

Investigation of the Synergistic Effects of the Extract of Neem Tree
and Caffeine on the Promoter Activity of *Cyp6a8* in *Drosophila melanogaster*

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Abstract

Cytochrome P450 genes are a family of genes known to perform a wide variety of functions. Many of these genes in *Drosophila melanogaster*, including *Cyp6a2*, *Cyp6g1*, and *Cyp6a8*, have been linked to development of pesticide resistance. The most resistant strains of *D. melanogaster* show over-expression of these genes compared to the susceptible strains. However, the molecular mechanisms by which these genes become over-expressed are not well understood. By creating transgenic flies containing a portion of the upstream DNA promoter region from *Cyp6a8* and the *luciferase* enzyme reporter gene, the promoter activity for *Cyp6a8* could be studied quantitatively. In the present study, larvae and adult flies of two different transgenic strains were treated with caffeine, extract of neem tree, and caffeine and neem together. Caffeine, as discovered in previous studies, induced transcription in both developmental stages of both strains. However, neem only induced promoter activity in the larvae. According to the study, larvae and adults were found to have somewhat different patterns of promoter activity, which could be caused by differences in developmental stages or simply by including male and female larvae in the study. The results of the synergistic treatments indicate that caffeine and neem induce by the same mechanism in larvae, but by a different mechanism in adults. While this is not entirely impossible, it necessitates further study to determine whether this difference in developmental expression is accurate. The activity of *Cyp6a8* is undoubtedly affected by xenobiotics in the environment including caffeine and neem, and the mechanisms by which this occurs are slowly being identified.

I. Introduction

Cytochrome P450 genes make up a superfamily of hemoproteins that are found in nearly all organisms and play diverse roles throughout the organism's life cycle. In *Drosophila melanogaster*, P450s are often involved in metabolism of foreign substances, but they have also been found to serve many other functions, such as assisting in activation of fatty acids such as lauric acid (Helvig, 2004). Many of these metabolic P450 genes have been linked to development of insecticide resistance in *Drosophila*, since the most resistant strains have been found to show over-expression of specific genes. These genes may include *Cyp6g1*, *Cyp12d1*, *Cyp6a2*, and the gene in the present study, *Cyp6a8* (Le Goff, 2003). It has been found that inhibition of cytochrome P450 genes by PBO, or piperonyl butoxide, results in the susceptibility of previously insecticide resistant strains (Brandt, 2002). Not only does the absence of these genes cause susceptibility, but heterologous expression of these proteins in cell culture, bacteria, and yeasts confers the ability to metabolize insecticides. However, the molecular mechanisms through which over-expression of these genes occurs in *Drosophila* is not well understood. Specific xenobiotics, such as caffeine and barbiturate compounds, have been found to induce expression of certain P450 genes, and further study of such xenobiotics could greatly increase the understanding of the regulatory mechanism for these genes. It is complicated by the fact that, while *Cyp6a2* and *Cyp6a8* genes are located on the second chromosome, their levels of expression are affected by other regions found on the third chromosome (Maitra, 2000).

Caffeine is a naturally occurring chemical to which most humans are exposed regularly. It is found naturally in certain plants, and then is manufactured into certain

medications, foods, and drinks, such as soft drinks. Caffeine has been found to induce expression of a wide variety of genes and to affect a number of cellular processes in all types of cells and organisms. In human cell lines, it has even demonstrated the ability to alter cell cycle regulation and promote apoptosis at very high concentrations (Bode 2007). In a previous trial, a microarray assay discovered the caffeine-based induction of four specific P450 genes, including *Cyp6a8*, at higher than 10-fold induction (Willoughby, 2006). Another experiment found the same induction of *Cyp6a2* and *Cyp6a8* by directly measuring specific gene activity after treatment with caffeine (Bhaskara, 2006).

Neem is also naturally occurring; however, it has a much more complex composition with more than one dominant compound. The Neem tree *Azadirachta indica*, native to India, has many medicinal and household uses that come from various parts of the plant. The bark has been found to have analgesic properties, while the leaves have been used for leprosy or skin ulcers. Neem has also been used as an agrochemical to promote nitrogen fixation and as a safe natural pesticide (Brahmachari, 2004). One of the dominant insecticidal chemicals in neem extract is azadirachtin, which has been found to induce antifeedancy, and growth and reproductive problems in various invertebrates. Azadirachtin is an even more effective insecticide because of its high toxicity to insects and extremely low toxicity to mammals (Robertson, 2007). The direct effects of neem extract on specific P450 gene expression have not been studied in depth.

Since caffeine and neem have previously been found to impact cellular mechanisms of various organisms, they can be used as tools to study the mechanisms of *Cyp6a8* expression and development of pesticide resistance. While it has already been

demonstrated that caffeine induces the activity of the *Cyp6a8* promoter, neem has not yet been studied to the same extent. After determining the inductive effects of neem and comparing with those of caffeine, the synergistic effects of the two chemicals combined were studied. By comparing the levels of expression of each chemical alone with treatment of both chemicals simultaneously, it is possible to determine whether the substances act on the gene through the same pathway or a different pathway. The induction by neem and the possible synergistic relationships were examined in two different stages of development of two *D. melanogaster* strains. It is possible that constitutive expression and inductive effects may differ between developmental stages, so adult flies and third instar larvae were studied. The two strains used were constructed to contain a portion of the promoter region from *Cyp6a8* attached to the *luciferase* enzyme gene from fireflies. Thus, the level of induction of *Cyp6a8* promoter was quantified through the amount of *luciferase* produced.

II. Materials and Methods

Transgenic Fly Strains

The two transgenic lines of flies used in the experiment had been created for previous studies on the *Cyp6a8* gene. The creation of the lines involved the construction of a plasmid containing part of the promoter region from *Cyp6a8* originally from *Drosophila* and the *luciferase* reporter enzyme from the firefly. *0.2-luc 30-4 (H-ry)* flies contain the fragment of the promoter region from -11 to -199 base pairs upstream from the initiation site, while the *0.8-luc 110 (H-ry)* strain contains the region from -11 to -761 base pairs (Fig. 1). The plasmids also contained a gene for dominant wild type eye color, which would help distinguish those flies that had been transformed from those with the mutant eye color of the *ry*⁵⁰⁶ strains. The flies were infected with the plasmids, and the flies that had been transformed were distinguishable by eye color, as described above (Maitra, 2002). The flies were later made homozygous for the transgene so the culture could be more easily maintained. The stocks were kept at 25°C on a cornmeal-agar-molasses fly media.

Saturating Concentration and Media Preparation

To determine the synergistic effects of two chemicals, it is necessary to use at least one of the chemicals in a saturating concentration. Since it is impossible to dissolve enough neem extract to obtain maximal luciferase activity, it was necessary to determine the saturating concentration of caffeine. Flies were fed instant fly food treated with 0, 4, 8, and 16mM caffeine solutions in deionized water, and the level of luciferase activity, to be described later, was measured. The saturating concentration was determined to be 16mM, which is the caffeine concentration at which the level of gene expression reaches

Constructed Segments Inserted into *0.2-luc 30-4 (H-ry)* and *0.8-luc 110 (H-ry)*

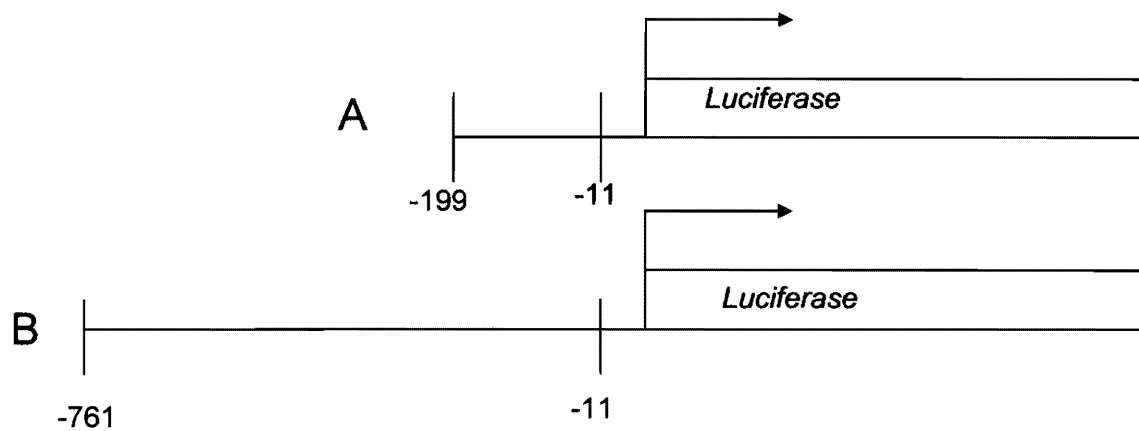


Fig. 1 *0.2-luc 30-4 (H-ry)* (A) contains an insertion from -11 to -199 from the promoter region of *Cyp6a8*. *0.8-luc 110 (H-ry)* (B) contains the region from -11 to -761 base pairs from the promoter. Both lines carry the same *luciferase* reporter gene.

a plateau and additional caffeine will no longer cause an increase in expression.

This saturating concentration agreed with the optimum concentration of 16mM found in a previous experiment (Maitra, 2002)

The adult flies and larvae of both strains were treated with deionized water, a 16mM caffeine solution, a 5% neem solution by mass, or both caffeine and neem solutions. To prepare the caffeine solution, 0.1474g of caffeine was added to 50mL of deionized water. The solution was then slowly added to a vial containing amount of dry instant fly food flakes. The food was allowed to absorb the solution until it was moist, and then the food was packed down to create a flat surface. The neem treatment vial was prepared in the same way, but with 2.5g of neem powder in 50mL of deionized water. The caffeine and neem solution contained both 0.1474g of caffeine and 2.5g of neem in 50mL water, and the control vial was treated with deionized water alone.

Treatment Procedure and Preparation of Extracts

To treat adult flies of either strain, a bottle of flies was etherized, and the flies were sorted by sex. At least 45 female flies were placed into each prepared treatment vial. After 24 hours, the females were removed from the vials and sorted into 3-1.5mL eppendorf tubes on ice, with each tube containing 10 females. Thus, each treatment was measured in triplicate. Larvae for both strains were collected at 3 days old, during the 3rd instar stage. Both male and female larvae were used, since it is difficult to distinguish sex characteristics during larval stages. The larvae were also allowed to feed on the treated food for 24 hours and were then transferred to 1.5mL eppendorf tubes containing 10 larvae each.

Fly extracts were prepared from each tube of 10 flies or larvae by adding 200uL of cold 1X CCLR buffer (Promega) and thoroughly homogenizing the flies on ice. The tubes were then centrifuged at 13K for 8 minutes at 4°C. Without removing lipid or parts of the pellet, as much supernatant as possible was removed and placed in a fresh tube on ice, while the pellets were discarded. The centrifugation was repeated using the same conditions, and 30uL of the supernatant was placed in a fresh tube to use later for a protein assay, and another 20uL was placed in a second tube for a luciferase assay. The extracts were stored at -80°C until the assays were performed.

Luciferase Assay

The amount of luciferase gene activity was determined by the bioluminescence produced by the reaction of luciferase enzyme and luciferase assay reagent, LAR, from a commercially available kit (Promega). After completely thawing both the extract and the LAR, 5uL extract was added to 50uL of LAR, the mixture was mixed by flicking, and the luminescence was measured in a luminometer (Zylux). The procedure was carried out in the dark to reduce spontaneous luminescence of luciferin without the interaction with luciferase. A reading for each extract, recorded in RLU per 5uL, was taken 15 seconds after adding the extract.

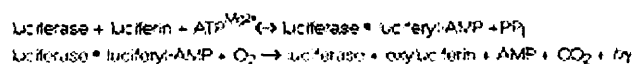


Fig. 2 Light producing reaction of luciferase enzyme with luciferin (LAR).
Promega Notes Magazine Number 44, Nov. 1993, p.24

Protein Assay

The concentration of protein in the extracts was determined using a commercially available BCA protein assay kit (Pierce). The extracts used for protein assays were

diluted two-fold by adding equal amounts of deionized water to each tube. The samples, tested in duplicate, were prepared by adding 10uL of diluted extract to 40uL of 0.5X CCLR buffer (Promega) in a small glass test tube. Standards of Bovine Serum Albumin were prepared, also in duplicate, by adding 0, 12.5, 25, 37.5, or 50 ul of 1 mg/ml BSA in 0.5X CCLR buffer and filling with enough 0.5X CCLR to a total volume of 50 ul. These standards represent protein concentrations of 0, 25, 50, 75, and 100 mg/ml, respectively. Two assay reagents were mixed in a 50:1 ratio, and 1mL of reagent mixture was added to each tube of samples and standards. After a 30 minute incubation at 37°C, the absorbances were read at 562nm, and protein concentrations were determined using linear curve from the BSA standards.

The results from the luciferase and protein assays were then combined to create units of RLU per ug of total protein (RLU/ug). Then analysis was done using Student's t-test.

III. Results

According to the data, caffeine induced gene expression of *Cyp6a8* in larval and adult stages of *0.2-luc* and *0.8-luc*. This corresponds to the caffeine induction found in previous experiments (Maitra, 2002), and the findings of our results were verified by analysis with Student's t-test. However, neem was only found to cause induction in the larvae of both strains. Gene expression was not induced in the adults of either strain by neem, which conflicts with an earlier experiment that found induction by neem of both larvae and adults. The concentration used in the present experiment was slightly lower than the previous experiment due to difficulties in dissolving the neem, so the reduced induction could be the result of decreased concentration.

Overall expression was found to be higher in larvae than adults for all three treatments and the control. Luciferase activity was generally two-fold higher for the larval stages than in the adults; however, fold-induction for larvae was not necessarily higher than adults. While larvae of *0.8-luc* showed the highest constitutive and overall gene expression, it was adults of *0.2-luc* that showed the highest fold-inductions.

0.8-luc of both developmental stages showed higher gene expression than *0.2-luc* both constitutively and after treatments. This was expected since *0.8-luc* contains the 0.2kb promoter region in *0.2-luc* as well as an additional 0.56kb more of the promoter region. *0.8-luc* would exhibit the same promoter activities as *0.2-luc* plus any activities that occur in the 560 base pairs further upstream. Although the overall measure of gene expression was higher for *0.8-luc*, fold-induction was actually higher in *0.2-luc*. Since the constitutive expression in *0.2-luc* was so low, the addition of different xenobiotics was able to cause a much higher level of induction.

Strain	Stage	Treatment	RLUs/ug	St Dev	Fold Induction
<i>0.2-luc</i>	Larvae	Water	1.33E+05	3.00E+04	1
		Caffeine	6.05E+05	2.42E+05	4.549
		Neem	3.11E+05	1.27E+04	2.338
		Caff + Neem	5.40E+05	5.18E+04	4.060
<i>0.2-luc</i>	Adults	Water	3.73E+04	4.02E+04	1
		Caffeine	2.72E+05	1.95E+04	7.292
		Neem	5.68E+04	9.97E+03	1.523
		Caff + Neem	5.30E+05	2.46E+05	14.209
<i>0.8-luc</i>	Larvae	Water	3.86E+05	6.95E+04	1
		Caffeine	8.76E+05	1.38E+05	2.269
		Neem	9.40E+05	1.74E+05	2.435
		Caff + Neem	1.50E+06	6.82E+05	3.886
<i>0.8-luc</i>	Adults	Water	2.23E+05	2.65E+03	1
		Caffeine	4.08E+05	6.03E+04	1.830
		Neem	2.07E+05	3.40E+04	0.928
		Caff + Neem	5.21E+05	2.65E+04	2.336

Table 1. Gene expression was measured in RLUs/ug of total protein and fold induction for treatment of larvae and adults of *0.2-luc* and *0.8-luc* strains with Caffeine, Neem, and Caffeine and Neem together. Distilled water treatment was used as a control.

The synergistic effects of caffeine and neem were difficult to study, since neem alone did not significantly induce gene expression in adults. While neem alone and caffeine alone both induced expression in larval stages, caffeine and neem combined did not show a significant increase in induction over that of caffeine alone, which was the saturating factor ($p=0.33$ for *0.2-luc* and $p=0.10$ for *0.8-luc*). These results indicate that caffeine and neem likely act through the same pathway in larvae. The combination of caffeine and neem in fact did cause increased expression in adults, although only the results from *0.8-luc* adults were statistically significant ($p=0.02$). These results were unexpected since neem alone was not found to induce expression in adults. Adults of *0.2-luc* also appear to show increased expression in the combined treatment of caffeine and neem compared to caffeine alone; however, a high standard deviation resulted in data that was not statistically significant ($p=0.07$).

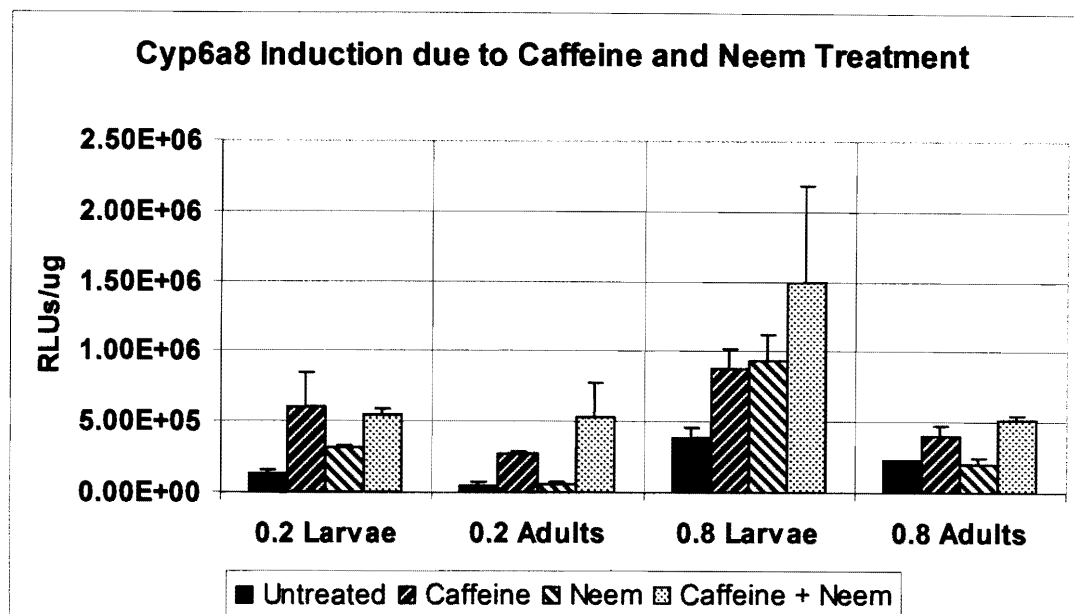


Fig. 3 The *luciferase* gene expression (measured in RLUs per ug of total protein) was measured after treatment with water, caffeine, neem, and caffeine and neem combined for both larval and adult stages of *0.2-luc 30-4 (H-ry)* and *0.8-luc 110 (H-ry)*.

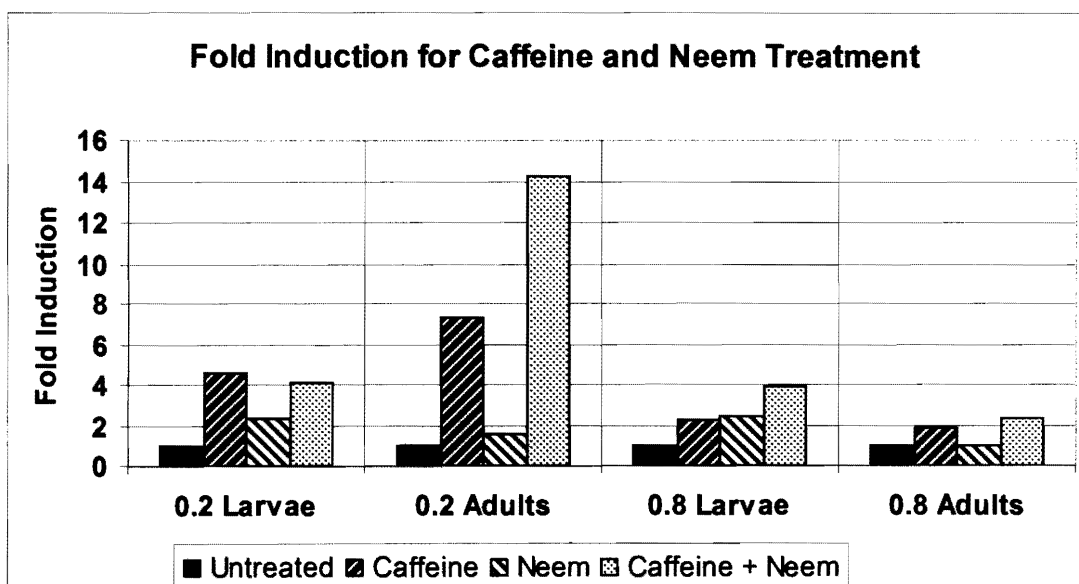


Fig. 4 Gene expression measured in Fold-Induction for larvae and adults of *0.2-luc 30-4 (H-ry)* and *0.8-luc 110 (H-ry)*. Fold induction of the untreated was considered 1.

IV. Discussion

While the data indicate that larvae and adults of both strains were induced by at least one chemical, it is clear that the levels of expression are not equal between the two developmental stages. In all cases, larvae show a greater level of induction than the adults of the same strain, for constitutive expression as well as chemically induced transcription. The causes for this could include many factors. It is possible that the promoter region is more exposed or certain transcriptional factors are available during a certain stage of development, which would confer increased transcription. The larvae may also be receiving a higher dose of the xenobiotics than the adults, since the larvae are constantly in the food and may absorb the chemicals through the cuticles as well as through ingestion. A more probable reason for the increased expression in larvae is that both male and female larvae were used, while only female adult flies were treated. In a previous study, male flies were found to show significantly higher constitutive expression of P450 genes. For *Cyp6a8*, the level of induction due to one chemical was also higher for males than females, but the induced expression after treatment with another was fairly similar to that of females (Le Goff, 2006). In order to determine whether induction due to caffeine or neem varies by sex, it would be necessary to repeat the same procedure using only female larvae.

The second noticeable trend in the data indicates that *0.8-luc110 (H-ry)* flies show higher overall activation of the *Cyp6a8* promoter than that of *0.2-luc 30-4 (H-ry)* flies. As previously discussed, the decreased constitutive expression in *0.2-luc* flies results from the presence of a smaller portion of the promoter region than *0.8-luc*. Maitra (2002) found that the most significant portion of the promoter is contained in the region from

199 to 761 base pairs sequence included in *0.8-luc 110 (H-ry)*, but absent from *0.2-luc 30-4 (H-ry)*. This was demonstrated through a 9-fold increase in constitutive expression between *0.2-luc* and *0.8-luc*, but a fragment containing a 3.1kb promoter sequence showed only 1.2-fold increase in constitutive expression from that of the 0.8kb sequence. Although *0.8-luc* shows higher overall expression of *Cyp6a8*, the *0.2-luc* strain gives higher fold-inductions after xenobiotic treatments. This could indicate that, although portions of the promoter are absent in the 200bp upstream DNA, the fragment actually contains the regions for binding and responding to caffeine and neem. In another study of the same strains, this same phenomenon was found, and caffeine caused a six-fold induction in *0.2-luc* but a four- to six-fold induction in *0.8-luc* (Bhaskara, 2006). Based on these findings and a study on the location of the reporter gene, Bhaskara concluded that caffeine specifically activates a particular region on the *Cyp6a8* promoter.

In the present study, caffeine was found to induce transcription in all four trial groups. However, the levels of induction for *0.8-luc* larvae and adults were lower than those reported previously. Bhaskara (2006) observed a nearly five-fold induction for *0.8-luc* adults, while this study found only two-fold induction. In contrast, the induction of *0.2-luc* flies and larvae were nearer the expected values. Since all of the fold-induction values for *0.8-luc* adults were fairly low, it is possible that it is the result of simple experimental error.

The treatment of adults with neem actually resulted in lower induction than expected. In preliminary experiments, flies and larvae were fed varying neem concentrations to determine its inductive effects. The 10% neem solution used in the earlier trials gave a two-fold induction for larvae of both strains and adults of *0.2-luc*.

However, neem was not actually soluble at such a high concentration, so 5% neem solution was used in the present study. The larvae showed expression similar to that with 10% neem solution, but the adults showed little or no induction by the neem solution. As discussed above, the adults may have received a lesser dose than the larvae so the concentration might not have been high enough to induce gene activity in adults. The adults also may have avoided exposure to food due to the bitter taste and proven antifeedant effect of neem (Robertson, 2007). In contrast, the larvae are unable to avoid contact with the xenobiotic-treated media.

No synergistic effects were found with caffeine and neem treated larvae of either strain. The results indicate that caffeine and neem induce transcription of the *Cyp6a8* gene through the same pathway, since the combination of caffeine and neem does not increase the induction from the saturating caffeine alone. Treatment of the adult flies, however, resulted in increased expression when treated with caffeine and neem compared to caffeine treatment alone. This induction was unexpected since neem alone was unable to increase transcription in adults. One possibility is that caffeine and neem actually do act by the same mechanism, but the 16mM caffeine solution was not fully saturating in adult flies. The flies may have been more willing to feed on the caffeine and neem treated food compared to the bitter tasting neem alone. A second potential explanation is that they work by different mechanisms but there is a difference between the developmental stages such as chromosome structure or another molecule present that prevents larvae from responding to both chemicals at the same time. It could also be the result of using male and female larvae but only female adults. In order to draw conclusions about the synergistic relationship between caffeine and neem, it would be necessary to perform the

same procedures using only female larvae and adults, or study the relationship in adults with higher concentrations of caffeine and neem. These experiments could provide further insight into the seemingly complex activation mechanism for *Cyp6a8*. By further study of the *Cyp6a8* promoter activity, the mechanism of pesticide resistance development in *Drosophila melanogaster* might be better understood.

V. References

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