50 for any individual (Kaplan *et al.,* 1997).

Table 1.1 Caffeine Content in Common Beverages (in mg)

Item	Typical	Range
Coffee (150ml cup)		
Brewed, drip method	110	60-180
Brewed, percolator	80	40-170
Instant	60	30-120
Decaffeinated	3	2-5
Espresso (30ml cup)	40	30-70
Teas (150ml cup)		
Brewed	40	20-90
Instant	30	25-50
Soft drinks (250ml)		**20-80**
Coke, Diet Coke	45	
Pepsi, Dr. Pepper	39	
Red Bull	80	
Cocoa beverage (150ml)	5	2-20
Chocolate milk (240ml)	6	2-7

(Barone and Roberts, 1996; Stavric *et al.,* 1988)

(i) Behavioral and Physiological Response

Consumption of caffeine can cause several behavioral and physiological responses in humans. The principal effects of caffeine come from its action as a central nervous system stimulant. These include those behavioral effects commonly experienced by its consumers, such as alertness and increased energy (Fredholm *et al.,* 1999). Caffeine consumption can also cause physiological changes such as increases in heart rate and blood pressure and an increase in blood flow towards muscles and a decrease towards the skin and internal organs (Berne *et al.,* 1998). In addition to this, caffeine is also a strong diuretic and appetite suppressant, and is therefore often included in diet and weight loss pills (Mandel, 2002). Consumption of large doses of caffeine can cause feelings of anxiety and nervousness as well as insomnia (Fredholm *et al.,* 1999) and lead to negative physiological effects such as rapid heart rate, diuresis (excessive urination), nausea, vomiting, and tremors (Berne *et al.,* 1998). Caffeine intake must also be reduced gradually to prevent withdrawal symptoms, which can include headaches, muscle pains, drowsiness, lethargy, irritability, and depression among others (Dews *et al.,* 2002).

Caffeine can also affect plant and mammalian cells grown in culture, and is known to be mutagenic. Under normal circumstances, cells do not proceed from S phase in mitosis until all the DNA has been replicated (Alberts *et al.,* 1994). In the presence of caffeine however, the feedback control that prevents cells from dividing before DNA replication is complete is disrupted (Alberts *et al.,* 1994). This allows the cells to finish S phase without completion of DNA replication, leading to chromosome loss and abnormalities (Timson, 1977). Caffeine has also been shown to have mutagenic effects in *E. coli* and other bacteria (Timson, 1977) and to increase the frequency of chromosome loss and mutations in *D. melanogaster* larvae (Mittler *et al.,* 1967;

Clark and Clark, 1968). The addition of caffeine to the diet of both larvae and adult *Drosophila* can also have severe consequences. In moderate to high doses, caffeine is lethal to larvae and adult *Drosophila melanogaster* (Zimmering *et al.*, 1977), and in smaller concentrations decreases longevity and fecundity in *Drosophila prosaltans* (Itoyama *et al.*, 1998). Caffeine sensitivity has been shown to vary among populations and between males and females in adults (Zimmering *et al.,* 1977), but no sex differences have been observed in larvae (Nigsch *et al.,* 1977).

(ii) Biochemistry of Response

The major effects of caffeine on the central nervous system occur by its interaction with the receptors of the neuromodulator adenosine (Snyder *et al.,* 1981; Fredholm, 1995). Neuromodulators are compounds that can vary the level of neuronal activity by increasing or decreasing the rate at which the nerve cell fires. Unlike neurotransmitters, neuromodulators are not stored in presynaptic vesicles and can act pre- or post-synaptically before being metabolized (Alberts *et al.,* 1994). The four identified adenosine receptor subtypes are members of a class called the purtinergic receptors that function primarily through G-protein signaling pathways, although there is also evidence for coupling to ion channels for some of the subtypes (Berne *et al.,* 1998). These receptors are located throughout the body in nerve cells in the brain, blood vessels, kidneys, heart, and the gastro-intestinal tract (Purves *et al.,* 2001; Berne *et al.,* 1998).

Adenosine receptors coupled to G-proteins can stimulate or inhibit the activity of adenylyl cyclase, depending on the receptor subtype. The activation of adenosine receptors can therefore affect the level of cyclic AMP in the cell, which is a common secondary messenger in G-protein signaling

pathways (Purves *et al.,* 2001; Berne *et al.,* 1998). In the central nervous system, this can alter the amount of neurotransmitter release and thus affect overall neuronal activity. Adenosine concentrations are regulated mainly by ATP metabolism, and increase during periods of wakefulness and decrease during sleep (Huston *et al.,* 1996). It is in fact this increase in adenosine concentration while awake and the resulting decrease in neuronal activity that is believed to cause the feelings of drowsiness observed prior to sleep (Snyder *et al.,* 1981; Fredholm, 1995). Therefore, adenosine is important as a modulator of neuronal excitability that inhibits the release of most excitatory neurotransmitters (Fredholm *et al.,* 1999).

Caffeine is a non-selective adenosine antagonist that can bind to the adenosine receptors because it has a similar molecular shape to adenosine (Figure 1.1). However while caffeine binds to the same receptors, it does not elicit any of the biochemical responses that adenosine does and actively blocks the binding of adenosine to these receptors. The pharmacological actions of adenosine and caffeine are summarized in Table 1.2 (Garrett and Griffiths, 1997). The effects of caffeine are believed to occur primarily from binding with two adenosine receptor subtypes, A1 and A2A (Ferre, 1997). These subtypes are present in different regions of the brain, with the A1 receptors being widely distributed while the A2A receptors are concentrated in the striatum (Ferre, 1997; Fredholm, 1995). This latter region of the brain also contains the highest level of dopamine receptors in the brain (Yung *et al.,* 1995) and plays an important role in the behavioral response to motivational stimuli (Ferre, 1997). The A2A receptors are in fact typically colocalized with dopamine D2 receptors on neurons (Yung, 1995). Studies have also shown that the adenosine A1 receptors can modulate dopamine release by interacting with dopamine D1 receptors (Cass and Zahniser, 1991).

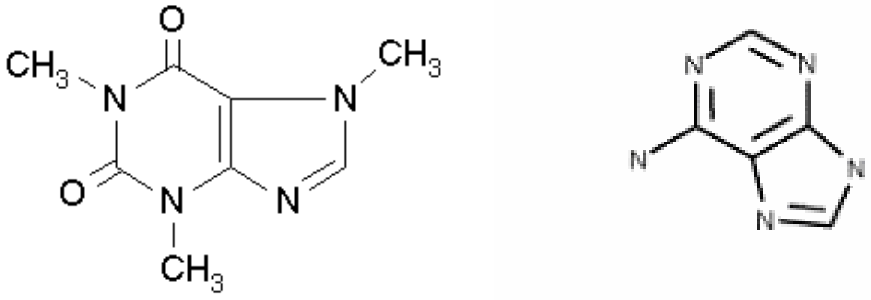


Figure 1.1 Molecular structures of caffeine and adenosine.

Table 1.2 Pharmacological Effects of Caffeine and Adenosine

System	Caffeine	Adenosine
CNS	Increases spontaneous electrical activity Enhances neurotransmitter release Stimulates locomotor activity Increases operant response rate Convultant activity	Decreases spontaneous electrical activity Inhibits neurotransmitter release Depresses locomotor activity Decreases operant response rate Anticonvulsant activity
Heart	Positive inotropic effects Positive chronotropic effects	Negative inotropic effects Negative chronotropic effects
Vasculature		
Peripheral	Dilation	Constriction
Central	Constriction	Dilation
Respiratory	Relaxes bronchial smooth muscles	Constricts bronchial smooth muscles
Renal	Diuresis, stimulates rennin release	Anitdiuresis
Adipose	Stimulates lipolysis	Inhibits lipolysis
GI	Increases gastric secretions	Inhibits gastric secretions

(Garrett and Griffiths, 1997)

Under normal conditions, adenosine binds to A1 receptors resulting in the inhibition of adenylate cyclase activity, and therefore a decrease in cyclic AMP levels and a corresponding decrease in neuronal firing. After the consumption of caffeine however, caffeine binds to the A1 receptors, blocking adenosine from doing so and thus preventing its decrease of neuronal firing (Snyder *et al.,* 1981). This results in an increase in neuronal activity in some regions of the brain, which signals the adrenal glands to produce adrenaline (Berne *et al.,* 1998). It is this release of adrenaline that increases heart rate and blood pressure after the consumption of caffeine. Adrenaline also causes glucose to be secreted from the liver, resulting in the release of sugar into the bloodstream and the feeling of increased energy (Berne *et al.,* 1998). After the caffeine is metabolized, adenosine can again bind to the A1 receptors, leading to a decrease in neuronal activity and adrenaline release. Once the adrenaline wears off, the user in most cases experiences a feeling of fatigue. After long-term consumption of caffeine, nerve cells can become oversensitive to adenosine (Fredholm, 1995), resulting in increases in blood pressure, headaches and other symptoms experienced during withdrawal. Adenosine A1 receptors have also been implicated in the development of tolerance to caffeine. Tolerance to caffeine has been noted after regular consumption, and in rats is associated with an increase in A1 receptor activity and a shift in these receptors to a higher affinity state that has increased sensitivity to adenosine (Fredholm, 1995).

Contrary to the A1 receptor subtype, adenosine A2A receptors stimulate the activity of adenylate cyclase after adenosine binds to it (Berne *et al.,* 1998). The behavioral effects of the A2A receptor subtype have been demonstrated to involve the neurotransmitter dopamine; adenosine plays a role opposite to dopamine in the striatum (Ferre *et al.,* 1993; Heffner *et al.,* 1989).

This is presumably a result of the D2 and A2A receptors being located on the same neurons but having opposite effects on adenylate cyclase activity and cyclic-AMP levels (Ferre *et al.,* 1992). The stimulatory effects of caffeine are therefore enhanced by the blockade of A2A receptors producing behavioral effects similar to those of dopamine in the striatum. This accounts for the observed involvement of dopamine in the behavioral and physiological effects of caffeine, even though caffeine does not bind to dopamine receptors (Ferre *et al.,* 1992; Daly and Fredholm, 1998). The effect of caffeine on dopamine release is biphasic, i.e. high doses decrease and low doses increase dopamine release (Ferre, 1997; Garrett and Griffiths, 1997). In the brain, low doses of caffeine stimulate spontaneous motor activity (Heffner *et al.,* 1989) and both aversive and appetitive behaviors (Ferre, 1997) by increasing the release of dopamine in specific regions. Higher doses of caffeine however produce the opposite effect, such as motor depression, by blocking the A1 adenosine receptors and thereby the release of dopamine (Ferre *et al.,* 1992).

After consumption, caffeine does not accumulate in the body and is easily metabolized into substances with varying pharmacological activity before being excreted (Mandel 2002). In humans, approximately 80% of caffeine is metabolized by demethylation to paraxanthine in the liver by cytochrome P-450 (Howard *et al.,* 2002). Levels of these metabolites increase in blood plasma with repeated caffeine consumption, and these substances are subsequently further broken down in the liver. This process is however frequently different in other species. In humans, the half-life of caffeine varies between four and five hours with modest intake, but increases at higher levels of intake or with liver damage (Mandel 2002). The behavioral activity of caffeine, and possibly its metabolites, can therefore persist for several hours after its ingestion.

(B) Nicotine

Nicotine, 3-(2-(N-methylpyrrolidinyl))pyridine, is a naturally occurring liquid alkaloid found in over sixty plant species. The species most widely cultivated for its nicotine however is *Nicotiana tobacum*, which is originally from South America and is grown for use in several products including cigarettes. In tobacco, nicotine is concentrated in the leaves, where it makes up approximately 5% of the plant by weight (Henningfield *et al.,* 1999). In its pure form, nicotine is a clear liquid sensitive to both light and oxygen that quickly oxidizes upon contact with air, resulting in a color change to pale yellow or brown. Pure nicotine is lipid soluble and easily absorbed through the skin, mouth and lungs (Berne *et al.,* 1998).

Once inhaled, nicotine diffuses through the lungs and mucous membranes and into the small blood vessels that line these tissues (Berne *et al.,* 1998). From there, nicotine travels quickly through the bloodstream and across the blood-brain barrier, resulting in behavioral and physiological effects within one minute (Balfour and Ridley, 2000). However, nicotine is also quickly broken down and removed from the body, with a half-life of approximately one hour (Yildiz, 2004). The nicotine content of cigarettes and other products is regulated because nicotine is one of the most toxic drugs known, acting with almost as much speed as cyanide (Berne *et al.,* 1998). The lethal dose of nicotine, LD50, for humans has been established at 60 mg in a single dose, at which it is lethal within minutes of consumption. Although the nicotine content in cigarettes is highly variable, ranging from 6 to 20 mg per cigarette, only 1 mg is usually absorbed (Henningfield *et al.,* 1998). This is however sufficient to produce behavioral and physiological effects. If all of the nicotine in each cigarette were absorbed, three or four cigarettes would be lethal and the content in just one would cause severe illness.

(i) Behavioral and Physiological Response

Nicotine has a wide range of behavioral and physiological responses, depending primarily on the dose consumed. In a small dose, such as that in cigarettes, nicotine has a stimulating effect (Balfour and Ridley 2000). Behaviorally, this is experienced as an increase in alertness and energy, and sometimes a sense of mild euphoria, while chronic users report a relaxing effect (Malin, 2001). Nicotine also causes several physiological responses, including increasing heart rate and blood pressure and reducing appetite (Berne *et al.,* 1998). At higher doses nicotine can cause negative effects that become more severe as the dose increases. Behavioral effects can include confusion, agitation, restlessness and in some cases depression (Balfour and Ridley, 2000). Physiological effects from increasingly higher doses of nicotine usually begin mildly with headaches and nausea, but can quickly become severe, with convulsions, breathing difficulties, rapid heartbeat, and elevated blood pressure. Consumption of high doses of nicotine can also lead to erratic heart rate and decreased blood pressure and even death (Berne *et al.,* 1998). Nicotine dependence and addiction can occur within a week of moderate consumption (Malin, 2001). After this, withdrawal symptoms are similar to those experienced upon withdrawal from caffeine, including anxiety, irritability, headaches, and insomnia. These symptoms are persistent and may last for months or years (Malin, 2001).

The physiological and behavioral changes resulting from both short and chronic nicotine use have been extensively characterized in mice, nematode worms and fruit flies. In *C. elegans*, nicotine has been reported to modify locomotion, feeding and egg laying (Trent *et al.,* 1993; Waggoner *et al.,* 2000). Application of nicotine or its antagonists to nematodes results in body muscle contractions, paralysis and increased egg-laying (Lewis *et al.,* 1980). Flies

exposed to volatilized nicotine show behavioral responses that are dependant on the dosage, much as in mammals. At low doses, flies can become hyperactive, but at higher doses flies can become sluggish or even paralyzed (Wolf and Heberlein, 2003). In mice, the behavioral and physiological effects of nicotine are analogous to those in humans, including the development of tolerance and addiction (Balfour and Ridley, 2000; Di Chiara, 2000). In cell cultures, some evidence suggests that nicotine can inhibit the ability of cells to repair DNA damage (Berne *et al.,* 1998).

(ii) Biochemistry of Response

Nicotine acts as an agonist for nicotinic receptors, a type of acetylcholine receptor to which both acetylcholine and nicotine can bind (Figure 1.2). There are two types of nicotinic receptors, neuronal and muscular, with distinct subunit compositions and several subtypes, but both are ligand gated ion channels and predominantly located pre-synaptically (Purves *et al.,* 2001). At low doses, nicotine binding to acetylcholine receptors results in a conformational change that opens a cation channel, depolarizing the nerve cells and thereby stimulating downstream neurons or muscle fibers (Berne *et al.,* 1998). At high doses, nicotine inhibits the function of acetylcholine receptors, blocking incoming signals and therefore neuronal information from being transmitted (Berne *et al.,* 1998). This blockage of acetylcholine receptors is also responsible for the toxicity of nicotine and therefore its effectiveness as an insecticide. Nicotine is known to increase the level of several neurotransmitters and chemicals that modulate behavior, including GABA, noradrenaline, glutamate, and endorphins (Seth *et al.,* 2001). For example, the production of endorphins, small proteins that relieve pain and lead to feelings of euphoria, is known to increase in the brain following nicotine consumption (Purves *et al.,* 2001).

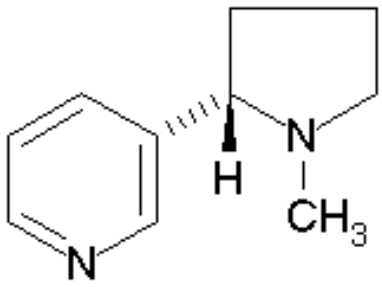


Figure 1.2 Molecular structure of nicotine.

The primary effects of nicotine however occur through the dopaminergic and serotonergic system (Di Chiara, 2000; Seth *et al.,* 2001), by either suppressing or increasing their release, depending on the brain region (Balfour and Ridley 2000). Stimulation of cholinergic neurons promotes the release of dopamine in the reward centers of the brain (Pietila and Ahtee, 2000). Stimulation of these neurons is believed to cause the euphoric and relaxing effects experienced from nicotine use and to contribute to its reinforcing and addictive properties (Balfour and Ridley, 2000). Nicotine also increases dopamine levels in the brain by repressing the production of monoamine oxidase, an enzyme responsible for metabolizing dopamine (Di Chiara, 2000). Furthermore, reduction in dopamine levels, either by pharmacological or genetic means, has been shown to decrease the response to nicotine in flies (Bainton *et al.,* 2000) and its reinforcing properties in mice (Di Chiara, 2000). Dopamine has even been implicated in the addictive properties of several drugs, including nicotine, cocaine, heroin, and ethanol and has therefore been suggested to play a central role in drug addiction (Betz et al., 2001)

Nicotine administration also increases serotonin release in various regions of the brain. The mechanism by which nicotine affects serotonin neurotransmission however is not fully understood, and there is no evidence for presynaptic nicotinic receptors on serotonergic neurons (Seth *et al.,* 2002). Interactions between cholinergic and serotonergic neurons, analogous to those observed with dopaminergic neurons, have been suggested to account for the effects of nicotine on serotonergic systems. Nicotine has been observed to reduce the density of serotonin receptors in some brain regions (Balfour and Ridley, 2000) and several serotonin receptor subtypes have been linked to the behavioral responses typically associated with nicotine use. For example, the irritability and anxiety that occurs during nicotine withdrawal are the result of

stimulation of serotonin 5HT-1a and 5HT-3 receptors, and can be alleviated by the administration of serotonin receptor antagonists (Malin, 2001; Seth *et al.,* 2002). Although the behavioral and physiological effects that result from nicotine activity on serotonin receptors have been characterized, many questions remain as to how nicotine modifies this system. Furthermore, although serotonin has been implicated as a factor in addiction to nicotine, the biochemical pathways through which serotonin receptors influence nicotine addiction are not known.

Long-term nicotine treatment can cause a long-lasting inactivation of some nicotinic receptors (Waggoner, 2000), with chronic exposure leading to either up-regulation (Barantes *et al.,* 1995) or down-regulation (Messing, 1982), depending on the cell type. This occurs through transcriptional and post-translational modifications, but not much is known about the pathways that regulate these processes. The process through which nicotinic receptors can become desensitized to nicotine and the development of tolerance are also not well understood and are areas of ongoing research. Once nicotine is absorbed into the bloodstream, it is quickly metabolized. Approximately 80 percent of nicotine is broken down in the liver, primarily into cotinine, with smaller amounts metabolized in the lungs into cotinine and nitric oxide (Yildiz, 2004). The rate of nicotine metabolism is however highly variable and defects in the enzyme that metabolizes nicotine into cotinine have been shown to affect nicotine addiction and dependence (Pianezza *et al.,* 1998). Any remaining nicotine in the bloodstream is filtered in the kidneys and excreted (Berne *et al.,* 1998; Yildiz, 2004).

1.4 Drosophila as a Model System

For the last hundred years, *Drosophila melanogaster* has been used as a model system in genetic and biochemical research. This is because it offers many advantages, including a short generation time of approximately 14 days and it is easy and economical to maintain in large quantities and under controlled conditions. Fruit flies also posses a relatively small genome with only four chromosomes and many easily observable mutant phenotypes. Recent advances in fruit fly biology and the development of resources such as the complete genome sequence, SNP databases and the availability of mutant lines have only increased the importance of *Drosophila* as a model organism. Model systems are also important in the study of multifactorial traits such as behavior and pharmacological response, where the phenotypic contribution of individual genes can be small relative to the variance, and environmental influences can be substantial. Therefore, model systems such as *Drosophila* in which the environmental and genetic background can be controlled are extremely beneficial.

(A) Nervous System and Neurotransmitters

Invertebrate model organisms, with their relatively simple and well characterized nervous system are useful in studying neurobiological phenomena and have already led to insights into neurobiology, neurochemistry and behavior. Even though *Drosophila* possesses a simple nervous system comprised of 300,000 neurons, it is able to mimic many of the neurobiological processes and behaviors observed in mammals. *Drosophila* is also useful in that it lacks the substantial blood-brain barrier present in vertebrates (Leal and Neckameyer, 2002), making it easier to introduce chemicals into the brain.

In other aspects, such as the basic architecture of the nervous system, as well as the neurotransmitters, receptors and secondary messenger systems, vertebrates and invertebrates are comparable (Hewes and Taghert, 2001; Yoshihara *et al.,* 2001). Given that the neurotransmitter receptors are highly conserved across animal taxa, with similar structures and a moderate rate of mutation (Hen, 1993; Fryxell, 1995), it is reasonable to suppose that there will be some parallels in their activity between flies and mammals.

Furthermore, the majority of the genes implicated in studies of drug response and addiction, including the nicotinic, dopamine, and serotonin receptor and transporter genes are present in the *Drosophila* genome (Hewes and Taghert, 2001; Yoshihara *et al.,* 2001). In humans, five types of dopamine receptors and seven types of serotonin receptors have been identified; with several subtypes also present (Purves *et al.,* 2001). In the *Drosophila* genome, five serotonin and two dopamine receptors have been identified, along with ten nicotinic acetylcholine receptors (Yoshihara *et al.,* 2001). In *Drosophila* however, nicotinic acetylcholine receptors are nervous system specific and not present at neuromuscular junctions (Wolf and Herberlein, 2003). The *Drosophila* genome also contains genes for synaptic vesicle formation, trafficking and reuptake as well as for secondary messenger pathways that are homologous to those in vertebrates for the dopaminergic, serotonergic and other neurotransmitter systems (Yoshihara *et al.,* 2001). In addition to this, the cellular processes and architecture of the nervous system and synapses are highly conserved between *Drosophila* and vertebrate species, and similarities in several neurological processes and diseases have been noted.

(B) Drug Response and Resistance

Genetic approaches have been used to isolate mutations affecting several neurological processes in flies, including learning (Dubnau and Tully, 1998), grooming ability (Phillis *et al.,* 1992) and reflex behaviors in decapitated flies (Hirsh, 1998; Ashton *et al.*, 2001). Invertebrates have also been used to study the activity of toxic substances (Salanki, 2000), and *Drosophila* in particular has been used to test the activity of several common drugs of abuse. *Drosophila melanogaster* can respond to several drugs, including cocaine (McClung and Hirsh, 1998; Bainton *et al.,* 2000), LSD (Nichols *et al.,* 2002), nicotine (Bainton *et al.,* 2000), caffeine (Nigsch *et al.,* 1977; Zimmering *et al.,* 1977), ethanol (Herberlein, 2000; Wolf *et al.,* 2002), and biogenic amines (Hirsh, 1998). Several of these studies have implicated neurotransmitters in the effects of these drugs. Drugs can be introduced into *Drosophila* by feeding, volatilization or direct application through injection (Manev et al., 2003). These methods differ in the speed and extent of response, and each has distinct advantages and disadvantages. Therefore, the appropriate method of drug delivery used in a study must be selected based on the specific questions to be answered.

Drug resistance has been studied extensively in invertebrates, with the majority of studies focusing on the development of resistance to insecticides. Pesticide resistance typically involves only one or a few dominant genes of large effect (McKenzie and Batterham, 1995). These studies have typically focused on agricultural pesticides introduced by humans and not chemicals that occur naturally and are encountered by the organisms tested for resistance. Differences in the selective pressures experienced in response to “natural” toxins as opposed to agricultural pesticides could in all probability result in divergent genetic architectures.

The genetic sources of variation for resistance to toxic substances have also been previously examined in *Drosophila*, albeit briefly. These studies have investigated the toxic effects of caffeine in *D. melanogaster* (Nigsch *et al.,* 1977; Zimmering *et al.,* 1977) and *D. prosaltans* (Itoyama *et al.,* 1998) and of octanoic acid in *D. sechellia* (Jones, 1998). In *D. sechellia* at least three loci in larvae and five loci in adults have been identified for resistance to octanoic acid, the primary toxin in the fruit of its host plant (Jones, 1998; Kern *et al.,* 2001). Exposure to low doses of caffeine decreases fecundity and longevity in another species of fruit flies, *D. prosaltans* (Itoyama *et al.,* 1998). Studies in adult *D. melanogaster* have shown significant genetic variation for resistance on caffeine media between wild type and mutant strains as well as between males and females (Zimmering *et al.,* 1977). *D melanogaster* larvae fed caffeine also exhibited variation for survival time between lines, but not between males and females (Nigsch *et al.,* 1977). These studies were not however able to determine the genetic architecture of the variation for drug resistance between lines or sexes, or the biochemical basis of resistance to caffeine.

To investigate the basis of genetic variation for drug resistance, we used adult *Drosophila melanogaster* flies to study resistance to the toxic effects of several drugs. The primary drugs used in this study were nicotine and caffeine, but resistance to the biogenic amines dopamine, octopamine and tyramine was also examined. To analyze resistance to these drugs, adult flies were placed on drugged food and survival time was measured until all of the flies were deceased. This assay was used to study the genetic architecture of natural variation for drug response, to screen mutant lines in order to identify loci conferring increased resistance and to perform an association test between two serotonin receptor genes and drug resistance.

Chapter 2:
Genetic Architecture of Drug Resistance

2.1 Introduction

Dissecting the genetic architecture of complex multifactorial traits, such as drug response is a complicated task. The relative contributions of genetic, environment and genotype-environment interactions must be partitioned for the traits of interest. This can be complicated for behavioral traits, where evidence suggests the effects of individual genes are small relative to the variance. Nevertheless, the analysis of these traits is an important area of research and techniques are being developed to overcome these limitations.

Genetic approaches have already been used to study several behaviors in flies (Sokolowski, 2001), including learning (Dubnau and Tully, 1998), reflex behaviors in decapitated flies (Hirsh, 1998; Ashton *et al.*, 2001), heart rate (Johnson *et al.*, 1998; Robbins *et al.*, 1999), alcohol-induced behavior (Heberlein, 2000) and drug response (Zimmering *et al.*, 1977). Most of these studies have adopted Mendelian genetic strategies, but given anecdotal reports of the effect of genetic background, it is also important to characterize the genetic architecture of naturally occurring variation for behaviors such as drug susceptibility. The drugs that we have studied include the biogenic monoamines dopamine, octopamine and tyramine, as well as caffeine and nicotine. Biogenic monoamines are neurotransmitters involved in synaptic transmission that are highly conserved in most animals (Walker *et al.*, 1996) and are believed to modify and regulate moods, personality traits and environmental responses, as well as having several physiological effects.

Previous studies have shown that monoamines affect locomotor activity (Hirsh, 1998) and heart rate (Ashton *et al.*, 2001) and are lethal when added to the diet. In addition, complete loss of monoamine production is also lethal (Bainton *et al.*, 2000). Caffeine is also lethal to adult *Drosophila melanogaster* (Zimmering *et al.*, 1977), and in smaller concentrations decreases longevity and fecundity in *Drosophila prosaltans* (Itoyama *et al.*, 1998). Furthermore, caffeine sensitivity has been shown to vary among populations and between males and females (Zimmering *et al.*, 1977), but the source of these differences is not known. The effects of nicotine in Drosophila have not been studied in detail, but in the nematode worm, *Caenorhabditis elegans*, it has been shown to affect locomotion and egg laying (Trent *et al.,* 1993; Waggoner *et al.,* 2000).

Studies associating genes with behaviors such as depression and alcoholism have been undertaken in mice and humans (McLeod and Evans, 2001), but have had only mixed success. The fruitfly, *Drosophila melanogaster*, offers many advantages as a model system for pharmacogenetic analysis because of the availability of resources such as the genome sequence and mutant lines. Flies can also be grown in controlled environments and their genetic background can be manipulated. In the absence of receptor mutants in flies, we have initiated a quantitative genetic analysis of pharmacological variation in flies. Here we present an initial characterization of the architecture of survival time upon chronic drug exposure in *Drosophila* and show that sex, genotype, and interaction effects are prevalent for drug resistance, and that the genetic effects are largely independent for each drug.

2.2 Materials and Methods

(A) Drosophila Stocks

Parental lines used in this study consisted of sixteen isofemale lines of *Drosophila melanogaster.* These flies were collected from the Kerrytown Fruit Market in Ann Arbor, Michigan in 1996. The stocks were maintained in 10 mL vials on standard cornmeal medium with yeast and kept at 25^0C on a 12-hour light/dark cycle throughout the experiment. All flies were reared at a density of 50 to 100 larvae per vial.

Crosses of the extreme lines for caffeine resistance were produced to study the genetic architecture of drug resistance. F1 and F2 generations of the extreme parental lines (i.e., high x low) as well as the reciprocal crosses were assayed. Crosses were also made of high x high (A3 and A6), low x low (A2 and A19), no sex effect x no sex effect (A7 and A16), and sex effect (A2 and A3) x no sex effect. In each case, one male and one virgin female were used to found five independent replicates, from which two sets of 10 males and 10 females were assayed for time to mortality. Replicates were established over several months, involving independently prepared food and drug batches.

(B) Drug Resistance Assay

Five drug treatments were used to test for drug sensitivity. Drugs were ordered from Sigma, and octopamine O-0250 (20 mg/ml), tyramine T-7255 (20 mg/ml), dopamine H-8502 (40 mg/ml), nicotine N-3876 (3 μl/ml) and caffeine C-0750 (10 mg/ml) were directly dissolved in molten fly food just prior to pouring into empty vials. Drugged food was used between one and four days after preparation. Starvation resistance on agar medium was also measured as a control for variation in overall fitness between the lines and sexes.

Flies screened for drug resistance were collected between one and three days after emergence and were kept on standard cornmeal media for three days prior to placing on drugged food. These flies were then separated by sex, and ten flies of each sex were placed separately in vials with drugged food. The number of live flies was counted every twelve hours until all of the flies were dead. Ten replicate vials of each line and sex were scored.

(C) Statistical Analysis

Analysis of variance was performed using SAS Proc GLM on the survival time for each individual fly, computed as the midpoint of the 12-hour interval in which the fly died. This ensured that among fly variability is the source of residual error, but Vial effects were also included in the model for response to each drug. In the model, Vial and Line were treated as random effects and Sex as a fixed effect:

$$Age\ at\ death = \mu + Line + Sex + Sex{\times}Line + Vial(Sex{\times}Line) + Error$$

Genetic correlations between the drug treatments reported in Table 2.2 for each sex separately were calculated according to Robertson (1959) as:

$$r_{drug1,drug2} = (MS_L - MS_{D{\times}L})/(MS_L + MS_{D{\times}L} - 2.MS_{error})$$

where MS represents the mean square in a two-factor ANOVA for the residual error, line (L), drug (D) or drug x line (D*L) interaction.

Further analysis of caffeine resistance was performed by generation means analysis, implementing the methods of Kearsey and Pooni (1996) and Gilchrist and Partridge (2001) in JMP software Version 3 (SAS Institute, 1995). The observed generation means for survival times were used to estimate genetic parameters. First, a regression model was constructed containing only the mean survival time. To this model, other regression terms were added, starting with additive (a) and dominance (d) effects, then digenic epistatic (aa, ad, or dd), maternal (am or dm), and sex-linked effects. Each parameter was added stepwise and estimated parameter values were used to calculate expected generation means. The expected and observed generation means were compared using a χ^2 test, and only parameters that improved the model were kept at each step. Table 2.3 indicates the parameters that significantly improved the model when added, as well as the effect and significance of the indicated parameters. Note that some parameters improve the overall fit of the model without themselves being significant.

2.3 Results

(A) Genetic Variation for Drug Response

Sixteen isofemale lines of *Drosophila melanogaster* were assayed to gauge the amount of genetic variation present for survival time on five drug treatments. Flies between three and six days of age were separated by sex and placed in vials containing standard cornmeal media mixed with one of the drugs. For some of the drugs, behavioral changes such as grogginess (nicotine and dopamine) or hyperactivity (caffeine) were observed within 12 hours of transfer to the drugged food. The number of flies that were alive in each vial was counted every twelve hours until all of the ten flies in each vial were deceased. Drug concentrations were chosen on the basis of preliminary titration experiments (data not shown) such that the mean survival time for most lines ranged between 24 and 96 hours. Line means are shown for starvation media, tyramine, octopamine, and dopamine, and by sex for nicotine and caffeine, in Figure 2.1. The range of variation was clearly greater for the latter three drugs. Very similar mean survival times for each line and sex were inferred from the point of inflexion of Kaplan-Meier survival plots. Age at death was approximately normally distributed within lines for all drugs.

Analysis of variance was used to assess the significance of the contributions of genotype, sex, genotype-by-sex interaction, and within and among vial effects, to the variation. The *F*-ratios associated with each effect and associated significance levels are indicated along with the estimated variance component for the random effects, in Table 2.1. For nicotine and caffeine, genotype (Line) and sex as well as the interaction between these factors, were highly significant. In general, females are twice as resistant to nicotine as males and 50% more resistant to caffeine, so that the absolute

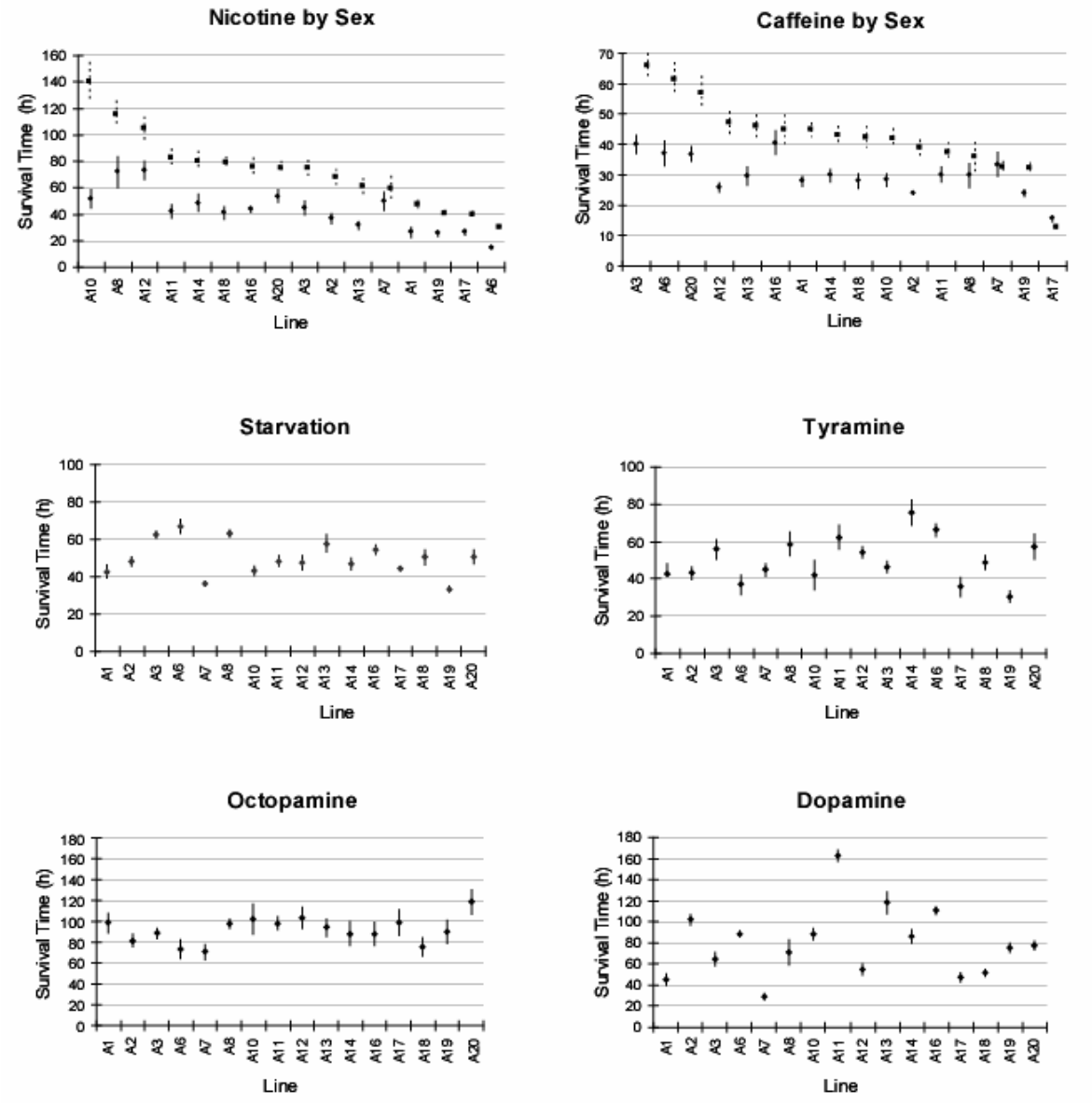


Figure 2.1 Differences among lines for time to mortality upon chronic drug exposure. All plots show the mean time of death for 16 isofemale lines. Error bars indicate two standard deviation units. The top two panels show the nicotine and caffeine responses by sex (females squares and dashed error bars; males circles and solid error bars), in rank order of the overall mean survival time for each line. The bottom four panels show the mean survival time for both sexes pooled (since no overall effect was observed) for response to starvation, tyramine, octopamine, and dopamine, in numerical order to allow visual comparison of the profiles.

Table 2.1 Variance Components for Drug Effects

	Nicotine	Caffeine	Dopamine	Tyramine	Octopamine	Starvation
Line	5.46** (0.10)	4.47** (0.04)	15.10*** (0.41)	5.76** (0.29)	3.23* (0.05)	11.87*** (0.21)
Sex	40.86***	39.45***	0.47[n.s.]	0.12[n.s.]	0.01[n.s.]	44.80***
Line×Sex	12.76*** (0.36)	13.44*** (0.25)	11.34*** (0.05)	6.88*** (0.10)	1.88* (0.02)	10.32*** (0.18)
Vial(L×S)	1.66*** (0.03)	0.81[n.s.] (0)	0.96[n.s.] (0)	3.70*** (0.13)	2.94*** (0.15)	0.79[n.s.] (0)
Error	(0.51)	(0.71)	(0.54)	(0.48)	(0.78)	(0.61)
Heritability	0.23	0.15	0.23	0.26	0.11	0.20

[n.s.] non significant * 0.05 > P > 0.01 ** 0.01 > P > 0.001 *** P < 0.001

differences between the sexes tend to increase with overall levels of resistance. There was no overall effect of sex on the response to the other drugs, although a small interaction effect, largely attributable to a few lines, was observed for dopamine and tyramine. Genotype differences were only marginally significant for the monoamines tyramine and octopamine, partly as a consequence of relatively large among vial differences for these drugs. Heritability of survival time on each drug was estimated in Table 2.1 as half the proportional contribution of the genotype and genotype-by-sex variance components, and ranges up to 0.23. As reported by others (Hoffmann *et al.*, 2001; Kennington *et al.*, 2001; Harshmann *et al.*, 1999), starvation resistance also shows considerable genetic variation, the heritability of which is increased relative to that of drug resistance in our experiments by the low among individual variance, despite the similar distributions of line means.

A source of potential experimental error in these studies is the consistency of drug delivery to flies. For dopamine, nicotine, and caffeine, vial effects were either non-significant or contributed just a few percent of the total variance. This suggests that these drugs were reproducibly dissolved in the different batches of cornmeal medium prepared. Vial effects were higher for tyramine, consistent with the low solubility of this compound and for octopamine, reflecting the general absence of sex and genotype effects for this drug. The residual error term in each treatment indicates differences among flies within vials and presumably includes effects of differential ingestion as well as physiological responses to the drugs. There is no simple way to tease these apart, but we note that these error terms are in the same range as those observed for many morphological traits. Even for drugs such as octopamine with marginally significant line effects and high error rates, clear differences can still be observed between extreme lines for survival.

(B) Correlations Among Drug Responses

To determine whether genotype-specific drug responses merely reflected generalized differences in fitness among lines, for example due to fixation of deleterious alleles, the genetic correlations among lines were examined. Line means normalized to a standard deviation of one and mean of zero are plotted in Figure 2.2, which is dominated by the crossing of line means. Genetic correlation coefficients are given for each sex in Table 2.2, with females above the diagonal and males below it. In general, correlations among treatments are low, further implying that the genetic differences among lines that contribute to extreme drug resistance or sensitivity are different for each drug. A remarkable example of this is line A11, which is hyper-resistant to dopamine alone among the drugs tested.

However, there are also trends that suggest some common susceptibility factors. First, caffeine and tyramine sensitivity are correlated with starvation resistance, possibly indicating that some of the response is due to avoidance of food laced with these drugs. Starvation is not however the sole cause of caffeine-induced mortality since several of the lines survive for longer on the drugged food than on agar while others have reduced mortality. Second, two of the lines, A17 and A19, are among the most sensitive to nicotine, caffeine, tyramine, and dopamine as well as starvation, suggesting poor general metabolic performance. In fact, one of these lines has since been lost due to low fecundity. At the other end of the spectrum, it is noteworthy that the two lines most resistant to caffeine (and starvation), A3 and A6, are relatively sensitive to nicotine, while those most resistant to nicotine have intermediate sensitivity to the other drugs. Third, a relatively high correlation was observed between nicotine and tyramine or octopamine, suggesting that these drugs may operate through related physiological systems.

Table 2.2 Genetic Correlations among Drug Treatments by Sex

	NIC	CAF	DOP	TYR	OCT	STA
NIC		0.062	0.078	0.282	0.330	0.052
CAF	0.111		0.159	0.492	-0.046	0.659
DOP	-0.026	0.041		0.225	0.058	0.169
TYR	0.488	0.265	0.268		0.149	0.453
OCT	0.442	-0.036	0.159	0.089		-0.007
STA	0.057	0.473	0.117	0.039	-0.238	

Top right: Female Bottom left: Male

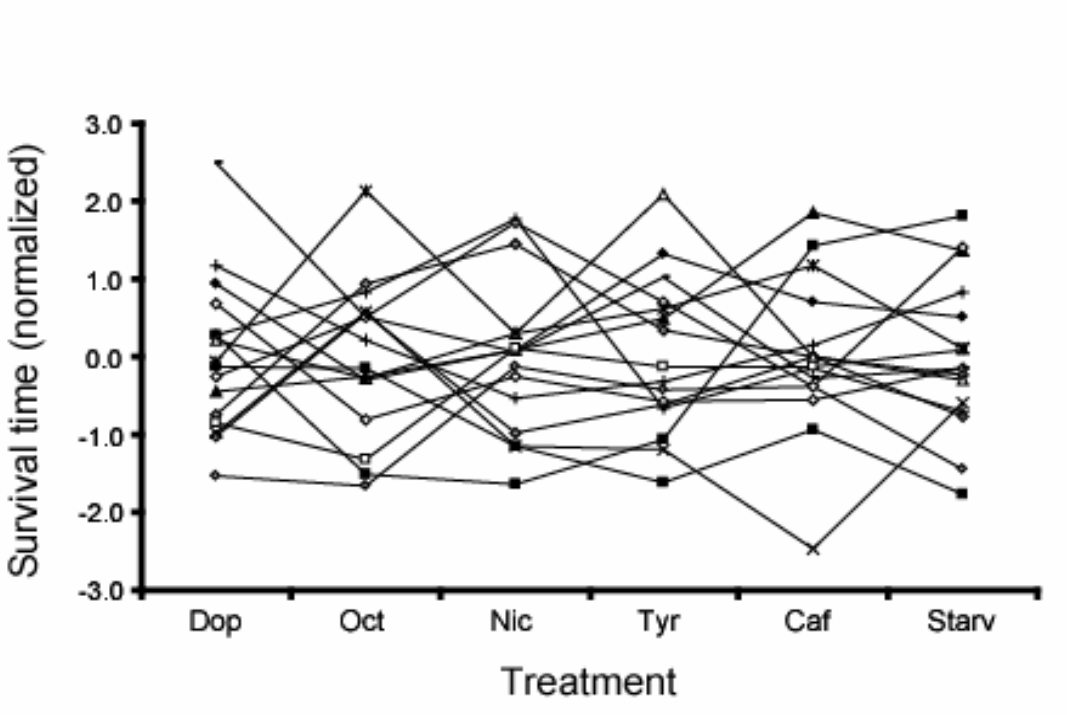


Figure 2.2 Phenotypic correlation among lines. Lines join points representing the mean time of death for both sexes pooled of each isofemale line, normalized by subtracting the total mean and dividing by the standard deviation for each drug. Genetic correlations computed from the variance components are indicated in Table 2.2

(C) Additivity and Dominance of the Caffeine Response

To begin to dissect the genetic architecture of drug sensitivity, we performed a series of crosses between lines with extreme responses to caffeine. Caffeine was chosen for further study due to the highly significant genotype, sex, and interaction effects and the absence of vial effects for response to this drug. Crosses were designed to assess the degree of dominance for both the overall resistance and sensitivity to caffeine, and for the sex-specificity of the response. Additive, dominance, epistatic, and maternal effects were all tested by generation means analysis. Sex-linked effects were also included, but did not improve the fit of the multiple regression in any of the crosses.

As expected, the survival time of F1 progeny of crosses between resistant (A3 or A6) and sensitive (A19 or A2) isofemale lines was intermediate between that of the two parents, as shown in Figure 2.3. After addition of reciprocal backcross and F2 data, generation means analysis of these resistant by sensitive crosses was performed for males and females separately (Table 2.3), but no consistent explanatory model was observed. Among individuals variance increased in the F2 relative to the F1 for some crosses, but the effect was too inconsistent to provide a reliable estimate of the number of genes that may be contributing to the variation. This may reflect insufficient power of the analysis given the high individual variability, or an effect of residual genetic variation in the inbred isofemale lines. It is also consistent with the possibility that drug sensitivity is influenced by a large number of loci of small effect.

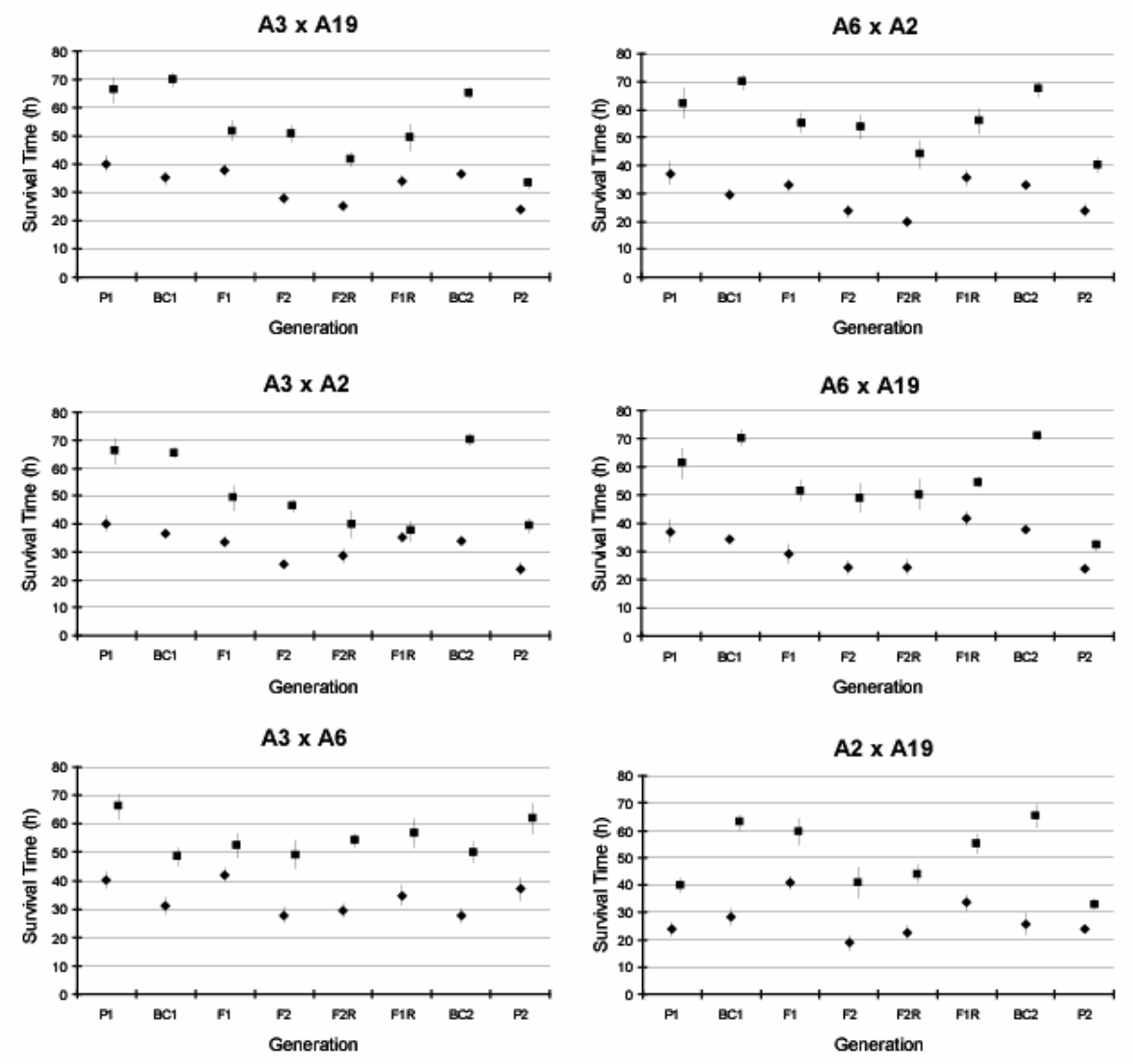


Figure 2.3 Generation means of caffeine sensitivity in crosses between lines showing a difference between the sexes. Female survival times in hours are plotted as squares (error bars indicate two standard deviation units from a total of 10 replicate vials for each generation), males as diamonds. Note the increase in survival time of Backcross females irrespective of the BC parent (except in the cross involving two resistant lines, A3 and A6); and the general decrease in survival time F2 males.

Table 2.3 Significance of Genetic Effects from Generations Means Analysis of Caffeine Resistance

Males

	A3 / A19	A6 / A19	A6 / A2	A3 / A6	A3 / A2	A2 / A19
m	35.2 ±1.7 ***	30.8 ±3.4 ***	11.6 ±5.6 *	27.4 ±2.2 ***	31.6 ±1.9 ***	23.6 ±1.9 ***
a	10.4 ±2.6 **	10.4 ±4.9 NS		4.1 ±2.5 NS	-8.1 ±3.8 *	
d		6.0 ±6.2 NS	23.8 ±7.8 **			11.3 ±3.6 **
aa			18.6 ±7.0 *			
ad	-28.6 ±7.8 **	-20.6 ±10.5 NS			10.8 ±10.7 NS	
dd				9.8 ±6.2 NS	2.9 ±3.6 NS	
am		-3.5 ±2.9 NS				1.0 ±1.4 NS
dm	-5.9 ±2.3 *					-2.6 ±2.2 NS
RSq	0.66 **	0.65 *	0.46 *	0.33 NS	0.37 NS	0.57 *

Females

m	60.0 ±3.4 ***	86.6 ±7.3 ***	71.4 ±8.1 ***	64.2 ±2.7 ***	66.5 ±5.4 ***	35.3 ± 4.0 ***
a	23.8 ±7.4 **	19.6 ±8.9 *	-14.8 ±8.7 NS		-20.4 ±9.5 *	
d				-58.6 ±9.3 ***		87.8 ±16.8 ***
aa		-32.7 ±14.0 *	-15.1 ±13.8 NS			
ad	-28.1 ±23.4 NS	-29.5 ±21.6 NS	23.7 ±22.5 NS		49.9 ±25.4 NS	
dd		-31.4 ±10.6 *	-14.7 ±14.2 NS	48.5 ±8.4 ***	-18.7 ±14.7 NS	-67.2 ±18.8 **
am						
dm				4.5 ±1.3 **		
RSq	0.52 *	0.78 **	0.49 NS	0.81 ***	0.35 NS	0.76 ***

NS non-significant *p<.05 **p<.01 ***p<.001

Crosses between the two resistant and the two sensitive lines also indicate that different loci contribute to survival time on caffeine even in isofemale lines with similar phenotypes. In both cases, F1 progeny failed to reproduce the extreme phenotype of the two genetically distinct parents, such that a cross between resistant lines (A3 and A6) gave rise to relatively sensitive F1 means while a cross between two sensitive lines (A2 and A19) gave rise to resistant F1 flies. The possibility of epistatic interactions contributing to drug sensitivity is suggested by the observation that F1 female progeny of the two sensitive lines actually have resistance levels similar to those of the most resistant inbred lines. This is observed in the multiple regression models for these crosses, where the dominance by dominance parameters improved the model, and were highly significant (Table 2.3).

(D) Sex Specificity of the Caffeine Response

Characterization of the genetic interactions affecting caffeine-induced mortality is further complicated by highly unusual sex-specific effects in the F2 and backcross generations of all crosses involving at least one sensitive (low survival time) parent. As can be seen in Figures 2.3, backcross females in both directions are uniformly more resistant than even the resistant parent. Just as strangely, the F2 males are uniformly more sensitive to the drug treatment than even the most sensitive parent. These results were repeatably observed in replicates set up at different times, and cannot be attributed to a batch effect of the food since in each case the opposite sex behaved as predicted. In separate analyses, the food batch was also found not to significantly affect survival times (not shown).

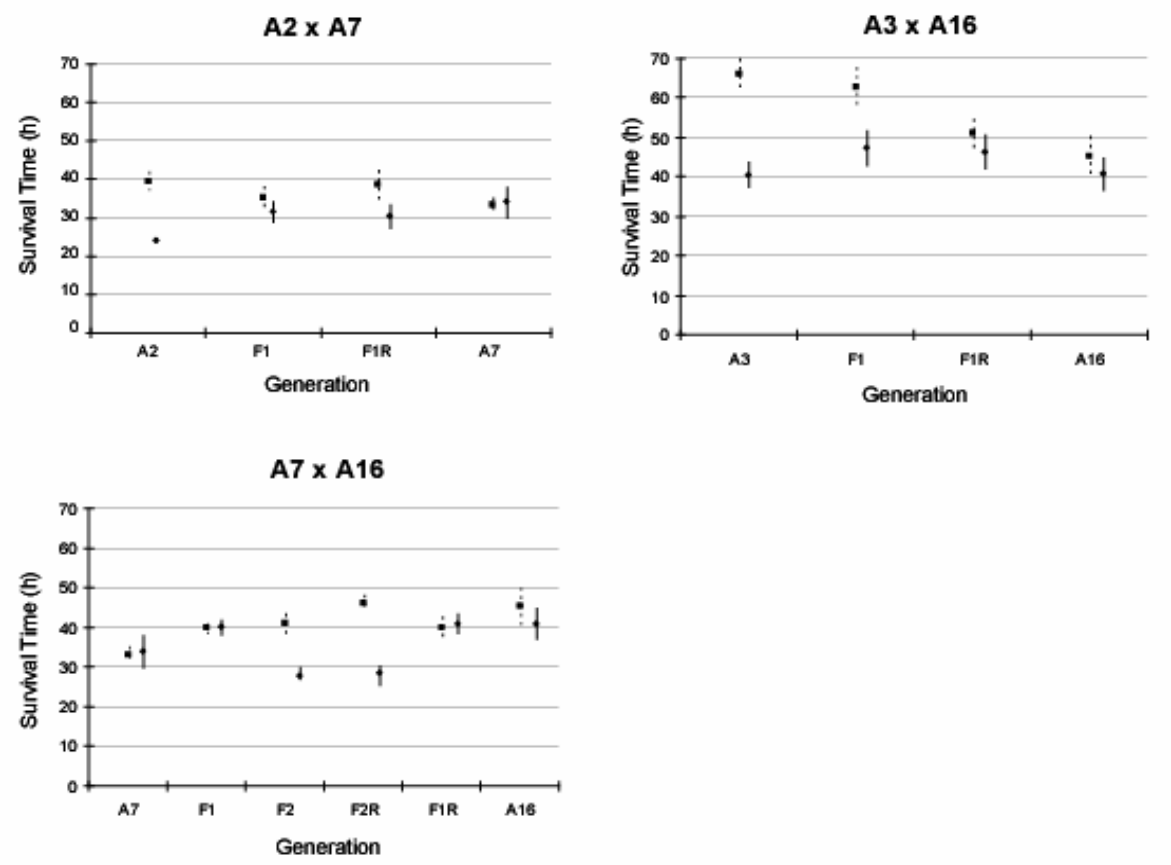


Figure 2.4 Generation mean of caffeine sensitivity in crosses between lines in the absence of a sex difference for at least one parent. The plots are the same as in Figure 2.3, except that the F2 and BC generations are removed to highlight the features discussed in the text.

The unusual sex-specific nature of the response in F2 and backcross individuals was also observed in crosses designed to explore the nature of the genotype-by-sex interaction, as shown in Figure 2.4. Three lines of evidence imply that the degree of sex-specificity is superimposed on the overall drug response. The first is that overall the two sexes are genetically correlated for all drug responses, as can be seen in Figure 2.1. The second is that a few of the lines have almost no sex effect on caffeine response, while the remainder have a large difference (a similar claim could be made for the nicotine response). This may imply that one or a few loci independently regulate the degree of sex-specificity. The third is that the sex difference is lost in the F1 of four of the crosses between sex-specific (A2 or A3) and phenotypically similar non-sex-specific (A7 or A16) lines. The directional loss of sex-specificity in the A3 by A16 cross may imply the presence of an X-linked factor affecting the dominance of the female resistance to caffeine in A3. However, the unusual female backcross and male F2 effects also appear in these crosses, though for simplicity these results are not included in Figure 2.4. We do not have a good explanation for these effects, which defy standard quantitative genetic models.

2.4 Discussion

(A) Genetic Architecture of Drug Resistance in Drosophila

Given the recent surge in interest in human pharmacogenetics, there is a pressing need for the development of model systems for the study of the genetic basis of pharmacological variation. Although the biochemical pathways through which monoamine neurotransmitters are metabolized are fairly well characterized in *Drosophila melanogaster*, remarkably little is known about the genetics of neurotransmitter receptor function, or more generally drug activity in flies. This is perhaps because much of the behavioral research on this organism has been driven by forward genetic screens for perturbation of specific behaviors such as learning and vision. Nevertheless, the demonstration that there is genetic variation in flies for behaviors such as foraging and ethanol tolerance (Sokolowski, 2001; Bainton *et al.*, 2000), and for pharmacological traits such as heart rate and autonomic "headless" behaviors (Ashton *et al.*, 2001), has encouraged us to initiate a genetic dissection of response to chronic drug exposure. In the absence of Mendelian mutants, we have started by characterizing the levels of naturally occurring variation, as this will form a baseline for the interpretation of the effects of gene knockouts.

A basic question in behavioral genetics is whether specific phenotypes can be disrupted by single mutations of large effect, or whether many mutations of small effect have diverse and pleiotropic effects on a variety of traits. Several mutations have been uncovered in key genes involved in signal transduction that have remarkably specific consequences such as disruption of a specific step in learning or switching larval feeding behavior (Goodwin *et al.*, 1997; Osborne *et al.*, 1997). On the other hand, mutations in enzymes that are

involved in the biosynthesis and degradation of monoamines are known primarily from their effect on pigmentation (for example *ebony*, which encodes β -alanyl dopamine synthase, and *pale*, which encodes tyrosine hydroxylase) and have not been shown to disrupt pharmacology. Similarly, only a couple of the neurotransmitter receptor genes that have been identified have been associated with lesions, and these were isolated by molecular rather than phenotypic screens, suggesting that receptor mutant phenotypes are likely to be subtle. One report has implicated the biological clock pathway in modification of cocaine sensitivity (Andretic *et al.*, 1999), but these results may be confounded with the effects of genetic background in the experiment. One of the aims of this study has been to define a trait that may be suitable for genetic screens for aberrant response to drug exposure.

Our key findings can be summarized as follows. (i) There is ample naturally occurring genetic variation for survival time upon chronic ingestion of several drugs including nicotine, caffeine, dopamine and tyramine, although the evidence in relation to octopamine was equivocal. (ii) Survival time may not be the most biologically meaningful trait, but it is easy to score and has moderate heritability and good repeatability, all of which make it simpler (though not necessarily more desirable) for genetic analysis than assays that involve measurement of behavioral responses. (iii) The correlations among drug responses are moderate to non-existent, indicating that much of the genetic variation is specific for one or a few of the drugs. (iv) Females tend to be more resistant than males to nicotine and caffeine, and sex-by-genotype interactions are also seen for these drugs and for the response to dopamine. (v) Preliminary dissection of differences in caffeine sensitivity suggests a complex genetic architecture with many genes of small effect and some dominance for resistance.

Dissection of the genetic architecture of behavioral responses in line crosses is complicated by relative large vial and among individual variance. Our results for caffeine resistance, similar to those of Kennington *et al.* (2001) in their analysis of the correlated trait of starvation resistance, failed to reveal a consistent picture of the extent or nature of epistatic effects, though these certainly seem to be present. Further dissection of this phenomenon is probably best approached by fine structure QTL mapping of the loci that are responsible for modulation of survival times on each drug. Unfortunately, though, the most direct interpretation of our data is that drug sensitivity is affected by many genes of small effect. This conclusion is supported by the preliminary results of a screen for P-element insertions in an isogenic background that suggests that mutations in at least 5% of the genome may affect drug sensitivity, often in a sex and drug-specific manner (A.P. Wagoner and G.G., unpublished data). Cloning of the genes associated with these insertions and analysis of interactions among the loci will complement classical quantitative genetic dissection of drug resistance.

The sex-specificity of the response to caffeine is particularly intriguing, in so far as F2 progeny of crosses between any pair of lines always resulted in much reduced male survival times, while backcross females showed elevated resistance. This was true even in the case of a cross between two isofemale lines that did not show any difference between the sexes. Sex effects for caffeine resistance have been previously reported, with females of some mutant strains also living longer than males (Zimmering *et al.*, 1977; Ityoyama, 1998). Various explanations have been proposed for this, including differences in body size and repair efficiency between males and females. Caffeine is known to cause an increase in the frequency of chromosomal loss in both

males and females, but no evidence has been found that caffeine can induce any sex-linked lethal mutations (Clark and Clark, 1968). Our interpretation is that the difference in response between the sexes may be superimposed on the general ability of the flies to resist the drugs. Nevertheless, for all of the drugs, there is a high correlation between the sexes, indicating that common factors influence the response within a line. Investigation of how sex-specific factors interact with these loci will be an essential element of dissection of drug responses, and has implications for understanding the evolutionary dynamics of variation affecting neurotransmitter activity.

(B) Sources of Variation Affecting Drug Response

Comparison of variation among wild type isolates can only provide indirect information as to the sources of variable drug response. More direct approaches will include cloning of mutations that produce discrete responses, survey of gene and protein expression differences among lines, and characterization of the interactions among candidate loci that affect various processes impinging on pharmacology. Nevertheless, our results provide some insight into the likely sources of variability.

The most obvious source is behavioral variation, namely avoidance of drugged food. The strong correlation between survival on caffeine and on starvation media, for example implies that flies may simply not like the taste of caffeine and starve themselves to death. In this case, variation in genes involved in taste perception and/or processing of the behavioral response could contribute to the enhanced survival times in several lines. Alternatively, the behavioral response could be more remote, since flies become hyperactive upon exposure to the drug. Food avoidance might therefore relate to an altered desire to eat or from the appetite suppressing properties of caffeine.

It is just as likely that variation in metabolic pathways for drug absorption, action and elimination (Evans and Relling, 1999) are involved in differential survival. There is a large literature dealing with evolved resistance to insecticides, which often reflects the emergence of detoxifying enzymes. Similarly, human multidrug resistance in cancer patients often involves modification of drug uptake and transport or catabolism. Some of these factors would be expected to be pleiotropic, some drug-specific, and little can be concluded about their involvement in naturally occurring variation from this study.

An arguably more interesting source of variation would be specific pharmacological responses relating to the processing of drug ingestion at multiple cellular levels, from the synapse to modification of gene expression to neural connectivity. Some of these drugs, notably octopamine, also act as hormones in systemic regulation of metabolism, so it is possible that effects on survival are mediated independent of the nervous system. Furthermore, we do not know what happens to the drugs either in the food or after ingestion, so it is impossible to say where their site of action may be at this time.

Microarray studies of exposure of mice and fibroblast cultures both indicate that common signal transduction pathways are affected by chronic exposure to nicotine, and we are currently investigating whether this is also the case in flies. Dopamine and serotonin are believed to have a role in the effects of nicotine in mice (Pietila and Ahtee, 2000; Balfour and Ridley, 2000), and may indicate a common biochemical pathway to drug addiction and response (Betz *et al.*, 2000). Intriguingly, octopamine and tyramine both cause a dramatic reduction in transcription throughout the genome, commencing as

soon as 12 hours after transfer of flies to the drugged food (unpublished data), which undoubtedly leads to mortality. Starvation, nicotine, and caffeine do not have this effect, and it will be important to establish the cause of mortality induced by these treatments.

Finally, survival is also likely to be affected by general "fitness" of the flies. This is clear in the case of those lines that are sensitive to all treatments, and so are likely to have fixed deleterious recessive alleles. An extreme interpretation of our results would be that the specific drug responses bear little relation to processing of particular drugs, and rather reflect chance interactions between drugs and fitness factors that segregate in the lines. However, flies that feed on vegetative matter are undoubtedly exposed to a wide range of metabolites, some of which will be directly toxic and some of which will produce metabolites that lie in monoamine biosynthesis pathways. Clearly we are a long way from being able to document any relationship between variation for drug sensitivity and ecological variables. The first step in that direction will be identification of the quantitative trait loci underlying pharmacological differences. This will be a daunting task, but is as feasible as any other complex trait, be it morphological or behavioral.

Chapter 3:
P-element Mutagenesis

3.1 Introduction

The development of model systems to study the genetics underlying pharmacological variation has gained importance with the rise of human pharmacogenetics. This has created interest in the biochemical pathways and targets on which drugs and other bioactive substances function. One area of active research is in how these substances affect neurobiological processes, including behavior. Invertebrates have previously been used in these studies to test for the effects and mechanisms of action of toxic substances (Ballatori and Villalobos, 2002). *Drosophila* in particular is useful in that the biochemical pathways through which several neurotransmitters are metabolized are fairly well characterized and many of the genes have been identified (Yoshihara *et al.,* 2001). *Drosophila* is also useful because it responds to psychoactive substances such as alcohol (Heberlein, 2000; Rodan *et al.,* 2002) and cocaine (Andretic *et al.*, 1999) as well as others with biochemical and behavioral responses comparable to those observed in mammals.

Although important, the genetic architecture of multifactorial traits can be difficult to investigate. This is often the case for behavioral traits such as drug response, where the environmental variance and environment-genotype interactions can be quite large relative to the genetic effects. These factors can confound the characterization of naturally occurring variation for these traits, as we observed in our analysis of caffeine resistance. Therefore, techniques such as mutagenesis screens and candidate gene analysis may prove more successful in the study of genetic factors involved in these traits. *Drosophila melanogaster* offers many advantages for these studies because of the availability of resources such as the complete genome sequence and the existence of an extensive collection of mutant lines.

One concern with applying a classical mutagenesis screen to behavioral traits is whether the effects of any single mutation are large enough to detect or if many mutations of small effect are required to observe a phenotypic change. Studies of mutations in neurotransmitter receptor and transporter genes have suggested that their phenotypic effects are usually subtle, with only a few implicated in behavioral responses (Yoshihara *et al.,* 2001). Furthermore, mutations in the enzymes involved in the production and degradation of monoamines are known largely for their effects on pigmentation and not behavior. Classical forward genetic screens have however already identified genes affecting several behaviors in *Drosophila* including learning (Dubnau and Tully, 1998), grooming (Phillis *et al.*, 1992) and reflex behaviors (Hirsh, 1998). In fact, mutations with very specific effects on behavior, such as the disruption of a single step in the learning process have been identified (Goodwin *et al.*, 1997).

Forward genetic screens for drug and pesticide resistance in insects have revealed a complicated architecture that is dependent on the class of pesticide. Resistance to agricultural pesticides, *i.e.* those either not found in nature or at least not in the environment of the insect, involves only a single or a few genes with large phenotypic effects (McKenzie and Batterham, 1995). Resistance to natural plant toxins in contrast usually involves a few genes of small effect, but is neither simple Mendelian nor highly polygenic (Jones, 1998). Generally, resistance to toxins that are encountered naturally by an insect appears to be more complex than resistance to externally applied agricultural pesticides. This is thought to occur due to the higher dose and shorter time span in which these agricultural pesticides are introduced and resistance develops compared to the adaptation to natural toxins.

Although studies associating genes with drug response have met with success in mice and humans as well as *Drosophila*, no clear picture has yet emerged as to how organisms respond and develop resistance to pesticides and other toxins. Because of the difficulties in characterizing the genetic architecture of natural variation for drug response, we have begun a screen for mutations involved in drug resistance and sensitivity. Forward genetic screens using P-element insertions have been successful in identifying novel genes affecting body size (Currie *et al.,* 1998), sensory bristle number (Lyman *et al.,* 1996) and neural development (Norga *et al.,* 2003). P-element mutagenesis screens have also been used to identify novel loci affecting behavioral traits such as olfaction (Fanara *et al.,* 2002; Ganguly *et al.,* 2003) and starvation resistance (Harbison *et al.,* 2004). Here we present a genetic screen for P-element insertion mutations affecting survival time upon chronic exposure to nicotine or caffeine in *Drosophila melanogaster.*

3.2 Materials and Methods

(A) Drosophila Stocks

Drosophila melanogaster lines used for the mutagenesis screen were constructed in the lab of Dr. Hugo Bellen at the Baylor College of Medicine, Houston Texas. These lines were generated by the insertion of a single P-element transposon into a Samarkand background lacking the white gene. The transposon used, PGT1, contains a mini-white gene to confirm the presence of the P-element by the restoration of eye pigmentation. It also contains a Gal4 gene, which can activate transcription of sequences under the control a UAS promoter (Lukacsovich *et al.,* 2001). All lines used in the mutagenesis screen contained the same Samarkand line and P-element. For the initial screen, approximately one thousand mutant lines were assayed for drug resistance. All stocks were reared in 10 mL vials at a density of 50 to 100 larvae per vial. These stocks were maintained on standard cornmeal medium with yeast at 25^0C on a 12-hour light/dark cycle.

(B) Drug Resistance Assay

Flies were collected between one and three days after emergence and kept on standard cornmeal media for three days before being scored for drug response. After three days, the flies were separated by sex and ten flies of each sex were placed in vials containing drugged food. The number of live flies was counted every twelve hours until all of the flies were dead. The drugged food was prepared fresh prior to each assay and used within five days of preparation. The two drugs used in this screen were ordered from Sigma and mixed directly into molten fly food at a final concentration of 3 μl nicotine (N-3876) and 10 mg of caffeine (C-0750) per ml of food.

(C) Mutagenesis Screen

For the initial mutagenesis screen 970 lines, each homozygous for a single P-element insertion, were assayed for survival time on nicotine and caffeine media. These lines were tested in batches of approximately seventy five lines along with a control consisting of the parental Samarkand line lacking the P-element insertion. The effect of each insertion on drug resistance was calculated for each line as the deviation of the line mean from the mean of the control line in that batch as described in Harbison *et al.* 2004. This was performed separately for males and females and lines significant for resistance or sensitivity in either sex were isolated for further study. Lines were selected for increased resistance using the 95% confidence limit, which was calculated as $\pm z_{\alpha}\sigma/(n)^{\frac{1}{2}}$, where z_{α} is the critical value of the normal distribution (1.96 for the 95% confidence limit), σ is the standard error of the total variance and n is the number of replicate vials performed (Harbison *et al.,* 2004). The total variance, σ^2, was estimated for the drug treatments from the sum of the Line and Error variance components for each sex from the ANOVA analysis. The number of replicate vials performed, n, for each sex was three. Sensitive lines were selected on the basis of drug-specific sensitivity (lines sensitive to only one drug) to eliminate lines that have low overall fitness. These lines were in the lowest 25 percent of survival time for one drug, but average or above average survival time on the other.

P-element lines selected for increased resistance or sensitivity were isolated and two replicates of each line and sex were retested. The lines were tested in a single batch with the Samarkand control line, and the top 10 lines resistant to each drug were selected. These lines were backcrossed to the parental Samarkand line for five generations to reduce the background genetic

variation between the lines. After five generations, the lines were remade homozygous for the region surrounding the insertion by selecting for the P-element. This was accomplished by selecting for the white gene within the insertion, which confers a red eye phenotype to the white eyed Samarkand parental line. These backcrossed lines were tested alongside the original insertion lines that were used to create the crosses to verify the effect of the insertion on drug resistance. Again, two replicates of each sex and line were tested for each insertion and each backcrossed line.

(D) Statistical Analysis

Analysis of variance was performed using SAS Proc GLM on the average survival time for each vial. This was computed as the average of the midpoints of the 12-hour interval in which each fly within a vial died. Vial effects were included in the model for response to each drug. In the model, Vial and Line were treated as random effects and Sex as a fixed effect:

$$Age\ at\ death = \mu + Line + Sex + Sex \times Line + Vial\ (Sex \times Line) + Error$$

Genetic correlations between the drug treatments and sexes were calculated according to Robertson (1959) as:

$$r_{drug1,drug2} = (MS_L - MS_{D \times L})/(MS_L + MS_{D \times L} - 2.MS_{error})$$
$$r_{sex1,sex2} = (MS_L - MS_{S \times L})/(MS_L + MS_{S \times L} - 2.MS_{error})$$

where MS represents the mean square in a two-factor ANOVA for the residual error, line (L), drug (D), sex (S) or interaction term.

3.3 Results

(A) Variation for Drug Response

To identify genes involved in drug response, we assayed approximately 1000 P-element insertion lines for survival time on two drug treatments. Nicotine and caffeine were selected based on previous work that showed significant genetic variation between lines and sexes for survival time on these drugs (Carrillo and Gibson, 2002). For each replicate, ten adult flies (3-6 days old) of each sex were scored separately for survival time on standard cornmeal food mixed with one of the drugs. Males and females were not separated until immediately before being placed on the drug media and were therefore generally likely to have mated. The number of flies alive in each vial was counted every twelve hours until all of the flies in the vial were deceased.

Drug concentrations were selected from pilot experiments (data not shown), and were titrated so that the mean survival time ranged from 18 to 54 hours in those trials. The P-element lines screened were on average more sensitive than those from the pilot experiments on near-isogenic lines, with mean survival time ranging from 14 to 24 hours as shown in Figure 3.1. Survival time on both drugs is approximately normally distributed among lines, with higher variation present between replicates for caffeine compared to nicotine. This is consistent with our observations of natural variation for survival time in isofemale and near-isogenic populations of *Drosophila melanogaster*. Also consistent with previous studies (Zimmering *et al.,* 1977; Carrillo and Gibson, 2002) is that females tended to be more resistant than males to both of these drugs (Figure 3.1). Overall, females lived 50% longer than males on both drugs, so that the absolute difference in survival time between the sexes increases with resistance.

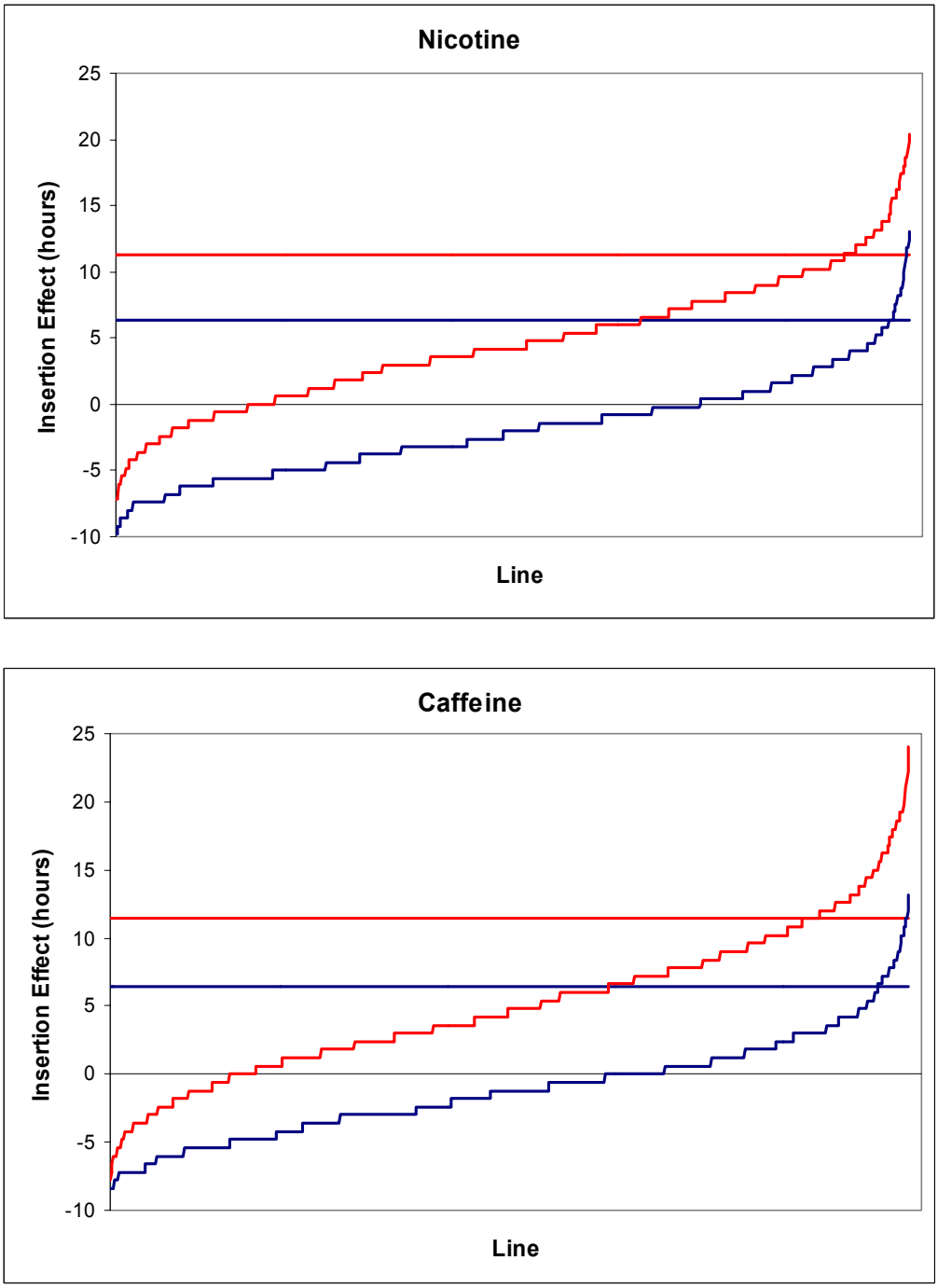


Figure 3.1 Distribution of insertion effects on drug resistance. Plots show the effect of insertions on survival time in all 970 P-element lines screened for males (blue) and females (red) in rank order for each sex. The 95% confidence intervals used as thresholds for the selection of resistant lines are also shown.

The significance of the line, sex, line-by-sex interaction, and within and among vial effects to the variation in survival time was measured by analysis of variance. The *F*-ratios and significance levels associated with each effect are indicated in Table 3.1. For the insertion lines tested, both the line and sex effects, but not the interaction between these factors, were highly significant. One possible source of experimental error, and thus a major concern with this assay, is the consistency of drug delivery. The amount of drug available to the flies can vary as a result of differences in the concentration and distribution of the drug within and between the independently prepared batches of drug media. Vial effects were measured by comparing survival time between replicates, which contained food batches prepared separately and at different times. For both nicotine and caffeine, these vial effects were either non-significant or contributed little of the total variance, suggesting that there was little variation in drug distribution between food batches. Differences among the flies within vials are reflected in the residual error term. These differences could include variation in the ingestion of the drug media or in the physiological responses to the drugs.

(B) Correlations among Drug Responses

To determine whether the line effects observed are a result of drug specific factors or generalized fitness differences among the lines, the genetic correlation coefficient was calculated for survival time between the drug treatments. This is necessary to verify that the variation for survival time between the lines is a result of the insertion specifically affecting drug response and not simply general fitness. This is particularly important in the selection of lines for drug sensitivity, as insertions with deleterious effects on overall lifespan must be differentiated from those that only affect survival in response to the drug treatment.

Table 3.1 Variance Components for Drug Response

	Nicotine	Caffeine
Line	3.46***	3.93***
Sex	104.70***	117.95***
Line×Sex	1.623*	1.79[n.s.]
Vial (L×S)	0.95[n.s.]	1.24**

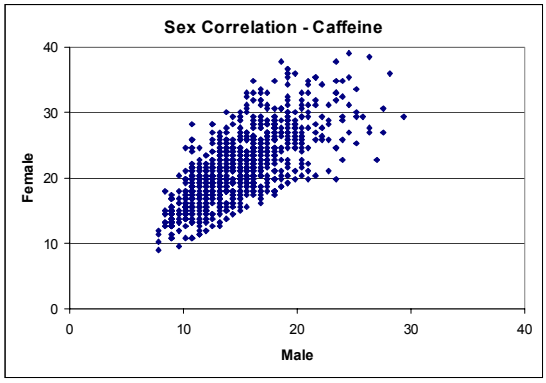
[n.s.] non significant * 0.05 > P > 0.01 ** 0.01 > P > 0.001 *** P < 0.001

The genetic correlation between the treatments was low (R=0.25), indicating that the genetic differences among the lines contributing to variation in survival time are distinct for the drugs (Figure 3.2). Furthermore, there was little overlap between lines resistant to the two drugs, with less than ten percent of the lines selected for resistance being resistant to both drugs. This does not however mean that there are no insertions affecting general fitness traits. There were in fact lines sensitive to both nicotine and caffeine that also exhibited low overall fitness and fecundity. This suggests that some insertions affect general fitness, and must be separated from those sensitive only in response to the drug treatment during the selection process.

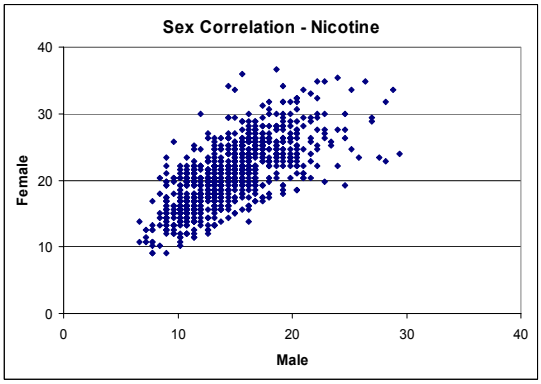
The correlation between males and females was also calculated to determine if the insertions affect drug response similarly between the sexes. Contrary to the low correlation between drug treatments, the correlation between males and females was relatively high (R=0.71). This suggests that the insertions have similar effects on drug response in both sexes, although females tend to be 50% more resistant on average to both drug treatments compared to males. The overall result is that as resistance increases, the absolute difference in survival time between the sexes increase as well. Exceptions to this do occur however, with some lines exhibiting sex specific effects, namely an increase or decrease in resistance in only one sex.

(C) Selection of Lines

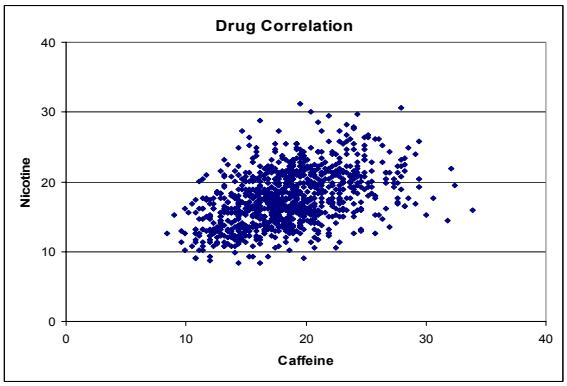
Insertion lines that exhibited increased resistance or sensitivity to either drug were isolated for further study. These lines were selected by comparing the survival time of the insertion lines within each treatment batch to the control line tested in that batch. The control and insertion lines share an identical genetic background and should differ genetically only by the presence



r = .712



r = .710



r = .222

Figure 3.2 Genetic correlations among drug treatments and sexes. All plots are of survival time in hours. Correlations between the sexes were calculated independently for the two drugs. The correlations between nicotine and caffeine treatments are for the average of each line.

of the P-element insertion at a random location in the genome. Any genetic differences in drug response between them should therefore be a result of the insertion. Overall, approximately two-thirds of the insertion lines exhibited greater sensitivity to the drug treatment than the control line (Figure 3.3). This is not surprising, as the probability that a random mutation would have deleterious as opposed to advantageous effects on fitness is likely higher.

In the initial screen, two replicates of each sex were tested for the approximately 1000 insertion lines. As described previously, these lines were tested in smaller batches of seventy to seventy five lines with a parental control lacking the insertion. Insertion lines were selected separately for males and females from each batch by comparison to the control line within that batch. Lines were chosen for increased resistance by selecting lines above the 95% confidence interval (Figure 3.1). In the initial screen, 91 lines were selected for nicotine resistance and 107 lines were selected for caffeine resistance (Table 3.2). Of these, 182 were resistant to only one drug while 16 were resistant to both drugs. This can be expected from the estimate of correlation between the nicotine and caffeine treatments. The correlation between drug treatments was found to be greater than zero but much less than one (R=0.22), indicating that there is genetic variation between the lines but that they are not having the same effect in both treatments. This suggests that different mechanisms or pathways are responsible for resistance to these two drugs. This is likely, as these drugs function through different metabolic and physiological pathways, even though they have some common intermediaries (Betz *et al.,* 2000) and can cause similar behavioral and physiological effects.

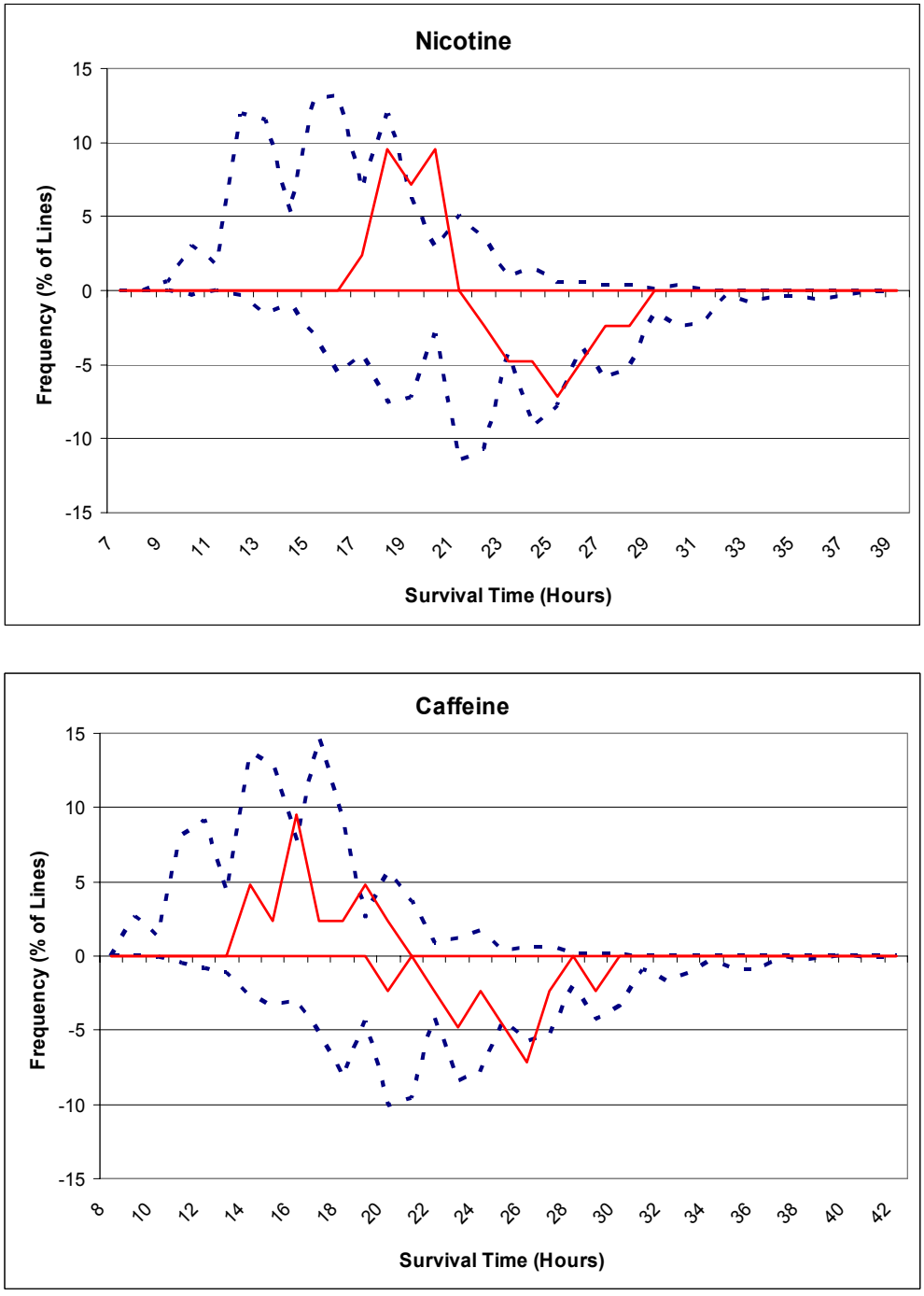


Figure 3.3 Distribution of survival time for insertion lines in the initial screen. The frequency of the insertion lines (dashed blue) and the control lines (solid red) are plotted, with males plotted above the Y-axis and females below the axis.

Table 3.2 Lines Selected for Drug Resistance and Sensitivity

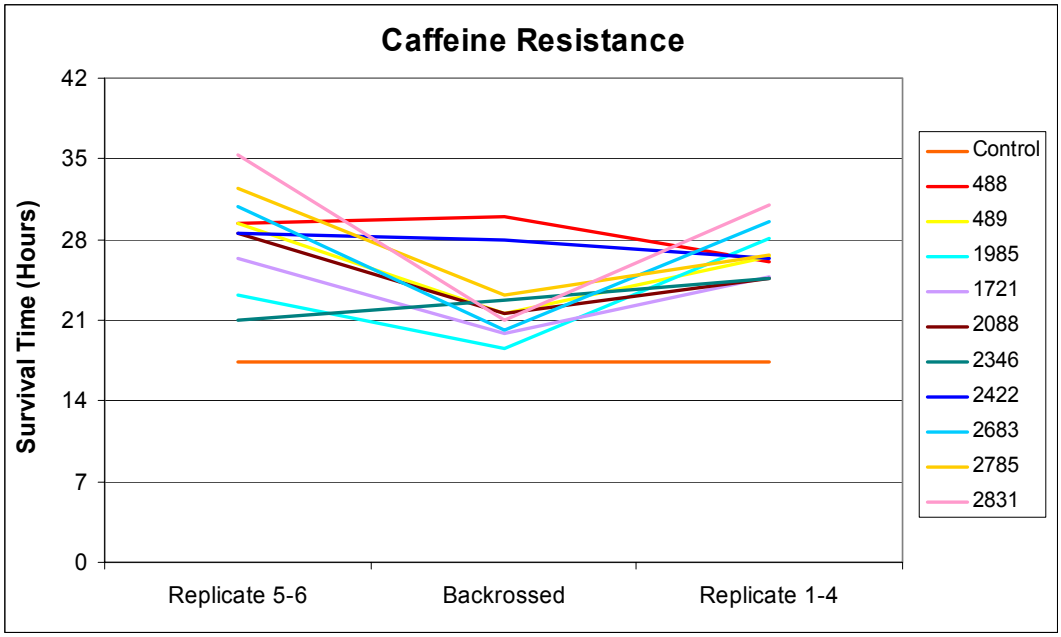
	Nicotine	Caffeine
First Screen:		
Resistant to One Drug	75	91
Resistant to Both Drugs	16	16
Drug-specific Sensitive	93	81
Second Screen:		
Resistant to One Drug	9	9
Resistant to Both Drugs	1	1
Backcross Screen:		
Resistant	9	4

The 182 lines selected for drug resistance were separated into two groups according to the drug for which they were resistant, and re-screened only on that drug. All of the lines in each group were tested simultaneously on the same batch of drug food to minimize the environmental variation. As in the initial screen, two replicates of each sex were performed for each line. The same methods used in the initial selection were applied to these replicates to select lines resistant to either drug. From this, 19 lines were chosen for increased survival time, 9 resistant to each nicotine and caffeine and 1 resistant to both drug treatments (Table 3.2).

Lines selected for drug resistance were backcrossed to the parental control line for five generations to reduce the genetic variation between lines. After backcrossing, the only genetic difference between the insertion lines and the control should be the genetic interval encompassing the insertion. Any genetic variation in survival time between the backcrossed lines and the control should consequently result from the insertion. Lines in which genetic factors other than the P-element, or interactions between other genetic factors and the P-element, contribute to the majority of the variation should as a consequence exhibit survival times similar to the control and significantly different to those observed in the initial selected lines not backcrossed. Lines in which the P-element contributes to all or most of the genetic variation in drug response however should have no significant difference in survival time between the backcrossed and the initial replicates. To test the effect of the P-element on survival time, two replicates of each sex and line were scored for survival time for the 19 selected lines and the backcrossed lines derived from them. These lines were tested for drug response only on the drug to which they exhibited resistance.

Comparison of the backcrossed and initial lines reveals different sources for the variation in survival time in the two drug treatments (Figure 3.4). In the lines selected for caffeine resistance, the majority (7 out of 10) exhibited a significant difference in survival time between the backcrossed and initial lines (Table 3.3). In fact, the survival time in these lines were not significantly different from those of the control line lacking the P-element. This suggests that the variation for survival time in these lines is not a direct result of the P-element but rather from other differences, i.e. spontaneous mutations, in the genetic background of the initial lines or from interactions between these and the P-element. For nicotine resistance however, only one of the backcrossed lines exhibited a significant difference in survival time from its initial line (Table 3.3). This line, 1721, was the only line selected for resistance to both drugs. The other 9 backcrossed lines on the other hand did not differ significantly in survival time from the initial lines, suggesting that the insertion did contribute to the variation for survival time from the control in these lines. However, this difference in response between treatments could also result from the higher variability observed in the caffeine assay. Based on these results, 11 lines were selected to map the location of the insertions: 3 for resistance to caffeine and 8 for nicotine resistance.

In addition to increased resistance, lines were also selected for sensitivity to nicotine and caffeine. The selection process for lines sensitive to the drug treatments was complicated by the occurrence of insertions affecting overall fitness. Lines with low overall fitness and hence low survival time must be distinguished from lines sensitive only in response to the drug treatment. This was accomplished by selecting lines that exhibited drug-specific sensitivity, i.e. those lines that were sensitive to one drug treatment while being of average or above average resistance to the other treatment. Utilizing the



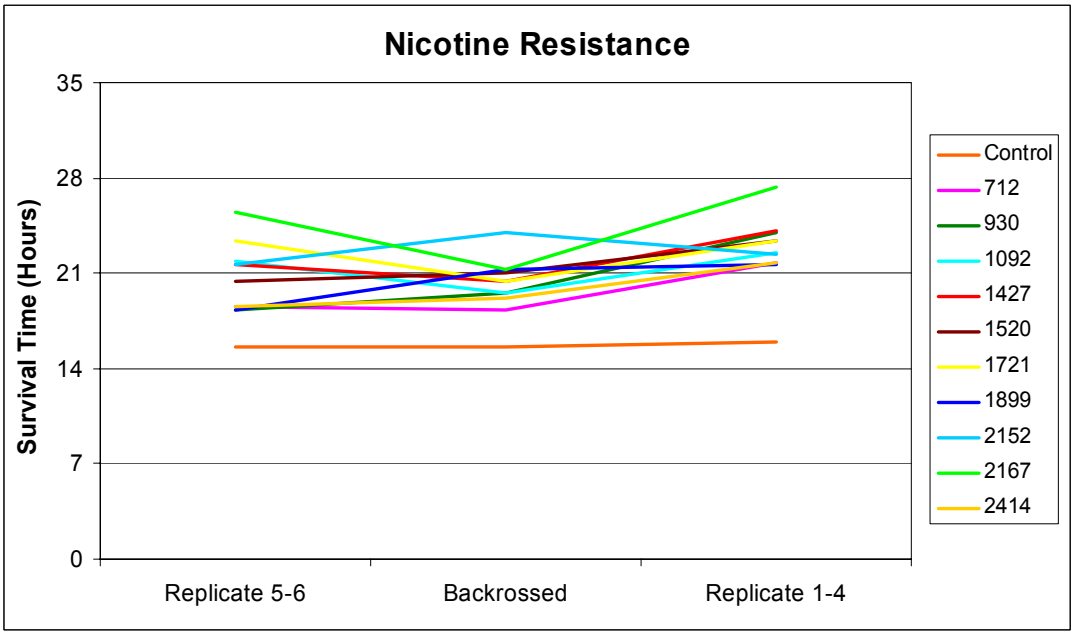


Figure 3.4 Survival times for insertion lines selected for increased resistance and the backcrossed lines generated from them. Plots are of lines averages and include all ten liens selected for resistance to each drug. Replicates 1-4 indicate the initial replicates used during the screening process. Replicates 5-6 are those performed simultaneously with the backcrossed lines.

Table 3.3 Significance of Backcrossed Lines

Line	P-value	Line	P-value
Caffeine		*Nicotine*	
488	.284	712	.388
489	.027	930	.396
1721	.019	1092	.416
1985	.044	1427	.354
2088	.144	1520	.430
2346	.500	1721	.037
2422	.427	1899	.246
2683	.029	2152	.179
2784	.012	2167	.051
2831	.002	2414	.336

P-values represent the significance of the difference in survival time between the initial lines and the backcrossed lines derived from them.

same methods described for drug resistance, the lines with the lowest 25% of survival time were selected from each drug treatments. The lines that were selected as sensitive to both drug treatments were then discarded from further study. In this manner a total of 170 lines were selected for drug-specific sensitivity (Table 3.2). Of the 170 lines selected for sensitivity to one drug, 43 were also selected in the resistance screen for the other drug.

After the selection of lines for drug-specific sensitivity, two replicates of each sex per selected line were tested for survival time on both drug treatments. Replicate vials for each line were assayed on the two treatments simultaneously to reduce the environmental variation. These replicates were analyzed with the same methods used in the initial selection process, but few of the lines met the criteria for drug-specific sensitivity. In this screen, the majority of the lines exhibited sensitivity to both drug treatments, while some had average resistance compared to the control line on both drug treatments, including the drug they were chosen for sensitivity to. The absence of lines with reproducible drug-specific sensitivity could be a result of environmental variation and/or the scoring and selection process. Because lines were scored at 12 hour intervals, distinguishing between lines with low survival times is complicated. These lines do significantly differ in survival time compared to the control, but a rank order of sensitivity for the lines can not be clearly established. A small variation in survival time between replicates can also alter the classification of these lines as sensitive, as their range of survival times all lie within the first two scoring intervals (12 and 24 hours). Therefore, it was not possible to select lines for further study of increased drug sensitivity. In order to investigate drug-specific sensitivity, more replicates and either shorter time intervals between scoring or lower drug concentrations will be necessary.

(D) Characterization of Insertions

To further discern the basis of genetic variation affecting drug response, the location of the P-element insertions were mapped in lines with a significant increase in resistance to caffeine and nicotine. The lines that exhibited a significant increase in resistance after backcrossing, and therefore where the resistance was likely caused by the insertion were selected for mapping. Therefore, a total of eleven lines, three for caffeine resistance and eight for nicotine resistance were selected. These lines were sent to Dr. Eric Spana at the Model System Genomics facility at Duke University, North Carolina. The insertions were cut out of the genomic DNA, amplified and then sequenced, along with some of the neighboring region, which allowed for the location of the insertion to be mapped (Table 3.4).

Out of the eleven lines submitted, two did not yield any genomic DNA or usable sequence, and it was therefore not possible to map their location. Furthermore, two of the lines selected for nicotine resistance were mapped to genes with no known function. Therefore, a total of seven lines, two for caffeine and five for nicotine resistance, were mapped to previously identified genes. Three of the insertions lines selected for nicotine resistance were mapped to the same gene, which encodes the *E2f* transcription factor. One of the insertions selected for caffeine resistance was also mapped upstream of a transcription factor gene, *corto*. Another insertion selected for nicotine was mapped to a gene with transmembrane receptor activity called *roundabout* (*robo*). In the last two insertion lines, which were selected for resistance to different drug treatments, the insertions were localized to the same gene, *6-phosphofructo-2-kinase* (*Pfrx*), involved in fructose metabolism. This was even though the two insertions were created independently.

Table 3.4 Location of P-element Insertions

Line	Gene
Caffeine	
488	500 bp upstream of corto
2346	located in Pfrx
2422	no sequence
Nicotine	
712	1.2 kb downstream of E2f
930	upstream of CG15316
1092	located in robo
1427	located in Pfrx
1520	located in E2f
1899	located in E2f
2152	no sequence
2414	6 kb upstream of CG 32737

3.4 Discussion

(A) Variation for Drug Response

While many of the biochemical pathways through which drugs and neurotransmitters function and the behavioral responses they elicit are well characterized, many aspects of drug response remain unclear. The ongoing development and promise of human pharmacogenetics to tailor therapeutic drugs to an individual's genetic background has raised interest in the identification of novel loci involved in drug response. This task is particularly suited for the use of model organisms such as *Drosophila melanogaster*, with relatively simple nervous systems, completed genome sequences and the availability of mutant strains. In fact, forward genetic screens in *Drosophila* have already identified genes affecting several behaviors including olfaction (Fanara *et al.,* 2002; Ganguly *et al.,* 2003), learning and memory (Goodwin *et al.*, 1997; Dubnau and Tully, 1998) and reflex behaviors (Hirsh, 1998) as well as genes involved in neural development (Norga *et al.,* 2003).

Genetic screens for mutations involved in drug and pesticide resistance in insects have shown that the genetic architecture of drug resistance is dependant on the type of pesticide under study. While resistance to agricultural pesticides not typically encountered by an insect involve only a single or a few genes of large effect (McKenzie and Batterham, 1995), resistance to natural plant toxins involves several genes of small effect, but is neither simple Mendelian nor highly polygenic (Jones, 1998). These studies however have not yet identified any specific loci responsible for conferring drug resistance or sensitivity and very little is known about the genetics of drug response in flies. Therefore, we have begun a screen to locate genetic loci involved in drug response. For our phenotype, we assayed survival time

upon chronic drug exposure as it has proved to be a robust trait with moderate heritability and genetic variation in natural populations (Carrillo and Gibson, 2002). Moreover, the detection of loci affecting genetic variation in flies for behaviors such as ethanol tolerance (Bainton *et al.,* 2000), starvation resistance (Harbison *et al.,* 2004), drug resistance (Jones, 1998) and autonomic reflex behaviors (Ashton *et al.,* 2001), and the complexity of dissecting the genetic architecture for drug response in natural populations (Carrillo and Gibson, 2002) has also encouraged us to initiate a screen for single loci affecting drug response.

Our main findings from the mutagenesis screen can be summarized as follows. (i) There is genetic variation between insertion lines for survival time upon chronic ingestion of nicotine and caffeine. (ii) On average females tend to be 50% more resistant than males to nicotine and caffeine, but survival time between the sexes is highly correlated (R=0.71) for both drug treatments. (iii) The correlation between treatments was low (R=0.22), suggesting that there is genetic variation for survival time, but that resistance to the two drugs occurs through different mechanisms. (iv) The genetic architecture of drug resistance appears to differ between drug treatments, with greater variation and epistatic effects for response to caffeine than nicotine. (v) Drug resistance appears to be a more robust and reproducible trait than drug-specific sensitivity for both nicotine and caffeine treatments.

Results of the analysis of variance show significant genetic variation among the 970 insertion lines (Table 3.1), which is evident in the survival time estimates between extreme lines in both drug treatments (Figure 3.1; 3.3). This is supported by our previous work in isofemale lines (Carrillo and Gibson, 2002) and P-element insertion lines (Wagoner and Gibson, unpublished data)

for drug resistance. Genetic variation for resistance to chronic caffeine exposure has also been demonstrated between different wild-type and mutant strains of *Drosophila* (Nigsch *et al.,* 1977; Zimmering *et al.,* 1977). Sex effects for caffeine resistance have also been previously reported in these studies, with females in most mutant and wild-type strains living longer than males (Zimmering *et al.*, 1977; Ityoyama *et al.*, 1998). Although females tended to be 50% more resistant to both drug treatments than males, there is a high correlation between males and females (R=0.71), indicating that the same genetic factors affect drug response in both sexes.

Comparison of overall drug resistance and sensitivity reveals distinctions between the two traits. These may be due to the differences in the genetic architecture between resistance and sensitivity or as an artifact of the scoring and selection process. In both the initial and subsequent screens, only 10 percent of lines selected for increased resistance to one drug were also resistant to the other, indicating that resistance between nicotine and caffeine is drug-specific. This is supported by correlation estimates between the drug treatments (R=0.22). Lines with increased sensitivity to both drugs on the other hand were quite common and are likely the result of insertions affecting general fitness. To overcome this, lines were selected for decreased resistance on the basis of drug-specific sensitivity. Further replicates of these lines however did not produce consistent results. The majority of lines selected for drug-specific sensitivity in the initial screen were sensitive to both drugs in the second, with the remainder not sensitive to either drug or to the one it was resistant to in the initial screen. After the two screens, a third replicate of all the lines still available from the initial 970 was performed. Comparison of survival time between the third replicate and the initial screen reveals a high correlation among lines selected for resistance, but not drug-

specific sensitivity. These results suggest that drug resistance is a more robust and reproducible trait than drug specific-sensitivity, but this could also be a result of the testing method.

(B) Independent Response to Drug Treatments

To identify genetic factors responsible for variation in drug resistance, insertion mutations affecting drug response will be cloned and localized. Comparison of the response to the two treatments can however provide some insights on the architecture of response to nicotine and caffeine. The correlation between drug treatments (R=.22) indicate that genetic variation is affecting these responses, but that resistance to nicotine and caffeine occurs through independent mechanisms. This is likely if the insertions are affecting resistance through the metabolic pathways for drug metabolism and elimination. These detoxifying enzymes have been suggested as a major player in the evolution of resistance to insecticides and antibiotics (Vesell, 1991; Tenover, 2001; Howard *et al.,* 2002), yet they differ for the metabolism of nicotine and caffeine. Another source of potential genetic variation would be in the absorption of the drug at the cellular level throughout the organism. This can have effects on various pathways from signal transduction to neurotransmitter release at synapses and the modification of gene and protein expression (Balfour and Ridley, 2000; Pietila and Ahtee, 2000). These pathways, while sharing some neurotransmitters and other intermediaries (Betz *et al.,* 200), also differ between nicotine and caffeine and could result in distinct effects on the two treatments.

These drugs also have effects on metabolism and physiology separate from those on the central nervous system. Caffeine, for example, is also a powerful diuretic and appetite suppressant. Its role as an appetite suppressant

might in part account for the high correlation between caffeine and starvation resistance (Carrillo and Gibson, 2002). Alternatively, this correlation could result from the flies undergoing starvation by choosing not to eat the drugged media, either because of its taste or the induced hyperactivity after exposure to caffeine. If this is the case, drug response would also be affected by genetic variation not only in genes involved in the absorption, activity and metabolism of caffeine, but also in genes involved in taste perception. This overlap between resistance to caffeine and starvation may contribute to the higher variation between replicates for caffeine compared to nicotine. Analysis of natural variation for both caffeine and starvation resistance have shown abundant genetic variation and epistatic interactions exist for these traits, but have failed to reveal the genetic architecture underlying them (Kennington *et al.*, 2001; Carrillo and Gibson, 2002).

Differences in the response to these two drugs can also be observed in the backcross experiment. In this experiment, ten lines selected for resistance to each drug were backcrossed to the parental control line to eliminate background genetic variation. Comparison of survival time in these backcrossed lines to the lines they were derived from and the parental control reveals differences between the caffeine and nicotine treatments. For the majority of lines selected for nicotine resistance (9 out of 10), there was no significant difference in survival time between the initial and backcrossed lines, and a significant difference in survival time between the backcrossed and control line (Table 3.3; Figure 3.4). This suggests that in these lines the insertions contribute to the majority of the genetic variation for nicotine resistance. Nicotine response is also a robust trait with reproducible results and low variance between replicates.

For caffeine response however, in the majority of lines (7 out of 10) there was a significant difference between the original and backcrossed lines (Table 3.3; Figure 3.4). These backcrossed lines also had no significant difference in survival time from that of the parental control. This suggests that in these lines, caffeine resistance is affected by genetic variation other than the insertion or interactions between the insertion and other sources of variation in the genetic background. One interpretation of this is that caffeine response is affected by many genes of small effect, which is supported by results on the genetic architecture of caffeine resistance in natural populations (Carrillo and Gibson, 2002). This is also supported by preliminary results in another screen for P-element insertions that suggest that mutations in at least 5% of the genome may affect sensitivity to these drugs (Wagoner and Gibson, unpublished data).

(C) Candidate Genes for Drug Resistance

To dissect the genetic basis of nicotine and caffeine resistance, insertions that conferred an increase in survival time on these treatments were mapped to identify the genes potentially responsible. A total of eleven insertion lines with significant increases in drug resistance, three on caffeine and eight on nicotine treatments, were selected based on the results of the backcrossing tests. In these lines, resistance to the drug treatments did not significantly change after the reduction of variation in the genetic background, suggesting that the insertions were responsible for the majority of the variation in survival time. Of the eleven lines selected four of the insertions could either not be localized or where mapped to genes of unknown function that have not been characterized. Therefore, a total of seven lines, two for caffeine resistance and five for nicotine resistance were mapped to previously identified genes (Table 3.4).

The two insertions that were mapped for caffeine resistance were located in the *corto* and *Pfrx* genes. The *corto* gene encodes a protein that is localized in the nucleus and acts as a general transcription factor. Mutations in the *corto* gene have been isolated that affect several tissues, including the imaginal discs, larval salivary gland and the development of the adult head, abdomen and brain among others. The other insertion was mapped to a gene called *6-phosphofructo-2-kinase*, abbreviated as *Pfrx*. This gene encodes a protein with fructose-2,6-bisphosphate 2-phosphatase activity involved in the metabolism of fructose. The most intriguing aspect of this gene is that an independent insertion line selected for resistance to nicotine was also located in this gene. The potential role of this gene in drug resistance is not clear, but may be related to avoidance of drugged food, perhaps because of its taste or odor, and the resulting starvation response. In fact, in our initial screen for drug response, caffeine resistance was highly correlated with starvation resistance (R=0.57); therefore genes involved in starvation response and metabolism may be involved in resistance to caffeine as well.

In addition to *Prfx*, four other insertions selected for nicotine resistance were mapped to previously identified genes. One of these insertions was located in the *roundabout* gene, abbreviated as *robo*, while the other three were all located in the gene *E2f*. The *robo* gene encodes a protein that acts as a transmembrane receptor involved in axon guidance during development (Zlatic *et al.,* 2003). *Robo* is expressed in the developing embryo in the axons and growth cones of several neurons, including the embryonic and M1 neurons, where it is required to stop axon migration across the CNS midline (Kidd *et al.,* 1998). The last gene identified for nicotine resistance, *E2f*, was the target of three independent insertions isolated for increased resistance. As with *corto,*

the *E2f* gene encodes an RNA polymerase II transcription factor present in the nucleus. *E2f* is also a general transcription factor, and is expressed in the embryo primarily in the midgut, brain and central nervous system where it is involved in the regulation of cell proliferation and DNA replication during S phase (Brook *et al.,* 1996). Although it is expressed largely in the nervous system and brain, *E2f* is a general transcription factor with diverse activity, therefore its specific contribution to drug response is not easy to determine.

Overall, the role of the genes identified for resistance to nicotine and caffeine on drug response is not clear-cut. These genes ranged from general transcription factors expressed during the development of various tissues, including the nervous system and one involved in neuronal differentiation to a gene involved in metabolic processing. This assay however proved quite robust, with one of the genes identified for nicotine resistance being the insertion site of three independent P-elements. For the genes involved in neuronal development or differentiation, possible mechanisms can be theorized, but further work is necessary to verify their specific role in drug resistance. Important aspects of these studies will be to characterize interaction effects between mutations to determine pathways by which they influence drug response and, for the transcription factors, to determine the downstream targets that they regulate. A noteworthy finding of this study is that the genes identified are not those traditionally studied as candidate genes for drug response, which usually focus on synaptic transmission and drug metabolism. Therefore, although synaptic transmission and drug metabolism are clearly important in drug response, this study shows that variation in other unrelated genes can also contribute to phenotypic variation for pharmacogenetic traits.

Chapter 4:
Serotonin Receptor Association

4.1 Introduction

Identifying the segregating genetic basis of multifactorial traits such as drug response is complicated. These traits are typically regulated by multiple genes that individually have very small phenotypic effects. Furthermore, the genes underlying these traits often have substantial interactions with each other and the environment. Therefore model organisms, where the genetic background and environment can be controlled, are very useful. Invertebrates, with their relatively simple nervous system are particularly useful in the study of neurobiological traits, including drug response. Invertebrates have in fact already been used to test for the mechanism of toxicity of several substances (Salanki, 2000; Ballatori and Villalobos, 2002). *Drosophila melanogaster* is particularly suitable as a model system for studying drug response because the basic architecture of its nervous system, as well as the biochemical pathways for drug response, including the neurotransmitter receptors and transporters are similar to those in vertebrates (Hewes and Taghert, 2001; Yoshihara *et al.,* 2001). Also, *Drosophila* responds to psychoactive substances such as ethanol (Heberlein, 2000; Rodan *et al.,* 2002), cocaine (McClung and Hirsh, 1998; Andretic *et al.*, 1999), LSD (Nichols *et al.,* 2002), caffeine (Nigsch *et al.,* 1977; Zimmering *et al.,* 1977), and nicotine (Bainton *et al.,* 2000).

The development of genomic and statistical resources has also increased the utility of model organisms for the study of polygenic traits. Association tests between genetic and phenotypic variation for example can confirm the contribution of candidate genes to a trait, and have already been used to study genetic variation for sensory bristle number in *Drosophila* (Mackay, 1996). Complete sequencing of large regions of candidate genes has also been used to test for associations between single nucleotide

polymorphisms (SNPs) in candidate genes and phenotypic variation, such as in *Egfr* for wing shape (Palsson *et al.,* 2003) and serotonin receptors for heart rate (Nikoh *et al,* 2004). These studies often employ chromosome substitutions into a common background or inbreeding to reduce variation in the genetic background and thereby increase the relative contribution of candidate genes to phenotypic variation, which is important in efforts to understand the genetic basis of drug response.

Among the numerous candidate genes for drug response, those for neurotransmitters such as serotonin have received much interest. In *D. melanogaster*, five serotonin receptor genes, along with genes for synaptic vesicle formation, trafficking and reuptake homologous to those in the vertebrate serotonergic system have been identified (Hewes and Taghert, 2001; Yoshihara *et al.,* 2001). Given that the serotonin receptors are highly conserved across taxa (Hen, 1993; Walker *et al.*, 1996), it is reasonable that there are parallels in their activity between flies and mammals. In humans, SNPs in serotonin receptors and transporters have been associated with several physiological and personality traits, such as depression (Frisch, 1999; Choi *et al.,* 2004), bipolar disorder (Ranade *et al.,* 2003) and schizophrenia (O'Donovan and Owen, 1999; Jonsson *et al.,* 2001) as well as others. Serotonin has also been linked to spatial and incentive learning, as well as the modulation of response to environmental stimuli following experience in nematode worms (Sawin *et al.,* 2000; Saeki *et al.,* 2001) and aggression and social dominance in lobsters (Kravitz, 1988).

More importantly, serotonin has a central role in the behavioral and physiological response to several drugs. This includes the behavioral changes observed in *Drosophila* following the consumption of the hallucinogenic drug

LSD, which is a powerful serotonin receptor agonist (Nichols *et al.,* 2002). Specific serotonin receptor subtypes have also been linked to several of the behavioral responses typically associated with nicotine use (Malin, 2001; Seth *et al.,* 2002). In addition, long term consumption of nicotine and other drugs can alter serotonin release and serotonin receptor density in several brain regions (Gawin, 1991, Balfour and Ridley, 2000) and the destruction or inhibition of serotonergic neurons can reduce drug response (Koob *et al.,* 1998). Furthermore, the behavioral and physiological effects associated with drug withdrawal are believed to result from the unmasking of compensatory adjustments to the serotonergic system during chronic drug use (Seth *et al.,* 2002). Although serotonin has been linked to the addictive and toxic effects of several drugs including nicotine, the mechanism by which serotonin influences these responses is not yet understood.

While several studies have been successful in associating polymorphisms in specific genes, including serotonin receptors and transporters, with behavioral traits, these associations have not always held up. Overall, these studies have met with mixed success and have at times been contradictory, and much is still unknown about the genetic basis of variation for drug response and resistance. The majority of studies have however revealed that serotonin is an important intermediary of several behavioral processes, including drug response. Therefore, to complement our forward genetic screens for genes involved in drug response, we performed a reverse quantitative genetic study in *Drosophila* with the serotonin receptor genes as candidates for drug resistance. Here we present the results of an association test between SNPs in three serotonin receptor genes, 5-HT1A, 5-HT1B and 5-HT2, from two populations of *Drosophila melanogaster* and resistance to nicotine and caffeine.

4.2 Materials and Methods

(A) Drosophila Stocks

Lines used in this study came from two North American populations of *Drosophila melanogaster*. These lines were inbred between fifteen and fifty generations by sib-pair mating of isofemales collected from West End, North Carolina (Palsson *et al.,* 2003) and from Wolfskill, California by Dr. Sergey Nuzhdin at University of California, Davis (Yang and Nuzhdin, 2003). A total of 204 near-isogenic lines were tested, 121 from the North Carolina population and 83 from the California population. For each of these lines, five replicate vials of each sex were phenotyped in independent batches. Each replicate contained ten flies, bringing the total number of flies tested per line to one hundred, namely fifty per sex. All stocks were kept on standard cornmeal medium with yeast in 10 mL vials. Stocks were maintained at 25^0C on a 12-hour light/dark cycle and at a density of 50 to 100 larvae per vial.

(B) Drug Resistance Assay

Drug response assays were performed on adult flies collected between one and three days after emergence. These flies were kept on standard cornmeal media for three days before being separated by sex and placed on drugged food. Ten flies were placed in each vial of drug media for each replicate, with a total of five replicates performed per line. Every twelve hours the number of live flies was counted until all of the flies were dead. For this assay, response to nicotine N-3876 (3 μl/ml) and caffeine C-0750 (10 mg/ml) from Sigma was tested by directly dissolving the drug in molten fly food. Drug media was prepared fresh immediately prior to its use, and a new batch of drug medium was prepared for each replicate trial. Drug medium was always used between one and seven days after preparation.

(C) Sequencing

DNA used for sequencing and restriction digests was isolated using the same protocol. The DNA was extracted from a single male of each near-isogenic line by homogenizing the fly in 50 µl of squishing buffer (10 mM Tris pH 8.2, 25 mM NaCl, 1 mM EDTA and 200 mg/ml Proteinase K). Flies were incubated in the squishing buffer for 30 minutes at 37^0C and then 95^0C for 2 minutes before being stored at -20^0C. Near-isogenic lines used in the association tests were sequenced by Dr. Naruo Nikoh and April Duty (Nikoh *et al.,* 2004) for approximately 13 kb in three serotonin receptor genes, 5-HT1A, 5HT1B and 5-HT2.

(D) Sequence and Statistical Analysis

Common polymorphic sites (less common allele frequency greater than 5%) were selected for association tests using TASSEL (http://www.maizegenetics.com). Only common polymorphic sites were used since rare sites do not offer a large enough sample size to detect significant effects. The three serotonin receptor genes yielded a total of 200 polymorphic sites for analysis. Association tests between the polymorphic sites and survival time on the drug treatments were conducted using SAS Proc MIXED according to the model:

$$Drug\ Response = G + S + P + G*P + G*S + P*S + G*S*P + L + Error$$

where SNP genotype (G), sex (S) and population (P) represent fixed effects. The line term was included as a random effect to control for the pseudo-replication that occurs between the sexes.

The percent variance explained for drug resistance by a particular SNP was inferred from the r-squared value in a one-way ANOVA by sex and population according to the model:

$$Drug\ Response = SNP + Error$$

4.3 Results

(A) Serotonin Sequence Variation

Near-isogenic *Drosophila melanogaster* lines from two populations, one from Wolfskill, California (UCD) and the other from West End, North Carolina (WE), were sequenced for three serotonin receptor genes by Dr. Naruo Nikoh and April Duty (Nikoh *et al.,* 2004). The genes sequenced were the 5-HT1A and 5-HT1B receptor genes, located on chromosome 2R, and the 5-HT2 receptor gene, which is located near the centromere of chromosome 3. A total of approximately 13 kilobases was sequenced from 204 UCD and WE lines (Table 4.1). Complete sequence coverage was not obtained for all of the lines however. This was due to the loss of some lines prior to sequencing and difficulties in the amplification and sequencing of some regions. For the 5-HT1 genes, sequence data was obtained from 83 UCD and 124 WE lines, while for the 5-HT2 gene, sequence was obtained from 83 UCD and 95 WE lines.

For the association test, common variants that occurred in at least five percent of the sequenced lines were selected. Rare sites that occurred in less than five percent of the population were not used as the sample size is not large enough to detect significant effects. This resulted in 187 common SNPs in the two 5-HT1 genes and 13 in the 5-HT2 gene (Table 4.1). The reduction in sequence diversity in the 5-HT2 gene compared to the 5-HT1 region is likely to be a consequence of the 5-HT2 gene's proximity to the centromere. Most of the common polymorphic sites segregate two nucleotide variants, but a handful of sites have three or even all four nucleotides segregating.

Table 4.1 Sequence Length and SNPs

Gene	Sequence Length	SNPs
5-HT1A:	6414 Bases	187 SNPs
5-HT1B:	3454 Bases	
5-HT2:	3030 Bases	13 SNPs

(B) SNP Association Test

Adult flies from both the North Carolina and California populations were assayed for resistance to chronic drug exposure. Resistance to nicotine and caffeine was quantified as survival time on vials containing drugged food, with the number of live flies in each vial counted every twelve hours until all of the flies were deceased. Five replicates of each sex were performed per line, with each replicate containing ten flies for a total of fifty males and fifty females per line. From this, the average survival time for each line was calculated for males and females separately. A total of 187 lines were tested for resistance to each drug, 116 from the North Carolina (WE) population and 71 from the California (UCD) population. Survival time on nicotine and caffeine in both populations is plotted by sex in Figure 4.1. All distributions are platykurtic, particularly for females, but are sufficiently close to normal that no scale transformation was warranted.

Association tests between the polymorphic sites and survival time on nicotine and caffeine were conducted using a mixed model with genotype, sex and population as fixed effects and line as a random effect. The interaction terms between each of the random effects were also included in the model. For the 13 SNPs identified in the 5-HT2 gene, there were no significant SNP or SNP interaction effects at the $p<0.05$ for either drug treatment (data not shown). In fact, the only significant effects after Bonferroni correction for multiple comparisons in the 5-HT2 gene were for sex and sex-by-population interactions. This was expected since previous experiments revealed that females tend to live approximately 50 percent longer than males on both nicotine and caffeine (Carrillo and Gibson, 2002).

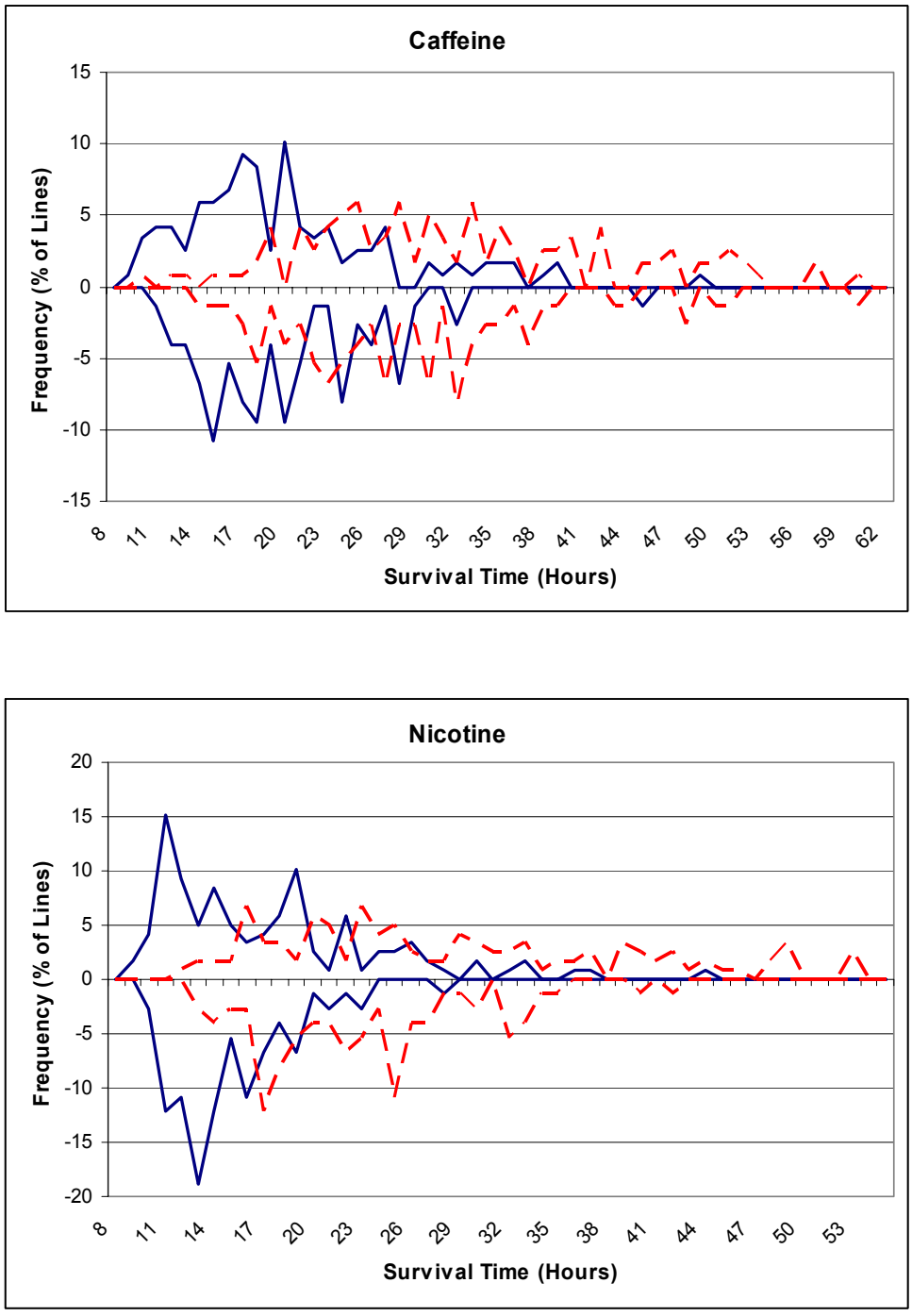
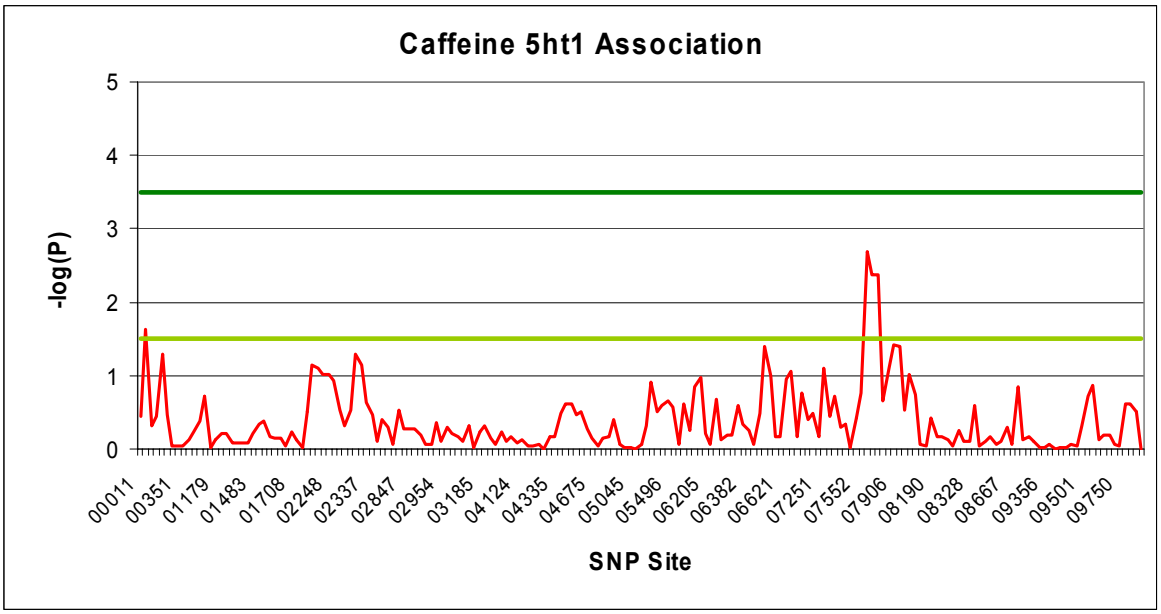


Figure 4.1 Distribution of survival time for WE and UCD lines on nicotine and caffeine treatments. WE lines are plotted above the Y-axis and UCD lines are below the axis, for both males (solid blue) and females (dashed red).

The 187 polymorphic sites in 5-HT1A and 5-HT1B were combined and tested for associations with drug resistance as a single region. At $p<0.05$, significant associations were detected between several SNPs, as well as interaction effects with sex and population, and both caffeine and nicotine resistance (Figure 4.2). However, Bonferroni correction for multiple comparisons and two treatments requires a significance threshold of .00013 (.05/374). There were no significant associations between caffeine resistance and any sites in 5-HT1. The only significant association with caffeine resistance after Bonferroni correction was with sex, which was highly significant ($p<.0001$) for both drug treatments.

However, a highly significant association ($p=2.1*10^{-5}$) with nicotine resistance was detected for one SNP (Site 2R:14183552 GenBank Sequence). This site is a G to A polymorphism in the 3' noncoding region of the 5-HT1A gene (Figure 4.3) that is present in both the UCD and WE lines, with the A allele in approximately 20 percent of the individuals in each population (Table 4.2). The full ANOVA for this site is shown in Table 4.2, and indicates a significant SNP-by-population effect. Subsequently, a separate ANOVA was performed for each population separately, which showed that the association with nicotine resistance is highly significant in the WE population ($p=4.5*10^{-11}$) but not in the UCD population ($p=.3$). This SNP effect therefore appears to be population specific, with the allele present at this locus contributing to variation in nicotine resistance in only the WE population. In the WE population, lines with the A allele at this site are on average more resistant to nicotine than lines with the G allele, while in the UCD lines there is no significant difference in survival time between lines with the two allelic variants (Figure 4.4).



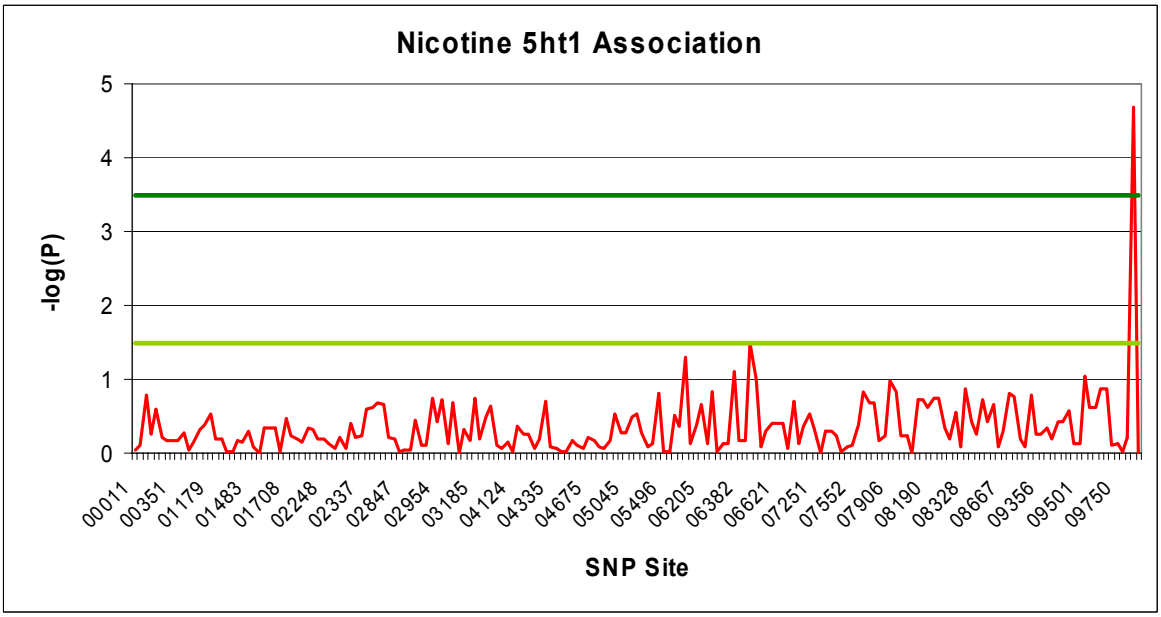


Figure 4.2 Association between SNPs in the 5-HT1B and 5-HT1A genes and nicotine and caffeine resistance. Significance values plotted as the negative logarithm of the p-values against location in the 5HT1 gene region. Plots are of mixed model analysis for genotype effects, and include the threshold at $p<.05$ (1.3) and after Bonferroni correction (3.87).

Table 4.2 Allele Frequency at SNP Site

Population	G Allele	A Allele
UC Davis	.77 (62/81)	.23 (19/81)
West End	.79 (94/119)	.21 (25/119)
Combined	.78 (156/200)	.22 (44/200)

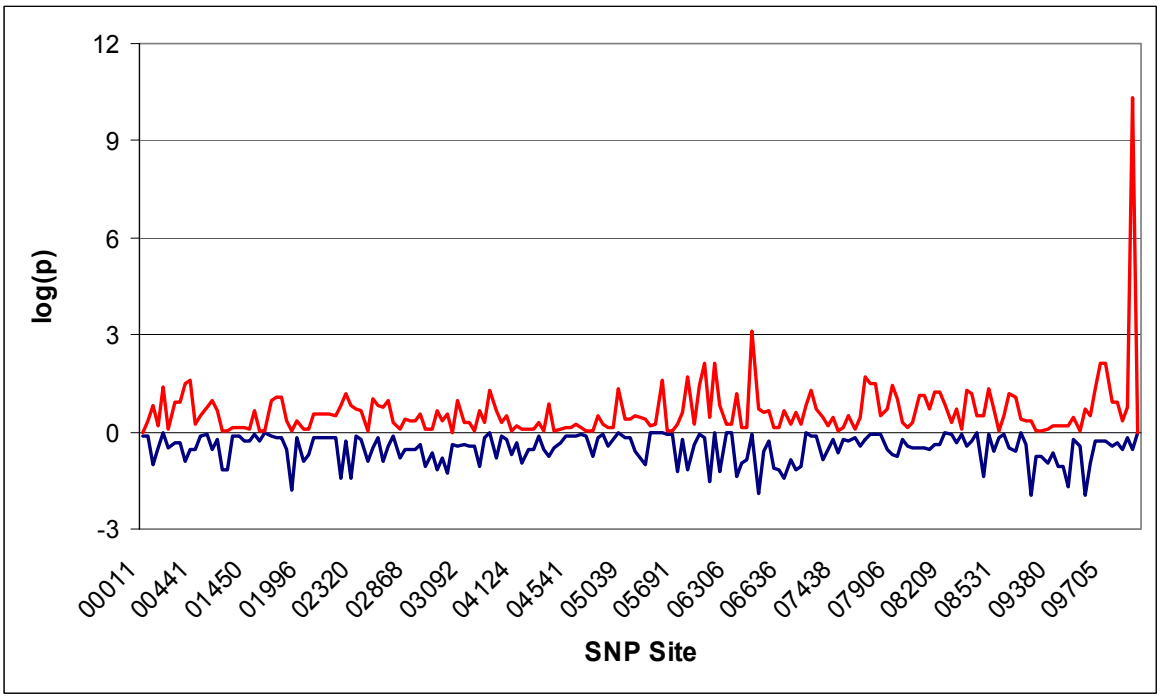


Figure 4.3 Association between SNPs in the 5-HT1B and 5-HT1A genes and nicotine resistance, separated by population. Significance values plotted as the negative logarithm of the p-values against location in the 5HT1 gene region. Plots are of mixed model analysis for genotype with WE lines (red) above the Y-axis and UCD lines (blue) below the axis.

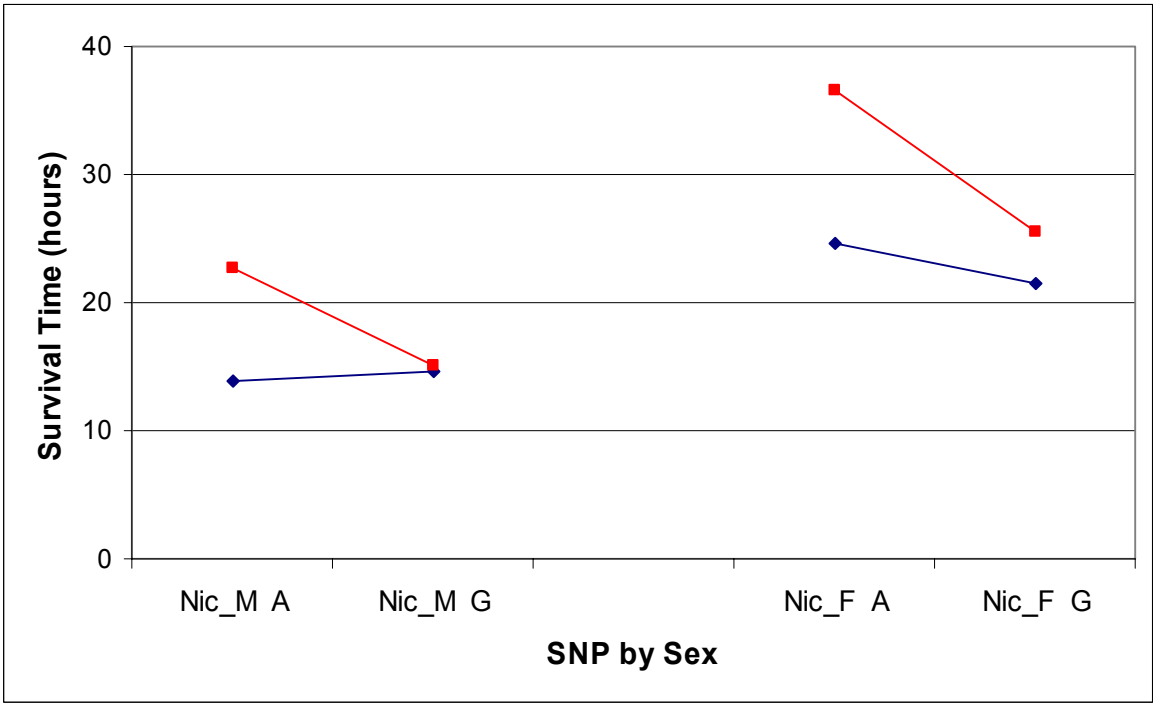


Figure 4.4 Average survival time on nicotine for the two allelic variants in UCD (blue) and WE (red) populations. Average survival time was calculated separately for males (left) and females (right).

4.4 Discussion

(A) Drug Response and the Serotonin Receptors

The components of the serotonergic system, in particular the serotonin receptors and transporters, have long been suggested to influence several behavioral and physiological responses. In humans, polymorphisms in serotonin receptors and transporters have been associated with depression (Frisch, 1999) and schizophrenia (O'Donovan and Owen, 1999; Malhotra *et al.,* 1998). In model organisms, serotonin has been linked to associative and incentive learning (Saeki *et al.,* 2001), and response to several drugs, including cocaine (Gawin, 1991), LSD (Nichols *et al.,* 2002) and nicotine (Seth *et al.,* 2002). Five serotonin receptor genes have been identified in the *Drosophila* genome (Yoshihara *et al.,* 2001) that are highly conserved, with similar molecular structures and activity, as those in vertebrates (Hen, 1993; Walker *et al.*, 1996). In our study, we focused on three serotonin receptors, 5-HT1A, 5-HT1B and 5-HT2, that are known to mediate response to hallucinogens such as LSD (Aghajanian and Marek, 1999) and the anxiolytic and locomotor stimulant effects of nicotine (Seth *et al.,* 2002). These subtypes have also been suggested to affect the development of sensitization following chronic nicotine exposure (Balfour and Ridley, 2000; Seth *et al.,* 2002). The role of these receptors in the addictive and toxic properties of nicotine and in the response to caffeine however has not been extensively examined.

The most compelling evidence for association between the serotonin receptor genes and drug resistance is a polymorphic site in the 3' UTR of 5-HT1A (Figure 4.2). Although the physiological effects of this SNP are not known, studies in *Drosophila* and *C. elegans* have linked *cis*-regulatory elements in the 3' UTR with the regulation of translation (Macdonald, 2001;

Kuersten and Goodwin, 2003; Wilkie *et al.,* 2003). Of the 3' UTRs that function as regulatory elements, most regulate the initiation of translation, while others inhibit protein elongation (Macdonald, 2001). These regulatory elements can affect many biological processes, including body patterning, embryonic axis establishment, sex and cell-fate determination, and neurogenesis (Kuersten and Goodwin, 2003). Furthermore, small micro RNAs (miRNAs) ~20 nucleotides in length have been identified that can repress translation of mRNAs by binding to complementary sequences also located in the 3' UTRs of target genes (Finnegan and Matzke, 2003; Bartel, 2004). Therefore, although the activity of some of these systems has only recently been characterized, they are proving to be common regulators of protein coding genes. These regulatory sequences are conserved between species, as was the 3' UTR surrounding the SNP associated with nicotine resistance in the 5-HT1A gene. The nucleotide sequence for ~300 bases surrounding this SNP was compared with that available from two closely related species, *D. simulans* (2-3 million years divergence) and *D. yakuba* (10 million years divergence). This region was highly conserved between the three species, sharing 95% sequence identity with *D. simulans* and 85% with *D. yakuba* (Table 4.3).

Unlike nicotine, which is known to interact with serotonergic neurons and to alter serotonin release, no link has been established between the behavioral and physiological effects of caffeine and serotonin. These effects of caffeine occur through distinct pathways that primarily involve the adenosine and dopamine systems (Fredholm, 1995; Garret and Griffiths, 1997). Therefore, there was no expectation of associations between polymorphisms in the serotonin receptors and caffeine resistance. Serotonin, as well as dopamine, has nonetheless been suggested as a potential mediator of the addictive and reinforcing properties of many drugs (Koob *et al.,* 1998; Betz *et*

Table 4.3 Sequence Alignment with *D. simulans* and *D. yakuba*

Drosophila simulans

Score = 517 bits (261), Expect = e-145
Identities = 304/317 (95%), Gaps = 1/317 (0%)
Strand = Plus / Plus

```
 Query: 1   ctttaacttatttacatggctttatctagcagactttcgttattctagcaattgtttact
            |||||||||| ||| ||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 92   ctttaacttacttatatggctttatctagcagactttcgttattctagtaattgtttact

Query: 61   tatattgtttaaaaaatatgaaaattgttttccatgcaccttgtccttgtgtgccgcagt
            |||||||| | | ||| ||||||||||||||| |||||||||||||||||||||||||
Sbjct: 152  tatattgtatgataaacatgaaaattgttttccgtgcaccttgtccttgtgtgccgcagt

Query: 121  tattgttattgagttagacaatttagccgacaaccattggaaacattttgaatcgattca
            ||| |||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct: 212  tatggttattgagttagacaatttagccgacagccattggaaacattttgaatcgattca

Query: 181  acgcacacgcaattgcattctttttctgctattgtccctagctgacaagtttcaattttc
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct: 272  acgcacacgcaattgcattctttttctgctattgtccctagctggcaagtttcaattttc

Query: 241  aatgctcgaagcca-gacccgaaattgatattgcctgagttgagtggaaagtgcagcagt
            |||||||||||||| |||||||||||||||||| |||||||||||||||||||||||||
Sbjct: 332  aatgctcgaagccaggacccgaaattgatattgcttgagttgagtggaaagtgcagcagt

Query: 300  ttgcaaaggaaccgaaac 317
            |||||||||| |||||||
Sbjct: 392  ttgcaaaggagccgaaac 409
```

Table 4.3 Continued

Drosophila yakuba

```
        Score = 285 bits (144), Expect = 1e-37
        Identities = 246/292 (85%), Gaps = 14/292 (5%)
        Strand = Plus / Plus


Query:    20 ctttatctagcagactttcgttattctagcaattgtttactta---tat-tgtttaaaaa
             ||| |  | ||| | |||  ||||| ||   ||| | ||| ||   ||  ||  | | |
Sbjct: 41396 cttaaggtcgcaaagttttttattataatgattatgtacgtaatgtaaatgcatgagat

Query:    76 atatgaaaattgttttccatgcaccttgtccttgtgtgccgcagttattgttattgagtt
             ||||||| ||||||| || || ||||||||||||||||||||||||| ||||||||||
Sbjct: 41456 atatgaatattgtttgccgtgtaccttgtccttgtgtgccgcagttatggttattgagtt

Query:   136 agacaatttagccgacaaccattggaaacattttgaatcgattcaacgcacacgcaattg
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 41516 agacaatttagccgacagccattggaaacattttgaatcgattcaacgcacacgcaattg

Query:   196 cattcttttctgctattgt--------ccctagctgacaagtttcaattttcaatgctc
             ||||||||| ||| ||||||        ||||||||| |||||||||||| ||||||||
Sbjct: 41576 cattcttttgctgttattgtgttattgtccctagctggcaagtttcaatttgcaatgctc

Query:   248 gaagccagacccgaaattgatattgcctgagttgagtggaaagtgcagcagt 299
              |||||||||||||||||||||||  |||||||||||||||||||||||
Sbjct: 41636 caagccagacccgaaattgatattgctcgagttgagtggaaagtgcagcagt 41687
```

al., 2000). Our analysis shows that at least in the sequenced segments of the serotonin receptors, and for the available polymorphisms, there was no significant association with caffeine resistance. Likewise, no significant associations were detected between polymorphisms in the 5-HT2 gene and resistance to nicotine or caffeine. However, as was mentioned previously, testing was limited by the low frequency of common polymorphisms in this gene, probably as a consequence of its proximity to the centromere.

(B) Sex and Population Effects

Significant variation was also observed for resistance to nicotine and caffeine between the two populations and sexes. This variation between males and females was expected, as it was present in previous studies in natural populations (Carrillo and Gibson, 2002), mutant and wild type lines (Zimmering *et al.,* 1977; Itoyama *et al.,* 1998) and P-element insertion. In all of these, females tended to live longer than males on both nicotine and caffeine media (Figure Not Shown). However, resistance was highly correlated between males and females (R=0.69 for caffeine; R=0.66 for nicotine), suggesting that the same genetic variants are contributing to the phenotypic variation. Furthermore, after Bonferroni correction no significant SNP-by-sex or three way interactions between SNP, sex and population were present, which supports the argument that the SNP effects are not sex-dependent. Even though there is clearly variation for resistance to these drugs between males and females, the genetic basis of this variation could not be determined.

As stated previously, significant population effects were also observed for nicotine and caffeine resistance. This was most evident in the SNP-by-population interaction for the SNP associated with nicotine resistance (Figure 4.3). This SNP was clearly population specific, with the allelic variant present

at this locus contributing to variation in nicotine resistance in the WE lines but not the UCD lines. In the WE population, lines with the A allele at this site are on average more resistant to nicotine than lines with the G allele, while in the UCD lines there is no significant difference in survival time between lines with the two allelic variants (Figure 4.4). Furthermore, although the A allele is present in only 20% of the WE lines sequenced, it is present in approximately 50% of the lines in the top 25% for nicotine resistance. In the UCD lines however, the A allele is present in approximately 25% of both the total lines sequenced and lines in the top 25% for nicotine resistance. Therefore, for the WE lines the frequency of the A allele is enriched in lines resistant to nicotine. In the WE population, this SNP explained 22% of the variance for nicotine resistance in males and 17% in females. These percentages are likely to be inflated by the Beavis effect of overestimation of the magnitude of effect due to random sampling in discovery screens, but nevertheless are remarkably high for the contribution of a single nucleotide.

The basis of this interaction effect, as well as other SNP-by-population interactions, is likely the result of variation in the genetic background between the two populations. Analysis of multiple sequences of the 5-HT1A gene revealed definite population structure between the two populations for an in intronic haplotype (Nikoh *et al.,* 2004), though this polymorphism is not associated with either heart rate or drug sensitivity in our assays. This divergence between the WE and UCD populations is not consistent with admixture or strong selection, and is more likely the result of weak selection caused by different selection pressures in the two environments (Nikoh et al., 2004). It has been demonstrated that variation in genetic backgrounds can modify the phenotypic expression of some mutations by either suppressing or enhancing their effects. Therefore, this SNP-by-population interaction could

result from an interaction between the SNP and some other site in the genome at different frequencies in the WE and UCD populations because it is responding to unique selection pressures in each environment. Alternatively, the divergence in population at this site could result from linkage between this region and some other site under selection. To resolve this, further tests are required to determine the exact source of this population divergence and its effect on nicotine resistance.

Chapter 5:
Thesis Conclusions

5.1 Project Aims

As discussed previously, dissecting the genetic basis of variation in complex, multifactorial traits such as drug response can be difficult. Furthermore, the contribution of each of the many genes that affect these phenotypic traits is often small and complicated by environmental effects and genotype-environment interactions. Therefore, the analysis of these traits requires robust, reproducible assays and model organisms for which the environment and genetic background can be controlled. With this in mind, we began a study of the basis of genetic variation for drug resistance in *Drosophila melanogaster* by measuring survival time upon chronic drug exposure. Using this assay, we used several methods to investigate aspects of drug resistance and sensitivity. Here I review the main aims of this study and the accomplishments towards each, as well as summarize the general conclusions of this thesis.

(A) Genetic Architecture of Natural Variation

The first aim of this study was to examine the genetic architecture of natural variation for response to the neurotransmitters dopamine, octopamine and tyramine as well as to nicotine and caffeine. This was accomplished by analyzing resistance to these substances in sixteen isofemale lines, which revealed significant genetic differences for resistance to all of these drugs with relatively small or non-significant vial effects. For nicotine and caffeine resistance, significant sex and sex-by-genotype interactions were also observed. This proved that ample genetic variation existed for drug resistance, and that survival time was a robust and reproducible assay for testing this resistance. Furthermore, genetic correlation coefficients between drug treatments were also small, suggesting that the genetic differences

among lines that contribute to extreme drug resistance are different for each drug. To further analyze the genetic architecture of drug resistance, we performed reciprocal crosses of lines with extreme responses to caffeine (i.e., high x low resistance), and measured survival time in the F1, F2 and backcross generations. Analysis of the additivity, dominance and epistatic effects of caffeine resistance by generation means analysis proved to be quite complex however. These results were inconclusive, and did not fit any of the standard quantitative genetic models.

(B) P-element Mutagenesis Screen

The second goal of this study was to identify genes affecting survival time upon chronic exposure to nicotine and caffeine. Approximately 1000 P-element insertion lines, each homozygous for a single insertion, were screened for resistance to nicotine and caffeine. Lines exhibiting significant increase in resistance were backcrossed to the parental lines to reduce variation in the genetic background, which revealed differences in the architecture of caffeine and nicotine resistance. While the insertion lines selected for nicotine resistance were still resistant after backcrossing, lines selected for caffeine resistance were not, suggesting the increase in resistance was due to other variation in the genetic background or interactions between the insertions and the genetic background. Lines that still had a significant increase in resistance to these drugs after backcrossing were characterized to determine the location of the insertions. Overall, nicotine resistance proved to be a reproducible trait, with three independent insertions selected for nicotine resistance located in the same gene. Caffeine resistance and sensitivity to both drugs however, appears to be complex or at least difficult to interpret using these methods.

(C) Candidate Gene Association

Finally, we tested for associations between single nucleotide polymorphisms in three candidate genes and nicotine and caffeine resistance. Three serotonin receptor genes, 5-HT1A, 5-HT1B and 5-HT2, were selected as candidate genes for drug resistance based on previous studies linking serotonin neurotransmission with response to nicotine and other drugs. Approximately 200 lines from two populations, one in NC and the other from CA, were sequenced and phenotyped for drug resistance. Association tests were performed using common polymorphic sites that were present in at least 5% of the lines, resulting in 200 sites for analysis. No significant associations were detected between polymorphisms in any of the serotonin receptor genes and caffeine resistance. For nicotine resistance however, a highly significant association was detected with a SNP in the 3' UTR of the serotonin 5-HT1A receptor gene. This site was also highly significant for SNP-by-population interactions, and showed different responses in the two populations.

5.2 General Conclusions

(A) Genetic Variation for Drug Resistance

Analysis of drug resistance using several experimental designs revealed that abundant genetic variation exists for this trait in *Drosophila melanogaster*. Our study in isofemale lines revealed that natural variation was present for resistance to several neurotransmitters as well as to nicotine and caffeine. A screen of P-element insertion lines also demonstrated that the disruption of specific genes could result in significant increases in drug resistance. Determining the genetic basis of this variation, especially with the relatively large environment and environment-genotype interactions for pharmacological traits, is far more complicated. Furthermore, although genetic variation for drug resistance was evident in these studies, the architecture of this resistance was different between nicotine and caffeine. In fact, genetic correlation estimates showed little correlation between drug treatments (r=.087 in isofemale lines; r=.22 in insertion lines). This is not surprising, as the primary behavioral and physiological effects of these drugs occur through different neurotransmitter systems (Chapter 1.3). Similarities, such as the involvement of dopaminergic neurons and the metabolic enzymes for the removal of these substances do however exist.

These differences in resistance are intriguing in that they may reveal features about the genetic architecture of the response to these drugs. For example, resistance to nicotine proved to be quite robust, with low variation between replicates. Meanwhile, caffeine resistance had higher variation between replicates and also appeared to be more susceptible to genetic background effects. A specific example of this was in the screen of P-element lines that were backcrossed to the parental Samarkand line lacking the

insertion, which had distinct results for resistance to the two drugs. These lines were backcrossed to reduce variation in the genetic background and thus confirm the role of the insertions on drug resistance. For nicotine resistance, the role of the insertions on drug resistance was confirmed and shown to contribute to the majority of the increase in survival time observed. For caffeine resistance in contrast, significant genetic background or background-insertion interactions were observed.

Similar results were observed in our characterization of the genetic architecture of natural quantitative variation for caffeine resistance. Generation mean analysis of survival time revealed that caffeine resistance did not fit any of the standard models, although epistatic interactions between only two or three genes can usually be tested. The complexity of this response is therefore likely to result from the interaction of many genes, each with a relatively small effect on resistance. This could however also result from the numerous targets of caffeine activity, which not only acts as a central nervous system, but also as a diuretic and appetite suppressant among others. In fact, a high correlation was found in the isofemales used for the generation means analysis between resistance to caffeine and to starvation ($r=.57$). Therefore, resistance to caffeine may be a combination of its toxic and neurological effects and to starvation, either from the appetite suppressing properties of caffeine or from avoidance of caffeine media due to its taste or odor.

In addition to the large and robust effects of single P-element insertions on nicotine resistance, candidate gene association studies were also able to associate specific polymorphisms with variation in nicotine resistance. Three serotonin receptor genes were used as candidate genes for nicotine response, since serotonin has been identified as the primary neurotransmitter through

which nicotine exerts its behavioral and physiological effects. Even after Bonferroni correction and in the presence significant SNP-by-population effects, one polymorphic site was significantly associated with nicotine resistance. This showed that genes and even SNPs of large effect contribute to variation for nicotine resistance. In fact, in the WE population, this SNP explained 20% of the variance for nicotine resistance, even though these percentages are likely to be inflated by the Beavis effect of overestimation of the magnitude of effect due to random sampling in discovery screens. Therefore, compared to caffeine resistance, which appears to be affected by many genes of small effect, variation in nicotine resistance appears far less sensitive to variation in the genetic background.

(B) Candidate Genes for Drug Resistance

Although genetic variation for drug resistance was observed in studies of natural quantitative variation and mutagenesis screens of insertion lines, the identification and characterization of candidate genes responsible for this variation is far more difficult. For this reason, many studies on pharmacological traits such as drug response have focused on candidate genes involved in synaptic transmission, such as neurotransmitter receptors and transporters, and the metabolism and excretion of drugs, such as cytochrome P450 and other detoxifying enzymes. Most association studies for drug response, including our own, have focused on neurotransmitters linked to drug response, especially dopamine and serotonin. Candidate gene approaches can be quite successful, as was our association test between polymorphisms in the serotonin receptor genes and nicotine resistance. These approaches however leave unstudied other sources of genetic variation that could influence pharmacological traits but that are not involved, or at least not directly so, in these biochemical pathways.

In addition to those candidate genes and pathways previously identified or suspected of involvement in drug response and resistance, there are several other potential sources of genetic variation for this trait. This can include changes in drug consumption caused by genes governing the ability to taste or smell these compounds or in an organism's response to them. It can also include genes involved in varies changes of drug activity, including absorption, as well as other unrelated pathways. In our P-element mutagenesis screen for example, the genes identified for effects on drug resistance were not in genes typically associated with drug resistance, such as synaptic transmission or drug metabolism and excretion. The genes that were identified ranged from general transcription factors involved in general development as well as the development of the brain and nervous system, to neuronal development and metabolism. These genes are not directly involved in pathways typically associated with drug response, and have not been previously identified for involvement in drug resistance. Strong evidence suggests that these genes are involved in drug response, with multiple independent insertions selected for drug resistance mapped to the same gene in two cases. Therefore, although candidate gene studies can be very useful and practical for the study of complex multifactorial traits, they can miss other sources of genetic variation that can have significant contributions to phenotypic variation. Furthermore, analysis of these unrelated genes and pathways could result in novel targets for pharmacogenetic studies.

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Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
Control	16.4	26	21.2	17.2	23.6	20.4
Control	16	26.8	21.4	15.6	23.2	19.4
Control	17.6	25.6	21.6	17.6	24.8	21.2
Control	15.6	24.4	20	17.2	26.8	22
Control	14	22.4	18.2	14.4	20.8	17.6
Control	14.4	22.8	18.6	15.2	21.2	18.2
Control	16	24	20	15.2	22.4	18.8
Control	13.2	19.6	16.4	15.6	22.4	19
Control	19.8	25.8	22.8	18	25.8	21.9
Control	16	22	19	15.2	21.2	18.2
Control	18.8	24.4	21.6	17.6	24.8	21.2
Control	18.4	28.2	23.3	16.4	23.6	20
80	16.2	26.4	21.3	13.2	24.6	18.9
111	12	15	13.5	10.8	13.2	12
131	15	22.8	18.9	12.6	18.6	15.6
151	13.2	18.6	15.9	14.4	26.4	20.4
177	11.4	21	16.2	22.8	34.8	28.8
180	10.8	24.6	17.7	13.8	19.2	16.5
200	10.8	15.6	13.2	12.6	20.4	16.5
228	18	18	18	16.2	16.2	16.2
291	16.2	22.8	19.5	13.2	15.6	14.4
336	13.2	16.8	15	10.2	15.6	12.9
356	15	19.8	17.4	14.4	25.2	19.8
357	11.4	21.6	16.5	19.8	22.2	21
358	15.6	26.4	21	18	25.8	21.9
369	16.2	22.8	19.5	18.6	28.2	23.4
370	19.2	22.2	20.7	17.4	25.8	21.6
371	13.2	19.2	16.2	13.2	18.6	15.9
372	23.4	24.6	24	17.4	24.6	21
373	15	22.8	18.9	16.2	21.6	18.9
375	10.8	18	14.4	16.8	25.2	21
376	20.4	22.2	21.3	18.6	23.4	21
377	19.2	32.4	25.8	10.2	15	12.6
378	16.2	21.6	18.9	16.2	21.6	18.9
382	13.8	19.8	16.8	15	22.8	18.9
383	14.4	22.8	18.6	16.2	23.4	19.8
386	14.4	28.2	21.3	18	27	22.5
389	17.4	25.8	21.6	18	22.8	20.4
391	16.8	21.6	19.2	17.4	25.2	21.3
459	16.8	20.4	18.6	14.4	22.2	18.3
484	21	30	25.5	15	21.6	18.3
485	14.4	22.8	18.6	12.6	20.4	16.5
488	19.2	36.6	27.9	26.4	34.8	30.6
489	25.2	33.6	29.4	22.2	29.4	25.8
490	13.8	18	15.9	13.8	19.2	16.5

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
498	13.8	25.2	19.5	13.2	18	15.6
501	17.4	28.2	22.8	16.8	24.6	20.7
524	20.4	33	26.7	16.8	24	20.4
525	10.8	19.8	15.3	13.2	21	17.1
528	13.2	15.6	14.4	15	19.2	17.1
604	21.6	35.4	28.5	12.6	22.8	17.7
637	18	27.6	22.8	19.8	25.8	22.8
663	15.6	25.8	20.7	10.2	10.8	10.5
664	15.6	23.4	19.5	16.8	25.2	21
668	14.4	22.8	18.6	21	24.6	22.8
669	13.8	18	15.9	12.6	12	12.3
670	15	18	16.5	16.2	20.4	18.3
683	11.4	12.6	12	15.6	19.2	17.4
712	10.8	28.2	19.5	16.2	28.2	22.2
735	16.8	25.2	21	9	19.2	14.1
737	13.2	17.4	15.3	15.6	22.2	18.9
759	13.2	25.2	19.2	19.8	23.4	21.6
760	13.8	15	14.4	13.2	16.8	15
764	21.6	20.4	21	15	16.8	15.9
766	15.6	24	19.8	13.8	19.2	16.5
767	27	22.8	24.9	23.4	25.8	24.6
780	13.8	18.6	16.2	8.4	15	11.7
789	10.8	15.6	13.2	24	22.2	23.1
790	10.2	15.6	12.9	11.4	17.4	14.4
792	12	19.2	15.6	19.2	26.4	22.8
799	15	24	19.5	14.4	18.6	16.5
816	17.4	27	22.2	21.6	26.4	24
829	13.2	22.8	18	19.8	27	23.4
846	16.2	21	18.6	15.6	19.8	17.7
863	16.2	25.2	20.7	17.4	24	20.7
864	17.4	26.4	21.9	13.2	16.8	15
877	15	22.8	18.9	11.4	15.6	13.5
878	13.8	18.6	16.2	15.6	21.6	18.6
927	24.6	27	25.8	14.4	16.2	15.3
929	9	13.8	11.4	9	13.8	11.4
930	15.6	22.8	19.2	21	28.2	24.6
985	17.4	28.2	22.8	15	20.4	17.7
986	10.8	13.2	12	13.8	16.2	15
987	15.6	21	18.3	16.2	21	18.6
990	12	18	15	13.2	20.4	16.8
992	11.4	15.6	13.5	14.4	19.8	17.1
1006	15	29.4	22.2	13.2	19.8	16.5
1007	16.2	21.6	18.9	19.8	24.6	22.2
1008	21.6	27	24.3	18	26.4	22.2
1009	23.4	24.6	24	16.2	27	21.6

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1010	24	34.8	29.4	15.6	22.8	19.2
1011	25.8	29.4	27.6	13.8	19.8	16.8
1012	13.2	23.4	18.3	10.8	15	12.9
1013	15.6	22.8	19.2	16.2	21	18.6
1014	15.6	18.6	17.1	11.4	15	13.2
1016	15.6	32.4	24	10.8	14.4	12.6
1017	24	25.8	24.9	18.6	27.6	23.1
1018	19.8	27	23.4	19.8	28.2	24
1019	15.6	18.6	17.1	13.2	21.6	17.4
1020	15	20.4	17.7	15	20.4	17.7
1024	21.6	28.2	24.9	18	23.4	20.7
1025	15	16.8	15.9	13.8	19.2	16.5
1026	12.6	12.6	12.6	11.4	15.6	13.5
1027	15	24	19.5	10.8	19.8	15.3
1028	16.2	23.4	19.8	16.8	27.6	22.2
1037	16.2	19.8	18	11.4	16.2	13.8
1043	19.2	27	23.1	16.8	26.4	21.6
1045	15.6	20.4	18	15	21.6	18.3
1046	15.6	19.8	17.7	12	15.6	13.8
1047	16.2	21	18.6	15.6	17.4	16.5
1048	11.4	21	16.2	15.6	21.6	18.6
1049	12	19.8	15.9	11.4	16.2	13.8
1056	16.2	18.6	17.4	15	21	18
1062	18	29.4	23.7	20.4	31.8	26.1
1063	19.2	31.8	25.5	15	23.4	19.2
1065	21	34.8	27.9	18	28.2	23.1
1066	25.2	29.4	27.3	15.6	25.2	20.4
1067	24	32.4	28.2	18.6	28.2	23.4
1068	19.2	29.4	24.3	15.6	26.4	21
1080	14.4	22.8	18.6	16.8	25.8	21.3
1081	13.2	17.4	15.3	15	22.2	18.6
1091	18.6	24.6	21.6	10.8	20.4	15.6
1092	17.4	22.8	20.1	16.2	30	23.1
1097	17.4	19.8	18.6	15	21.6	18.3
1099	13.2	26.4	19.8	10.8	23.4	17.1
1127	16.2	20.4	18.3	18	20.4	19.2
1128	15	21	18	15	19.2	17.1
1129	16.8	21	18.9	14.4	22.2	18.3
1130	14.4	14.4	14.4	21	21	21
1132	17.4	24	20.7	13.2	20.4	16.8
1135	14.4	16.2	15.3	12	13.8	12.9
1136	16.2	19.2	17.7	10.8	19.8	15.3
1137	21	21	21	12.6	12.6	12.6
1138	13.2	18	15.6	15	20.4	17.7
1139	16.2	18.6	17.4	20.4	28.8	24.6

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1142	13.8	18	15.9	16.2	21.6	18.9
1144	14.4	24	19.2	16.2	22.8	19.5
1172	12.6	14.4	13.5	10.8	15.6	13.2
1173	13.8	16.2	15	15	21.6	18.3
1179	14.4	19.2	16.8	15	20.4	17.7
1189	15.6	22.8	19.2	13.2	19.8	16.5
1194	16.8	28.2	22.5	19.2	28.8	24
1195	13.2	19.2	16.2	12.6	19.2	15.9
1196	9	16.8	12.9	9	19.2	14.1
1198	16.2	22.8	19.5	14.4	27	20.7
1214	15.6	19.2	17.4	13.8	19.8	16.8
1215	12.6	22.2	17.4	14.4	22.2	18.3
1216	15.6	31.2	23.4	19.2	30	24.6
1217	10.8	21	15.9	18.6	30	24.3
1218	10.8	18	14.4	16.8	27.6	22.2
1219	15.6	22.8	19.2	16.8	20.4	18.6
1221	18	25.8	21.9	10.8	13.8	12.3
1222	10.8	19.8	15.3	16.2	26.4	21.3
1223	15	23.4	19.2	13.2	20.4	16.8
1224	13.2	22.8	18	14.4	20.4	17.4
1226	20.4	26.4	23.4	24	27.6	25.8
1227	9.6	16.8	13.2	15	24	19.5
1228	15.6	33	24.3	18	30	24
1229	18	29.4	23.7	15.6	24.6	20.1
1231	17.4	33	25.2	19.2	34.2	26.7
1232	13.2	16.8	15	13.2	19.2	16.2
1233	16.8	24.6	20.7	12	16.2	14.1
1242	13.2	19.2	16.2	16.8	24.6	20.7
1243	14.4	21.6	18	21.6	25.8	23.7
1244	16.2	25.8	21	15	24	19.5
1245	13.2	24	18.6	16.2	27	21.6
1246	11.4	16.8	14.1	7.2	12.6	9.9
1247	17.4	26.4	21.9	14.4	24	19.2
1257	15.6	24	19.8	17.4	31.2	24.3
1258	15.6	23.4	19.5	17.4	24	20.7
1258	10.8	18.6	14.7	22.2	32.4	27.3
1259	13.2	24.6	18.9	14.4	21.6	18
1260	18.6	37.8	28.2	16.2	28.8	22.5
1272	17.4	27	22.2	18	30.6	24.3
1274	13.2	21.6	17.4	16.2	27	21.6
1277	10.8	17.4	14.1	9	14.4	11.7
1278	9	16.8	12.9	10.2	21	15.6
1279	13.8	21.6	17.7	13.8	19.2	16.5
1280	16.8	20.4	18.6	15.6	28.8	22.2
1290	18.6	30.6	24.6	14.4	24	19.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1291	12	23.4	17.7	12.6	21.6	17.1
1294	18	19.8	18.9	11.4	13.8	12.6
1295	22.2	25.8	24	18.6	36.6	27.6
1296	14.4	19.8	17.1	15.6	21	18.3
1297	18.6	28.8	23.7	16.2	25.8	21
1299	15.6	22.8	19.2	17.4	25.8	21.6
1314	15.6	27	21.3	19.2	29.4	24.3
1315	7.8	11.4	9.6	9.6	16.2	12.9
1316	13.2	18	15.6	18	22.8	20.4
1321	15.6	19.2	17.4	13.8	19.2	16.5
1324	9	13.2	11.1	18	22.2	20.1
1324	17.4	25.2	21.3	20.4	26.4	23.4
1325	11.4	19.8	15.6	10.8	17.4	14.1
1327	15.6	24	19.8	16.2	27.6	21.9
1330	13.8	20.4	17.1	13.2	22.8	18
1332	18	23.4	20.7	11.4	22.2	16.8
1334	12.6	19.8	16.2	16.2	22.2	19.2
1336	11.4	22.2	16.8	17.4	25.8	21.6
1339	15	22.8	18.9	17.4	23.4	20.4
1340	16.2	28.8	22.5	16.8	26.4	21.6
1341	12.6	25.8	19.2	13.2	22.2	17.7
1342	7.8	10.2	9	10.2	20.4	15.3
1352	9	13.2	11.1	6.6	13.8	10.2
1353	20.4	24	22.2	20.4	22.2	21.3
1353	12.6	18.6	15.6	15.6	19.8	17.7
1354	15.6	22.8	19.2	15	19.8	17.4
1359	19.2	25.8	22.5	9	12	10.5
1361	10.8	18	14.4	10.8	15.6	13.2
1363	18.6	26.4	22.5	18	24.6	21.3
1371	19.2	24.6	21.9	16.8	26.4	21.6
1372	12.6	22.8	17.7	9	12.6	10.8
1373	16.2	25.8	21	18	19.8	18.9
1374	19.8	28.8	24.3	13.2	18.6	15.9
1375	13.2	21	17.1	15.6	18	16.8
1376	15	21	18	15	21.6	18.3
1377	16.2	23.4	19.8	17.4	25.8	21.6
1378	15	23.4	19.2	13.8	18	15.9
1379	11.4	19.8	15.6	12.6	17.4	15
1379	18	28.2	23.1	15	22.8	18.9
1380	16.8	21	18.9	10.2	15.6	12.9
1383	15	17.4	16.2	17.4	25.2	21.3
1385	15.6	23.4	19.5	13.8	18.6	16.2
1387	9	17.4	13.2	13.8	21.6	17.7
1389	11.4	13.2	12.3	13.2	13.2	13.2
1398	19.2	29.4	24.3	24	35.4	29.7

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1402	13.8	13.8	13.8	15.6	15.6	15.6
1409	15	25.8	20.4	15.6	26.4	21
1411	14.4	23.4	18.9	15	22.8	18.9
1412	17.4	26.4	21.9	10.8	15.6	13.2
1413	15.6	19.8	17.7	16.2	17.4	16.8
1414	15.6	24	19.8	16.8	27.6	22.2
1416	15.6	22.8	19.2	20.4	30.6	25.5
1417	9.6	13.2	11.4	15	17.4	16.2
1419	20.4	29.4	24.9	22.8	27.6	25.2
1420	11.4	13.8	12.6	11.4	14.4	12.9
1422	10.2	13.8	12	9	17.4	13.2
1423	11.4	20.4	15.9	13.2	14.4	13.8
1424	15.6	21	18.3	13.8	15.6	14.7
1426	15	20.4	17.7	15.6	25.2	20.4
1427	16.8	22.8	19.8	19.2	28.2	23.7
1428	14.4	24.6	19.5	12	21.6	16.8
1431	14.4	24.6	19.5	16.2	23.4	19.8
1433	14.4	19.2	16.8	16.8	19.2	18
1437	13.2	20.4	16.8	15.6	20.4	18
1438	15	28.8	21.9	10.8	14.4	12.6
1442	15	22.8	18.9	19.2	25.8	22.5
1443	13.8	24	18.9	10.8	15	12.9
1445	18	28.2	23.1	15.6	22.2	18.9
1454	15	24.6	19.8	12	18	15
1464	10.8	19.8	15.3	15.6	18.6	17.1
1466	10.8	16.8	13.8	10.8	13.8	12.3
1468	13.8	18	15.9	12.6	14.4	13.5
1469	12	20.4	16.2	13.2	16.8	15
1471	11.4	16.8	14.1	13.8	18.6	16.2
1472	17.4	27	22.2	13.2	20.4	16.8
1476	16.8	29.4	23.1	13.2	19.2	16.2
1477	13.8	27.6	20.7	16.8	19.8	18.3
1478	13.2	20.4	16.8	10.8	17.4	14.1
1479	12	19.8	15.9	10.8	20.4	15.6
1480	11.4	21	16.2	14.4	18.6	16.5
1481	15	17.4	16.2	10.2	12	11.1
1484	15	22.2	18.6	16.2	22.8	19.5
1485	17.4	22.8	20.1	13.2	15.6	14.4
1486	10.8	16.8	13.8	15.6	20.4	18
1487	14.4	21	17.7	11.4	15.6	13.5
1488	16.8	20.4	18.6	10.2	15.6	12.9
1491	12.6	17.4	15	13.2	20.4	16.8
1493	15.6	24	19.8	17.4	26.4	21.9
1497	15	19.8	17.4	10.8	15	12.9
1499	15.6	25.8	20.7	15	24.6	19.8

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
1509	14.4	24.6	19.5	11.4	16.2	13.8
1510	10.8	17.4	14.1	16.2	24	20.1
1515	13.2	21.6	17.4	12.6	20.4	16.5
1518	17.4	21.6	19.5	13.2	18	15.6
1519	16.2	23.4	19.8	14.4	15.6	15
1520	22.2	29.4	25.8	24.6	27.6	26.1
1526	13.8	21	17.4	13.2	18	15.6
1533	16.2	23.4	19.8	16.2	25.8	21
1536	13.2	24	18.6	11.4	16.2	13.8
1537	10.8	10.8	10.8	12.6	12.6	12.6
1538	15	23.4	19.2	15	22.8	18.9
1540	17.4	27	22.2	15	19.2	17.1
1542	16.2	23.4	19.8	12.6	17.4	15
1543	21.6	35.4	28.5	19.8	30	24.9
1543	19.2	36	27.6	15.6	19.8	17.7
1546	13.2	20.4	16.8	10.2	16.8	13.5
1548	16.2	22.8	19.5	11.4	13.8	12.6
1556	19.2	20.4	19.8	11.4	17.4	14.4
1557	10.2	15	12.6	9.6	13.2	11.4
1561	16.2	26.4	21.3	16.8	22.2	19.5
1562	16.2	22.2	19.2	22.8	27	24.9
1563	19.2	26.4	22.8	19.8	31.8	25.8
1564	15.6	21	18.3	16.8	24	20.4
1565	16.8	24	20.4	13.2	22.2	17.7
1566	14.4	16.2	15.3	10.8	16.2	13.5
1567	15	19.8	17.4	21.6	29.4	25.5
1568	15	24.6	19.8	20.4	27	23.7
1570	15.6	21	18.3	16.2	26.4	21.3
1571	15.6	22.8	19.2	10.8	15	12.9
1572	19.2	28.8	24	15.6	25.2	20.4
1573	12.6	25.2	18.9	15	24	19.5
1575	16.2	22.8	19.5	16.8	28.8	22.8
1576	14.4	19.2	16.8	19.2	23.4	21.3
1582	11.4	16.2	13.8	20.4	21	20.7
1585	13.8	16.2	15	10.2	14.4	12.3
1586	15.6	22.2	18.9	15.6	25.2	20.4
1587	11.4	11.4	11.4	13.2	13.2	13.2
1593	15.6	30.6	23.1	20.4	22.8	21.6
1596	14.4	18	16.2	9	15	12
1597	15.6	21	18.3	18.6	24.6	21.6
1599	13.2	19.2	16.2	10.8	22.2	16.5
1600	17.4	22.2	19.8	15	19.8	17.4
1601	11.4	15.6	13.5	9	13.2	11.1
1602	19.8	19.8	19.8	9	9	9
1603	14.4	25.2	19.8	13.2	19.2	16.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1604	12.6	18	15.3	10.8	15	12.9
1605	15	15	15	11.4	11.4	11.4
1607	18	25.8	21.9	13.8	20.4	17.1
1608	19.2	28.8	24	11.4	22.2	16.8
1609	15	21	18	15	18.6	16.8
1611	10.8	13.8	12.3	13.2	19.2	16.2
1612	9.6	9.6	9.6	11.4	11.4	11.4
1613	12	19.2	15.6	10.8	16.2	13.5
1618	19.2	35.4	27.3	13.2	26.4	19.8
1619	16.8	24.6	20.7	10.8	15.6	13.2
1620	10.2	21	15.6	13.2	19.2	16.2
1623	11.4	17.4	14.4	13.2	20.4	16.8
1625	14.4	15.6	15	12	15.6	13.8
1626	12.6	20.4	16.5	11.4	13.8	12.6
1627	18.6	18.6	18.6	16.8	24.6	20.7
1628	13.2	21	17.1	12.6	21.6	17.1
1628	13.8	22.8	18.3	13.8	15.6	14.7
1629	19.2	25.2	22.2	14.4	21	17.7
1630	7.8	12	9.9	7.8	12.6	10.2
1631	13.2	16.2	14.7	10.2	13.8	12
1632	13.2	20.4	16.8	13.8	20.4	17.1
1633	16.2	22.8	19.5	12	15.6	13.8
1634	16.2	22.8	19.5	11.4	15.6	13.5
1635	12.6	16.8	14.7	10.8	16.2	13.5
1636	10.2	17.4	13.8	11.4	19.8	15.6
1637	10.8	20.4	15.6	10.2	20.4	15.3
1638	15	19.2	17.1	20.4	23.4	21.9
1641	13.8	25.8	19.8	14.4	21	17.7
1643	9	13.2	11.1	10.8	14.4	12.6
1644	10.8	18.6	14.7	12	19.2	15.6
1645	13.2	19.8	16.5	12.6	17.4	15
1646	13.2	16.8	15	13.2	18	15.6
1647	13.2	21.6	17.4	12	18	15
1649	21	33	27	10.8	16.2	13.5
1654	17.4	20.4	18.9	13.8	15.6	14.7
1656	13.2	12.6	12.9	19.2	24	21.6
1659	21	27.6	24.3	15	24	19.5
1659	15	30	22.5	16.8	28.2	22.5
1660	16.2	24	20.1	19.2	18	18.6
1661	16.2	24.6	20.4	15.6	24	19.8
1662	13.2	15.6	14.4	16.8	18	17.4
1664	15	19.8	17.4	13.8	18.6	16.2
1665	11.4	19.2	15.3	21	29.4	25.2
1665	20.4	25.8	23.1	15.6	24	19.8
1671	20.4	27.6	24	27.6	23.4	25.5

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
1672	11.4	14.4	12.9	15.6	19.8	17.7
1674	18.6	27.6	23.1	12.6	17.4	15
1683	14.4	16.8	15.6	10.8	16.2	13.5
1684	11.4	16.8	14.1	10.8	16.2	13.5
1685	20.4	29.4	24.9	18.6	27.6	23.1
1686	17.4	24	20.7	13.8	21.6	17.7
1687	15	26.4	20.7	12	18	15
1688	16.2	18.6	17.4	24.6	19.2	21.9
1689	16.2	26.4	21.3	16.2	24	20.1
1692	17.4	22.2	19.8	16.2	13.8	15
1693	10.2	10.8	10.5	11.4	22.2	16.8
1696	16.8	20.4	18.6	18.6	25.2	21.9
1697	12.6	16.8	14.7	18.6	20.4	19.5
1705	16.8	23.4	20.1	17.4	26.4	21.9
1709	13.2	22.8	18	16.8	20.4	18.6
1711	19.2	28.2	23.7	21	20.4	20.7
1712	16.8	24.6	20.7	13.2	18.6	15.9
1713	13.8	24	18.9	18.6	25.8	22.2
1714	15.6	25.8	20.7	19.2	25.8	22.5
1715	19.2	30	24.6	10.2	21.6	15.9
1716	10.8	15.6	13.2	12.6	18.6	15.6
1717	19.2	27	23.1	11.4	20.4	15.9
1719	10.8	21.6	16.2	14.4	20.4	17.4
1720	13.8	19.2	16.5	10.8	15	12.9
1721	24	28.8	26.4	21.6	24	22.8
1722	11.4	17.4	14.4	12.6	16.8	14.7
1724	12	16.8	14.4	13.2	17.4	15.3
1725	16.8	23.4	20.1	14.4	20.4	17.4
1726	10.8	21	15.9	10.8	21.6	16.2
1727	15	16.8	15.9	16.2	19.8	18
1728	16.8	27.6	22.2	18.6	24	21.3
1729	11.4	18.6	15	19.8	22.8	21.3
1730	9	12.6	10.8	10.8	12	11.4
1732	18	22.8	20.4	9.6	13.2	11.4
1733	15.6	18.6	17.1	14.4	16.2	15.3
1735	13.2	21	17.1	12	22.2	17.1
1736	16.8	24.6	20.7	12	30	21
1739	15	22.8	18.9	12.6	19.8	16.2
1740	15.6	19.8	17.7	12	16.8	14.4
1740	13.2	21	17.1	9.6	19.8	14.7
1741	10.8	19.8	15.3	9.6	15	12.3
1743	19.8	25.2	22.5	13.2	15	14.1
1744	12	19.2	15.6	8.4	10.2	9.3
1745	15	27.6	21.3	13.2	21	17.1
1748	20.4	26.4	23.4	18	26.4	22.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
1756	10.2	14.4	12.3	10.8	14.4	12.6
1757	16.2	20.4	18.3	20.4	18.6	19.5
1761	9	10.8	9.9	10.2	15	12.6
1763	12.6	21.6	17.1	11.4	15	13.2
1769	11.4	21.6	16.5	12	14.4	13.2
1770	12.6	18	15.3	22.8	30	26.4
1778	17.4	30.6	24	16.8	20.4	18.6
1779	10.8	15	12.9	10.8	19.2	15
1780	16.8	25.8	21.3	16.2	21	18.6
1783	13.2	25.8	19.5	17.4	27	22.2
1784	11.4	15	13.2	13.8	18	15.9
1785	12.6	19.2	15.9	15	22.2	18.6
1786	16.8	24	20.4	18.6	24	21.3
1787	18.6	22.8	20.7	10.2	13.8	12
1797	16.8	25.8	21.3	15.6	21.6	18.6
1798	16.2	25.2	20.7	10.8	17.4	14.1
1803	15	21.6	18.3	9	13.8	11.4
1818	18	17.4	17.7	10.8	15	12.9
1820	10.2	16.8	13.5	20.4	24.6	22.5
1821	15.6	19.2	17.4	7.8	13.2	10.5
1822	18.6	18.6	18.6	15	15	15
1828	8.4	14.4	11.4	9	12.6	10.8
1830	9	15	12	9	14.4	11.7
1831	12.6	20.4	16.5	10.2	15	12.6
1835	19.8	27.6	23.7	15.6	19.8	17.7
1836	12	22.2	17.1	11.4	15.6	13.5
1838	14.4	22.8	18.6	11.4	13.8	12.6
1839	16.2	24.6	20.4	13.2	22.2	17.7
1841	18	27.6	22.8	15	21.6	18.3
1845	19.2	34.2	26.7	12.6	19.8	16.2
1846	14.4	29.4	21.9	19.2	24.6	21.9
1848	13.2	26.4	19.8	10.8	15.6	13.2
1856	12	14.4	13.2	9.6	12	10.8
1857	16.8	24	20.4	11.4	16.8	14.1
1858	10.8	17.4	14.1	10.2	11.4	10.8
1859	20.4	31.2	25.8	15	20.4	17.7
1860	13.2	13.2	13.2	16.2	16.2	16.2
1862	13.8	24	18.9	12	22.2	17.1
1863	12	15.6	13.8	9	12.6	10.8
1878	16.8	33	24.9	20.4	22.2	21.3
1880	12.6	21.6	17.1	12.6	21	16.8
1888	10.8	24	17.4	9	15	12
1889	13.8	25.8	19.8	10.8	19.8	15.3
1889	22.2	26.4	24.3	22.8	19.8	21.3
1891	19.2	30	24.6	10.2	16.2	13.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
1892	10.2	19.8	15	10.2	15.6	12.9
1893	15	24.6	19.8	13.2	25.2	19.2
1896	15.6	21.6	18.6	7.8	12.6	10.2
1897	15	16.2	15.6	16.8	18.6	17.7
1898	15.6	15.6	15.6	18	18	18
1899	18	27.6	22.8	16.8	27	21.9
1900	9	16.8	12.9	10.2	15	12.6
1902	11.4	11.4	11.4	13.2	13.2	13.2
1903	10.2	18	14.1	15	21	18
1907	13.2	19.2	16.2	12	18.6	15.3
1908	22.8	28.8	25.8	16.8	19.2	18
1909	10.8	21.6	16.2	10.8	16.8	13.8
1911	10.8	22.2	16.5	10.8	19.2	15
1912	14.4	18	16.2	21.6	22.8	22.2
1913	19.2	31.2	25.2	16.2	23.4	19.8
1914	13.2	21.6	17.4	14.4	34.2	24.3
1915	12.6	17.4	15	9	16.8	12.9
1916	12	16.2	14.1	10.8	20.4	15.6
1925	10.8	20.4	15.6	13.2	19.8	16.5
1928	15.6	19.2	17.4	16.8	24	20.4
1947	13.2	19.2	16.2	11.4	16.2	13.8
1948	14.4	23.4	18.9	10.8	21	15.9
1949	9.6	14.4	12	10.8	12	11.4
1950	13.2	18	15.6	13.2	19.2	16.2
1959	12.6	13.8	13.2	15	23.4	19.2
1960	13.2	18.6	15.9	12.6	19.8	16.2
1968	10.8	18.6	14.7	16.2	24.6	20.4
1971	10.8	21.6	16.2	13.2	21	17.1
1972	13.2	19.8	16.5	12	24.6	18.3
1974	12.6	14.4	13.5	13.8	17.4	15.6
1976	16.2	17.4	16.8	18	22.8	20.4
1977	16.2	19.2	17.7	17.4	19.2	18.3
1979	18.6	30.6	24.6	13.2	21	17.1
1981	15.6	22.8	19.2	19.8	22.8	21.3
1985	21.6	31.2	26.4	21.6	28.8	25.2
1986	16.8	24	20.4	11.4	20.4	15.9
1989	12.6	22.8	17.7	11.4	21.6	16.5
1990	17.4	18	17.7	19.2	22.8	21
1993	15	19.2	17.1	16.2	19.2	17.7
2003	15.6	27	21.3	10.8	16.8	13.8
2009	13.2	19.2	16.2	13.2	16.2	14.7
2010	10.2	15	12.6	14.4	22.8	18.6
2014	9	11.4	10.2	11.4	19.8	15.6
2018	13.2	19.8	16.5	11.4	16.8	14.1
2019	29.4	29.4	29.4	14.4	26.4	20.4

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2021	16.2	26.4	21.3	13.2	23.4	18.3
2022	11.4	11.4	11.4	10.2	10.2	10.2
2023	18	24	21	10.8	15	12.9
2029	15	25.8	20.4	13.2	18	15.6
2031	16.8	22.8	19.8	16.8	19.2	18
2032	16.8	18	17.4	19.2	18.6	18.9
2034	15	27	21	11.4	25.2	18.3
2035	12	15	13.5	10.8	20.4	15.6
2042	11.4	13.2	12.3	9.6	12	10.8
2044	10.8	18.6	14.7	10.2	15	12.6
2045	10.8	15.6	13.2	10.2	14.4	12.3
2046	18	19.8	18.9	19.8	26.4	23.1
2049	18	28.8	23.4	11.4	20.4	15.9
2051	15	24	19.5	18.6	23.4	21
2051	19.2	25.2	22.2	16.2	19.2	17.7
2052	13.2	21	17.1	11.4	19.8	15.6
2053	11.4	12	11.7	17.4	24.6	21
2055	14.4	18	16.2	15.6	21	18.3
2058	8.4	12.6	10.5	9	12.6	10.8
2061	9.6	13.2	11.4	17.4	23.4	20.4
2062	10.2	16.8	13.5	12.6	21.6	17.1
2063	12	16.2	14.1	13.2	22.2	17.7
2064	15.6	18	16.8	18	26.4	22.2
2065	11.4	15.6	13.5	9	16.8	12.9
2067	9	12.6	10.8	12	19.2	15.6
2068	14.4	22.8	18.6	10.8	16.2	13.5
2069	15.6	18	16.8	16.8	27	21.9
2072	14.4	22.2	18.3	10.2	16.2	13.2
2077	13.2	19.2	16.2	13.2	22.2	17.7
2081	14.4	21	17.7	10.8	16.8	13.8
2082	15	18	16.5	15.6	21.6	18.6
2084	18	21	19.5	12.6	14.4	13.5
2086	10.2	18	14.1	13.2	22.2	17.7
2087	15.6	21.6	18.6	16.2	24	20.1
2088	15	29.4	22.2	14.4	19.8	17.1
2094	16.2	20.4	18.3	24.6	26.4	25.5
2095	13.2	20.4	16.8	13.2	22.2	17.7
2098	13.8	21.6	17.7	13.2	25.8	19.5
2100	11.4	16.2	13.8	10.2	13.8	12
2102	16.8	28.8	22.8	10.8	21	15.9
2104	12.6	19.2	15.9	22.2	25.2	23.7
2106	11.4	17.4	14.4	9	15.6	12.3
2107	10.8	18	14.4	14.4	16.2	15.3
2108	9	15	12	13.2	16.2	14.7
2109	19.2	28.8	24	21.6	20.4	21

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2111	9	12.6	10.8	9	16.2	12.6
2112	13.2	15	14.1	13.2	15.6	14.4
2113	12.6	19.2	15.9	13.2	20.4	16.8
2114	9.6	12	10.8	10.2	13.2	11.7
2115	9	12.6	10.8	10.2	16.2	13.2
2116	18.6	30.6	24.6	10.2	15.6	12.9
2117	12	21	16.5	9.6	20.4	15
2118	13.2	24	18.6	10.2	18.6	14.4
2121	9	13.2	11.1	9	15.6	12.3
2123	16.2	23.4	19.8	19.8	24	21.9
2125	10.2	13.8	12	10.2	15.6	12.9
2126	12	15.6	13.8	12	13.2	12.6
2127	12	23.4	17.7	18.6	21	19.8
2128	13.2	19.8	16.5	15	22.8	18.9
2130	12.6	19.2	15.9	9	15	12
2131	12.6	20.4	16.5	15	19.8	17.4
2132	9	13.2	11.1	13.2	19.2	16.2
2133	12	18.6	15.3	18	22.2	20.1
2135	13.8	18	15.9	9	14.4	11.7
2136	12.6	20.4	16.5	12.6	22.8	17.7
2138	10.2	15.6	12.9	11.4	15	13.2
2141	10.8	14.4	12.6	13.2	24	18.6
2143	11.4	18.6	15	13.2	18	15.6
2144	15.6	17.4	16.5	9.6	18	13.8
2152	15	27.6	21.3	18	24.6	21.3
2153	10.8	18	14.4	9	16.8	12.9
2154	13.2	16.2	14.7	10.8	16.2	13.5
2157	16.2	24.6	20.4	18	17.4	17.7
2158	15.6	26.4	21	9	14.4	11.7
2159	24	29.4	26.7	17.4	25.2	21.3
2160	13.8	19.8	16.8	10.8	15.6	13.2
2167	18.6	27	22.8	21.6	33	27.3
2169	12.6	16.2	14.4	10.8	21	15.9
2170	16.2	20.4	18.3	21	20.4	20.7
2171	14.4	19.2	16.8	10.8	17.4	14.1
2173	18.6	18.6	18.6	15	15	15
2174	9.6	16.2	12.9	12	18	15
2175	16.8	31.8	24.3	19.2	26.4	22.8
2178	15.6	20.4	18	12	15	13.5
2180	15.6	27	21.3	16.8	23.4	20.1
2181	14.4	22.2	18.3	16.2	18.6	17.4
2182	13.2	21	17.1	20.4	24	22.2
2183	16.8	16.2	16.5	15	20.4	17.7
2185	16.2	18	17.1	19.8	26.4	23.1
2188	10.2	19.8	15	17.4	22.8	20.1

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2189	9	13.2	11.1	11.4	18	14.7
2190	9	10.8	9.9	11.4	21	16.2
2192	18.6	21	19.8	17.4	29.4	23.4
2194	8.4	13.2	10.8	7.2	10.8	9
2195	11.4	19.2	15.3	15.6	21	18.3
2197	13.2	17.4	15.3	7.8	16.8	12.3
2198	10.2	15.6	12.9	11.4	19.8	15.6
2199	10.2	14.4	12.3	11.4	16.8	14.1
2200	13.2	13.2	13.2	13.8	13.8	13.8
2201	25.2	30	27.6	15	24.6	19.8
2202	13.8	22.2	18	15.6	22.2	18.9
2203	14.4	16.8	15.6	10.2	11.4	10.8
2204	13.8	15	14.4	15.6	18	16.8
2206	13.2	21	17.1	13.8	22.8	18.3
2207	13.8	18	15.9	13.2	16.8	15
2209	9	14.4	11.7	15	20.4	17.7
2210	11.4	17.4	14.4	13.2	19.8	16.5
2211	10.8	14.4	12.6	9	13.2	11.1
2212	10.2	12.6	11.4	12.6	19.8	16.2
2217	9.6	15	12.3	11.4	21	16.2
2219	11.4	17.4	14.4	15	21	18
2225	16.2	28.2	22.2	16.8	22.2	19.5
2227	11.4	13.8	12.6	9.6	14.4	12
2230	10.8	17.4	14.1	9.6	15	12.3
2233	16.2	27.6	21.9	15.6	19.2	17.4
2234	12	13.8	12.9	15.6	21	18.3
2236	11.4	19.8	15.6	16.2	24	20.1
2238	16.2	28.2	22.2	18.6	25.8	22.2
2239	14.4	21.6	18	19.2	28.2	23.7
2240	13.8	24	18.9	15	23.4	19.2
2241	9	14.4	11.7	15	19.8	17.4
2242	15	22.8	18.9	16.8	25.2	21
2242	10.8	16.8	13.8	15.6	21	18.3
2245	9	13.8	11.4	15	20.4	17.7
2246	12	21	16.5	12	16.8	14.4
2248	16.2	33	24.6	18.6	22.8	20.7
2249	11.4	16.8	14.1	19.8	23.4	21.6
2251	18	23.4	20.7	19.8	23.4	21.6
2252	15	18	16.5	18.6	22.2	20.4
2253	12.6	24	18.3	11.4	17.4	14.4
2257	20.4	30.6	25.5	20.4	25.2	22.8
2262	15	27	21	16.2	24.6	20.4
2263	13.2	24.6	18.9	10.2	17.4	13.8
2266	15.6	20.4	18	14.4	14.4	14.4
2268	13.2	24	18.6	13.2	19.2	16.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2271	15	16.8	15.9	13.2	19.8	16.5
2273	17.4	24.6	21	23.4	25.2	24.3
2275	16.2	22.2	19.2	16.8	21.6	19.2
2276	11.4	20.4	15.9	15	19.2	17.1
2279	15	23.4	19.2	19.2	31.8	25.5
2281	12	12	12	16.8	16.8	16.8
2282	13.8	30	21.9	10.8	14.4	12.6
2283	16.8	28.2	22.5	13.8	16.8	15.3
2284	19.8	27.6	23.7	22.2	22.2	22.2
2285	11.4	19.8	15.6	15.6	22.2	18.9
2286	11.4	20.4	15.9	13.2	18	15.6
2287	18	24.6	21.3	13.2	19.8	16.5
2289	23.4	19.8	21.6	18.6	25.2	21.9
2291	21.6	31.8	26.7	15.6	24	19.8
2292	13.2	24	18.6	18	22.2	20.1
2294	9.6	18	13.8	13.2	19.2	16.2
2295	12.6	17.4	15	10.2	15.6	12.9
2297	13.2	22.2	17.7	12	18	15
2299	10.2	16.2	13.2	16.8	24.6	20.7
2306	13.8	19.8	16.8	15	25.8	20.4
2309	10.8	18.6	14.7	10.8	16.8	13.8
2311	17.4	19.2	18.3	10.8	21	15.9
2311	16.2	18.6	17.4	17.4	16.8	17.1
2312	16.8	28.8	22.8	15	22.8	18.9
2314	13.8	15.6	14.7	13.2	21	17.1
2317	19.8	24.6	22.2	13.8	25.2	19.5
2320	18	28.8	23.4	12.6	22.8	17.7
2321	13.2	21.6	17.4	11.4	16.8	14.1
2326	10.2	14.4	12.3	15	20.4	17.7
2327	10.2	16.2	13.2	17.4	24.6	21
2328	7.8	9	8.4	9	16.2	12.6
2330	13.2	19.8	16.5	14.4	29.4	21.9
2334	10.2	19.2	14.7	11.4	22.8	17.1
2335	10.2	15.6	12.9	10.8	16.8	13.8
2337	9.6	16.2	12.9	9.6	15	12.3
2339	18.6	24.6	21.6	16.8	28.8	22.8
2340	15	19.8	17.4	15	19.8	17.4
2341	18	21	19.5	20.4	27.6	24
2342	9	15	12	9.6	15	12.3
2344	11.4	18.6	15	10.8	16.2	13.5
2345	13.2	23.4	18.3	12	20.4	16.2
2346	16.8	33.6	25.2	12.6	23.4	18
2347	13.2	14.4	13.8	11.4	16.8	14.1
2348	10.8	25.8	18.3	19.8	26.4	23.1
2350	10.2	15.6	12.9	10.8	16.2	13.5

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
2351	14.4	24.6	19.5	11.4	16.8	14.1
2352	17.4	23.4	20.4	14.4	20.4	17.4
2354	19.2	19.2	19.2	17.4	17.4	17.4
2355	11.4	24.6	18	14.4	19.2	16.8
2356	15	21	18	18.6	24.6	21.6
2358	18	34.8	26.4	13.2	16.2	14.7
2359	9	14.4	11.7	15	19.8	17.4
2361	13.2	27	20.1	12.6	21.6	17.1
2362	12	13.2	12.6	14.4	22.2	18.3
2365	17.4	19.8	18.6	13.8	17.4	15.6
2368	9.6	13.8	11.7	9.6	20.4	15
2369	16.2	19.2	17.7	16.8	22.2	19.5
2372	11.4	15.6	13.5	13.8	18.6	16.2
2372	11.4	17.4	14.4	10.8	13.2	12
2376	16.8	17.4	17.1	12.6	20.4	16.5
2377	16.2	22.8	19.5	21	22.2	21.6
2380	18	30.6	24.3	19.2	24.6	21.9
2384	18.6	20.4	19.5	28.8	33.6	31.2
2386	12.6	18.6	15.6	16.8	24.6	20.7
2387	12	16.8	14.4	12.6	18	15.3
2388	16.8	18	17.4	12.6	19.2	15.9
2391	17.4	28.2	22.8	13.8	19.8	16.8
2393	15.6	22.8	19.2	28.2	22.8	25.5
2394	12.6	20.4	16.5	16.8	25.2	21
2395	13.2	24	18.6	17.4	24	20.7
2398	13.2	18.6	15.9	13.2	19.2	16.2
2400	22.8	21	21.9	25.2	33.6	29.4
2406	12.6	19.8	16.2	7.8	9	8.4
2407	14.4	24	19.2	15	22.2	18.6
2408	10.2	14.4	12.3	13.2	13.2	13.2
2410	15	20.4	17.7	15.6	21.6	18.6
2412	12.6	20.4	16.5	15	17.4	16.2
2413	15	20.4	17.7	20.4	25.2	22.8
2414	15	25.8	20.4	15	29.4	22.2
2415	22.8	31.2	27	18.6	24	21.3
2416	19.2	27.6	23.4	27	29.4	28.2
2418	13.2	23.4	18.3	10.2	18	14.1
2419	13.2	18.6	15.9	16.2	21	18.6
2420	16.8	25.2	21	20.4	24	22.2
2422	27.6	30.6	29.1	20.4	27.6	24
2423	20.4	29.4	24.9	20.4	27	23.7
2434	22.8	25.8	24.3	16.8	19.8	18.3
2435	10.2	16.2	13.2	8.4	13.2	10.8
2436	11.4	20.4	15.9	10.2	16.8	13.5
2437	18	28.2	23.1	12.6	17.4	15

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2439	13.8	24.6	19.2	12.6	19.2	15.9
2440	12	21.6	16.8	10.2	16.8	13.5
2444	15.6	25.8	20.7	18.6	24.6	21.6
2447	15.6	22.8	19.2	15.6	20.4	18
2448	16.8	25.8	21.3	17.4	25.2	21.3
2449	15.6	22.8	19.2	13.8	21.6	17.7
2450	19.8	28.2	24	22.8	26.4	24.6
2452	18	26.4	22.2	13.2	16.8	15
2454	10.8	20.4	15.6	18.6	28.2	23.4
2456	17.4	22.8	20.1	12.6	19.8	16.2
2457	22.8	28.2	25.5	13.8	26.4	20.1
2459	10.8	18	14.4	10.8	16.8	13.8
2461	10.2	17.4	13.8	12	19.2	15.6
2462	20.4	20.4	20.4	19.2	19.2	19.2
2464	11.4	18.6	15	14.4	18	16.2
2465	10.8	18.6	14.7	11.4	15.6	13.5
2466	15.6	19.2	17.4	11.4	18	14.7
2469	16.2	24	20.1	10.8	20.4	15.6
2470	21	34.2	27.6	14.4	19.8	17.1
2471	13.2	23.4	18.3	13.8	19.2	16.5
2472	13.2	17.4	15.3	15	21.6	18.3
2473	10.8	10.8	10.8	17.4	17.4	17.4
2474	10.8	18	14.4	9.6	13.2	11.4
2478	9	15.6	12.3	9.6	15.6	12.6
2479	12	17.4	14.7	15	22.8	18.9
2480	11.4	19.2	15.3	15	16.8	15.9
2486	28.2	36	32.1	14.4	29.4	21.9
2487	19.2	36	27.6	17.4	27	22.2
2488	10.2	13.8	12	7.8	10.8	9.3
2489	13.2	15.6	14.4	25.2	24.6	24.9
2490	12.6	28.2	20.4	10.8	19.8	15.3
2491	11.4	18.6	15	15.6	22.2	18.9
2492	16.2	34.8	25.5	17.4	25.8	21.6
2493	15	19.2	17.1	17.4	25.2	21.3
2494	13.2	15.6	14.4	7.8	9	8.4
2495	18.6	27	22.8	15.6	19.8	17.7
2497	15	22.8	18.9	13.2	20.4	16.8
2498	16.2	19.2	17.7	16.2	25.8	21
2499	19.8	28.2	24	13.8	24.6	19.2
2501	18	27	22.5	15	27	21
2502	16.8	16.8	16.8	17.4	27	22.2
2503	18	24.6	21.3	21	33.6	27.3
2505	21	19.8	20.4	16.2	24	20.1
2505	18.6	18.6	18.6	16.2	19.2	17.7
2506	19.2	28.8	24	27	28.8	27.9

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf M	Caf F	Caf Avg	Nic M	Nic F	Nic Avg
2508	16.2	24.6	20.4	16.8	21.6	19.2
2509	13.8	24.6	19.2	9	14.4	11.7
2510	16.2	29.4	22.8	12	18	15
2511	19.8	30	24.9	15.6	22.2	18.9
2512	16.8	18.6	17.7	24.6	30	27.3
2513	9	15.6	12.3	11.4	22.2	16.8
2514	14.4	19.2	16.8	16.2	23.4	19.8
2514	16.8	18.6	17.7	15.6	19.2	17.4
2515	15	21.6	18.3	10.2	22.2	16.2
2518	21	24.6	22.8	10.8	12	11.4
2519	16.8	21	18.9	15.6	21.6	18.6
2520	20.4	21.6	21	12	16.2	14.1
2522	16.8	24	20.4	15	24	19.5
2523	17.4	24	20.7	10.2	19.2	14.7
2524	17.4	28.2	22.8	19.2	27.6	23.4
2527	18	18	18	21	21	21
2528	16.2	21.6	18.9	19.2	24	21.6
2529	16.2	19.2	17.7	18.6	24	21.3
2529	19.2	19.2	19.2	22.2	24.6	23.4
2531	23.4	31.8	27.6	16.2	26.4	21.3
2532	9	15	12	6.6	10.8	8.7
2534	8.4	13.2	10.8	7.8	10.2	9
2536	19.2	22.2	20.7	12	19.2	15.6
2537	13.2	22.8	18	12.6	19.2	15.9
2538	13.2	15.6	14.4	16.2	21.6	18.9
2539	13.2	19.2	16.2	16.2	22.8	19.5
2540	27.6	27	27.3	16.8	21.6	19.2
2542	13.2	15.6	14.4	9.6	12.6	11.1
2544	21	29.4	25.2	20.4	32.4	26.4
2545	16.2	25.8	21	22.2	34.8	28.5
2546	16.2	24	20.1	15.6	24	19.8
2547	18	25.8	21.9	15.6	25.2	20.4
2551	12.6	24	18.3	11.4	12.6	12
2553	13.2	19.2	16.2	16.2	24	20.1
2554	19.2	36.6	27.9	16.8	24.6	20.7
2555	12.6	18	15.3	11.4	21.6	16.5
2556	24	22.8	23.4	29.4	24	26.7
2560	16.8	22.2	19.5	25.8	23.4	24.6
2563	19.2	21.6	20.4	15.6	23.4	19.5
2564	14.4	19.8	17.1	9	15.6	12.3
2565	10.8	18	14.4	11.4	20.4	15.9
2566	14.4	17.4	15.9	10.8	21	15.9
2567	26.4	27	26.7	19.8	22.8	21.3
2570	16.8	25.2	21	12.6	26.4	19.5
2572	15.6	18.6	17.1	16.2	22.2	19.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2573	14.4	22.2	18.3	12	17.4	14.7
2574	10.2	19.8	15	11.4	24.6	18
2575	16.8	18	17.4	15.6	19.8	17.7
2578	13.2	16.8	15	11.4	19.2	15.3
2579	14.4	17.4	15.9	22.2	27.6	24.9
2580	12.6	28.2	20.4	18	25.8	21.9
2582	19.8	25.8	22.8	15	21.6	18.3
2583	15	15.6	15.3	12.6	13.2	12.9
2584	20.4	31.8	26.1	22.2	30	26.1
2585	22.2	27.6	24.9	20.4	32.4	26.4
2587	17.4	22.8	20.1	13.8	17.4	15.6
2588	15	18.6	16.8	15.6	21.6	18.6
2590	19.2	20.4	19.8	16.2	25.8	21
2592	18	18	18	16.2	15.6	15.9
2596	26.4	27.6	27	15	33.6	24.3
2601	15.6	21.6	18.6	16.8	22.2	19.5
2602	18	24.6	21.3	13.2	18	15.6
2605	16.8	31.2	24	12.6	18.6	15.6
2609	20.4	26.4	23.4	18.6	24	21.3
2610	13.8	18.6	16.2	13.8	23.4	18.6
2612	17.4	27	22.2	12.6	27	19.8
2615	13.8	25.2	19.5	15	22.2	18.6
2617	13.2	16.2	14.7	11.4	12	11.7
2620	16.8	18.6	17.7	19.2	22.2	20.7
2623	16.2	24.6	20.4	14.4	18.6	16.5
2624	12	18	15	19.8	20.4	20.1
2626	19.8	36	27.9	18	26.4	22.2
2630	13.8	22.8	18.3	13.8	21	17.4
2631	13.2	16.8	15	16.8	24.6	20.7
2634	20.4	25.2	22.8	15	24	19.5
2639	12	15	13.5	9	16.8	12.9
2640	10.8	18.6	14.7	12.6	15.6	14.1
2642	8.4	18	13.2	8.4	14.4	11.4
2643	19.8	24.6	22.2	13.8	22.8	18.3
2644	8.4	15	11.7	9.6	16.2	12.9
2645	13.2	24.6	18.9	16.2	27	21.6
2646	10.8	16.2	13.5	10.8	18	14.4
2647	12.6	21.6	17.1	8.4	20.4	14.4
2650	16.2	31.8	24	13.2	24.6	18.9
2651	20.4	25.2	22.8	13.8	21	17.4
2653	20.4	25.8	23.1	17.4	22.8	20.1
2657	13.2	20.4	16.8	12.6	19.8	16.2
2661	11.4	18	14.7	11.4	19.8	15.6
2663	11.4	19.8	15.6	15.6	25.2	20.4
2665	12.6	21.6	17.1	9	23.4	16.2

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2667	10.8	16.8	13.8	8.4	18	13.2
2670	11.4	19.2	15.3	15	21	18
2671	9.6	20.4	15	9	22.2	15.6
2673	13.2	25.2	19.2	16.2	25.8	21
2676	10.2	24.6	17.4	14.4	26.4	20.4
2679	16.8	31.8	24.3	15	24.6	19.8
2680	16.2	19.2	17.7	12	15	13.5
2682	18.6	19.8	19.2	15	21	18
2683	26.4	38.4	32.4	15	24	19.5
2689	15.6	19.8	17.7	20.4	18.6	19.5
2690	16.8	25.2	21	11.4	19.2	15.3
2692	15	17.4	16.2	15	19.8	17.4
2693	12	15.6	13.8	10.8	15	12.9
2696	15	24	19.5	11.4	16.8	14.1
2698	19.2	22.8	21	19.8	28.8	24.3
2702	15.6	22.8	19.2	13.2	18	15.6
2707	10.8	25.8	18.3	9.6	25.8	17.7
2708	18	27	22.5	16.2	26.4	21.3
2712	14.4	21	17.7	15.6	24	19.8
2713	9	16.8	12.9	9	19.8	14.4
2714	19.2	28.8	24	12	18	15
2715	19.2	25.8	22.5	11.4	21	16.2
2719	18	19.8	18.9	19.8	26.4	23.1
2720	18	23.4	20.7	14.4	24	19.2
2724	14.4	24.6	19.5	15	21.6	18.3
2725	13.8	23.4	18.6	9	17.4	13.2
2726	15.6	30	22.8	12	22.2	17.1
2727	13.8	27	20.4	12	16.8	14.4
2728	15	28.8	21.9	15.6	36	25.8
2731	13.2	20.4	16.8	7.2	11.4	9.3
2733	18	28.2	23.1	19.2	31.8	25.5
2733	19.2	27	23.1	13.8	20.4	17.1
2735	14.4	25.8	20.1	15.6	28.8	22.2
2736	10.8	13.2	12	9	15.6	12.3
2737	10.8	19.2	15	9	17.4	13.2
2744	19.8	21	20.4	28.2	31.8	30
2745	18	22.2	20.1	18	22.8	20.4
2747	16.2	21.6	18.9	15.6	25.2	20.4
2749	24.6	31.2	27.9	16.8	21	18.9
2754	25.2	25.2	25.2	19.2	19.2	19.2
2755	20.4	27	23.7	16.2	23.4	19.8
2758	16.8	16.8	16.8	16.8	16.8	16.8
2760	21	22.2	21.6	16.8	25.8	21.3
2762	21.6	25.8	23.7	13.8	24.6	19.2
2767	18.6	27	22.8	16.8	25.8	21.3

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2768	21	31.2	26.1	10.8	19.8	15.3
2769	12	18.6	15.3	7.8	10.8	9.3
2771	16.8	21.6	19.2	15	20.4	17.7
2777	13.2	23.4	18.3	12	15.6	13.8
2778	23.4	34.8	29.1	12.6	21	16.8
2779	23.4	33	28.2	12.6	20.4	16.5
2780	14.4	18	16.2	13.2	18.6	15.9
2783	15.6	22.8	19.2	12.6	19.8	16.2
2785	24.6	39	31.8	9	19.8	14.4
2786	23.4	33	28.2	13.2	19.8	16.5
2787	12.6	19.2	15.9	15	19.2	17.1
2788	15	26.4	20.7	12.6	20.4	16.5
2796	13.2	18	15.6	11.4	21.6	16.5
2797	19.8	26.4	23.1	11.4	21	16.2
2798	24.6	35.4	30	11.4	19.2	15.3
2801	13.8	21.6	17.7	12	20.4	16.2
2802	12	19.8	15.9	10.2	15.6	12.9
2805	23.4	37.8	30.6	13.2	22.2	17.7
2806	14.4	19.2	16.8	12	20.4	16.2
2809	27	40.8	33.9	12	19.8	15.9
2811	15.6	20.4	18	12	17.4	14.7
2812	12.6	21	16.8	11.4	16.8	14.1
2813	16.2	20.4	18.3	12.6	20.4	16.5
2814	16.8	27.6	22.2	15.6	25.8	20.7
2817	15	20.4	17.7	12	19.2	15.6
2818	15	20.4	17.7	12.6	18	15.3
2821	15.6	21.6	18.6	11.4	16.8	14.1
2823	15	21.6	18.3	12	15.6	13.8
2826	19.2	30	24.6	12	20.4	16.2
2829	18.6	24	21.3	16.2	23.4	19.8
2830	17.4	25.2	21.3	14.4	21	17.7
2831	22.2	34.2	28.2	15.6	27.6	21.6
2832	16.2	21.6	18.9	16.2	22.2	19.2
2835	17.4	31.2	24.3	15.6	25.8	20.7
2841	16.2	24.6	20.4	18	31.8	24.9
2842	13.2	21.6	17.4	13.8	22.2	18
2843	16.8	29.4	23.1	16.8	25.8	21.3
2844	16.2	22.2	19.2	13.8	18.6	16.2
2845	9	12.6	10.8	11.4	15	13.2
2846	16.8	24	20.4	14.4	24.6	19.5
2849	17.4	22.2	19.8	14.4	21.6	18
2851	15	21.6	18.3	14.4	25.2	19.8
2860	13.8	24	18.9	17.4	28.2	22.8
2862	17.4	28.8	23.1	16.8	26.4	21.6
2864	16.8	22.8	19.8	15	19.2	17.1

Appendix A: Survival Time of Insertion Lines (Initial Screen).

Line	Caf_M	Caf_F	Caf_Avg	Nic_M	Nic_F	Nic_Avg
2865	17.4	28.8	23.1	15	25.8	20.4
2870	9	13.8	11.4	10.2	19.2	14.7
2872	17.4	24	20.7	14.4	17.4	15.9
2873	19.2	30	24.6	15	25.2	20.1
2874	20.4	22.8	21.6	15	22.2	18.6
2875	20.4	25.2	22.8	16.2	26.4	21.3
2877	9	15	12	12.6	18	15.3
2878	9.6	14.4	12	10.8	19.2	15
2879	15	24.6	19.8	10.8	21	15.9
2881	12.6	20.4	16.5	9	21	15
2882	20.4	24.6	22.5	15.6	22.2	18.9
2883	12.6	27	19.8	15	24.6	19.8
2885	16.2	23.4	19.8	17.4	28.2	22.8
2886	10.2	15.6	12.9	17.4	19.2	18.3
2888	16.2	18	17.1	14.4	16.8	15.6
2889	16.8	20.4	18.6	13.2	17.4	15.3
2894	11.4	22.8	17.1	13.8	21	17.4
2895	10.2	16.8	13.5	13.2	18	15.6
2897	17.4	18.6	18	14.4	21	17.7
2898	11.4	17.4	14.4	10.8	16.8	13.8

Appendix B: Lines Selected for Drug Response.

1/3 C	4/6 C	7/9 C	10/12 C	13/15 C	16/18 C	19/21 C	22/24 C	25/27 C	28/30 C	31/33 C	34/36 C	37/39 C	40/41 C
488	524	489	1216	1438	1556	1374	1427	2486	927	1659	2778	377	1008
1353	604	1010	1228	1472	1602	1419	1689	2531	1016	1878	2779	1685	1226
2175	1543	1011	1231	1476	1979	1618	2233	2540	1359	2019	2786	2554	2051
2257	1748	1017	1260	1743	1985	1659	2262	2544	1540	2109	2798	2643	2449
2346	1845	1065	1290	1891	2088	1721	2437	2567	1649	2248	2805	2650	2771
2423	1859	1066	1295	2049	2102	2159	2452	2584	1787	2422	2809	2651	
2434	1908	1067	1520	2201	2116	2487	2456	2596	2320	2749	2831	2679	
	2358		1572			2726	2470		2683	2754		2785	
	2492						2883		2714				
	2626												
356	792	386	375	1480	1968	1383	1820	177	1587	1656	1426	151	1258
357	2133	391	1217	1486	1972	1417	2327	789	1976	2053	2796	712	2086
1564	2306	1007	1218	1491	2010	1464	2454	2489	2692	2104	2860	1278	2242
1582	2386	1130	1324	1785	2061	1627	2473	2512		2631		1316	
1638	2529	1139	1567		2062	2141	2513	2560				1387	
1697	2539				2063	2219		2579				2188	
1770	2553				2077	2299		2612				2217	
2348	2572					2330						2663	
												2670	
												2886	

Caffeine
107 Resistant
81 Sensitive

1/3 N	4/6 N	7/9 N	10/12 N	13/15 N	16/18 N	19/21 N	22/24 N	25/27 N	28/30 N	31/33 N	34/36 N	37/39 N	40/41 N
488	637	489	1231	1477	1714	1419	1427	177	1976	1671	816	151	668
1770	759	1062	1295	1786	1914	1721	1665	2400	1977	2094	1324	712	1008
2170	829	1092	1520	1893	1968	1899	1820	2416	2683	2192	1711	1043	1226
2273	930	1130	1562	2127	1985	2330	2279	2506	2689	2384	2167	1316	1258
2284	1543	1139	1563	2152	2061	2487	2454	2512		2393	2339	1336	1576
2348	2251	1194	1567	2201	2414			2545		2744	2841	1685	2182
2423	2524							2556				1783	2394
								2560				2188	
								2728				2580	
												2645	
1006	604	484	1247	1438	1556	735	1740	2457	663	1692	2768	377	2051
1607	864	1011	1259	1443	1602	1374	2246	2486	1359	2019	2778	766	2052
1608	1412	1091	1290	1488	2003	1619	2253	2518	1821	2157	2779	2715	2072
1674	1845	1137	1561	1743	2023	1717	2263	2523	1896	2499	2786	2725	2112
1687	2358		1565	1798	2029	2602	2321	2596	2158	2733	2798	2785	
1715	2469			1803	2068		2351	2623	2551	2762	2809	2879	
1732	2510			1818	2084		2437						
2282	2605			1888	2102		2440						
2283				1891	2116		2452						
2311													

Nicotine
91 Resistant
93 Sensitive

Legend
Resistant
Sensitive
Sen and Res
Res for Both
Line Lost

Appendix C: Survival Time of Insertion Lines (Second Screen).

Line	Caf M	Caf F	Caf Avg	Line	Nic M	Nic F	Nic Avg
Control	14.4	19.2	16.5	Control	12.8	18.4	15.6
377	18	19.2	18.6	151	15	21.6	18.3
488	20.4	28.2	24.3	177	13.8	25.2	19.5
489	22.8	24.6	23.7	488	12	25.8	18.9
524	11.4	15	13.2	489	15	21	18
604	12.6	24	18.3	637	13.2	15.6	14.4
927	15	19.8	17.4	668	14.4	20.4	17.4
1008	15	22.8	18.9	712	16.8	25.8	21.3
1010	14.4	18	16.2	759	16.2	19.2	17.7
1011	13.2	17.4	15.3	816	17.4	23.4	20.4
1016	13.8	18	15.9	829	12	20.4	16.2
1017	15	23.4	19.2	930	19.2	27.6	23.4
1065	14.4	21.6	18	1008	13.8	22.8	18.3
1066	11.4	15.6	13.5	1062	13.2	25.2	19.2
1067	13.8	21.6	17.7	1092	12.6	31.2	21.9
1216	11.4	14.4	12.9	1130	12	19.2	15.6
1226	21.6	22.8	22.2	1139	16.8	15	15.9
1228	16.8	21	18.9	1194	12	23.4	17.7
1231	18.6	23.4	21	1226	13.2	18.6	15.9
1260	12	18	15	1231	13.2	23.4	18.3
1290	15.6	18.6	17.1	1258	15	22.8	18.9
1295	16.8	26.4	21.6	1295	16.8	22.8	19.8
1353	13.8	18	15.9	1316	18	22.2	20.1
1359	15.6	18.6	17.1	1324	13.2	18.6	15.9
1374	16.2	21	18.6	1419	10.8	17.4	14.1
1419	12.6	24	18.3	1427	19.2	30	24.6
1427	13.2	18.6	15.9	1477	15	19.2	17.1
1438	12.6	15.6	14.1	1520	19.2	22.2	20.7
1472	15	19.8	17.4	1543	12.6	22.8	17.7
1476	13.2	19.8	16.5	1562	12	18.6	15.3
1520	18.6	24.6	21.6	1563	14.4	18.6	16.5
1540	13.2	15.6	14.4	1567	10.2	15.6	12.9
1543	15	25.8	20.4	1576	15	18	16.5
1556	15.6	20.4	18	1665	17.4	19.2	18.3
1572	16.2	22.2	19.2	1671	19.8	21.6	20.7
1602	12.6	17.4	15	1685	15	19.8	17.4
1618	18.6	19.8	19.2	1711	13.2	18.6	15.9
1649	15.6	21	18.3	1714	13.2	25.2	19.2
1659	15.6	21	18.3	1721	24.6	23.4	24
1685	15	22.2	18.6	1770	10.8	18	14.4
1689	17.4	22.2	19.8	1783	13.8	24.6	19.2
1721	28.2	31.2	29.7	1786	13.2	25.8	19.5
1743	13.8	16.8	15.3	1820	12	17.4	14.7
1748	15.6	21	18.3	1893	21.6	19.8	20.7
1787	13.2	19.2	16.2	1899	19.8	22.8	21.3
1845	15.6	24	19.8	1914	16.2	22.8	19.5
1859	13.2	16.8	15	1968	13.2	19.2	16.2
1878	13.2	19.2	16.2	1976	14.4	22.2	18.3
1891	12.6	16.2	14.4	1977	14.4	22.8	18.6

Appendix C: Survival Time of Insertion Lines (Second Screen).

Line	Caf M	Caf F	Caf Avg	Line	Nic M	Nic F	Nic Avg
1908	13.2	15.6	14.4	1985	16.8	24.6	20.7
1979	11.4	15.6	13.5	2061	16.8	19.2	18
1985	19.2	27	23.1	2094	16.8	19.8	18.3
2019	13.2	26.4	19.8	2127	15	23.4	19.2
2049	13.8	24	18.9	2152	20.4	26.4	23.4
2051	14.4	20.4	17.4	2167	25.2	29.4	27.3
2088	24	30	27	2170	12.6	16.8	14.7
2102	15	21	18	2182	15.6	17.4	16.5
2109	15.6	23.4	19.5	2188	18	21	19.5
2116	12.6	18	15.3	2192	17.4	25.2	21.3
2159	13.8	16.8	15.3	2201	15	22.2	18.6
2175	13.8	18.6	16.2	2251	11.4	19.2	15.3
2201	16.8	25.2	21	2273	15	21.6	18.3
2233	13.8	20.4	17.1	2279	11.4	17.4	14.4
2248	15.6	21.6	18.6	2284	13.2	17.4	15.3
2257	14.4	18	16.2	2330	13.8	17.4	15.6
2262	12.6	16.8	14.7	2339	13.8	21.6	17.7
2320	13.2	18	15.6	2348	12.6	12.6	12.6
2346	21.6	26.4	24	2384	17.4	22.8	20.1
2358	12.6	16.2	14.4	2393	15.6	15.6	15.6
2422	19.2	28.2	23.7	2394	12.6	17.4	15
2423	16.8	16.8	16.8	2400	16.8	23.4	20.1
2434	13.8	15.6	14.7	2414	13.8	28.8	21.3
2437	14.4	15	14.7	2416	18	22.8	20.4
2449	13.8	18	15.9	2423	12	19.8	15.9
2452	13.8	24	18.9	2454	16.2	19.8	18
2456	12	20.4	16.2	2487	13.8	19.2	16.5
2470	14.4	22.2	18.3	2506	10.8	16.8	13.8
2486	13.2	17.4	15.3	2512	19.2	17.4	18.3
2487	13.8	22.8	18.3	2524	14.4	17.4	15.9
2492	14.4	18	16.2	2545	12	22.2	17.1
2531	14.4	18	16.2	2556	13.2	18	15.6
2540	13.8	18.6	16.2	2560	15	21	18
2544	13.8	22.2	18	2580	14.4	19.8	17.1
2554	12.6	18.6	15.6	2645	15.6	19.8	17.7
2567	12.6	17.4	15	2683	14.4	23.4	18.9
2584	17.4	23.4	20.4	2689	16.2	19.2	17.7
2596	12.6	18	15.3	2728	13.2	19.2	16.2
2626	14.4	20.4	17.4	2744	15.6	24	19.8
2643	15	22.8	18.9	2841	15.6	25.2	20.4
2650	13.2	19.2	16.2				
2651	18.6	26.4	22.5				
2679	13.2	19.8	6.6				
2683	21.6	31.8	26.7				
2714	12.6	17.4	15				
2726	16.8	21	18.9				
2749	12.6	16.2	8.1				
2754	13.2	17.4	15.3				
2771	15.6	21.6	18.6				

Appendix C: Survival Time of Insertion Lines (Second Screen).

Line	Caf M	Caf F	Caf Avg	Line	Nic M	Nic F	Nic Avg
2778	13.2	19.2	16.2				
2779	15	17.4	16.2				
2785	23.4	27	25.2				
2786	13.2	19.8	16.5				
2798	13.8	21.6	17.7				
2805	12	15.6	13.8				
2809	13.2	16.8	15				
2831	27	33.6	30.3				
2883	13.2	19.2	16.2				

Appendix D: Lines Selected for Drug Resistance in Second Screen.

Caffeine Resistance		*Nicotine Resistance*	
Tray	Line	Tray	Line
1/3	488	4/6	930
1/3	2346	7/9	1092
7/9	489	10/12	1520
16/18	1985	13/15	2152
16/18	2088	16/18	2414
19/21	1721	19/21	1721
28/30	2683	19/21	1899
31/33	2422	22/24	1427
34/36	2831	34/36	2167
37/39	2785	37/39	712

Appendix E: Survival Time of Backcrossed and Original Lines.

Caffeine

Backcrossed Line	ST (M)	ST (F)	ST (Line)
Control	13.6	21.2	17.4
488	25.8	34.2	30
489	18	25.2	21.6
1721	16.2	23.4	19.8
1985	15	22.2	18.6
2088	15.6	27.6	21.6
2346	21	24.6	22.8
2422	25.2	30.6	27.9
2683	14.4	25.8	20.1
2785	22.2	24	23.1
2831	17.4	24.6	21

Original Line	ST (M)	ST (F)	ST (Line)
Control	13.6	21.2	17.4
488	21.6	37.2	29.4
489	25.8	33	29.4
1721	21	31.8	26.4
1985	19.2	27	23.1
2088	22.8	34.2	28.5
2346	18	24	21
2422	25.8	31.2	28.5
2683	25.8	36	30.9
2785	28.2	36.6	32.4
2831	31.8	39	35.4

Nicotine

Backcrossed Line	ST (M)	ST (F)	ST (Line)
Control	12.8	19.2	16
712	15.6	21	18.3
930	16.2	22.8	19.5
1092	15.6	23.4	19.5
1427	15.6	25.2	20.4
1520	16.8	25.2	21
1721	18.6	22.2	20.4
1899	16.8	25.8	21.3
2152	19.8	28.2	24
2167	18.6	24	21.3
2414	16.8	21.6	19.2

Original Line	ST (M)	ST (F)	ST (Line)
Control	12.8	19.2	16
712	16.8	20.4	18.6
930	14.4	22.2	18.3
1092	16.2	27.6	21.9
1427	18.6	24.6	21.6
1520	17.4	23.4	20.4
1721	21	25.8	23.4
1899	15.6	21	18.3
2152	18	25.2	21.6
2167	22.2	28.8	25.5
2414	16.2	21	18.6

Appendix F: Sequence and Map Position of Insertion Sites.

488

GCAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCGA
CGGGACCACCTTATGTTATTTCATCATGCCGTGACGTATTCAGCACACAC
ACAGTCGCAGGAGACGAGCGCTGTCTGTGGGTCCGCCGGTTATCATACCA
TTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTAT
ACCTCAAATGGTTGTCGTACCTCTCATGGTTCCGTTACGCCAACGAGGG
TCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTGC
ACATCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCATCCTGGAGAC
CTTTAACTTCAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

712

GCAACGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCG
ACGGGACCACCTTATGTTATTTCATCATGCAACAAGGGTAACGGCTGGC
AGTGTTTCACGCGATTTCGAAACGTTGAGATCGTTGCCGCGGTCGCCGT
GAATTGGAATTGTGAGTGTGTTCGTCGTGCGGAAAATCATCGCTGTCAA
ATAGAGGCCACAGTGAATTGCCGATACCTAATACTGTGCAAGGCGAAAT
TATGTGCCCCAGCATTTCGTGAATGAAAAGTGCGAAAATACAAAATACA
GAGCAATAGTGCGAGGTCGGTGGAGTTCGAATTAAAGAAATTCCACGAA
ATACAAGTGCGGGCGATTCTCGTGTGCTGGCGAGAGTGCGGCTTGTCCG
GGTGTGTGAGTAAAATTCACGGTAAATAAATAAATAAAATTTGTGTACA
CACACATACACACACACACGCGCNCTGTNTGTGGGNCCNCCGGNTATCA
TACCNTTCNNGCTCTNNGGNGGNTTNTTCNNNAACTCGGGCTCGGNGCC
ANTATACCNCAANTGGGTGTCNCNCCTCTCGTGGTNNCNNNACNCCAAC
GAGGGTNTGCNGATTAACCANTGNGCANACGTGGANCCGGGAAAAATTA
TNTGCANATCGTAAAACACCANG

930

GCAAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCG
ACGGGACCACCTTATGTTATTTCATCATGGACCAGCGGATGCTAGCGCT
GTCTGTGGGTCCGCCGGTTATCATACCATTCCTGCTCTTTGGCGGCTTC
TTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAAATGGTTGTCGTACC
TCTCATGGTTCCGTTACGCCAACGAGGGTCTGCTGATTAACCAATGGGC
GGACGTGGAGCCGGGCGAAATTAGCTGCACATCGTCGAACACCACGTGCC
CCAGTTCGGGCAAGGTCATCCTGGAGACGCTTNAACTTCANNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCNNNNNNNN
NNNNNNNNNNNNNNNNNNGNNNNNNNGNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Appendix F: Sequence and Map Position of Insertion Sites.

1092

GCAAACGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATC
GACGGGACCACCTTATGTTATTTCATCATGGTTGTATCTATAACTCCTT
GTAATATTTGCTTTACATCCGCAGCAGCTGCACTTTGTAATATCCGAAA
ACGAGTATCTACTTCAAATGGAAGCTTCGCGGTTGGATCGTACTTTCTT
GGGTGCAAACTTACTCCAATTTAACTGTTGGAAATTAACTATTTTCCTG
CTCTTTAGTGCGATTCACGGCGCTGTCTGTGGGTCCGCCGGTTATCATA
CCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGT
ATACCTCAAATGGTTGTCGTACCTCTCATGGTTCCGTTACGCCAACGAGG
GTCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTG
CACATCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCATCCTGGAGA
NTTNAAANTTTCAAANNNNNNNNNNNNNNNNGNNNNAAATTATGTGNN
CTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNANNNNNNNNNNNNNNNNNNNNNNANNNNNNNNNNNNNNNNNNN
NNNNNNNNNGNNNNANNNNNNNNNNNNNNNNNNGNNNNNN

1427

GCAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCGA
CGGGACCACCTTATGTTATTTCATCATGGTCCGTACCCCGTACCGTGCTC
GCCTATTGCGGCTATGCTGTTTTGTTTGATTTTTTGGTACTCGCTCGGC
GATCCGATCGGATGGTATCATATCGTGGGGGATCGTATGGGATCGGATC
GCTCGCGTGCCGTTTCGTGTCGTATCGCTTCGTTTCGCATCGCTTTCGC
AAACGGCGGTCCGGGGCTCCGCGCTGTCTGTGGGTCCGCCGGTTATCATA
CCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGT
ATACCTCAAATGGTTGTCGTACCTCTCATGGTTCCGTTACGCCAACGAGG
GTCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTG
CACATCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCATCCTGGAGN
NTTTAAATTTCAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

1520

GCAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCGA
CGGGACCACCTTATGTTATTTCATCATGGCCCAAATGAAGGAGAACGAG
AGCGCTGTCTGTGGGTCCGCCGGTTATCATACCATTCCTGCTCTTTGGC
GGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAAATGGTTGT
CGTACCTCTCATGGTTCCGTTACGCCAACGAGGGTCTGCTGATTAACCAA
TGGGCGGACGTGGAGCCGGGCGAAATTAGCTGCACATCGTCGAACACCAC
GTGCCCCAGTTCGGGCAAGGTCATCCTGGAGACGCNTNAACTTCAACGT
GCCCCAGTTCGGGCAAGGTCATCCTGGAGACGCTTNAACTTCANCNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Appendix F: Sequence and Map Position of Insertion Sites.

1899

GCAAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCG
ACGGGACCACCTTATGTTATTTCATCATGGNACGGCGTCTCTTGNANCN
NNNAATTTGAGGCGTCANCANCTGAGCNCTGTNGTGTGGGTCGCGCCGN
TTATCGATACNATTCCTGNTCTTTGGNGGCTTCTTCTTGAACTCGGGCT
CGGTGCCAGTATACCTCAAATGGTTGTCGTACCTCTCATGGTTCCGTTA
CGCCAACGAGGGTCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCG
AAATTAGCTGCACATCGTCGAACACCACGTGCCCCAGTTCGGGAAAGGTC
ATCCTGGAGACNCTTAAACTTCAAGCCNGNTTNGGGNANGNGNTCNNGN
NGACACNTNNATTNNACAANNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

2152

GCAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCGA
CGGGACCACCTTATGTTATTTCATCATGCGCTGTCTGTGGGTCCGCCGG
TTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTC
GGTGCCAGTATACCTCAAATGGTTGTCGTACCTCTCATGGTTCCGTTAC
GCCAACGAGGGTCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGA
AATTAGCTGCACATCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCA
TCCTGGAGACGCTTAAACTTCANCGNNNNNNNTNTNNNNNNGNNNNNN
NNGNNTNTNGNNNNNNNNNCGCCGNNNNNNNNNNNNNNNNNNNNTN
NGGNNNNNAGANNNNNNNNNNNNANNNNNNNANNNNAANGNNNNTNN
NGGGNNNNANGNANNNGNNNNNNNNNNNNNNGNGNNNNNNGNNNGNN
GNNNNNNNNNNNNNNNNNNNNGNNNNNNNNNNNNNNNANNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNANNNNNNNNNGNNNNNNNNNN
NNNNNNNNGNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN
NNNNGNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNNNC

2346

GCAAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCG
ACGGGACCACCTTATGTTATTTCATCATGCCCCGTACCGTGCTCGCCTAT
TGCGGCTATGCTGTTTTGTTTGATTTTTTGGTACTCGCTCGGCGATCCG
ATCGGATGGTATCATATCGTGGGGGATCGTATGGGATCGGATCGCTCGC
GTGCCGTTTCGTGTCGTATCGCTTCGTTTCGCATCGCTTTCGCAAACGG
CGGTCCGGGGCTCCGCGCTGTCTGTGGGTCCGCCGGTTATCATACCATTC
CTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACC
TCAAATGGTTGTCGTACCTCTCATGGTTCCGTTACGCCAACGAGGGTCT
GCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTGCACA
TCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCATCCTGGAGNCTTT
AAANTTCAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Appendix F: Sequence and Map Position of Insertion Sites.

2414

GCAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCGA
CGGGACCACCTTATGTTATTTCATCATGATTTGTGTGAAGCAAAGTGTG
CGAGTGTGTGTATCTGACAGAGTGTGCGTGTGTGTGTGTGCCTAGCAGC
GTGCTTGAATGAGTGACGCAGCGACGGAGAGACAAAAAGACGGACGGAC
TTGCCTGCGCTGTCTGTGGGTCCGCCGGTTATCATACCATTCCTGCTCT
TTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAAATG
GTTGTCGTACCTCTCATGGTTCCGTTACGCCAACGAGGGTCTGCTGATT
AACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTGCACATCGTCGAA
CACCACGTGCCCCAGTTCGGGCAAGGTCATCCTGGAGACCTTTAACTTCA
AANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNN

2422

GCAAAGTGCACTGAATTTAAGTGTATACTTCGGTAAGCTTCGGCTATCG
ACGGGACCACCTTATGTTATTTCATCATGGNCNGGACCCNAACNANNNC
TNATGNGAGNNACAAACAACATNTNTGNACTNNCTGTGGNGNCNCCNGT
AATCATACCAGTNCCNGCTNNNTGGNGGNTTCNTNTTGAACANCGACNN
TCGGTGCNNGTATACCTNAAATGGNTGTCTGACCTCTCATGGNTCCTTT
ACNCCNACCAGGGNNTGNTGATTAACCNATGGNANNACGTGTAGCCGGN
NGAANNTATCTGNNNNTNGTCGAACNNCNCNTGNCNCNGTTNNGGCAA
NGNCNTCCTGNAGANNCNTTAACTTCACGGACGTGGAGCCGGGCGAAAT
TAGCTGCACATCGTCGAACACCACGTGCCCCAGTTCGGGCAAGGTCATCC
TGGAGACGNTTNAACTTCAANNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

Appendix G: Survival Time of UCD and WE Lines.

Line	Caf_M	Caf_F	Nic_M	Nic_F
UD001	10.32	16.56	11.76	13.2
UD002	14.64	22.56	12.72	16.8
UD003	31.44	43.2	13.92	39.84
UD008	11.52	13.68	10.8	12.72
UD009	16.32	24	12.48	17.28
UD010	24.72	26.64	17.28	16.08
UD011	27.6	36.96	16.56	25.44
UD012	11.04	22.8	11.76	12.72
UD013	27.6	29.04	13.92	26.88
UD015	19.92	24	14.88	17.52
UD017	31.92	50.04	15.6	23.04
UD018	19.68	47.76	13.68	32.16
UD023	14.88	20.16	13.44	18.72
UD026	18.72	49.68	11.76	18.24
UD027	19.68	33.12	10.56	19.92
UD030	14.4	17.76	13.68	19.68
UD033	25.92	35.04	20.4	21.36
UD034	44.4	62.16	17.28	34.32
UD035	12.48	25.44	22.08	31.92
UD037	13.2	15.6	10.56	15.84
UD040	12.48	18.48	11.52	16.08
UD041	11.52	29.04	13.44	18
UD043	19.68	32.16	9.6	20.16
UD044	17.28	30.72	27.84	29.52
UD045	19.2	34.32	15.84	25.2
UD047	19.92	31.2	18.72	24.96
UD052	27.84	33.6	15.84	24.96
UD057	25.44	29.28	17.52	24.96
UD058	27.12	34.08	21.6	28.08
UD060	25.68	42.48	20.88	26.88
UD061	14.64	29.28	13.2	23.76
UD062	12.48	14.88	12.48	17.28
UD064	23.28	36.96	15.12	31.68
UD065	26.16	36.96	18.24	22.56
UD068	16.56	22.8	19.92	22.8
UD069	16.56	19.44	12.24	16.56
UD070	24.48	26.88	10.56	21.12
UD072	15.84	29.04	15.12	25.68
UD073	18.24	24.96	18.24	32.64
UD075	27.36	22.08	22.32	27.6
UD079	19.2	27.36	14.16	24.96
UD081	24	27.12	14.16	24.72
UD083	28.08	38.64	18.96	32.16

Appendix G: Survival Time of UCD and WE Lines.

Line	Caf_M	Caf_F	Nic_M	Nic_F
UD086	17.76	19.2	12.72	24.48
UD087	22.8	23.04	13.68	16.08
UD088	16.56	26.88	10.8	18.72
UD089	13.2	31.92	12.48	20.4
UD090	13.2	21.84	12.72	31.68
UD091	13.68	17.76	10.32	19.68
UD093	23.52	24	15.84	21.36
UD096	18.48	21.6	10.32	16.8
UD098	17.28	28.56	12	13.44
UD100	16.56	47.76	14.4	29.76
UD105	15.36	31.44	15.36	21.12
UD113	17.04	28.08	10.32	14.16
UD114	20.64	31.92	16.56	21.12
UD115	20.88	32.88	15.36	22.56
UD118	23.76	37.92	18.48	31.92
UD123	15.36	25.68	10.32	22.08
UD127	15.12	21.36	12.96	16.8
UD129	23.04	31.68	16.56	18.72
UD132	20.16	20.64	11.76	17.28
UD133	17.04	24.96	12.96	16.32
UD136	18	19.68	11.04	15.6
UD137	13.68	16.8	12.48	18
UD140	17.28	21.84	11.28	24.24
UD142	14.88	17.28	12.48	16.56
UD144	14.16	18	16.08	20.4
UD145	21.36	31.44	13.92	26.88
UD147	14.16	22.56	12.96	14.16
UD148	23.52	32.4	16.8	33.36
WE001	24.72	46.8	19.68	25.2
WE002	9.36	12.48	10.08	12.24
WE003	14.64	24.72	18.24	31.2
WE004	18	18.72	10.32	16.08
WE005	19.2	33.84	18.96	35.28
WE006	16.56	21.12	10.8	15.84
WE008	10.08	34.32	10.08	25.68
WE010	34.8	45.84	17.28	43.92
WE011	16.08	30.72	18.96	24.96
WE012	22.56	30	19.2	28.32
WE013	19.68	33.84	23.76	38.88
WE014	35.04	62.4	26.88	41.28
WE015	31.2	48.48	32.64	48.48
WE017	31.68	46.32	29.76	41.28
WE018	16.56	34.32	15.12	33.6

Appendix G: Survival Time of UCD and WE Lines.

Line	Caf_M	Caf_F	Nic_M	Nic_F
WE021	29.04	32.16	16.56	24.72
WE022	26.64	38.16	10.56	22.08
WE023	19.44	32.64	13.2	28.08
WE024	20.88	30.48	17.76	26.16
WE025	26.64	48.72	18.48	28.56
WE026	19.44	24.24	11.52	17.52
WE027	33.84	51.6	15.6	22.56
WE028	38.4	49.2	36.24	52.08
WE029	17.28	22.56	13.2	17.76
WE030	14.4	20.4	11.04	19.2
WE031	17.52	35.28	15.36	36.24
WE032	20.16	29.28	13.2	23.52
WE033	15.12	38.64	9.84	14.88
WE034	19.2	32.64	10.08	19.44
WE036	16.8	26.64	12.96	19.2
WE037	11.76	22.32	11.52	26.64
WE039	26.88	31.92	12.72	23.04
WE040	18.96	27.6	12.72	13.92
WE041	13.68	50.64	8.16	15.6
WE042	13.68	44.4	11.76	22.56
WE043	38.4	56.4	25.44	41.28
WE044	37.44	56.88	31.92	47.76
WE046	34.32	34.32	21.6	30.48
WE047	19.68	29.76	18.96	29.52
WE048	19.44	26.16	21.6	34.32
WE049	16.32	27.36	14.4	30.96
WE050	13.68	18.48	14.16	18.48
WE051	16.32	18.72	17.04	22.8
WE052	14.64	41.52	14.64	23.76
WE053	10.56	30.24	10.08	17.76
WE054	15.6	24	12.48	19.2
WE057	16.08	24	11.28	15.84
WE059	19.2	20.16	10.08	15.12
WE060	24.24	31.68	10.08	24.72
WE061	15.6	29.76	9.84	15.6
WE063	20.16	26.64	17.52	24.24
WE064	26.16	32.64	13.92	21.6
WE066	17.04	23.76	8.88	12.96
WE067	29.04	37.2	24.72	38.88
WE068	19.44	41.04	18.24	28.56
WE069	11.76	23.04	21.12	44.88
WE070	9.84	15.6	18.48	32.4
WE071	15.6	20.16	13.2	20.4

Appendix G: Survival Time of UCD and WE Lines.

Line	Caf_M	Caf_F	Nic_M	Nic_F
WE072	18.24	25.44	16.08	21.36
WE073	24.24	35.28	9.6	20.4
WE074	9.12	14.88	10.56	14.16
WE075	8.64	9.84	10.32	27.12
WE077	23.76	49.44	21.12	29.76
WE078	14.64	23.04	14.16	22.32
WE079	12.48	16.08	16.08	30.96
WE080	18.48	29.52	16.08	29.04
WE081	21.12	28.32	35.28	48.48
WE084	12.48	25.2	22.08	39.84
WE086	13.2	20.4	24.48	32.88
WE087	22.32	26.88	13.44	15.36
WE088	25.2	32.88	18.72	39.12
WE089	25.44	39.36	25.44	42.72
WE091	48.96	45.36	18	36.48
WE092	10.08	24.24	17.76	32.4
WE094	17.76	24.72	12.24	19.92
WE095	12.96	27.84	10.56	19.92
WE096	13.68	17.76	25.2	31.68
WE098	16.08	17.28	29.04	43.2
WE100	32.64	51.84	14.4	31.44
WE101	19.92	35.28	21.36	22.08
WE102	13.92	34.08	25.2	35.04
WE103	11.28	25.2	18.72	38.64
WE104	11.04	24.48	13.68	27.84
WE105	25.92	46.32	26.16	36.96
WE107	15.84	21.84	12.72	17.76
WE108	17.04	44.4	15.6	39.12
WE110	20.88	29.28	32.64	45.6
WE111	30.24	42	18	38.16
WE112	16.32	24.96	13.92	18.24
WE113	21.12	50.4	43.44	47.52
WE114	17.52	32.88	10.32	19.92
WE115	22.56	50.4	23.28	52.32
WE116	20.4	32.64	19.2	23.76
WE118	19.68	27.36	14.64	22.8
WE119	21.84	27.84	11.52	13.2
WE121	10.32	39.84	21.6	48.24
WE123	22.08	22.08	21.12	23.28
WE124	24	38.4	24.48	40.56
WE126	21.12	41.28	20.16	40.8
WE127	10.08	22.8	13.68	32.4
WE129	26.88	39.36	23.04	52.32

Appendix G: Survival Time of UCD and WE Lines.

Line	Caf_M	Caf_F	Nic_M	Nic_F
WE131	18	23.04	18.72	20.88
WE133	22.8	27.36	12	25.92
WE134	9.6	18.48	10.56	22.32
WE135	16.32	39.36	11.04	24.48
WE136	13.2	18.48	11.28	16.08
WE137	15.36	11.52	16.56	24.24
WE138	14.88	20.64	13.2	16.08
WE141	17.52	24.24	10.08	20.16
WE142	15.84	34.8	10.08	29.28
WE144	15.6	37.44	18.24	28.8
WE146	33.6	42	18.24	34.32
WE147	19.2	27.84	9.12	12
WE148	16.32	37.68	11.52	20.88
WE149	36	52.56	27.84	48.72
WE150	14.88	22.8	10.56	20.64

Appendix H: Allelic Variant in Each Line at SNP Site.

Line	SNP	Line	SNP	Line	SNP
WE001	G	WE047	G	WE100	G
WE002	G	WE048	A	WE102	G
WE003	A	WE049	G	WE103	A
WE004	G	WE050	G	WE104	G
WE005	G	WE051	G	WE105	A
WE006	G	WE052	G	WE107	G
WE007	G	WE053	G	WE108	A
WE008	G	WE054	G	WE109	G
WE010	G	WE057	G	WE110	A
WE011	G	WE058	G	WE111	G
WE012	G	WE059	G	WE112	G
WE013	A	WE060	G	WE114	G
WE014	A	WE061	G	WE115	G
WE015	A	WE064	G	WE116	G
WE017	A	WE066	A	WE118	G
WE018	G	WE067	G	WE119	G
WE021	G	WE068	G	WE121	G
WE022	G	WE069	G	WE123	G
WE023	G	WE070	A	WE124	G
WE024	G	WE071	G	WE125	A
WE025	G	WE072	G	WE126	A
WE026	G	WE073	G	WE128	A
WE027	G	WE074	G	WE129	G
WE028	A	WE075	G	WE131	G
WE029	G	WE077	G	WE133	A
WE030	G	WE079	G	WE134	G
WE031	G	WE080	G	WE135	G
WE032	G	WE081	A	WE136	G
WE033	G	WE084	G	WE137	A
WE034	G	WE086	G	WE138	G
WE036	G	WE087	G	WE139	G
WE037	G	WE088	A	WE141	G
WE038	G	WE089	A	WE142	G
WE039	G	WE091	A	WE144	A
WE040	G	WE092	G	WE146	G
WE041	G	WE094	G	WE147	G
WE042	G	WE095	G	WE148	G
WE043	G	WE096	A	WE149	G
WE044	G	WE097	G	WE150	G
WE046	A	WE098	G		

Appendix H: Allelic Variant in Each Line at SNP Site.

Line	SNP	Line	SNP	Line	SNP
UD001	G	UD047	G	UD090	A
UD002	A	UD048	A	UD091	G
UD003	A	UD052	G	UD093	G
UD008	A	UD055	G	UD095	A
UD009	G	UD056	G	UD096	G
UD010	G	UD057	G	UD098	G
UD011	G	UD058	A	UD100	G
UD012	G	UD060	G	UD105	A
UD013	G	UD061	G	UD113	G
UD015	G	UD062	G	UD114	G
UD017	G	UD063	G	UD115	G
UD018	G	UD064	A	UD118	G
UD023	A	UD065	A	UD120	G
UD026	G	UD066	G	UD126	G
UD027	G	UD068	G	UD127	G
UD030	G	UD069	A	UD128	G
UD031	G	UD070	G	UD129	G
UD033	G	UD072	A	UD130	G
UD034	G	UD073	A	UD132	G
UD035	G	UD075	A	UD133	A
UD037	G	UD079	G	UD136	A
UD040	G	UD081	G	UD137	G
UD041	G	UD083	G	UD142	G
UD043	A	UD086	G	UD144	G
UD044	G	UD087	G	UD145	G
UD045	A	UD088	G	UD147	G
UD046	G	UD089	G	UD148	G

	G	A
UCD	62/81	19/81
WE	94/119	25/119
Total	156/200	44/200

	G	A
UCD	0.77	0.23
WE	0.79	0.21
Total	0.78	0.22