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# Linkage Analysis of Caffeine Resistance and Circadian Rhythm in Caffeine-Treated DDT Resistant and Susceptible Strains of *Drosophila melanogaster*

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To the Graduate Council:

I am submitting herewith a thesis written by Chandrashis Bhowmick entitled "Linkage Analysis of Caffeine Resistance and Circadian Rhythm in Caffeine-Treated DDT Resistant and Susceptible Strains of *Drosophila melanogaster*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Ranjan Ganguly, Major Professor

We have read this thesis and recommend its acceptance:

Jae H. Park, Mariano Labrador

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have read this thesis
and recommend its acceptance:

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Vice Provost and Dean of the
Graduate School

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# Linkage analysis of caffeine resistance and circadian rhythm in caffeine-treated DDT resistant and susceptible strains of *Drosophila melanogaster*

**A Thesis**

**Presented for the Master of Science Degree**

**The University of Tennessee, Knoxville**

**Chandrashis Bhowmick**

**August 2007**

# Dedication

This thesis is dedicated to my beloved parents, wife and Dr. A. K. Banerjee.

**Acknowledgement**

In preparing this thesis I am indebted to many people both in the United States and India whose encouragement and support helped me to write this thesis, and it is a great pleasure for me to thank them all. First, I would like to thank the Department of Biochemistry & Molecular Genetics for giving me the opportunity to attend the University of Tennessee and for providing financial support.

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## Abstract

Caffeine is found in coffee, tea, soft drinks, many plant products and various drug preparations. It is the most consumed common psychoactive drug around the world. Consumption of caffeine causes several behavioral and physiological responses in humans and other mammals. Caffeine is also known to be an insect repellant and can be used as an insecticide. As observed in mammals, caffeine treatment increases the locomotor activity in insects including *Drosophila*. However, very little is known about genetic and molecular basis of caffeine sensitivity and action in insects. In the present study, I have used DDT resistant (91-R) and susceptible (91-C and $ry^{506}$) strains of *Drosophila melanogaster* to examine whether these strains also differ in caffeine resistance and locomotor activity following caffeine treatment. Results showed that time required for 50% mortality (LT-50) of the 91-R strain were at least 2-fold higher than the LT-50 of the 91-C and $ry^{506}$ strains. In all strains, caffeine LT-50 was found to be at least 1.5-fold higher in females than in males. I also used chromosome substitution stocks made between the DDT resistant 91-R and DDT susceptible 91-C and $ry^{506}$ strains. Caffeine-mortality tests on these stocks showed that the major resistance factors against caffeine are linked to the second chromosome and the factors on the X and the third chromosomes play a minor but positive role. Experiments on locomotor activity showed that on caffeine-free media both DDT resistant and susceptible strains were more active during light than dark cycle. While the both DDT susceptible strains showed increased locomotor activity on caffeine media during dark and light cycle, the DDT resistant 91-R strain did not show any change in locomotor activity on medium containing low dose

(1.5mM) caffeine. This refractoriness to low dose of caffeine appears to be linked to the second chromosome as deduced by examining the chromosome substitution stocks; strains carrying the second chromosome of 91-R displayed this behavior. On the other hand, locomotor activity of the DDT resistant strain decreased both during light and dark cycle when exposed to higher dose (3mM) of caffeine. This behavior is again found to be linked to the second chromosome because chromosome substitution stocks carrying the second chromosome of the 91-R strain showed decrease in locomotor activity on medium containing 3mM caffeine. The X and 3$^{rd}$ chromosomes also carry factors that modulate the effect of the second chromosome, but in a complex manner.

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## A. General Introduction

All living organisms are exposed to different chemicals that are present in the environment. Many of these chemicals, foreign to the living body (xenobiotics), are consumed via food, water and air. Different organisms respond to these compounds differently and show diverse physiological and behavioral responses. Organism's response to the xenobiotic substances is an important topic because xenobiotics can affect an individual's health, survival and fecundity (Carillo and Gibson, 2002). Response to xenobiotic compounds depends on various factors such as the rate of absorption, transport via circulation, metabolism and excretion. Since these factors are genetically determined and genetic polymorphisms are quite prevalent in the population of a given species, xenobiotics may not affect all individuals similarly. One of the most well known example is some strains of a given insect species is resistant to variety of insecticides while most strains are not. This has been observed in many insects viz., *Musca*, *Anopheles*, *Helicoverpa*, *Drosophila* and etc (Hemingway et al., 1998; Scott, 1999; Feyereisen, 2005).

Various chemicals present in natural and manufactured food and drinks may also affect the physiology and behavior. Again, not all individuals in a given population show similar effect to a xenobiotic because some individuals are resistant or tolerant whereas others are not. Caffeine is one such xenobiotic compound that is heavily consumed worldwide. The natural sources of caffeine are leaves, seed or fruit of more than sixty plant species, the most well known being coffee, tea and cocoa. The amount of caffeine necessary to produce any effect varies among individuals and it depends on the body size and metabolic rate. The direct effect of caffeine and gene expression has been widely

studied (Bhaskara et al., 2006) but a clear picture between the genetic and pharmacological variation is yet to be deciphered. Response to caffeine is a complex phenomenon because it is the outcome of activity of multiple genes that can interact with each other and the environment. Nevertheless, many studies have already been successful in identifying the genetic loci affecting caffeine response and other complex behaviors (Bhaskara et al., 2006; Chung et al., 1998).

## 1. Caffeine- its source, chemistry and consumption

Caffeine is a psychoactive drug widely consumed in the world through various beverages. In its pure state, caffeine is a bitter, white alkaloid and a xanthine derivative methylated at N-1, N-3 and N-7 positions. N-1, N-3, and N-7 demethylation reactions produce theobromine (TB), paraxanthine (PX), and theophylline (TP) respectively (Chung et al., 1998). Caffeine is present in dietary sources like coffee, tea, chocolate bars, soft drinks and cocoa beverages (Lorist and Tops , 2003) The word comes from the Italian term for coffee, caffè, German *Kaffein* or French *caffeine*. It was first extracted from coffee in 1821. It is believed that coffee originated in Ethiopia and by the end of 17th century A.D. it was introduced to the rest of the world for its stimulatory effect of temporarily warding away drowsiness and restoring alertness. Global consumption of caffeine has been estimated today at 120,000 tons per annum, which makes it the world's most popular pyschostimulant substance. This number corresponds to one serving of a caffeine beverage for every person, per day. In North America, 90% of adults consume some amount of caffeine daily. The U.S. Food and Drug Administration lists caffeine as a "Multiple Purpose GRAS [Generally recognized as safe] Food Substance".

Caffeine content in common beverages such as coffee and tea, as well as in many carbonated drinks is summarized in Table 1. Caffeine is a central nervous system and metabolic stimulant, and it is also used in various pharmacological preparations and medications related to heart diseases and cold/flu remedies.

Caffeine reduces physical fatigue and restores mental alertness when unusual weakness or drowsiness occurs. It is completely absorbed by the stomach and small intestine within forty-five minutes of ingestion. It gets rapidly absorbed into the blood stream and is distributed throughout all the tissues in 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) and eliminated by first-order kinetics. Caffeine is widely used and its content in consumer products is not restricted. In fact, certain beverages contain about approximately 8 mg per liquid ounce and energy drugs, like Vivarin, contain approximately 200 mg each.

**2. Behavioral and physiological effects of caffeine**

Because of its widespread use, effect of caffeine on various physiological processes in humans, rats, mice and other mammals have been studied (Svenningsson et al., 1995). Extensive studies have been done on the effect of caffeine on sleep, hypertension and cardiac physiology. A modest number of studies have also been made on the effect of caffeine on cell cycle checkpoints, DNA repair, apoptosis and carcinogen metabolism have also been examined (Porta et al., 2003). Consumption of caffeine can cause changes in behavioral and physiological responses in humans because the major effect of caffeine is observed on the central nervous system (Fredholm et al., 1999).

These include those behavioral effects commonly experienced by its consumers, such as restoring alertness and cognition.

Upon caffeine consumption, physiological changes such as tachycardia, hypertension and increased blood flow in muscles and decreased blood flow in skin and internal organs are observed (Berne et al., 1998). Caffeine is also a strong diuretic and appetite suppressant, and is therefore an important component in diets and weight loss medications (Mandel , 2002). Caffeine is also reported to have anti-carcinogenic effect by inhibiting abnormal cell growth by viral, chemical and physical agents (Porta et al., 2003). Caffeine can also be used to treat Parkinson's disease and offer neuroprotection due to its property to block A2A receptors (Chen , 2003). High dosage of caffeine causes feelings of anxiety and nervousness as well as insomnia (Fredholm et al., 1999) and lead to abnormal effects such as hypertension, tachycardia, diuresis, nausea and tremors (Berne et al., 1998). Withdrawal symptoms of caffeine intake include drowsiness, muscle spasm, headaches, lethargy and depression (Dews et al., 2002). Caffeine acts as a mutagen and affects plant and mammalian cell growth in culture. In mitosis, cells synthesis phase cannot proceed until the DNA has been replicated. However, in the presence of caffeine, the factor that prevents cell from dividing before DNA replication is disrupted (Alberts et al., 1994), thus, the cells finish S phase without DNA replication, leading to chromosomal loss and abnormalities (Timson, 1977). Caffeine is known to be mutagenic in *E. coli* and other bacteria (Timson, 1977). It also causes chromosomal loss and mutations in *D. melanogaster* larvae (Mittler et al., 1967; Clark and Clark et al., 1968).

Caffeine acts as central nervous system stimulant by blocking the receptors of neuromodulator adenosine (Snyder et al., 1981; Fredholm , 1995). Neuromodulators are compounds that modulate the regions or circuits of the brain. Though many neuromodulators do also act as neurotransmitters, yet unlike the latter they are not found in presynaptic vesicles and produce effect pre- or post-synaptically without being metabolized (Alberts et al., 1994). Adenosine modulates the brain function by central inhibitory actions. So far, four adenosine receptors, $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$ have been cloned and characterized in several species. The effects of caffeine are believed to occur primarily by antagonizing two adenosine receptor subtypes, $A_1$ (inhibitory adenosine receptor) and $A_{2a}$ (stimulatory adenosine receptor) receptors or by inhibiting the phosphodiesterase enzyme (PDE) (Ferre, 1997). $A_1$ and $A_{2a}$ are both G-protein coupled receptors, and present in different regions of the brain, with the A1 receptors being widely distributed while the $A_{2a}$ receptors are concentrated in the striatum of the brain (Ferre, 1997; Fredholm, 1995). $A_1$ is coupled to the inhibitory G-proteins like Gi-1, Gi-2, Gi-3, Go1 receptors and $A_{2a}$ receptor is coupled to the stimulatory G-protein like Gs (Fredholm et al., 1999; Fredholm et al., 2001). Binding of adenosine to the $A_1$ receptor causes inhibition of adenylyl cyclase and decreases the cAMP level, a common second messenger in G-protein signaling pathways. On the other hand, binding of adenosine to the $A_{2a}$ leads to stimulation of adenylyl cyclase and increases the intracellular cAMP level (Berne et al., 1998, Purves et al., 2001). In the central nervous system, this leads to change in neurotransmitter release, which affects the neuronal activity (Fig. 2) Adenosine concentration decreases during sleep and increases while awake. (Huston et al., 1996). Caffeine acts in two ways. First, because its structure is similar to adenine (Fig. 1), it

binds with and antagonizes the inhibitory A1 type adenosine receptor. Second, caffeine inhibits cAMP phosphodiesterase enzyme that breaks down cAMP. Both these actions increase the intracellular level of cAMP which initiates cascades of events leading to induction of transcription. In mammals, cAMP has been shown to regulate Cytochrome P450 transcription (Viitala et al., 2001; Guo et al., 2003). *Drosophila melanogaster* has putative adenosine receptor that is similar to that of A1 and $A_{2a}$ human adenosine receptors. The sequence alignment of these receptors is shown in Fig 3. In *Drosophila dunce* gene encodes cAMP phosphodiesterase enzyme and mutation of this gene increases the cAMP level.

Evidences in the literature show that caffeine and circadian rhythm often go hand in hand (Meadahl; 2000). Circadian rhythms are operationally defined as 24-hour biological rhythms that persist in the absence of daily light-dark and temperature cycles (Edery; 2000). Caffeine, being an adenosine antagonist, affects sleep and increase arousal (Fredholm et al., 1999). Caffeine when administered systematically **(**injected**),** has been shown to modulate circadian rhythm in Syrian hamsters in a dose dependent manner (Antle et al., 2001). It has been shown that caffeine mimics the effect of light on clock cells thereby reducing or completely blocking phase shifts. In mice, caffeine administration was shown to significantly influence the circadian rhythms of heart rate, temperature controls and motor activity under controlled conditions and 12-hour dark/light cycles (Pelissier et al., 1999).

The results of circadian research may hold the promise of significant medical applications. At the most basic level, circadian variations affect both the course of disease and the efficacy of medications. The first genetic links to circadian rhythms were

found in the fruit fly *Drosophila melanogaster*, **(**Konopka and Benzer, 1971; Edery., 2000**)**. *Drosophila* turns out to be an ideal model system to study neurobehavioral genetics because of their relatively simple central nervous system (CNS) and, complex enough behavioral phenotypes that are analogous to human behaviors (Carillo and Gibson, 2002). The identification of the clock gene, *Period* or *per*, which has been associated with the clock function in many organisms, was identified first in *Drosophila* by Ron Konopka and Seymour Benzer using *Drosophila* in 1971 (Edery., 2000). These *per* flies show conservation of behavioral changes in response to caffeine and to an adenosine agonist that produces sleep in mammals (Hendricks et al. 2000).

**3. Caffeine as insecticide**

Caffeine is a plant alkaloid, found in numerous plant species, where it acts as a natural pesticide that paralyzes and kills certain insects feeding upon them. Caffeine resistance has been studied extensively in invertebrates (Bard et al., 1980; Benko et al., 1997), with the majority of studies focusing on the development of resistance to insecticides. Researchers have shown that addition of caffeine to the diet for both larvae and adult Drosophila could lead to severe consequences. It has been shown that, in relatively high doses, caffeine is lethal to *Drosophila melanogaster* larvae (Zimmering et al., 1977), and in small doses 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 adult flies (Zimmering et al., 1977), but no sex differences have been observed in larvae (Nigsch et al., 1977). Caffeine is found to be effective in killing or repelling slugs and snails when applied to foliage (Hollingsworth et

al., 2002). Caffeine toxicity is shown to impair the larval growth and act as adult control in *Aedes aegypti* (Laranja et al., 2006).

**4. Research objectives:**

In *Drosophila melanogaster*, loci giving resistance to DDT and other insecticides have been mapped to the second chromosome. In the field collected *Drosophila*, the resistance locus maps close to ~64-67 cM on the right arm of $2^{nd}$ chromosome (Tsukamoto and Ogaki, 1953). Caffeine, a plant alkaloid, has also been reported to be a natural pesticide that paralyzes and kills certain insects feeding on the plants (Starr; 1999). Although Zimmering and his colleagues (1977) reported that different genetic stocks of *Drosophila* show differential survival rate when exposed to 1% caffeine solution. Preliminary observations also showed that DDT resistant and susceptible strains of *Drosophila* differ in caffeine resistance and circadian rhythm (Jae Park, unpublished observations). However, there is no published report showing the chromosomal linkage of the caffeine resistance and caffeine mediated change in circadian rhythm in *Drosophila*. Therefore the major focus of the proposed research is to use *Drosophila melanogaster* to elucidate the chromosomal linkage to caffeine resistance.

For this purpose, I used DDT resistant, 91-R and susceptible 91-C and $ry^{506}$ strains. I also used several chromosome substitution stocks made by using these parental strains. Since caffeine is known to cause hyperactivity (Barry et. al., 2005), in the second objective I examined whether caffeine has any effect on circadian rhythm in the three parental strains and chromosome substitution stocks mentioned above. These studies will demonstrate chromosomal linkage of caffeine resistance and whether there is any

difference between DDT resistant and susceptible strains with respect to effect of caffeine on circadian rhythm.

**B Materials and Methods:**

**1. Fly strains and culture condition**

In the present investigation three parental strains, *ry*$^{506}$, 91-R and 91-C, and twelve chromosomal substitution strains of *Drosophila melanogaster,* were used. The origin of 91-C and 91-R strains has been described previously (Maitra et al., 2002). Briefly, a large collection of flies caught in wild was split into two populations. One population (91-R) was selected on DDT medium for about 14 years while the other population (91-C) was never exposed to DDT (Dapkus and Merrell, 1977). In the *ry506* eye color mutant strain gene encoding xanthine dehydrogenase has a mutation. The population of 91-R strain used in the present investigation has not been on DDT selection since 1988. Periodic assay showed that DDT resistant phenotype is still maintained in the 91-R strain. 91-C and *ry506* strains on the other hand are susceptible to DDT.

To understand the chromosomal effects on caffeine resistance, chromosome substitution stocks carrying different combinations of the X, 2$^{nd}$ and 3$^{rd}$ chromosomes from the 91R and 91C strains were synthesized by Vita Lam, a graduate student in the lab. These strains are: RCC, CRR, RCR, CRC, RRC, and CCR (where R stands for the 91-R gene and C stands for the 91-C gene, and each arrangement follows the order of X, 2$^{nd}$, and 3$^{rd}$ chromosome positions) (Table 2). Similarly, another set of chromosome substitution stocks carrying different combinations of the X, 2nd, and 3rd chromosomes from the 91R and ry$^{506}$ strains were synthesized: Rrr, rRR, RrR, rRr, RRr, and rrR (where R stands for the 91-R gene and r stands for the ry$^{506}$ gene, and each arrangement follows the order of X, 2$^{nd}$, and 3$^{rd}$ chromosome positions) (Table 3). Both parental and chromosome substitution stocks were maintained in bottles on standard agar-cornmeal-

molasses medium with yeast and kept at $25^0$ C on a 12-hour light/dark cycle throughout the experiment.

Caffeine bioassays were performed on all twelve newly generated chromosome substitution stocks of $ry^{506}$-91R and 91C-91R as well as the controls to examine the effects of X, 2nd and 3rd chromosome on mortality and locomotor activity due to caffeine.

**2. Treatment with caffeine and mortality test**

Caffeine, anhydrous and powdered, ordered from Sigma was made into a 150mM aqueous stock solution and stored in $4^0$C. For the mortality test, required volume of aqueous caffeine solution was directly added to molten fly food containing 1% agarose-5% sucrose just prior to pouring into empty glass vials [disposable; 6mm x 50mm; Fisher] and stored in $4^0$C to solidify. The final concentration of caffeine in the media was 7.5mM in case of $ry^{506}$-91R substitution stocks and 15mM for 91C-91R substitution stocks.

**3. Statistical Analysis of Mortality test**

The number of live flies was counted every twelve hours until all of the flies were dead. The cumulative observation for the live flies was calculated and immediately recorded in Microsoft excel sheet. Based on the cumulative data, line graphs for each strain were plotted and the Lethal Time 50% was calculated. The lethal time 50% ($LT_{50}$) or the median lethal time is the time required to kill half the members of a tested population. Data were analyzed by SAS software. To compare the sexes between and within each strain, paired Student's T-test was performed. Analysis of variance

(ANOVA) was also 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.

**4. Treatment with caffeine and circadian rhythm assay**

The caffeine stock solution used for the mortality test was also used for the circadian rhythm assay. For this experiment, fly food was prepared in pyrex glass tubes [non-disposable; 5mm dia x 65mm length ; Trikinetics] with aqueous caffeine added to a required concentration in 1% agar-5% sucrose and stored in $4^0$C to solidify. For control, fly food without caffeine added to agarose-sucrose media was also prepared and stored in $4^0$C to solidify. In both the cases, fly food was used between 12 to 24 hours after preparation. For the circadian rhythm assay, only male flies were used to avoid interference from the eggs or the larvae. Sixteen male flies from both parental and substitution stocks were taken.

For mortality test, forty flies of each sex from both parental and substitution stocks were separated. Flies screened for caffeine resistance were collected between three and five days after emergence and were kept on standard cornmeal media for one day prior to placing on caffeine treatment. These flies were etherized and then separated by sex. Each fly was treated in separate vials. The parental stocks and chromosome substitution stocks of $ry^{506}$ and 91-R were treated on 7.5mM caffeine containing media for the mortality test. Similarly, the parental stocks and chromosome substitution stocks of 91-C and 91-R were treated on 15mM caffeine containing media for the mortality test. Starvation resistance on agar medium was also measured as a control for variation in

overall fitness between the lines and sexes. In both the experiments, all the flies in vials were kept at $25^0$C on a 12-hour dark-light chamber throughout the experiment.

Eight males were treated on agar-sucrose medium mixed with caffeine and the other eight males were treated on agar-sucrose medium without caffeine, as control. The parental stocks and chromosome substitution stocks of $ry^{506}$ and 91-R were treated on 1.5 mM caffeine containing media for the locomotor activity test. Similarly, the parental stocks and chromosome substitution stocks of 91-C and 91-R were treated on 3mM caffeine containing media for the locomotor activity test. At the beginning of the experiment, individual male flies were placed in the Drosophila Activity Monitor System (DAMS, Trikinetics, Waltham, MA, USA) inside glass tubes (one fly/tube) with enough food for 1 week of recording. All the flies in vials were kept at $25^0$C on a 12-hour dark-light chamber throughout the experiment. Monitors were housed inside environmental chambers (ThermoForma, Marietta, OH, USA) where temperature and humidity were kept constant. Each DAMS monitor contained 32 glass tubes. As each fly moved back and forth in the tube, it interrupted an infrared light beam that bisected the tube, and the accumulated count totals are reported to the host computer at the conclusion of each reading period and the number of counts/min was stored every 30 mins. Total period of observation was 7 days, including 1 day of adaptation.

**5. Statistical analysis of Circadian Rhythm**

Data analysis was performed using Matlab (Mathworks) software. The locomotor activity per thirty minutes for each fly was recorded on Microsoft excel sheet. Based upon the data, mean locomotor activity for each strain on normal food and caffeine

treated food for both dark and light cycle was calculated. Data was further analyzed by SAS software. To compare the circadian clock between each strain relative to control and caffeine treatment, paired Student's T-test was performed. Analysis of variance (ANOVA) was also performed using SAS Proc GLM to determine the significant difference among the strains.

## C. Results

### 1. Sexual dimorphism in caffeine resistance in DDT resistant and susceptible strains, and their chromosome substitution stocks

Three parental strains, $ry^{506}$, 91-C and 91-R and their chromosome substitution stocks were assayed to examine the effect of different chromosomes on caffeine resistance. While 91R is highly resistant, $ry^{506}$ and 91C are highly susceptible to DDT (Kuruganti et al., 2007). To examine sexual dimorphism in caffeine resistance, adult males and females between three and five days of age were separated and singly placed in vials containing agar-sucrose media mixed with caffeine. For $ry^{506}$ and 91-R parental strains and their chromosome substitution stocks, 7.5mM caffeine was used, whereas 15mM caffeine was used for 91-C and 91-R strains, and the chromosome substitution stocks synthesized using these stocks. Based on the preliminary observations made by Dr. Jae Park these concentrations of caffeine were chosen. In both experiments, the flies showed significant hyperactivity within 12 hours of transfer to the caffeinated food. The number of flies that were alive was counted every twelve hours until all flies in each vial died. Number of flies that die during the entire period of experiment on non-caffeine containing food was used as control. To determine the effect of caffeine on survival rate, cumulative percent death against time in hours was plotted. Results obtained for males and females of the chromosome substitution stocks made between $ry^{506}$ and 91-R strains were plotted as line graphs in Figures 4 and 5, respectively (subsequent figures and tables appear in the Appendix). Lethal time 50 (LT50) values for each strain and sex were determined from these line graphs, and the mean LT50 values obtained from three independent experiments are shown as bar graph in Figure 6 and the quantitative data are

shown in Table 4. LT50 values for males and females of 91-C and 91-R strains, and their chromosomal substitution stocks were also obtained by similar strategies. The line and bar graphs are shown in Figures 7, 8 and 9, and the quantitative data on LT50 of different stocks are shown in Table 5.

Figures 6 and 9, and Tables 4 and 5 show that the male flies are generally more sensitive to caffeine than female flies, especially for $ry^{506}$ and 91R, and the chromosome substitution stocks made from these stocks. The observed results cannot be attributed to a batch effect of the food because in all three independent experiments the same observations were made. In separate analyses, the food batches were found not to significantly affect the survival times (data not shown). To compare caffeine resistance between males and females of each stock, paired Student's T-tests were done. The *P* values for all stocks were <0.05 except for Mojito (rRR), Kamikaze (CCR) and Long Island (RCR). Therefore, it may be concluded that the females of the parental stocks are more resistant to caffeine than the males, and this is also true for most chromosome substitution stocks made between $ry^{506}$ and 91R, and between 91C and 91R strains.

Results also show that the DDT susceptible 91-C strain is more susceptible to caffeine than the DDT resistant 91-R strain (Table 5). The LT50 values of 91-C males and females were approximately 3-times lower than the LT50 values of the males and females of 91-R strain. Student's T-test showed that this difference is statistically significant ($P$ <0.001). The DDT susceptible $ry^{506}$ strains is also more susceptible than the DDT resistant 91R strains, but the difference could not be quantified because 7.5mM caffeine used to compare these two strains did not kill any 91R fly even after 216 hours exposure to caffeine (Table 4).

**2. Chromosomal effect on caffeine resistance**

Figures 6 and 9, and Tables 4 and 5 also show the effect of different chromosomes on the LT50 values. It was found that if the X-chromosome of the 91-C strain was replaced with the X-chromosome from the 91-R strain, no change in LT50 was observed in males or females (Fig. 9, and Table 5). This is evident if chromosome composition CCC is compared with RCC (chromosomes are written in the order X, 2nd and 3rd). However, substitution of the X chromosome of the *ry*$^{506}$ (rrr) with the X from 91R strain caused a significant increase in the LT50 values in both sexes (rrr vs Rrr, Fig. 6 and Table 4). If only the 3rd chromosome of *ry*$^{506}$ or 91C is replaced with that from the 91R strain, a differential effect was observed. A significant increase in LT50 was seen when the 3rd chromosome of the *ry*$^{506}$ was substituted with the 3rd chromosome from the 91R strain (rrr vs rrR, Table 4). In case of 91C strain, substitution of the 3rd chromosome showed significant increase in LT50 in males but not much in females (CCC vs CCR, Table 5). When both the X and the 3rd chromosomes of the *ry*$^{506}$ or 91C strain were substituted with the respective chromosomes from the 91R strain, a much higher increase in the LT50 value was observed in these two-chromosome substituted stocks (RrR vs rrr, and RCR vs CCC, Tables 4 and 5) compared to the stocks with only X or 3rd chromosome substitution. The most dramatic effect on LT50 was observed when the 2nd chromosome of 91-R was introduced in the genome. Thus, substitution of the 2nd chromosome of *ry*$^{506}$ and 91-C strain with the 2nd chromosome from the 91-R strain gave approximately a 3-fold increase in LT50 in both cases (rrr vs rRr and CCC vs CRC, Tables 4 and 5). Based on these observations it can be concluded that though substitution of 2nd chromosome with 91-R strain confer highest caffeine resistance, the genetic factors

present on the X and 3rd chromosomes may also play a minor positive role because RRR chromosome composition shows higher LT50 than CRC or rRr stocks.

### 3. Chromosomal linkage of circadian rhythm upon exposure to caffeine in *Drosophila melanogaster.*

Caffeine treatment has been shown to induce hyperactivity in mammals as well as in *Drosophila* (Matsuoka et al., 1987; Shaw et al., 2000)**.** In the present investigation three parental strains, $ry^{506}$, 91-R and 91-C and their twelve chromosome substitution stocks were used to determine whether the parental strains differ in the degree of caffeine-induced hyperactivity, and if they do which chromosome plays a major role in this regard. Therefore, adult male flies between three and five days of age were placed in vials containing agar-sucrose media mixed with caffeine and without caffeine, as control. For $ry^{506}$ and 91R parental and their chromosome substitution stocks, the final concentration of caffeine was 1.5mM, and for 91-C and 91R parental and their chromosome substitution stocks 3mM caffeine was used. Based on the observations for 7 consecutive days and nights, an average locomotor activity per 30 minutes for each strain during day and night on non-caffeine and caffeine food was calculated separately. The mean locomotor activity per 30 minutes for each strain on non-caffeine and caffeine food during 12: 12 hour light/dark cycles for 7 days and 7 nights was plotted and shown in Figures 10 and 11. The quantitative values of these results are presented in Table 6 and Table 7. Figures 12 – 15 show the activity profile of each strain listed in Tables 6 and 7.

In circadian rhythm assays (Fig. 10 and Fig. 11) the DDT susceptible strains, $ry^{506}$ and 91-C show consistency in caffeine hyperactivity, under both light and dark cycles

(Tables 6 and 7). However, the DDT resistant 91-R strain, under light conditions, showed no significant increase in activity upon 1.5mM caffeine treatment compared to the normal food (Table 6), but a significant decrease in activity was observed on 3.0mM caffeine-treated food under dark conditions (Table 7). The activity profiles (Figures 12-15) also show the similar trend. This proves that the arousal effect of caffeine during nighttime was evident in $ry^{506}$ and 91-C strains but not in 91-R strain.

The circadian data obtained for each strain (Tables 6 and 7) can be used to compare the activities between light and dark cycles, and between caffeinated and normal food. As expected, all parental strains show higher locomotor activity during light compared to the dark cycle. Similarly, they show higher activity when exposed to caffeinated food than the normal food. This is true for $ry^{506}$ and 91C strains, but the 91R strain does not show any increase in activity when exposed to caffeinated food. When compared between strains, activity of the 91R strain is found to be significantly higher than the activity of the 91C strain on caffeinated or normal food, and during light or dark cycle (Table 7). However, activity of the 91R strain is found to be similar to that found in the $ry^{506}$ strain. This may be because $ry^{506}$ and 91R strains and their chromosome substitution stocks were examined on 1.5mM caffeine whereas 91C and 91R stocks were examined on 3mM caffeine.

Tables 6 and 7 also show the chromosomal effects on the locomotor activities. To compare the effect of the chromosome, activity of the flies of different chromosome compositions exposed to the normal food needs to be compared. When the X-chromosome of the $ry^{506}$ strain was replaced with the X from the 91R strain, a significant increase in activity was observed on normal food both under dark and light cycles (rrr vs

Rrr, Table 6). This suggests the X-chromosome of the 91R strain makes the fly more hyperactive on normal food irrespective of the time of the day. Since caffeine also increased the activity (~2 fold) of the Rrr genotype both during light and dark cycles as it does in case of rrr genotype, it can be concluded that the X-chromosome of 91R does not alter the caffeine-induced hyperactivity. Substitution of the X-chromosome of the 91C with that from the 91R strain also increased activity of the flies on normal food only during dark cycle; no change in activity was observed under light cycle (CCC vs RCC, Table 7). Again, caffeine increased activity (~2 fold) of the RCC flies as expected. The differential effect of the X-chromosome of 91R in Rrr and RCC stocks could be due to the difference of chromosomal composition.

The effect of the 3$^{rd}$ chromosome of the 91R strain on the locomotor activity also depends on the composition of the other chromosomes. For example, on normal food the rrR genotype shows higher activity than the rrr genotype only in dark cycle; very little difference between the two genotypes is found in light cycle (Table 6). However, rrR genotype shows the usual increase in activity (~ 2- 2.5 fold) on caffeinated food at both times of the day. A different effect of the 3$^{rd}$ chromosome of the 91R strain is found when it is present in the same genome with the X- and 2$^{nd}$ chromosomes of the 91C strain (CCC vs CCR, Table 7). Although caffeine increases the activity of the CCR strain both during light and dark cycles, on normal food the activity of the CCC and CCR stock is more or less similar at both times of the day (Table 7).

Substitution of both the X and 3$^{rd}$ chromosomes of the *ry*$^{506}$ with those from the 91R strain did not alter the activity significantly on normal and caffeinated food under dark and light cycle (rrr vs RrR, Table 6). However, when similar substitutions were

made for the 91C strain, a significant increase in activity was observed both on caffeinated and normal food but in light cycle only (Table 7).

The effect of the 2nd chromosome of the 91R strain also shows some dependency on the source of the X and the 3rd chromosomes. When the 2nd chromosome of the *ry*$^{506}$ or the 91C strain was substituted with the 2nd chromosome from the 91R strain, a huge increase in the locomotor activity was observed both during light and dark cycles only when the flies were kept on normal food. This is evident if the activities of the rrr and CCC stocks are compared with the activities of the rRr and CRC stocks, respectively (Tables 6 and 7). Surprisingly, both these chromosomal substitution stocks, rRr and CRC, did not show any increase in activity at any time of the day when exposed to caffeinated food. Caffeine treatment either decreased or did not change the activity of these flies. It is also clear from the data (Tables 6 and 7) that if a stock carries the 2nd chromosome of 91R strain, it becomes refractory to caffeine-induced hyperactivity, although it (except RRr) shows much higher locomotor activity during dark cycle on the normal food compared to its parental stock (rrr or CCC). This becomes evident if rrr is compared with rRr, RRr or rRR, and CCC is compared with CRC, RRC or CRR (Tables 6 and 7). Although addition of the X or 3rd or both chromosomes of the 91R strain into the genome carrying the 2nd chromosome of the 91R changes the activity values, it does not make the flies sensitive to caffeine treatment; the stocks do not show increased activity following caffeine treatment during dark or light cycle. In keeping with this observation, 91R strain (RRR) also does not show caffeine-induced hyperactivity any time of the day. It can also be concluded that the other chromosomes influence the

specific effect of each chromosome to the circadian rhythm. Therefore, the interaction effect within chromosomes cannot be overruled.

**D. Discussion**

The goal of this research project was to determine the chromosomal linkage of caffeine-induced mortality in both sexes and phase shifts of the circadian clock in *D.melanogaster*. Our study shows that female flies are more resistant to caffeine than males, which is consistent with the proposed explanations by Zimmering *et al* (1977) and Ityoyama *et al* (1998) studies. The proposed explanations for such varied resistance was difference in body size and physiological repair efficiency between males and females. When treated with DDT, male *Drosophila* are known to show higher mortality rate than the females (Feyereisen 1995; Ganguly, unpublished data). It is believed that in females DDT is released in the hemolymph at a much slower rate than in the males because higher fat content in the females traps DDT longer causing it to be released more slowly. A similar mechanism may also explain the difference in LT50 values between males and females observed in the present study. The results presented in this project on caffeine resistance have similarities with DDT resistance in *Drosophila*. Studies by Tsukamoto and Ogaki (1953) showed that a locus at map position 64 cM on the $2^{nd}$ chromosome is responsible for resistance to DDT and few organophosphate insecticides. Resistance to imidacloprid insecticide in *Drosophila* has also been mapped to 64 cM on the second chromosome (Daborn et al., 2001). In addition to 64cM, other loci associated with DDT resistance have also been reported. Dapkus (1992) showed that in the 91R strain of *Drosophila melanogaster*, DDT resistance maps close to 56cM on the second chromosome. Although these studies suggest that the second chromosome has major resistance loci for DDT and other insecticides, loci at map positions 58.0 and 62.0 on the $3^{rd}$ chromosome have also been implicated in DDT resistance (Hallstrom, 1985;

Hallstrom and Blanck, 1985; Houpt et al., 1988). These suggest that DDT resistance in *Drosophila* is a multifactorial trait. Our study also shows that although the 2nd chromosome plays a major role in caffeine resistance, the X and 3rd chromosomes also have genetic factors that enhance the caffeine resistance. In view of this, like DDT resistance, caffeine resistance appears to be a multifactorial trait with 2nd chromosome playing a major role.

The locomotor activity results showed that flies carrying the second chromosome of the 91R strain make the flies hyperactive in the absence of caffeine. It could be that the 2nd chromosome may be responsible for the production of endogenous metabolite, which may antagonize the adenosine receptor. Caffeine-mediated hyperactivity is known to be mediated via adenosine receptor. Alternatively, there may be some other unknown genetic factors that make flies normally hyperactive and these genetic factors may be linked to the 2nd chromosome of the 91R strain. It is interesting to note that the same flies carrying the second chromosome of 91R strain are refractory to caffeine-induced hyperactivity. It is possible that the 91R strain is a fast-metabolizer of caffeine, and as a result caffeine is cleared quickly from the hemolymph of the flies. As a result, adenosine receptor may not be antagonized by caffeine in strains carrying the 2nd chromosome of the 91R strain.

Our approach about chromosomal linkage to both mortality rate and circadian rhythm provide indirect information as to the sources of variable caffeine response. Direct approaches like cloning of mutations that produce discrete responses; genetic mapping and protein expression studies among lines are needed to reveal the sources of variability. Nevertheless, our results provide some insight into the initial characterization

of the genetic architecture of survival time and behavioral response upon caffeine exposure in Drosophila and show that sex, genotype, and interaction effects that are prevalent in such response. The measurements are instrumental to the scientific study of fly neurobehavior and its genetic basis.

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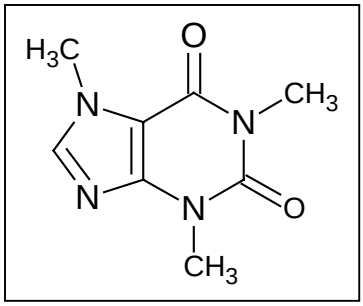
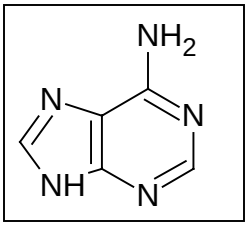
# APPENDIX

**Table1: Caffeine content in common beverages**

| Caffeine content of select common food and drugs | | |
|---|---|---|
| Product | Serving size | Caffeine content (mg) |
| Caffeine tablet -Vivarin | 1 tablet | 200 |
| Excedrin tablet | 1 tablet | 65 |
| Coffee, brewed | 240ml | 135 |
| Coffee, decaffeinated | 240ml | 5 |
| Coffee, espresso | 57ml | 100 |
| Dark Chocolate (Hershey's) | 1bar | 31 |
| Milk Chocolate (Hershey's) | 1bar | 10 |
| Red Bull | 250ml | 80 |
| Powershot | 30ml | 100 |
| Cocaine Energy drink | 250ml | 280 |
| Rockstar Energy drink | 473ml | 160 |
| Jolt Cola | 694ml | 150 |
| Soft drink 'Mountain dew' | 355ml | 54.5 |
| Soft drink 'Coca Cola classic' | 355ml | 34 |
| Green Tea | 240ml | 15 |
| Tea leaf- Bag | 240ml | 50 |

(Caffeine content of foods and drugs, 1996 and Erowid, 2006**)**




**Caffeine** **Adenine**

**Fig1: Molecular structures of caffeine and adenine**

**Table 2. Chromosome Substitution Stocks: Cross Between 91R$^{y}$800 vs. 91C-SK:**

| STRAIN NAME | CHROMOSOME ARRANGEMENT |
|---|---|
| Martini | R C C |
| Long Island | R C R |
| Kamikaze | C C R |
| Cosmo | C R R |
| Sea Breeze | C R C |
| Mudslide | R R C |

**Table 3. Chromosome Substitution Stocks: Cross Between 91R$^{y}$800 vs. ry$^{506}$:**

| STRAIN NAME | CHROMOSOME ARRANGEMENT |
|---|---|
| MaiTai | R r r |
| Pina Colada | R r R |
| Zombie | r r R |
| Daquiri | r R r |
| Bacardi | R R r |
| Mojito | r R R |

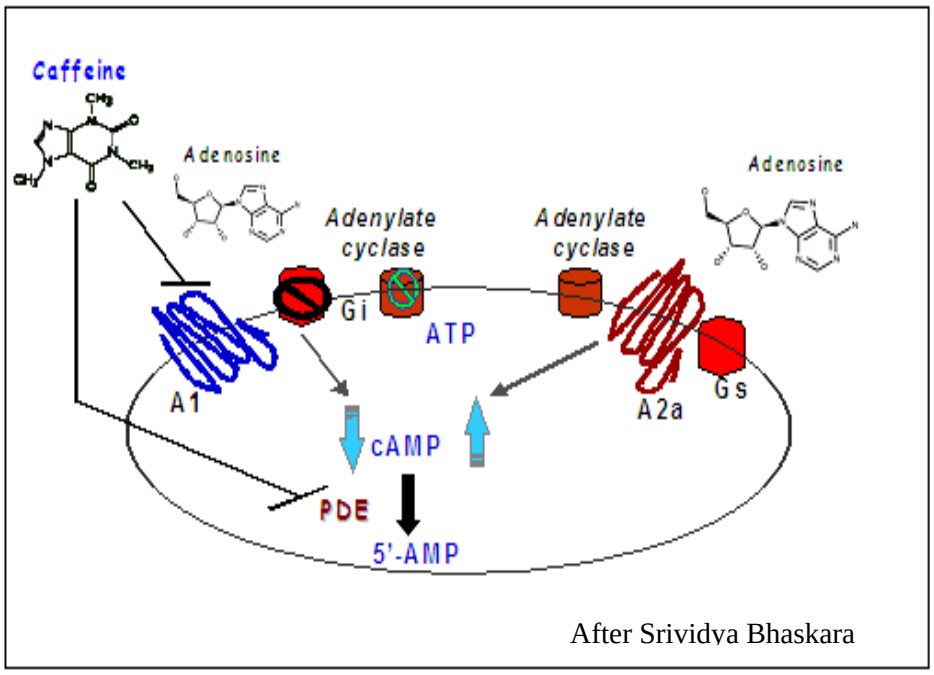



**Figure 2. Mechanism of Caffeine Action** – Adenosine plays an important role in biochemical processes, such as energy transfer - as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) - as well as in signal transduction as cyclic adenosine monophosphate, cAMP. cAMP is a second messenger derived from ATP by adenylate cyclase. In our active state, nerve cells release adenosine into our brain, which in turn binds to G-protein coupled receptors and thereby induce sleep. Caffeine is a non-selective adenosine antagonist that can bind to the adenosine receptors because it has a similar molecular shape to adenosine. Effects of caffeine are believed to occur primarily from binding with two adenosine receptor subtypes, A1 and A2A.Activation of A1 causes inhibition of adenylate cyclase and decreases the cAMP level. Activation of A2a leads to activation of adenylate cyclase and increases the intracellular cAMP level. Caffeine is a competitive inhibitor of an enzyme cAMP-dependent phosphodiesterase. Accumulation of increased cAMP causes metabolic stimulatory effect.

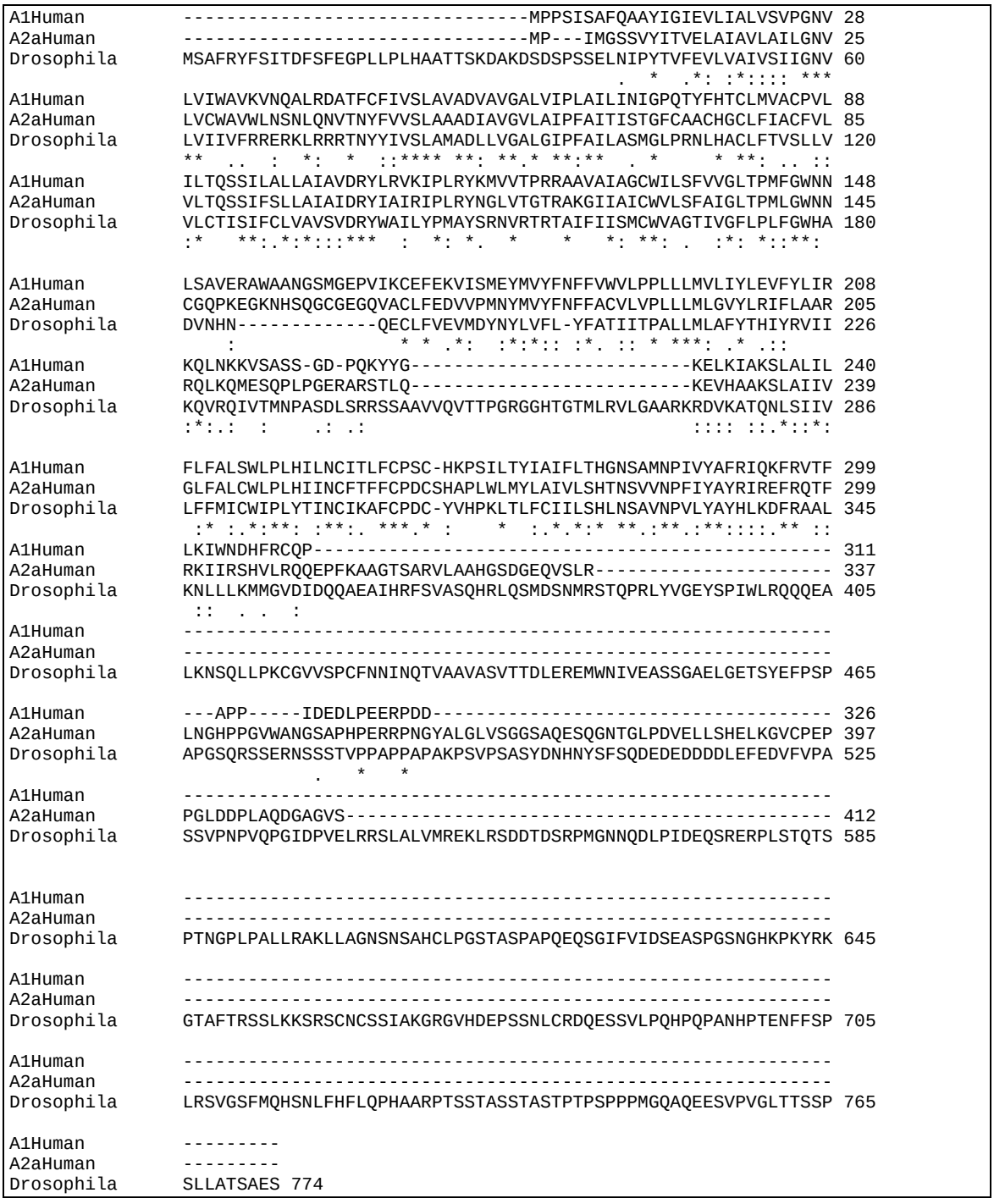



**Figure 3. Sequence alignment between A1 and A2a human adenosine receptor with the putative Drosophila adenosine receptor**.

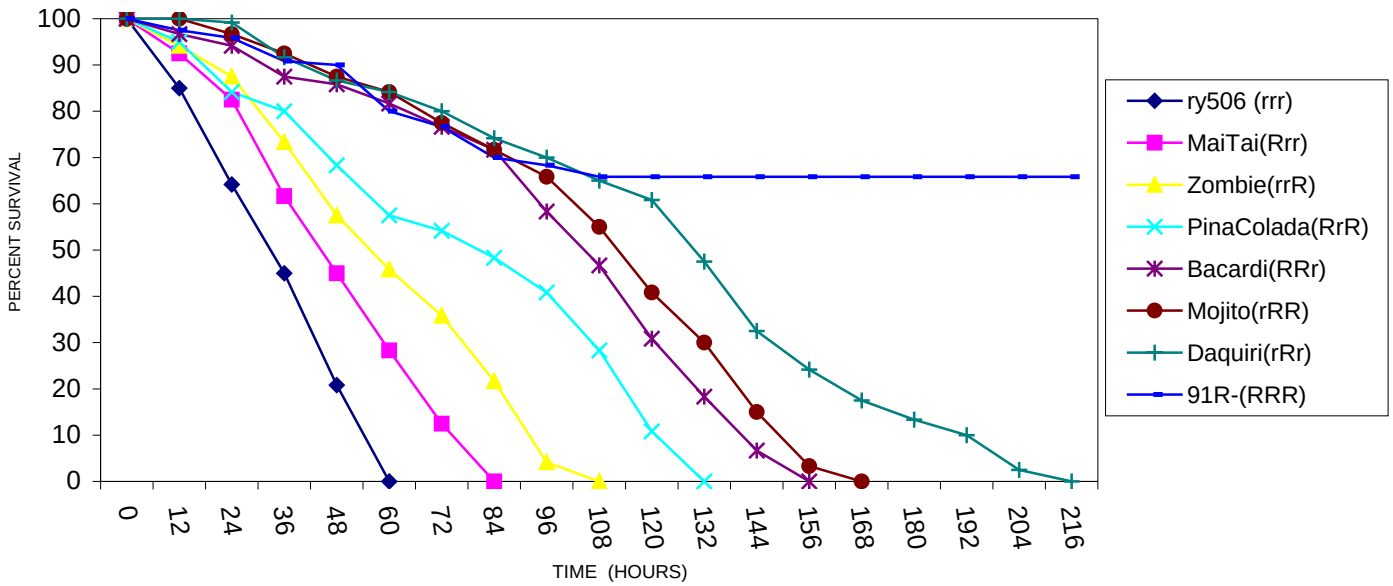



**Figure 4**. **The graph shows the effect of caffeine on cumulative percent mortality in male flies of *ry*$^{506}$ and 91-R strains, and their chromosome substitution stocks.** The adult male flies (3-5 days old) were exposed to 7.5mM caffeine containing food and mortality was determined every 12 hrs for 9 days. Time required for 50 % death or lethal time-50 (LT50) for each stock was determined and these data are presented in Figure 4. The data were analyzed using ANOVA analysis in SAS (SAS Institute, NC, 2000). For each strain, 3 replicates were done. ANOVA p-value <0.0001

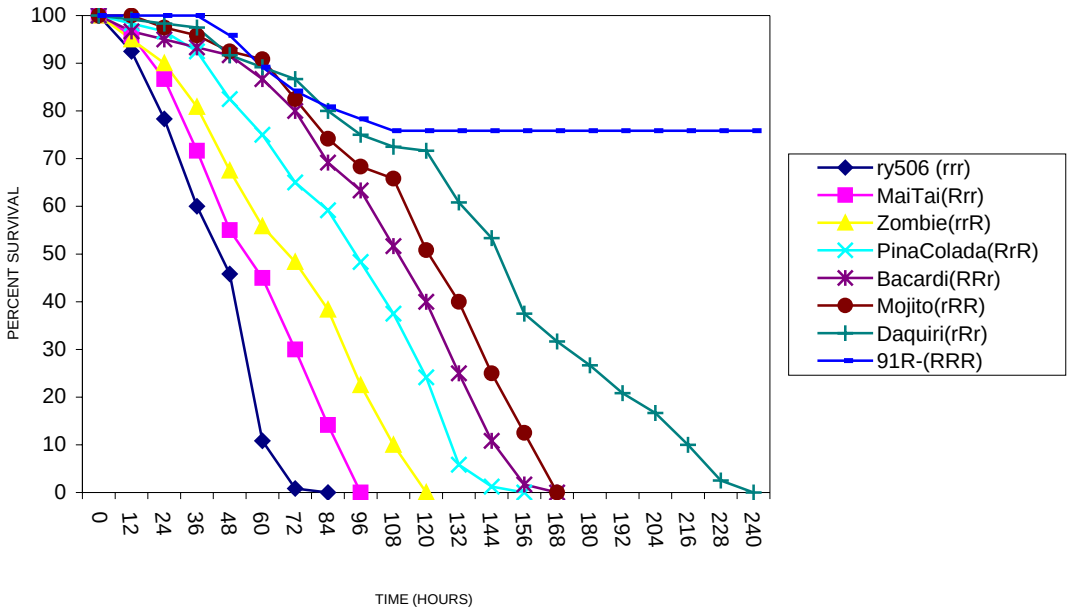



**Figure 5**. **The graph shows the effect of caffeine on cumulative percent mortality in female flies of *ry*$^{506}$ and 91-R strains, and their chromosome substitution stocks.** The adult female flies (3-5 days old) were exposed to 7.5mM caffeine containing food and mortality was determined every 12 hrs for 10 days. Time required for 50 % death or lethal time-50 (LT50) for each stock was determined and these data are presented in Figure 4. The data were analyzed using ANOVA analysis in SAS (SAS Institute, NC, 2000). For each strain, 3 replicates were done. ANOVA p-value <0.0001

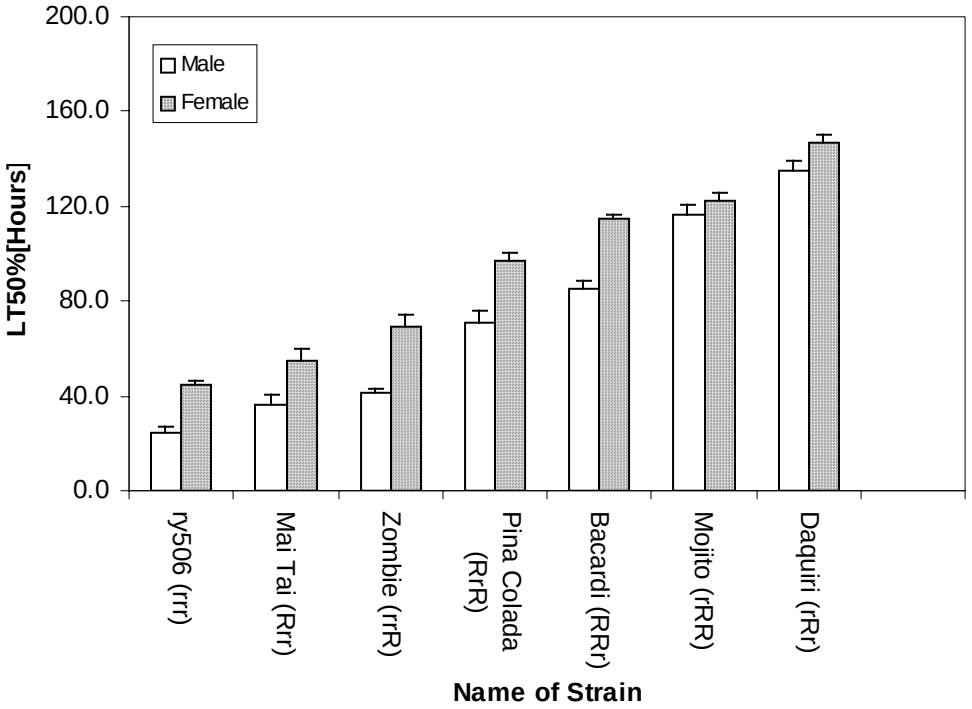



**Figure 6**. **Comparison of LT50 between males and females of *ry*$^{506}$ , 91-R and their chromosome substitution stocks.**

Each bar represents mean of triplicate experiments (+ standard deviation bars). All strains -ANOVA p-value < 0.0001; Male vs. Female within each stock- paired Student's T-test p-value < 0.05, except Mojito.

**Table 4: Mean LT50 of male and female flies calculated from Fig.2 and Fig.3 in 7.5mM caffeine concentration**

| | **Mean LT50 (HOURS) ± S.D** | |
|---|---|---|
| **Fly strain** | **Male** | **Female** |
| $ry^{506}$ (rrr) | 24.7 ± 2.08 | 45.0 ± 1.73 |
| MaiTai (Rrr) | 36.7 ± 4.16 | 54.7 ± 5.03 |
| Zombie (rrR) | 41.0 ± 2.00 | 69.0 ± 5.56 |
| PinaColada (RrR) | 70.7 ± 4.93 | 96.7 ± 4.04 |
| Bacardi (RRr) | 85.0 ± 4.00 | 115.0 ± 1.73 |
| Mojito (rRR) | 116.3 ± 4.04 | 122.0 ± 3.46 |
| Daquiri (rRr) | 135.1 ± 3.51 | 147.0 ± 3.00 |
| $91R^{y}800$ (RRR) | 100% alive after 216 hours | 100%alive after 240 hours |

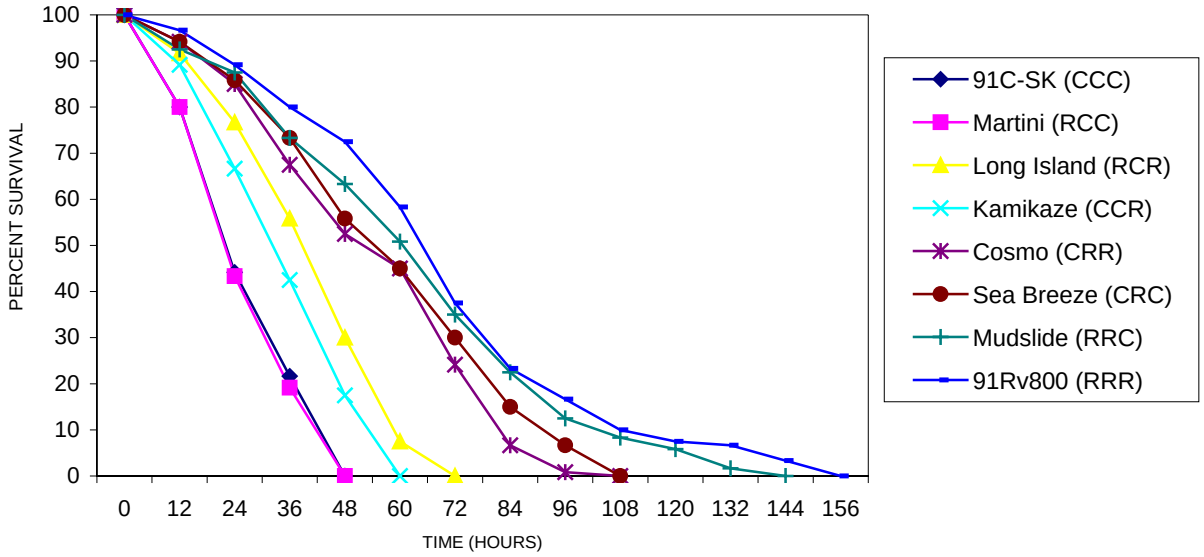



**Figure 7**. **The graph shows the effect of caffeine on cumulative percent mortality in male flies of 91-C and 91-R strains, and their chromosome substitution stocks.** The adult male flies (3-5 days old) were exposed to 15mM caffeine containing food and mortality was determined every 12 hrs until all the flies were dead. Time required for 50% death or lethal time-50 (LT50) for each stock was determined and these data are presented in Figure 7. The data were analyzed using ANOVA analysis in SAS (SAS Institute, NC, 2000). For each strain, 3 replicates were done. ANOVA p-value < 0.0001

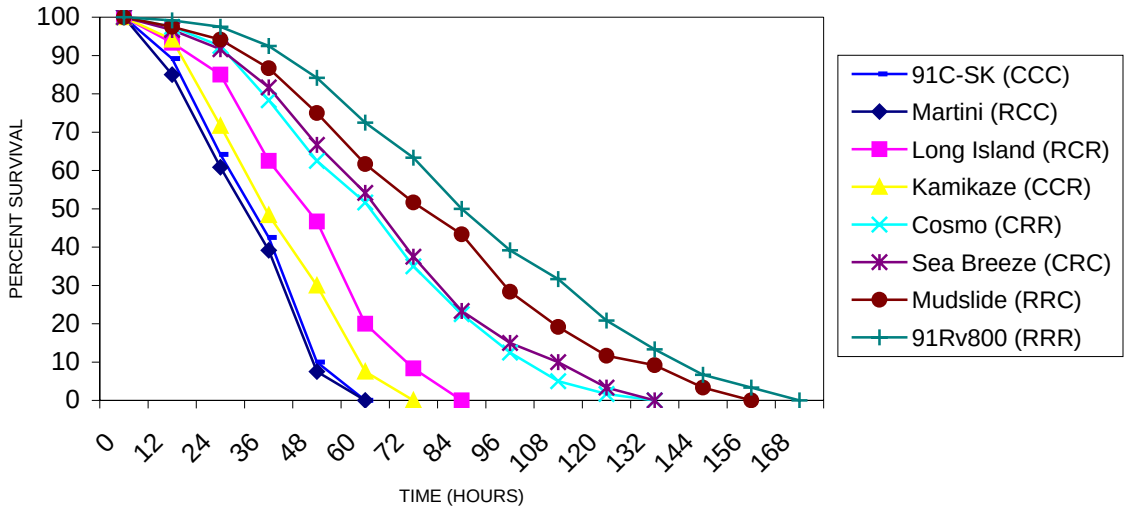



**Figure 8. The graph shows the effect of caffeine on cumulative percent mortality in female flies of 91-C and 91-R strains, and their chromosome substitution stocks.** The adult female flies (3-5 days old) were exposed to 15mM caffeine containing food and mortality was determined every 12 hrs until all the flies were dead. Time required for 50 % death or lethal time-50 (LT50) for each stock was determined and these data are presented in Figure 7. The data were analyzed using ANOVA analysis in SAS (SAS Institute, NC, 2000). For each strain, 3 replicates were done. ANOVA p-value < 0.0001

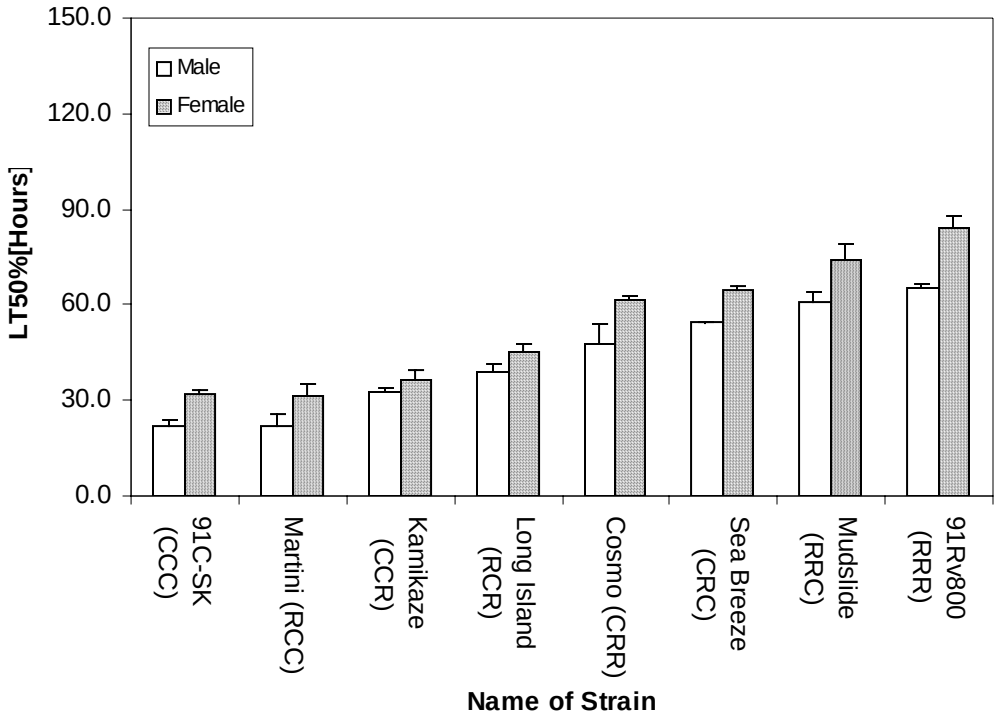



**Figure 9**. **Comparison of LT50 between males and females of 91-C, 91-R and their chromosome substitution stocks.**

Each bar represents mean of triplicate experiments (+ standard deviation bars). All strains -ANOVA p-value < 0.0001; Male vs. Female within each stock- paired Student's T-test p-value < 0.05, except Kamikaze.

**Table 5: Mean LT50 of male and female flies calculated from Fig.5 and Fig.6 in 15mM caffeine concentration**

| | Mean LT50 (HOURS) ± S.D | |
|---|---|---|
| **Fly strain** | **Male** | **Female** |
| 91C-SK (CCC) | 22.0 ± 1.73 | 32.0 ± 1.00 |
| Martini (RCC) | 22.0 ± 3.61 | 31.3 ± 4.04 |
| Kamikaze (CCR) | 32.3 ± 2.52 | 36.3 ± 2.08 |
| Long Island (RCR) | 38.7 ± 1.53 | 45.3 ± 3.51 |
| Cosmo (CRR) | 48.0 ± 6.00 | 61.3 ± 1.15 |
| Sea Breeze (CRC) | 54.3 ± 0.58 | 64.3 ± 1.52 |
| Mudslide (RRC) | 60.7 ± 3.21 | 74.3 ± 4.72 |
| $91R^{v}800$ (RRR) | 65.0 ± 1.73 | 84.3 ± 3.51 |

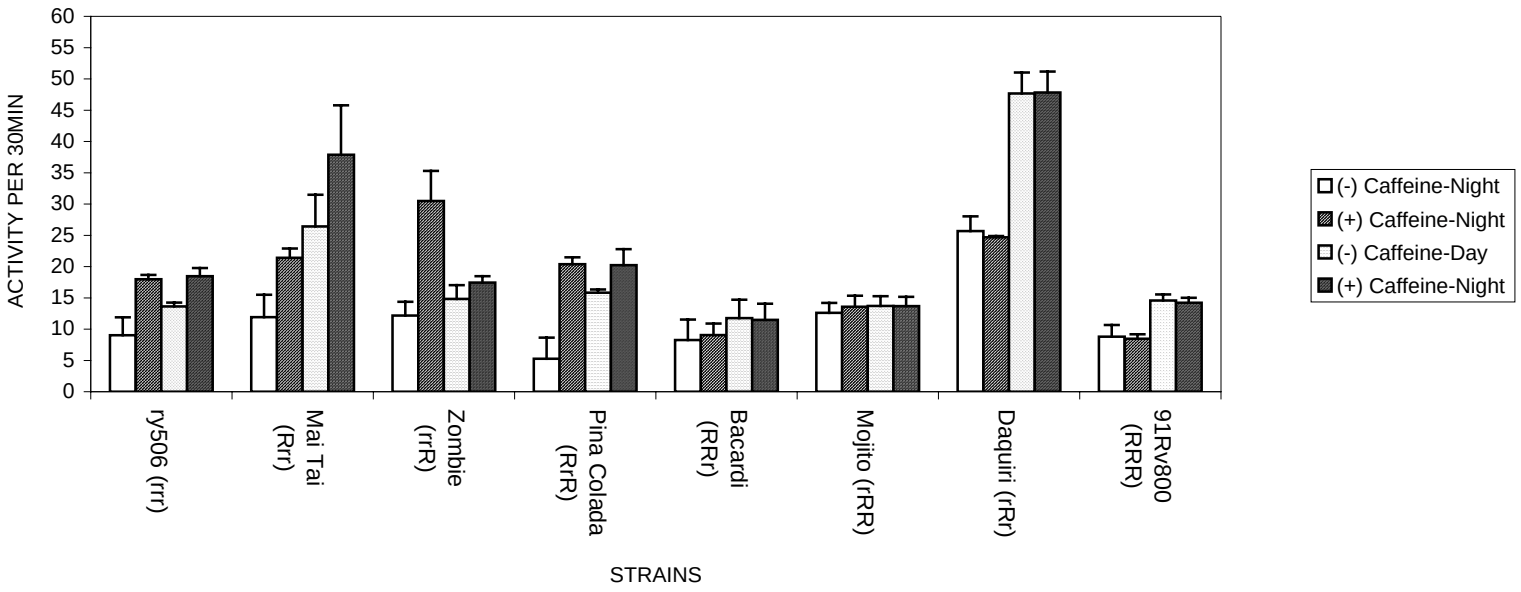



**Figure 10**. **The graph shows the effect of 1.5 mM caffeine on the circadian clock in male flies of *ry*$^{506}$ and 91-R strains, and their chromosome substitution stocks. Non-caffeine food was used as control.** The final concentration of caffeine was 1.5mM. Only male flies were used to avoid interference from the eggs or the larvae. All plots show the mean locomotor activity per 30 minutes for each strain during 12: 12 hour light/dark cycles for 7 days and 7 nights (+ standard deviation bars). All strains -ANOVA p-value < 0.0001; Non-caffeine food vs. Caffeine food (D/N) - paired Student's T-test p-value < 0.01.

**Table 6: Locomotor Activity counts of ry506 and 91R stocks during Night/Day in (-) and (+) 1.5 mM Caffeine food**

| | Night | | | Day | | |
|---|---|---|---|---|---|---|
| **Name of Strain** | (-) Caffeine | (+) Caffeine | Fold | (-) Caffeine | (+) Caffeine | Fold |
| $ry^{506}$ (rrr) | 9.03 | 17.99 | 1.992248 | 13.62 | 18.46 | 1.35536 |
| Mai Tai (Rrr) | 11.91 | 21.4 | 1.796809 | 26.43 | 37.89 | 1.4336 |
| Zombie (rrR) | 12.18 | 30.49 | 2.503284 | 14.84 | 17.43 | 1.17453 |
| Pina Colada (RrR) | 5.28 | 20.38 | 3.859848 | 15.83 | 20.22 | 1.27732 |
| Bacardi (RRr) | 8.26 | 9.05 | 1.095642 | 11.77 | 11.49 | 0.97621 |
| Mojito (rRR) | 12.6 | 13.58 | 1.077778 | 13.7 | 13.69 | 0.99927 |
| Daquiri (rRr) | 25.67 | 24.69 | 0.961823 | 47.67 | 47.82 | 1.00315 |
| $91R^{v}800$ (RRR) | 8.81 | 8.46 | 0.960272 | 14.57 | 14.22 | 0.97598 |

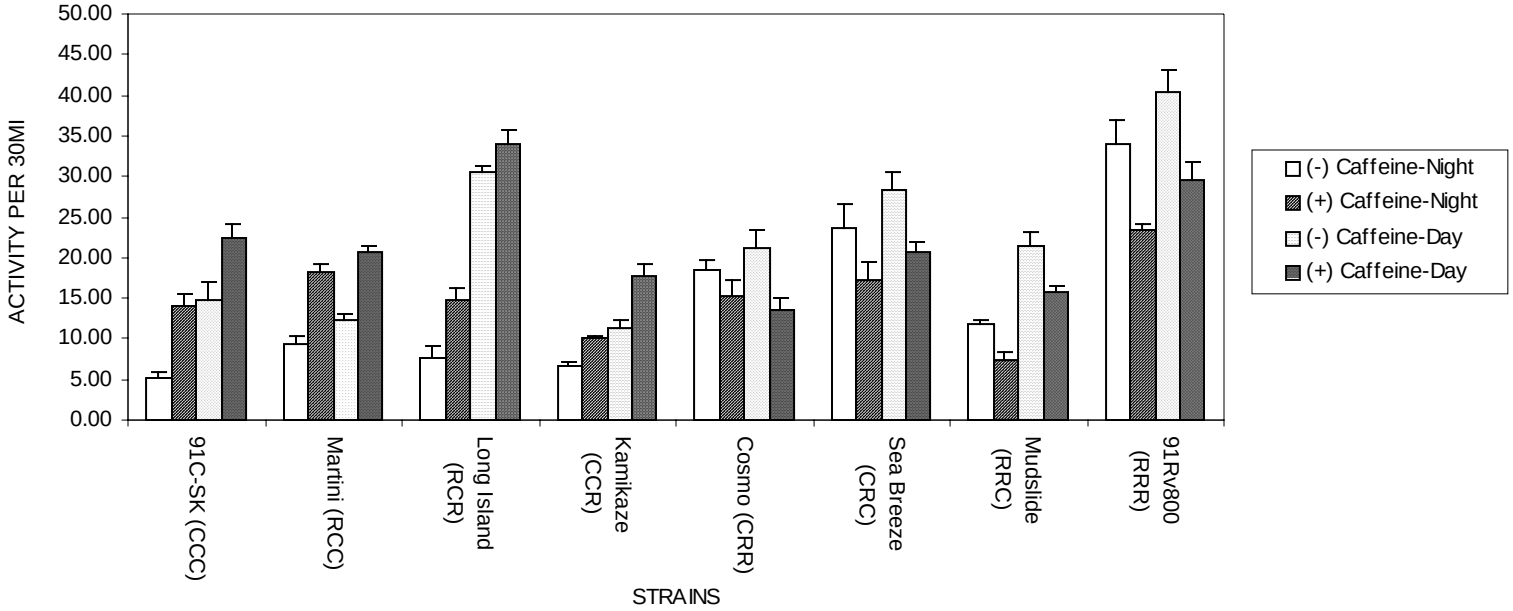



**Figure 11**. **The graph shows the effect of 3 mM caffeine on the circadian clock in male flies of 91-C and 91-R strains, and their chromosome substitution stocks. Non-caffeine food was used as control.** The final concentration of caffeine was 3mM. Only male flies were used to avoid interference from the eggs or the larvae. All plots show the mean locomotor activity per 30 minutes for each strain during 12: 12 hour light/dark cycles for 7 days and 7 nights (+ standard deviation bars). All strains -ANOVA p-value < 0.0001; Non-caffeine food vs. Caffeine food (D/N) - paired Student's T-test p-value < 0.01.

**Table7: Locomotor Activity counts of 91-C and 91-R stocks during Night/Day in (-) and (+) 3mM Caffeine food**

| | Night | | | Day | | |
|---|---|---|---|---|---|---|
| **Name of Strain** | (-) Caffeine | (+) Caffeine | Fold | (-) Caffeine | (+) Caffeine | Fold |
| 91C-SK (CCC) | 5.12 | 14.07 | 2.748047 | 14.73 | 22.35 | 1.51731 |
| Martini (RCC) | 9.43 | 18.33 | 1.943796 | 12.36 | 20.6 | 1.66667 |
| Long Island (RCR) | 7.72 | 14.77 | 1.913212 | 30.46 | 34.04 | 1.11753 |
| Kamikaze (CCR) | 6.56 | 10.09 | 1.53811 | 11.21 | 17.67 | 1.57627 |
| Cosmo (CRR) | 18.53 | 15.15 | 0.817593 | 21.06 | 13.66 | 0.64862 |
| Sea Breeze (CRC) | 23.59 | 17.17 | 0.727851 | 28.36 | 20.6 | 0.72638 |
| Mudslide (RRC) | 11.9 | 7.38 | 0.620168 | 21.43 | 15.74 | 0.73448 |
| $91R^{v}800$ (RRR) | 33.99 | 23.49 | 0.691086 | 40.3 | 29.51 | 0.73226 |

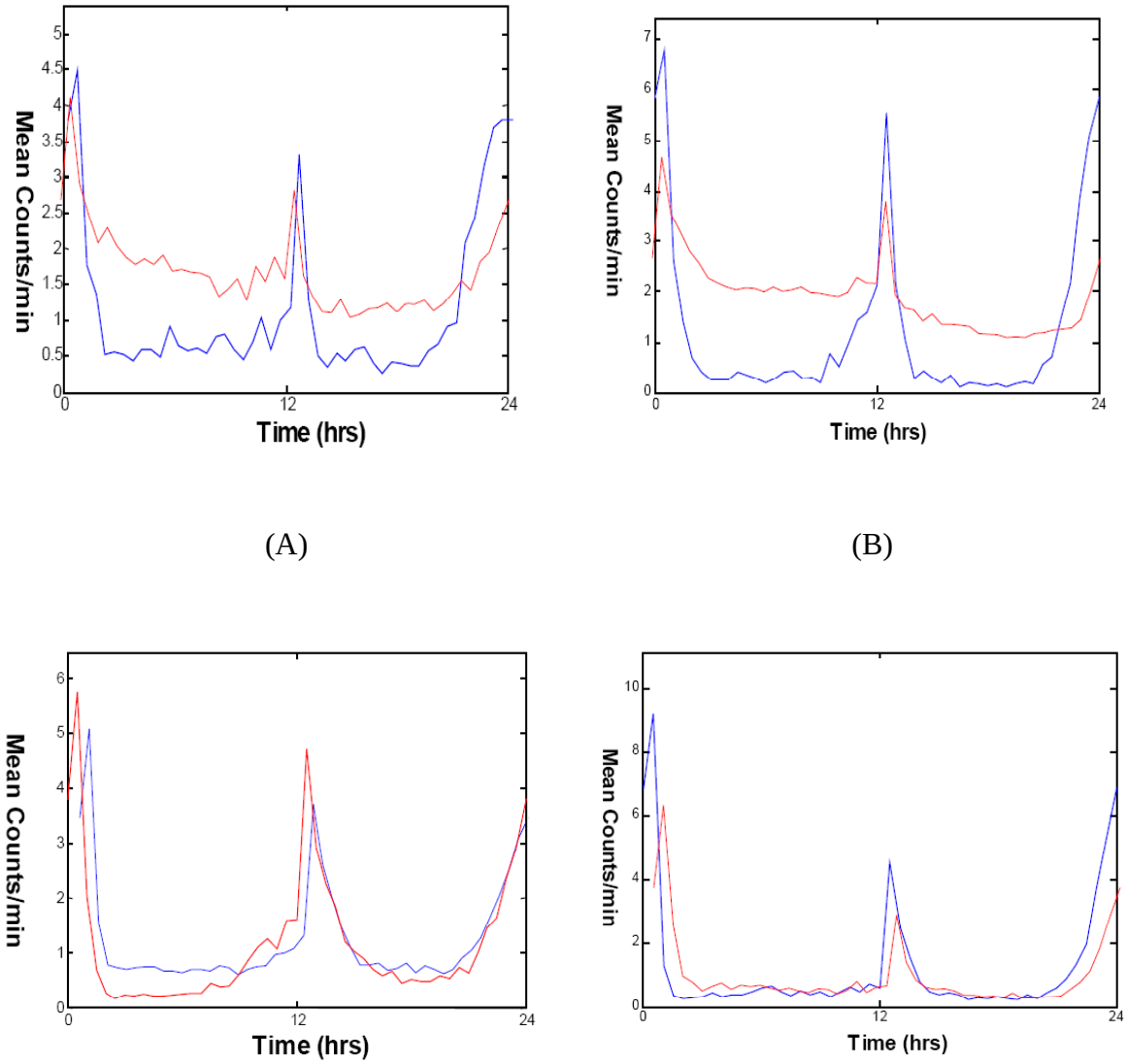



**Figure 12. Activity profile chart for (A) $ry^{506}$ (rrr), (B) Pinacolada (RrR), (C) 91-R (RRR), (D) Bacardi (RRr) fly strains in LD cycle.** Flies were entrained in 12:12 hour LD cycles for 7 days. X axis- Mean counts per minute; Y axis- Time in hours: 0 to 12 hours = Night; 12 to 24 hours = Day. The red line represents 1.5mM caffeine treatment and the blue line is non-caffeine treatment.

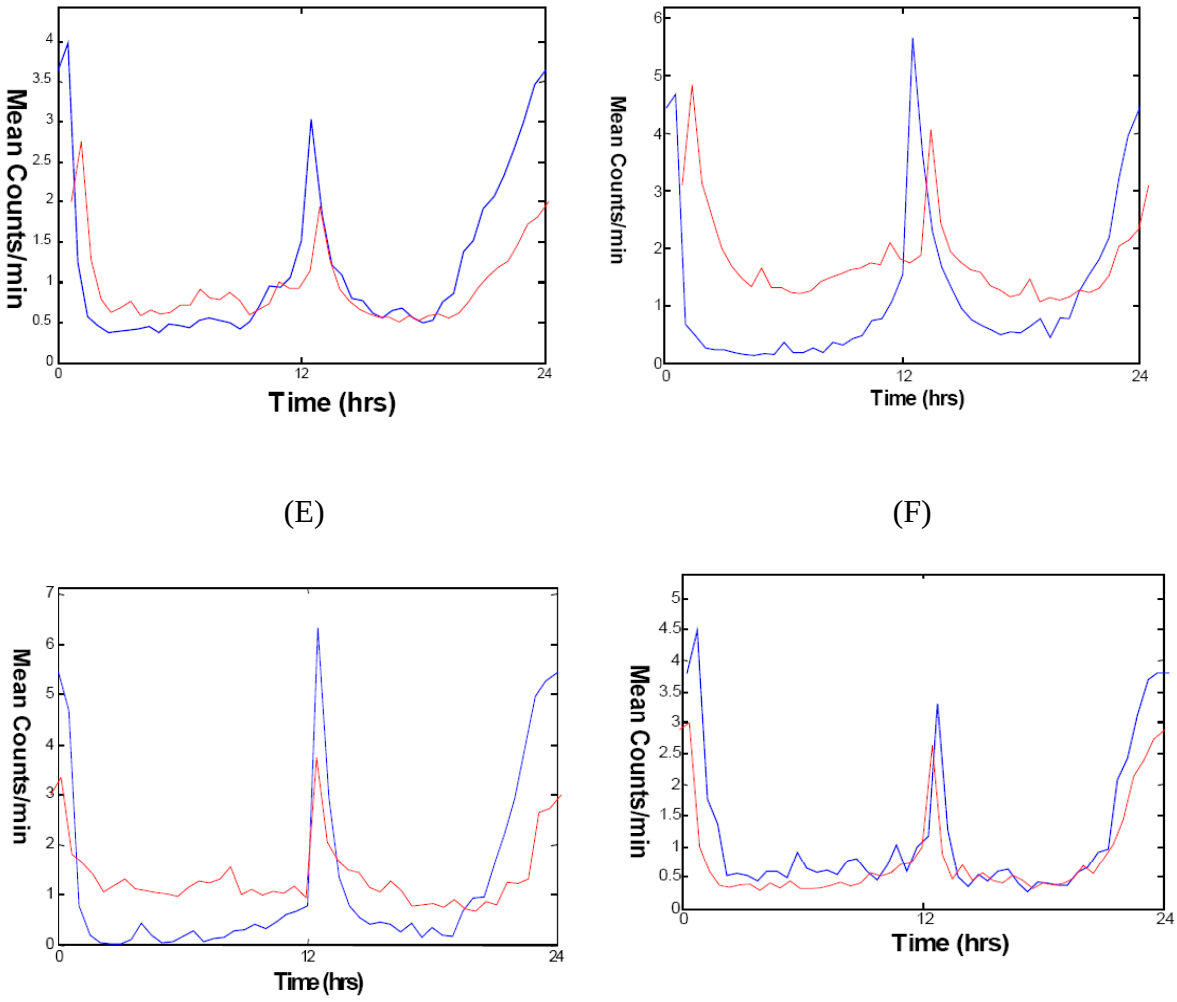



**Figure 13. Activity profile chart for (E) Mojito (rRR), (F) Zombie (rrR), (G) MaiTai (Rrr), (H) Daquiri (rRr) fly strains in LD cycle.** Flies were entrained in 12:12 hour LD cycles for 7 days. X axis- Mean counts per minute; Y axis- Time in hours: 0 to 12 hours = Night; 12 to 24 hours = Day. The red line represents 1.5mM caffeine treatment and the blue line is non-caffeine treatment.

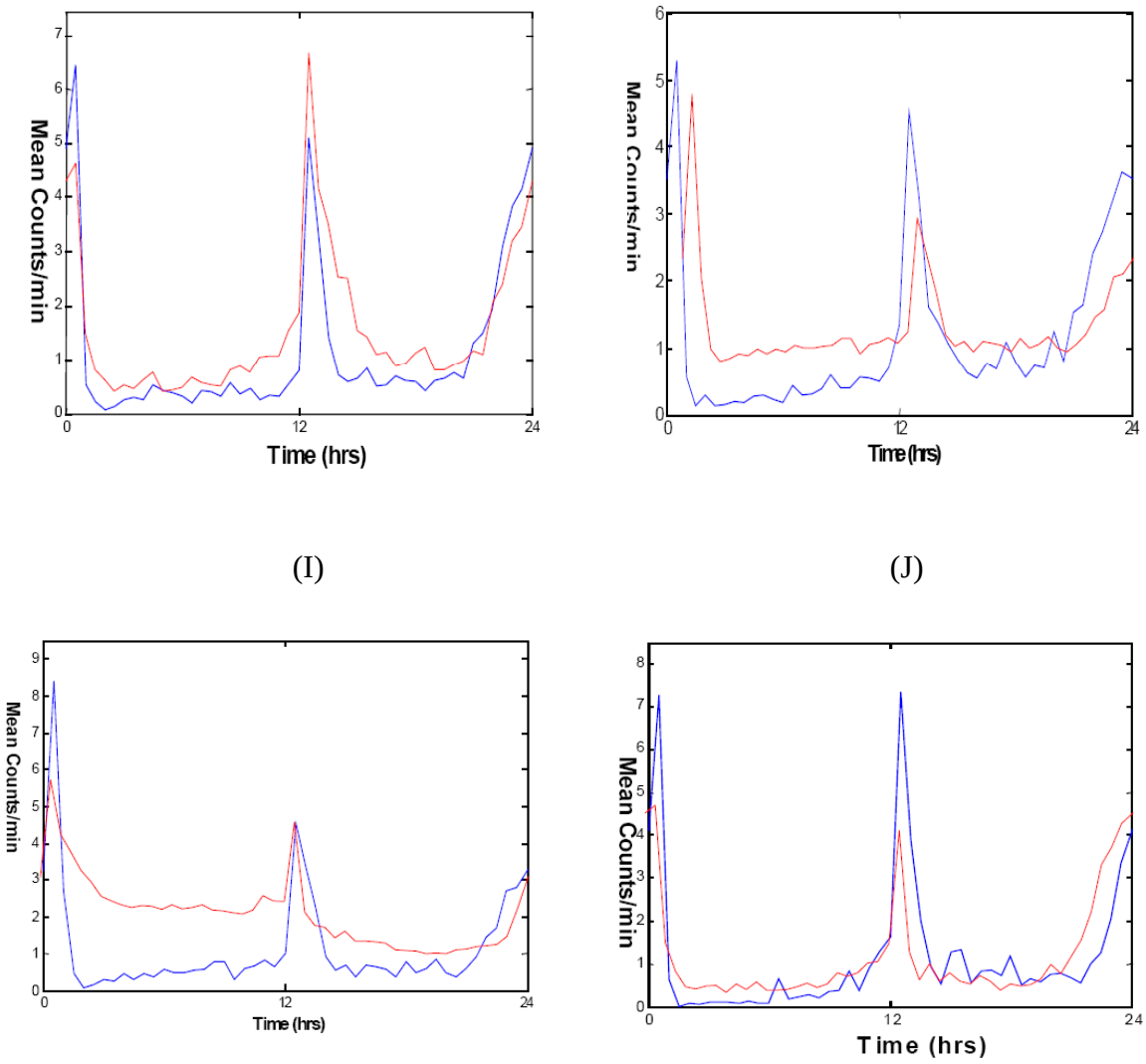
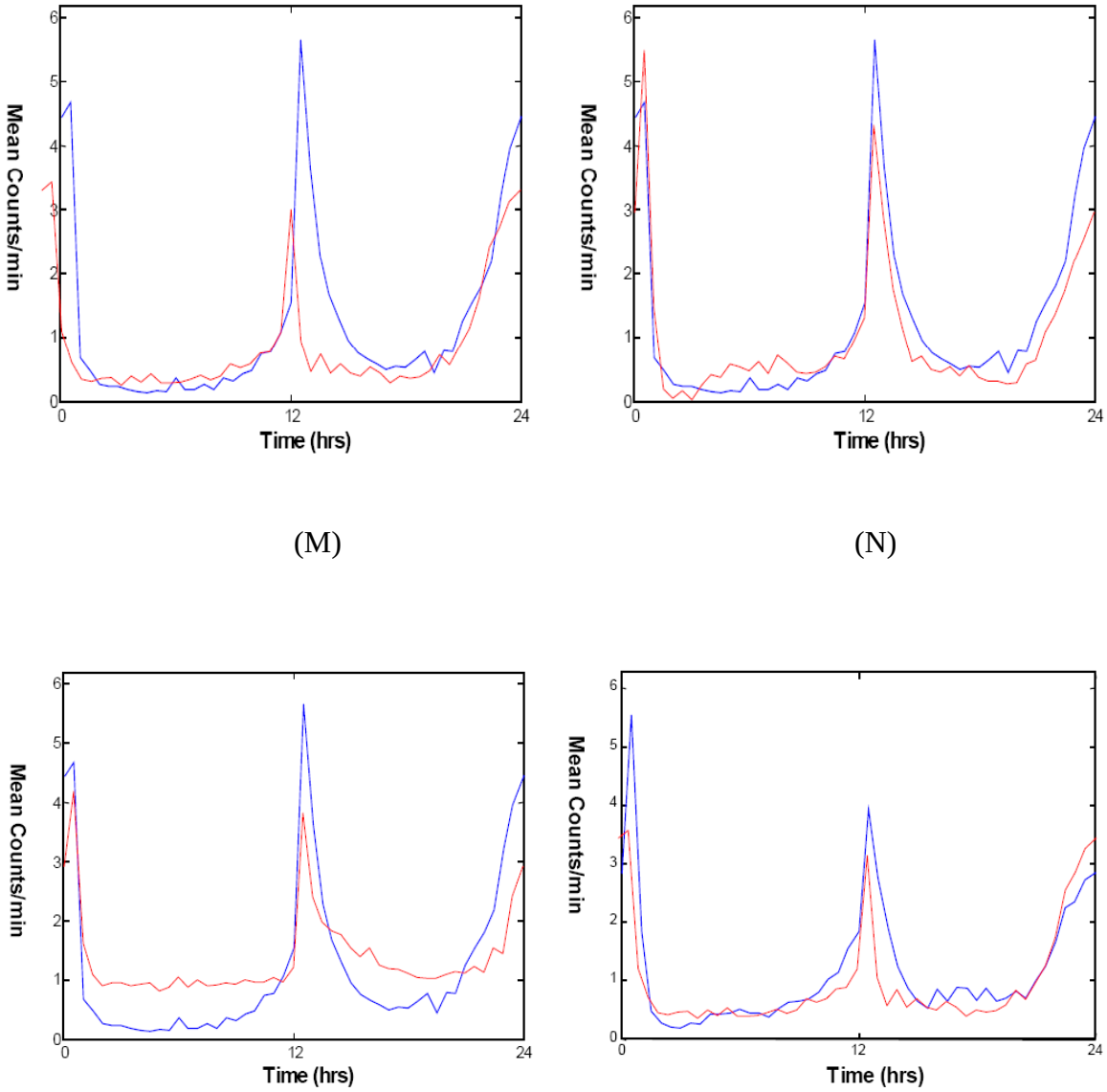



**Figure 14. Activity profile chart for (I) 91-C (CCC) (J) Martini (RCC), (K) LongIsland (RCR), (L) SeaBreeze (CRC) fly strains in LD cycle.** Flies were entrained in 12:12 hour LD cycles for 7 days. X axis- Mean counts per minute; Y axis- Time in hours: 0 to 12 hours = Night; 12 to 24 hours = Day. The red line represents 3mM caffeine treatment and the blue line is non-caffeine treatment.




(M) (N)

(O) (P)

**Figure 15. Activity profile chart for (M) Cosmo (CRR), (N) Mudslide (RRC), (O) Kamikaze (CCR), (P) 91-R (RRR) fly strains in LD cycle.** Flies were entrained in 12:12 hour LD cycles for 7 days. X axis- Mean counts per minute; Y axis- Time in hours: 0 to 12 hours = Night; 12 to 24 hours = Day. The red line represents 3mM caffeine treatment and the blue line is non-caffeine treatment.

## Vita

Chandrashis Bhowmick was born in Kolkata, India on August 19, 1977. He obtained a Bachelor of Pharmacy from the Department of Pharmaceutical Technology at Jadavpur University, Kolkata, India in August 2001. He then entered the Masters in Pharmacy in the Department of Pharmaceutical Technology at Jadavpur University, Kolkata, India in July 2002. He then came to the United States and joined the Department of Biochemistry and Cellular and Molecular Biology at the University of Tennessee. He successfully completed his M.S. in July 2007 from here. At the same time, he did a minor in statistics from the Department of Statistics at the University of Tennessee, Knoxville. He then left the University to begin a career in Health-Care Industry.