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Caffeine-Induced Promoter Activity of the CYP6A8 Gene in Male and Female Drosophila Melanogaste

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CAFFEINE-INDUCED PROMOTER ACTIVITY OF CYP6A8 GENE IN MALESAND FEMALE DROSOPHILA MELANOGASTER

by

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Abstract

Cytochrome P450 monooxygenases or CYPs comprise a superfamily of enzymes found in all living organisms, and they constitute an important metabolic system. One of many functions CYPs play is conferring insecticide resistance in many species of insects. Studying the regulation of *CYP* genes in *Drosophila melanogaster* is important because it may illuminate the mechanisms by which resistant insects detoxify insecticides, which may further the understanding of diseases and cancers in humans. In *Drosophila,* the *Cyp6a8* gene shows higher level of expression in insecticide-resistant strains compared to susceptible ones. Recently, it has been discovered that *Cyp6a8* is induced by caffeine and this is interesting because not much is known about caffeine-mediated gene induction. The ever-increasing popularity of caffeine in human consumption has raised questions on its physiological role in organisms. In a previous investigation the caffeine tablet Vivarin was shown to induce *Cyp6a8* expression in larvae. In the present investigation I examined whether caffeine induces *Cyp6a8* expression differentially in male and female flies. For this purpose, three transgenic lines, 0.8 *luc* 110, 0.8 *luc* 114, and 0.8 *luc* 123, carrying *luciferase* (*luc*) reporter gene were used. In these lines, the *luc* gene was under the control of a 0.8-kb (-11/-766) upstream promoter DNA of *Cyp6a8*. Adult flies of each line were allowed to feed on medium containing 16-mM caffeine for 24 hours. Inducibility of *Cyp6a8* promoter in each line was determined by assaying extracts of the flies for luciferase activity. The results showed that caffeine-treated flies of the 0.8 *luc* 110, 0.8 *luc* 114, and 0.8 *luc* 123 lines had about 1.3, 2.5, and 2.9 times higher luciferase activity respectively compared to the untreated flies. In the untreated control group, males consistently showed higher 0.8 *luc* activity than the females. However, a sex bias was not

seen when the caffeine induction level (fold-induction) was compared between the two sexes.

I. Introduction

Caffeine is probably the most frequently ingested pharmacologically active substance in the world. It is found in coffee, tea, soft drinks, medications, and numerous products containing cocoa and chocolate. Because of its wide consumption, the public and scientific communities have expressed interest in the potential adverse effects of caffeine on human health (Nawrot 2003). Following ingestion, caffeine is quickly and essential completely absorbed from the gastrointestinal tract into the bloodstream. The maximum caffeine concentration is reached 1-1.5 hours following ingestion, and the absorbed caffeine is easily distributed throughout the entire body. The liver is the primary site of caffeine metabolism.

Several clinical and epidemiological studies have suggested that caffeine consumption is associated with significant increases in total and low-density lipoprotein cholesterol levels, adversely affecting cardiovascular health, as shown by Nawrot (2003). Caffeine toxicity in humans presents a spectrum of clinical symptoms in humans, ranging from irritability, nervousness, insomnia, and gastrointestinal disturbances. It is now believed that habitual daily use of caffeine (>500 mg) represents a significant health risk and sustained abuse can result in the above adverse reactions. Caffeine appears to have multiple functions, including the inhibition of the adenosine receptor, the induction of calcium release, as well as the inhibition of certain protein phosphatases (Nawrot 2003). The main mechanism involves the antagonism of adenosine receptors that are present on the cell surface of the brain, blood vessels, kidneys, heart, and the GI tract. Adenosine normally acts on one of the isoforms (A1) of this receptor to decrease cellular concentrations of cyclic adenosine monophosphate or cAMP. Caffeine selectively blocks

adenosine A1 receptor and competitively inhibits the action of adenosine, directly causing an increase in cellular cAMP levels. Likewise, caffeine also inhibits cAMP phosphodiesterase, thus increasing cAMP levels (Scott 1998).

Studies with human cell lines have shown that caffeine in humans is metabolized by a cytochrome P450 monooxygenase (CYP) called CYP1A2. CYPs comprise a superfamily of enzymes found in all living organisms and they are extremely important for metabolic systems. CYPs are iron- or heme-containing proteins and they metabolize endogenous and exogenous compounds. Thus it is a part of the body's strategy to use CYPs to dispose of potentially harmful substances. CYPs add a hydroxyl group to the foreign compounds and make them water soluble for excretion or for conjugation to chemical groups such as glutathione by a second group of enzymes.

In insects, the CYPs have numerous functional roles in growth, development, and hormone biosynthesis. CYPs are also involved in conferring resistance to insecticides and tolerance to plant toxins. In resistant insects cytochrome P450s are expressed at a higher level than usual. In *Drosophila melanogaster,* a number of different P450 genes, *Cyp6a2, Cyp6a8, Cyp6g1* and *Cyp6w1* have all been documented to be overexpressed in resistant strains compared to susceptible strains. In the search for the mechanisms of insecticide resistance, *Drosophila melanogaster* has been used as a model organism to study *Cyp* gene regulation. *Cyp6a8* and *Cyp6a2* are located on the second chromosome, and they appear to be regulated by loci on the third chromosome. It is believed that the third chromosome produces repressors which downregulate the expression of *Cyp6a8* and *Cyp6a2* genes.

The *Cyp1A1* and *Cyp1A2* genes in rats are involved in the metabolism of caffeine, and they themselves have been previously shown to be induced by caffeine (Goasduff 1996). Pollock (1999) has also shown that estrogen inhibits *CYP1A2*-mediated caffeine metabolism in humans. In addition, a sex-biased effect of caffeine on the dopamine 2 receptor gene in mice has been observed by Stonehouse et al. (2003). In the present investigation I wanted to examine whether the cytochrome P450 genes in Drosophila also show differential expression in males and females following caffeine treatment. It has been shown previously by using reporter transgenic lines that the *Cyp6a8* promoter is induced by caffeine in female Drosophila (Dean and Ganguly, unpublished results). These transgenic lines were used in the present investigation.

II. Materials and Methods

Fly Strains and Culture Conditions

For this experiment, three transgenic lines, 0.8 *luc* 110, 0.8 *luc* 114, and 0.8 *luc* 123, carrying the firefly *luciferase* (*luc*) reporter gene were used. These transgenic lines were created by S. Maitra (2000). In these flies the *luc* gene has been placed downstream to the 0.8 kb promoter DNA of the *Cyp6a8* gene (Figure 1). In all of these strains one copy of the transgene is located on chromosome 2 of the ry^{506} host strain. The three transgenic lines were cultured at 24°C in medium containing of 0.65% bacto-agar, 5.5% cornmeal, 3% brewer's yeast, 5% unsulfered molasses, 2% light com syrup, 0.25% propionic acid. Every two weeks new cultures were made.

Treatment of Transgenic Flies

The transgenic lines used in the investigation were not homozygous for the transgene. So, the flies in each line could be homozygous or heterozygous for this transgene. To make sure that the flies to be used had the same number of transgenes, each transgenic line was crossed to the ry^{506} mutant strain, which has rosy eye color. The ry^{506} strain was used as the host strain for transformation. F_1 males and females showing ry^+ (red eye) color were collected. The ry^+ or red eye color indicates the presence of the transgene because it is attached to the ry^+ gene (Figure 2).

To treat the flies, instant fly food was reconstituted with a 16 mM caffeine solution. The F_1 female and male flies with ry^+ (red) eye color were selected, sorted, and allowed to feed separately on food with or without caffeine for 24 hours in the dark (see Figure 3).

Preparation of the Extracts

After treatment ten male and female flies were separately taken into 1.5-ml Eppendorf tubes and 20-µl 1X Cell Culture Lysis Reagent (Promega) was added. The flies were immediately homogenized for 20 seconds and placed on ice for 5 minutes. The homogenates were centrifuged at 4°C, for 5 minutes at 14,000 RPM. Following centrifugation, 100 µl of the clear supernatant was removed, carefully leaving behind the soluble and lipid layers, and placed into clean 1.5-ml Eppendorf tubes. These extracts were again centrifuged for 5 minutes at 4°C. Again leaving behind the lipid fraction, 50 µl was removed, divided into two 25 µl aliquots in clean 1.5-ml Eppendorf tubes and stored at -80°C.

Luciferase Assay

A commercially available kit (Promega, WI) was used to measure luciferase activity. The Luciferase Assay Buffer contained beetle luciferin and was prepared ahead of time according to the manufacturer's protocol. Single reaction aliquots of 100 µl were stored at -80°C in 1.5-ml Eppendorf tubes until use. For the luciferase assay, one aliquot of the buffer could be used for one extract. These assay buffers were kept on ice in darkness for 45 minutes to thaw and then incubated at room temperature for 15 minutes prior to use. The fly extracts were also allowed to thaw on ice for 15 minutes. Once the extracts and aliquots were thawed, 5 µl of the fly extract was added to 100 µl of assay buffer, mixed gently and placed in a luminometer (Zylux). The reading process took about 1 minute with 15-second intervals. Three readings were recorded as Registered Light Units (RLUs).

Protein Assay

The BCA protein assay kit (Pierce) was used to assay the amount of protein in the extracts used for the luciferase assay. Bovine Serum Albumin (BSA) at 2mg/ml was diluted with 1X Cell Culture Lysis Reagent (CCLR) to a BSA concentration of 1mg/ml and O.5X CCLR. Extracts were thawed on ice for 15 minutes and then diluted to O.5X CCLR. The dilution of the BSA standard and the extracts to 0.5X CCLR reduces the interference during the reaction. All reactions were performed in triplicate and according to the manufacturer's protocol. Absorbances from different quantities of BSA were used to draw a standard curve and absorbance was read at 562 nm. The three readings for each sample were averaged, and this value was used to calculate the total protein content using the standard curve. The final values were expressed in RLUs per μg of total protein.

Statistics (T-test)

A student's t-test compares two averages. The certainties with which these averages are measured are expressed in the standard deviation and are related to the number of cases observed. The t-test gives the probability that the difference between the two means is caused by chance. It is customary to say that if this probability is less than 0.05, then the difference is significant, meaning the difference is not caused by chance. Multiple student's t-tests were performed to examine the statistical significance of the data.

III. Results

This investigation of caffeine induction of *Cyp6a8* in adult male and female flies was performed using transgenic lines created by S. Maitra (Maitra 2000). The 0.8 *luc* lines used were either homozygous or heterozygous for the transgene. These lines were then crossed with the ry^{506} strain. This cross produced red-eyed (ry^+) and rosy-eyed (ry) F_1 progeny. The red-eyed flies were used because the 0.8 *luc* transgene is attached to ry^+, which acts as a selectable marker (Figure 1).

The difference in constitutive (control) expression of the reporter *luc* gene between males and females varied. Males in all lines males showed higher expression than females (Figure 4). Moreover, this was statistically significant in the 0.8 *luc* 110 transgenic line (Figure 4).

Table 1 and Figure 5 show that in each of the lines, 0.8 *luc* 110, 114, and 123, caffeine treated flies had higher activity compared to the control, in both males and females. In some cases caffeine induction was very pronounced and visible. In each of the three transgenic lines, t-tests showed that the increase in *Cyp6a8* activity caused by caffeine induction is statistically significant in all cases (Figure 5).

The level of induction between males and females was also compared in this examination, and the difference in fold-induction is shown in Figure 6. A comparison of the fold-induction between both sexes shows a common theme in each line. The difference in fold-induction between sexes is insignificant throughout all of the lines. The 0.8 *luc* 114 line has nearly no difference in fold-induction between sexes, and the 0.8 *luc* 110 and 123 lines also show very similar fold-induction between males and females.

In the present investigation I examined the sex-biased effects of caffeine-induced expression of *Cyp6a8*. Overall the data show an equal level of caffeine induction in both males and females, with no bias towards either. Furthermore, only the 0.8 *luc* 110, 114, and 123 transgenic lines were used in this experiment. Dean (2002) addressed the concern that induction by caffeine may be a peculiarity of these transgenic lines by testing additional lines. These additional transgenic lines showed a similar level of induction as the original transgenic lines (Dean 2002).

IV. Discussion

Caffeine induced expression of the 0.8 *luc* reporter gene is present in all of the transgenic lines used in this investigation. Generally, males show a higher constitutive (basal) expression compared to females. The transgenic lines showed variation in basal expression. The fold-induction varied between the three lines, but is found to be similar in lines 114 and 123 (Figure 6). These observations suggest that no sex bias is present in caffeine induction. However, the fold-induction was lower in the 0.8 *luc* 110 line, and consistent with the other lines, no sex bias was found. Nevertheless, it is clear that caffeine induces the *Cyp6a8* promoter.

Wild-type flies exhibit a low level of *Cyp6a8* expression due to repressor molecules rather than a weak promoter (Maitra 2000). *Cyp6a8* is located on the second chromosome while the repressor genes are located on the third chromosome. The DNA sequences between -199 and -761 are necessary for maximum constitutive expression (Maitra et al., 2002), as these sequences contain six barbie-boxes and other transcription factor binding sites. Additionally, trans-regulatory factors from the ry^{506} genome had an effect on the region between -11 and -761 (Maitra et al., 2002). The presence of these regulatory sequences seems to indicate that this region is the most important promoter region. Although sequences required for caffeine induction have not been discovered, the results of this investigation show that such sequences are present in the 0.8 kb DNA of the *Cyp6a8* gene.

However, the differences in fold change in expression due to caffeine induction between the three 0.8 transgenic lines are not explained by the presence of regulatory regions in the -199/-761 DNA sequence. Fleming (2003) stated that the mechanism by

which caffeine induces may interact with a regulatory sequence present in the -11/-199 region. While various lines of males and females showed differential induction by caffeine, overall I did not find evidence to conclude that caffeine had sex-biased effects in *Drosophila*. These results indicate that caffeine inducibility of the *Cyp6a8* promoter has no sex bias.

The mechanism of caffeine induction is unknown and should be explored further. Clearly, caffeine induces greater activity of the *Cyp6a8*-luciferase trans gene in both males and females. Several factors could be causing these results, including an increase in transcription, a post-transcriptional stabilization of the mRNA, a chromosomal position effect, or a post-translational change in luciferase activity or stability. Increased activity due to a post-translational change in luciferase or to a chromosomal position effect can be ruled out. In a recent investigation, Dean (2002) compared the levels of luciferase activity and CYP6A8 mRNA in caffeine-treated 0.8 *luc* transgenic lines. The results showed that the endogenous *Cyp6a8* gene was also induced by caffeine. So it can be concluded that caffeine induction involves either an increase in transcription levels or a stabilization of mRNA.

Goasduff et al. (1996) demonstrated in rats that caffeine increases the level of the homologous mammalian genes CYP1A1/1A2 mRNA and ruled out the possibility that mRNA stabilization led to these increased levels. Gonzales et al. (1993) states that CYP1A1 enzymes can only be regulated through activation of transcription, not through stabilization of mRNA levels. Similar studies to identify this mechanism have been done with the dopamine 2 receptor (D2R) gene in mice. Stonehouse et al. (2003) revealed that mice treated with a dose of caffeine showed 1.94- and 2.07-fold increases in D2R mRNA

and protein expression, respectively. The results conclude that caffeine increases D2R protein expression by stimulating transcription of the D2R gene. As similar mechanisms are likely at work in mice and *Drosophila melanogaster,* caffeine induction of *Cyp6a8* is very likely due to transcriptional activation, not stabilization of mRNA. Thus, caffeine causes changes in gene transcription in the body that may be related to the adaptive changes that occur after caffeine administration.

Since the actual mechanism of caffeine induction is unknown, Dean (2002) and Fleming (2003) have reviewed possible mechanisms for caffeine induction of *Cyp6a8.* Svenningsson et al. (1997) illustrated how caffeine upregulates c-fos and junB, two early gene products. The data show that a dose of caffeine induces these proteins, followed by Activating Protein 1 (AP-1). These proteins heterodimerize to form the AP-1 complex, a DNA binding transcriptional activator. Dean (2002) proposed that caffeine induction of *Cyp6a8* may be caused by *Drosophila* homologues of the c-fos and junB proteins. Interestingly, several putative binding sites for AP-l have been identified in the -11 /-761 region by Maitra et al. (2002). Therefore, caffeine induced expression of *Cyp6a8* could be caused by these putative AP-1 binding site sequences.

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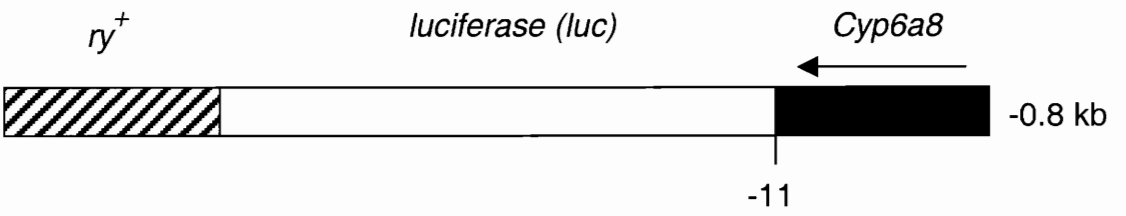

Figure 1. Diagram of the 0.8 *luc* transgene. It has the 0.8 kb upstream promoter DNA of *Cyp6a8*, from bases -11 to –761. This DNA has the basal promoter and transcription start signal.

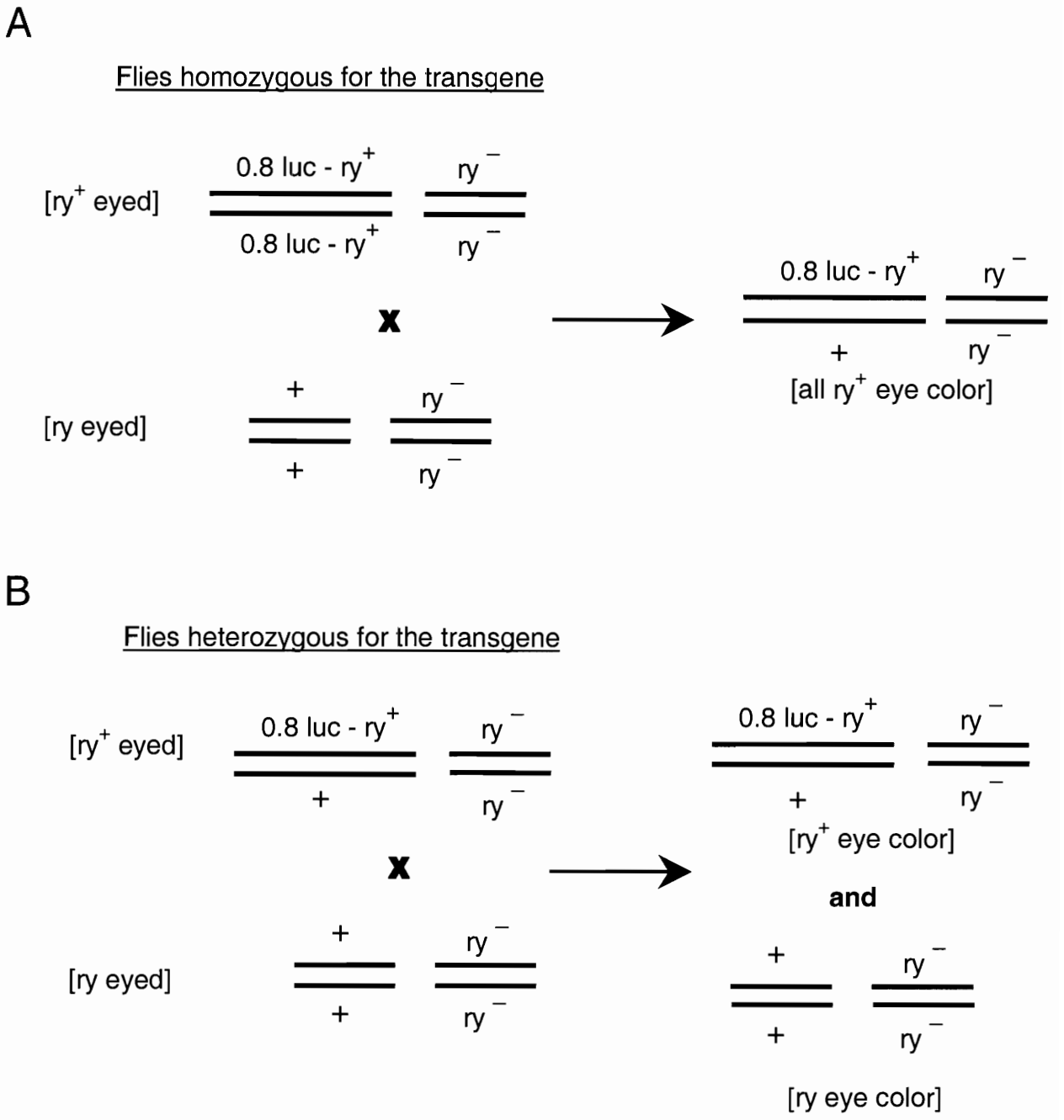

Figure 2. Crossing of transgenic flies to ry^{506}. The F_1 flies with red eyes (ry^+) have one copy of the transgene. Flies homozygous for the transgene when crossed will only produce progeny with one copy of the transgene (A). Flies heterozygous for the transgene will produce progeny with no transgene or with one copy of the transgene (B). The red eye color aids selection.

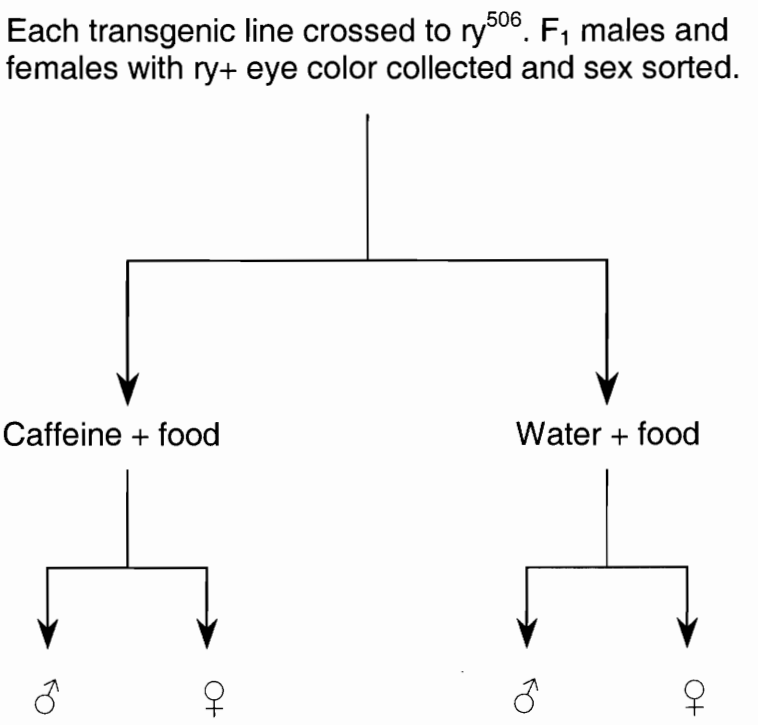

Figure 3. Flow chart of method used to treat flies with caffeine

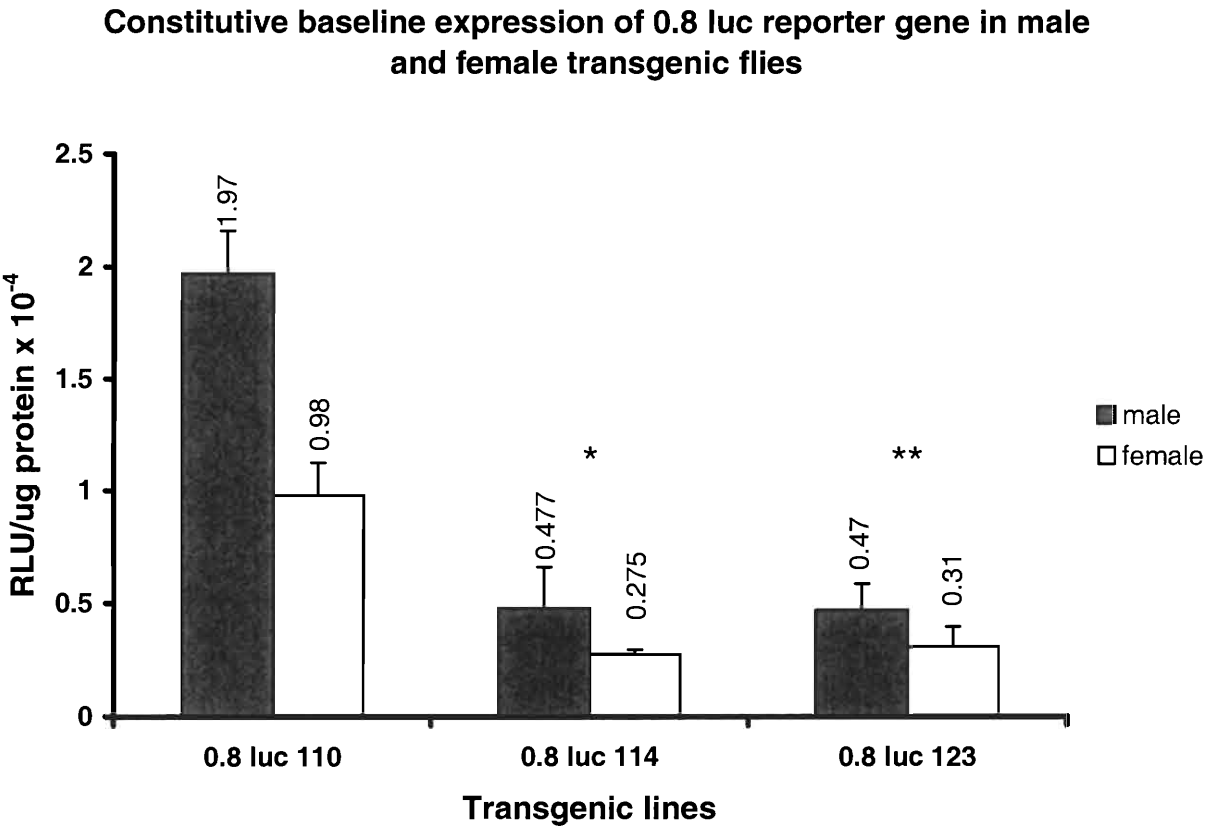

Figure 4. Graph showing difference in baseline expression of *Cyp6a8* in male and female 0.8 *luc* transgenic lines. T-test performed on 0.8 *luc* 114 (*, P ≤ 0.219) and 0.8 *luc* 123 (**, P ≤ 0.275)

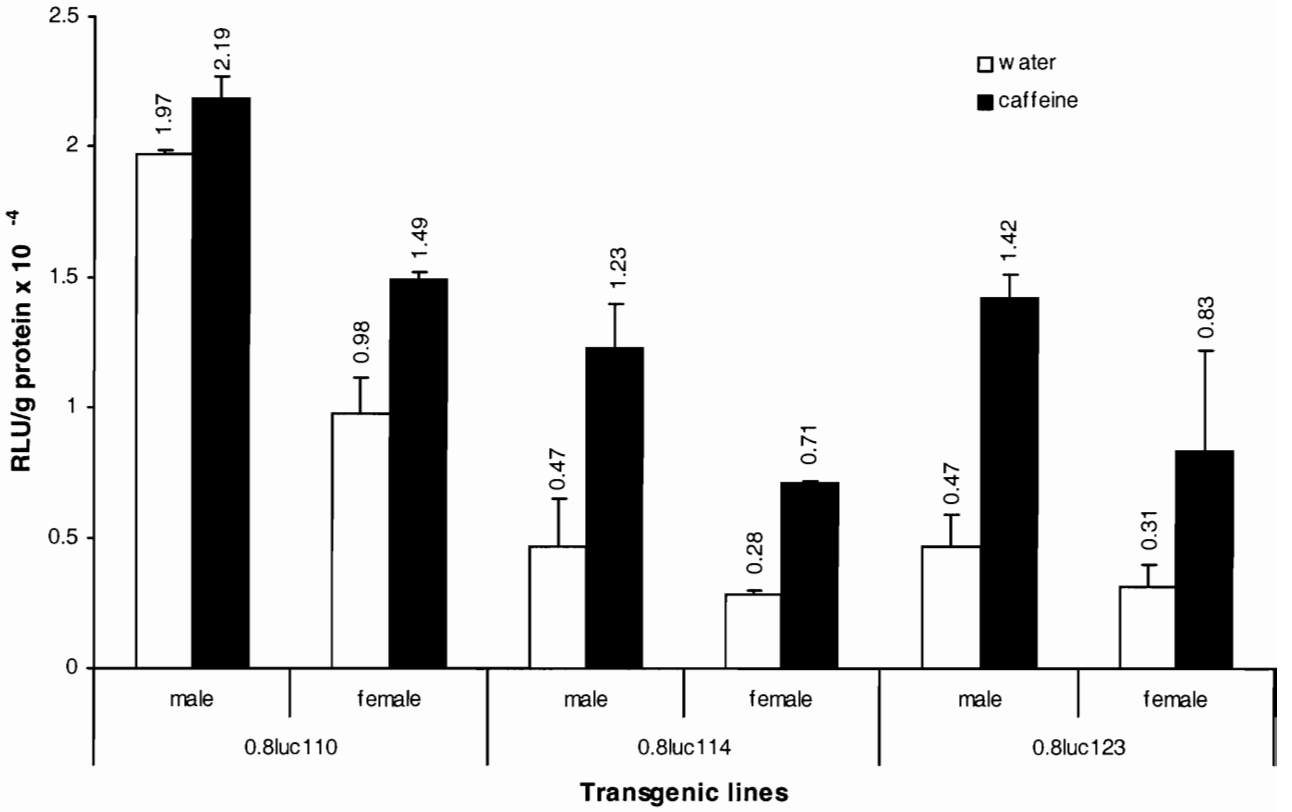

Figure 5. Graph showing caffeine induction of *Cyp6a8* in male and female 0.8 *luc* transgenic lines. P value for 0.8 untreated versus treated was less than 0.05 in all cases.

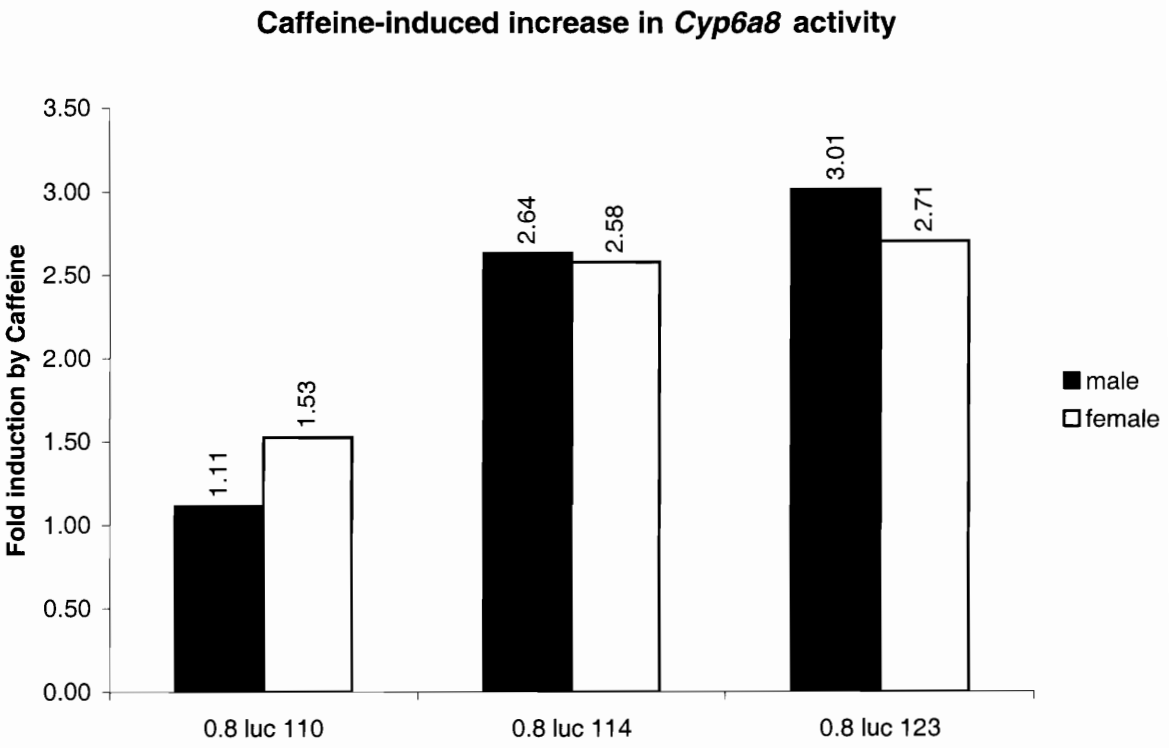

Figure 6. Graph showing the fold increase of caffeine-induced activity in male and female 0.8 *luc* transgenic lines.

Table 1. Table showing results of caffeine induction in transgenic lines

Strain	Sex	Treatment	Sample #	Protein conc. (mg/ml)	Avg. Protein conc. (mg/ml)	Luciferase Activity (RLUs/5µl)	Activity (RLUs/µg protein)	Standard Dev. (RLUs/µg)
0.8 luc 110 (Original)	Male	Water	1	2.00E+01	1.64E+01	1.60E+07	1.97E+05	1.89E+03
			2	1.28E+01		1.62E+07		
		Caffeine	3	1.38E+01	1.59E+01	1.69E+07	2.19E+05	8.22E+03
			4	1.80E+01		1.78E+07		
	Female	Water	5	2.78E+01	2.39E+01	1.29E+07	9.75E+04	1.45E+04
			6	1.99E+01		1.04E+07		
		Caffeine	7	2.53E+01	2.48E+01	1.82E+07	1.49E+05	2.70E+03
			8	2.42E+01		1.87E+07		
0.8 luc 114 (Original)	Male	Water	9	1.63E+01	1.60E+01	5.34E+06	4.66E+04	1.83E+04
			10	1.60E+01		2.50E+06		
			11	1.56E+01		3.32E+06		
		Caffeine	12	1.63E+01	1.62E+01	9.00E+06	1.23E+05	1.67E+04
			13	1.61E+01		1.09E+07		
	Female	Water	14	2.84E+01	2.45E+01	3.47E+06	2.75E+04	2.12E+03
			15	2.55E+01		3.57E+06		
			16	1.96E+01		3.08E+06		
		Caffeine	17	2.37E+01	2.31E+01	9.03E+06	7.10E+04	1.01E+04
			18	2.25E+01		7.38E+06		
0.8 luc 123 (Original)	Male	Water	19	2.13E+01	1.90E+01	5.56E+06	4.78E+04	1.19E+04
			20	1.75E+01		3.32E+06		
			21	1.81E+01		4.72E+06		
		Caffeine	22	1.56E+01	1.61E+01	1.05E+07	1.42E+05	9.85E+03
			23	1.60E+01		1.19E+07		
			24	1.67E+01		1.17E+07		
	Female	Water	25	2.21E+01	2.23E+01	2.37E+06	3.06E+04	8.71E+03
			26	2.06E+01		3.58E+06		
			27	2.40E+01		4.28E+06		
		Caffeine	28	1.94E+01	2.02E+01	3.79E+06	8.29E+04	3.93E+04
			29	1.92E+01		1.06E+07		
			30	2.20E+01		1.07E+07		