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Effects of different brands of coffee on the transcriptional activity of *Drosophila* Cyp6a8 gene promoter

Senior Honors Thesis

Chancellor's Honors Program

University of Tennessee, Knoxville

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Abstract

Enzymes are found in all living organisms, and they are the main workhorses of the cells. Cytochrome P450 monooxygenases (CYPs) are enzymes that comprise a superfamily of enzymes found in all living organisms. They are best known for their ability to metabolize and detoxify hundreds of xenobiotic (foreign) compounds such as drugs or other toxic chemicals encountered by organisms on a daily basis. These enzymes are important because they help protect the organism from xenobiotic compounds that can cause disease. One of these foreign compounds that CYPs metabolize is caffeine. Previous research has shown that caffeine induces Cyp6a8 gene of *Drosophila.* This study focuses on the effects of different brands of commercially available caffeinated and decaffeinated coffee on the transcriptional activity of the Cyp6a8 gene promoter. For this purpose a transgenic strain of Drosophila carrying firefly *luciferase* reporter gene under the control of 0.8-kb promoter DNA of the Cyp6a8 gene were exposed to different brands and coffee. I found that Starbucks Christmas blend did not induce the Cyp6a8 gene as much as Kroger Classic Roast. Kroger Classic Roast caused more induction of Cyp6a8 than did 10mM caffeine. Also, when comparing decaf coffee treatments, the Cyp6a8 gene was induced more by Kroger decaf than by Starbucks decaf.

I. Introduction

Enzymes are found in all living organisms, and they are the main workhorses of the cells. They synthesize various proteins, hormones, and a variety of biomolecules, and also catalyze biochemical reactions to generate energy necessary to carry out hundreds of cellular functions. Enzymes belonging to the same family are similar chemically and functionally, but they are not identical.

Cytochrome P450 monooxygenases (CYPs) are such enzymes that comprise a superfamily of enzymes found in all living organisms. They are best known for their ability to metabolize and detoxify hundreds of xenobiotic (foreign) compounds such as drugs or other toxic chemicals encountered by organisms on a daily basis. These enzymes are important because they help protect the organism from xenobiotic compounds that can cause disease. These enzymes are the reason insects are able to detoxify insecticides and become insecticide resistant. The problem of insecticide resistance has led to many negative consequences for farmers and crop production. This ability to detoxify is conferred by increased expression of P450 genes, such as CYP6a8 in *Drosophila*.

Another foreign compound that CYPs metabolize is caffeine. Caffeine is the most commonly used psychostimulant (Bhaskara et all, 2008). It is found naturally in berries, seeds and leaves of plants such as tea, cocoa, and coffee. Previous research has shown that caffeine induces Cyp6a8 in *Drosophila* (Bhaskara et al, 2006). Caffeine can cause numerous medical problems and many people try to stay away from caffeine by drinking decaffeinated coffee. For example, consuming extreme amounts of caffeine can cause psychomotor agitation, insomnia, headache, and gastrointestinal problems. Consuming caffeine becomes even more of a health risk for pregnant

women and children and adolescents. In pregnant women, caffeine metabolism is slowed down and caffeine and its metabolites pass freely across the placenta into the fetus. In children and adolescents, consumption of caffeine influences the central nervous system and can have a negative effect on the period of rapid growth and the final stage of brain development, calcium balance, and sleep duration (Wierzejska, 2012).

In *Drosophila*, it is known that caffeine induces the expression of the CYP6a8 gene promoter but the details of the exact mechanism have yet to be discovered. It is also known that caffeine content in coffee varies, but by how much? The objective of my research is to determine the relative amounts of caffeine present in different brands of commercially available coffee, specifically Starbucks brand and Kroger brand, by measuring the induction of CYP6a8 gene promoter of 0.8-*luc* 110 H-ry transgenic flies.

Materials and Methods

Fly strains and culture conditions:

Drosophila melanogaster transgenic strains of 0.8 luc 110 H-ry were used and cultured at room temperature on medium consisting of agar, yeast, cornmeal, unsulfured molasses, corn syrup, and propionic acid. This strain is homozygous for a *luciferase* (luc) reporter gene under the control of a 0.8 kb upstream DNA (-11/-761) of the Cyp6a8 gene of Drosophila (Maitra et al, 2002). In the present investigation male flies were used and special attention was taken to use flies that looked skinnier and hungrier than others.

Preparation of coffee solutions:

Two different brands each of caffeinated (Starbucks Christmas blend and Kroger classic roast) and decaffeinated (Starbucks and Kroger) coffee grinds were purchased from the local grocery stores. To make coffee solution, 4 grams of each coffee grind was individually placed in 50mL of boiling water in a beaker and brewed for 10 minutes at room temperature. A strainer was used to strain the coffee into a clean 150mL beaker and 50mL of the coffee solution was stored in a sealed container at 4^0C.

Treatment of Transgenic Flies:

Instant fly food was saturated with water, 10mM caffeine, caffeinated or decaffeinated coffee solution. Roughly the same amounts of instant fly food and treatment solutions were used to prepare each vial. The flies (0.8-luc 110 h-ry strain) were then etherized and 15-20 male flies were transferred into each treatment vial. A “force feeding” method was used in which cotton plugs were inserted into the vials so that the flies only had half as much space to roam around.

Using this method, the flies were restricted to an area closer to the food and more likely to go down to the food and drink. The vials were left in a dark cabinet for 24 hours.

Preparation of fly extracts:

Following the 24-hour treatment, flies were etherized and group of three flies were sorted into 1.5-ml Eppendorf tubes. To allow the effect of ether shock to wear off, the flies were allowed to wake up first but they were knocked down again by placing the Eppendorf tubes on ice. To each tube 150uL of 1X CCLR buffer (Promega) was added and the flies were homogenized thoroughly using a hand-held power homogenizer. During homogenization the tubes were placed on ice for 20-30 seconds intermittently to avoid protein denaturation. After homogenization, the tubes were centrifuged for 8 minutes at 13,000 RPM at room temperature. After centrifugation 75uL of clear supernatant from each tube was transferred into a fresh 1.5-ml Eppendorf tube. These tubes were centrifuged again as described above. An aliquot pf 35uL of clear supernatant was removed from each tube and placed in a clean 0.5-ml Eppendorf tube. The cell lysates were then stored at -20°C until further analysis.

Luciferase assay:

This assay was done at room temperature under diffused light. Cell lysates were removed from the freezer and placed on ice to thaw. LAR (luciferase assay reagent, Promega) was also thawed on ice in diffused light. For luciferase assay 25ul of LAR was added to the bottom of a clean1.5-ml Eppendorf tube. Then 5ul of cell extract was added directly to the LAR. The tube was closed quickly, agitated gently by flicking it 5 times, then placed in the luminometer for a 15-sec readout. The RLU (relative light reading) displayed by the instrument for each extract was recorded. This procedure was repeated for all extract samples.

Protein assay:

BCA protein assay was done by using a BCA protein assay kit (Pierce). Different amounts (0, 12.5, 25, 37.5, and 50ug) of Bovine Serine Albumin (BSA) were used to generate a protein standard curve. The stock BSA (2mg/ml) was diluted to 1mg/ml by mixing 150ul stock BSA with 150ul 1X CCLR buffer. Each of the fly extracts was assayed in duplicates. 40ul of fly extract were added to each tube, then 10ul 0.5X CCLR were added. 0.5ml of BCA reagent was added to each tube. The tubes were mixed gently, incubated at 37°C for 30 minutes, and then analyzed with a spectrophotometer at 595 nm wavelength. The duplicate values were then averaged to determine the ug of protein per ul of extract.

Statistical Analysis:

Standard deviations were calculated using Excel and a student's t-test was performed on all data to determine statistical significance.

II. Results and Discussion

Analyzing the Starbucks data revealed some significant results. In Starbucks (SB) trial 1, induction of the CYP6a8 gene promoter was significant for those flies treated with 10mM caffeine (p value = 0.056) and those treated with SB Christmas blend (p value = 0.018) as shown in Tables 1. Table 2 shoes that SB trial 2 did not yield any significant results, but the same pattern of CYP6a8 gene promoter induction can be seen. This pattern, shown in Figure 1, is that the CYP6a8 gene promoter of flies treated with 10mM caffeine and SB Christmas was induced more than that of flies treated with water. Also, flies treated with decaf SB showed similar induction to that of flies treated with water.

The data collected from the Kroger treatment groups revealed more significant data compared to the SB treatment groups. As shown in Table 3, the CYP6a8 gene promoter induction was significant in those flies treated with 10mM caffeine (p value = 0.007) but not technically significant in those flies treated with Classic Roast (p value = 0.057) and Kroger decaf (p value = 0.072). Table 2 shows the results from Kroger treatment trial 2. CYP6a8 gene promoter induction was not significant in flies treated with caffeine (p value = 0.091). However, it was significant in flies treated with Classic Roast (p value= 0.004) and Kroger decaf (p value = 0.0016). Comparing the amount of CYP6a8 gene promoter induction found in both Kroger trials, although not all results were significant, a similar pattern of induction is seen. The control group (water) is induced the least. Induction increases for flies treated with 10mM caffeine and then increases even more in flies treated with Kroger Classic Roast. For flies treated with Kroger decaf, induction decreases to a level similar to that shown in flies treated with 10mM caffeine.

Overall, when comparing CYP6a8 gene promoter induction patterns of flies treated with Starbucks brand coffee and flies treated with Kroger brand coffee, it seems as though Kroger brand coffee contains more caffeine. This conclusion can be seen by comparing relative CYP6a8 gene promoter fold induction patterns shown in Figures 1 and 2. In Starbuck treatment groups, the induction shown in flies treated with 10mM caffeine (fold induction = 4 and 4) is similar to that of flies treated with SB Christmas blend (fold induction = 3 and 4) while induction shown in flies treated with water (fold induction = 1 and 1) is similar to that of flies treated with SB decaf (fold induction = 2 and 1). In Kroger treatment groups, the induction shown in flies treated with 10mM caffeine (fold induction = 2 and 3) is similar to that of flies treated with Kroger decaf (fold induction = 3 and 2) while induction shown in flies treated with Kroger Classic Roast (fold induction = 16 and 6) is much higher than any other treatment group.

This finding is significant when discussing the adverse affects of caffeine. Eighty-nine percent of the adult US population consumes caffeine and 64% of this caffeine consumption is from coffee. The mean caffeine intake is 211 mg/day and some people consume as much as 1,066 mg/day (Fulgoni 2015). On the other hand, many other people try to avoid or minimize their caffeine consumption in order to prevent negative health related issues. Based on my results, Kroger Classic Roast induced the CYP6a8 gene promoter 3.5 times more than did Starbucks Christmas blend. Since this induction is directly related to the amount of caffeine, these results also show that Kroger Classic Roast contains 3.5 times that amount of caffeine. This can lead to people consuming more caffeine than they intend to and possible result in health issues. This same problem can arise from decaffeinated coffee. It is known that decaf coffee is not truly decaf, but just how much caffeine is present? Based on my results, Kroger decaf coffee contains two times that amount of caffeine compared to SB decaf. Some people are more

sensitive to caffeine or have pre-existing health issues that put them at a higher risk for palpitations, tremors, agitation, or gastrointestinal upset (Brown, 2012). Caffeine content should be labeled on all brands of coffee to make people aware of how much caffeine they are actually consuming.

One possible explanation for the presence of varied amounts of caffeine in decaf coffee is the way the coffee beans are processed. Currently, there are three different methods used to remove the caffeine from coffee beans. Each method begins by moistening the coffee beans, making the caffeine soluble, and drawing it out with a solvent (Clydesdale, 1999). The different methods use different solvents – water, methylene chloride, and carbon dioxide. Water, the "natural" way, removes 94-96% of the caffeine. Methylene chloride removes 96-97% and carbon dioxide removes 96-98%. The various levels of CYP6a8 gene promoter expression could be due to the different brands of decaf coffee being processed differently so that different amounts of caffeine are successfully removed. It is also possible that the method using methylene chloride might add a chemical to the coffee beans and that chemical might be causing CYP6a8 induction. Another possible explanation is that CYP6a8 induction might be caused in part from other chemicals present in the coffee, apart from chemicals that might be added during the decaffeination process. These chemicals might be acting through the CYP6a8 pathway or through another pathway.

More extensive research needs to be done in order to determine if caffeine is solely responsible for the CYP6a8 gene promoter induction or if there are other chemicals having an effect. More research also needs to be done to determine the exact mechanism of CYP6a8 gene promoter induction. Future research could focus on using these transgenic flies to test the effect of caffeine like molecules on the induction of P450 genes. This would reveal more details about

the mechanism of the pathway and could lead to the revelation of information about this mechanism in other eukaryotes.

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Table 1. Effect of Starbucks coffee on the promoter activity of CYP6a8 gene (Trial 1)

Sample	Treatment	RLU	ug of protein	RLU per ug of protein	Average RLU per ug of protein	S.D.	P-value*
1	H2O	440,734	4.468	98642			
2	H2O	349,547	2.9305	119278	89576.66667	28677.54008	
3	H2O	161,119	3.171	50810			
4	10mM Caffeine	2,522,829	4.4115	571875			
5	10mM Caffeine	2,094,640	4.4925	466252	406128.6667	165432.5326	.056
6	10mM Caffeine	693,728	3.8485	180259			
7	SB Christmas	1,325,935	4.578	289631			
8	SB Christmas	2,084,724	4.8715	427942	319620.3333	79096.77201	.018
9	SB Christmas	778,154	3.225	241288			
10	SB Decaf	791,366	3.3325	237469			
11	SB Decaf	583,231	3.064	190349	185597.6667	44419.72824	.062
12	SB Decaf	350,426	2.717	128975			

*Relative to water

Table 2. Effect of Starbucks coffee on the promoter activity of CYP6a8 gene (Trial 2)

Sample	Treatment	RLU	ug of protein	RLU per ug of protein	Average RLU per ug of protein	S.D.	P-value*
1	H2O	1,688,800	1.4055	1201565			
2	H2O	929,585	0.332	2799954	1726752	758925.6349	
3	H2O	1,477,548	1.2535	1178737			
4	10mM Caffeine	8,138,616	2.1855	3723914			
5	10mM Caffeine	7,434,829	1.314	5658165	6932221	3266396.106	.093
6	10mM Caffeine	7,505,089	0.6575	11414584			
7	SB Christmas	3,804,435	4.3285	878926			
8	SB Christmas	4,105,741	1.045	3928938	7942774.667	7931455.417	.33
9	SB Christmas	4,051,358	0.213	19020460			
10	SB Decaf	2,983,654	0.449	6645109			
11	SB Decaf	2,776,959	2.004	1385708	3151975.333	2470070.879	.48
12	SB Decaf	2,689,181	1.887	1425109			

*Relative to water

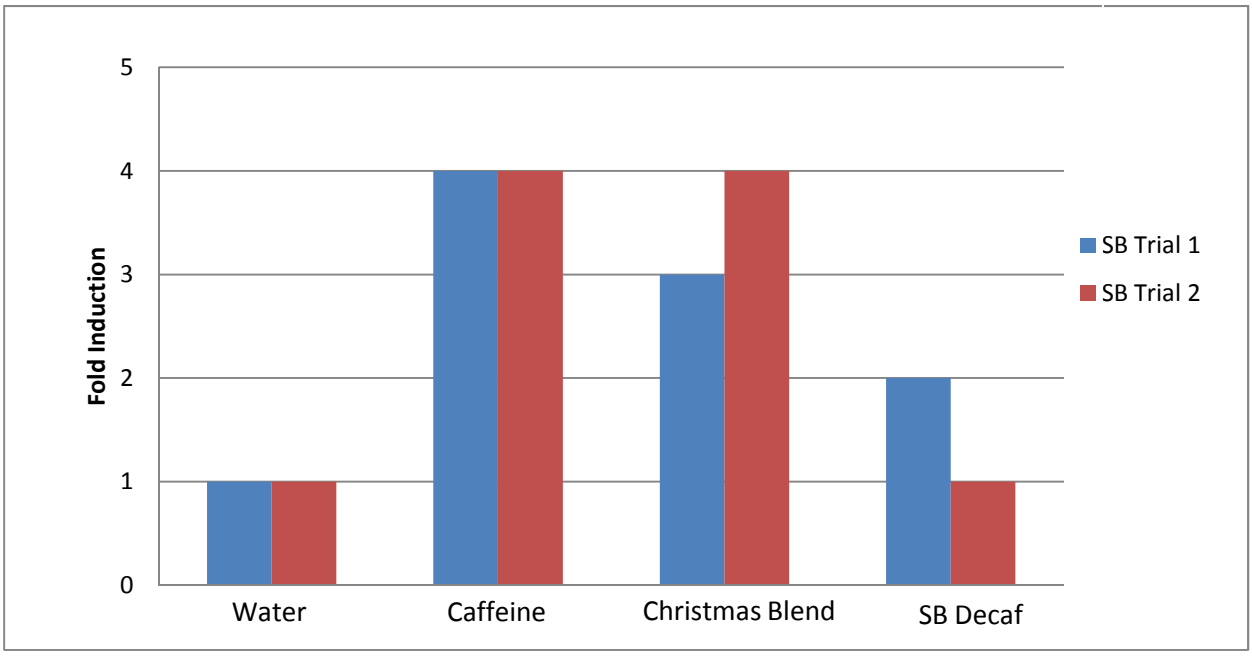

Figure 1. Induction of CYP6a8 gene promoter in flies treated with Starbucks coffee. Fold induction was measured compared to water.

Table 3. Effect of Kroger coffee on the promoter activity of CYP6a8 gene (Trial 1)

Sample	Treatment	RLU	ug of protein	RLU per ug of protein	Mean RLU per ug of protein	S.D.	P-value*
1	H2O	3,692,250	2.261	1633016			
2	H2O	3,138,372	1.3005	2413204	1589227.67	463599.64	
3	H2O	1,334,708	1.85	721463			
4	10mM Caffeine	13,837,356	2.2335	6195368			
5	10mM Caffeine	11,149,346	1.7475	6380169	5636142.00	924622.14	.007
6	10mM Caffeine	16,252,670	3.751	4332889			
7	Kroger Classic Roast	14,240,246	0.677	21034336			
8	Kroger Classic Roast	17,741,228	0.4135	42905025	25683044.33	12600189	.057
9	Kroger Classic Roast	13,299,864	1.0145	13109772			
10	Kroger Decaf	3,980,018	0.952	4180691			
11	Kroger Decaf	2,573,412	0.6355	4049428	5569723.667	2057903.6	.072
12	Kroger Decaf	5,117,108	0.6035	8479052			

*Relative to water

Table 4. Effect of Kroger coffee on the promoter activity of CYP6a8 gene (Trial 2)

Sample	Treatment	RLU	ug of protein	RLU per ug of protein	Mean RLU per ug of protein	S.D.	P-value*
1	H2O	1,431,039	2.7415	521991			
2	H2O	1,420,078	2.107	673981	724528.6667	189410.28	
3	H2O	1,655,101	1.693	977614			
4	10mM Caffeine	6,631,308	5.2875	1254148			
5	10mM Caffeine	3,673,766	2.876	1277387	1673057.333	576072.93	.091
6	10mM Caffeine	5,855,898	2.354	2487637			
7	Kroger Classic Roast	10,904,026	3.384	3222229			
8	Kroger Classic Roast	11,660,522	2.229	5231279	4364438	842987.2	.004
9	Kroger Classic Roast	11,089,138	2.39	4639806			
10	Kroger Decaf	5,296,170	3.038	1743308			
11	Kroger Decaf	3,550,496	1.903	1865736	1787509.333	55470.39	.0016
12	Kroger Decaf	4,448,591	2.537	1753484			

*Relative to water

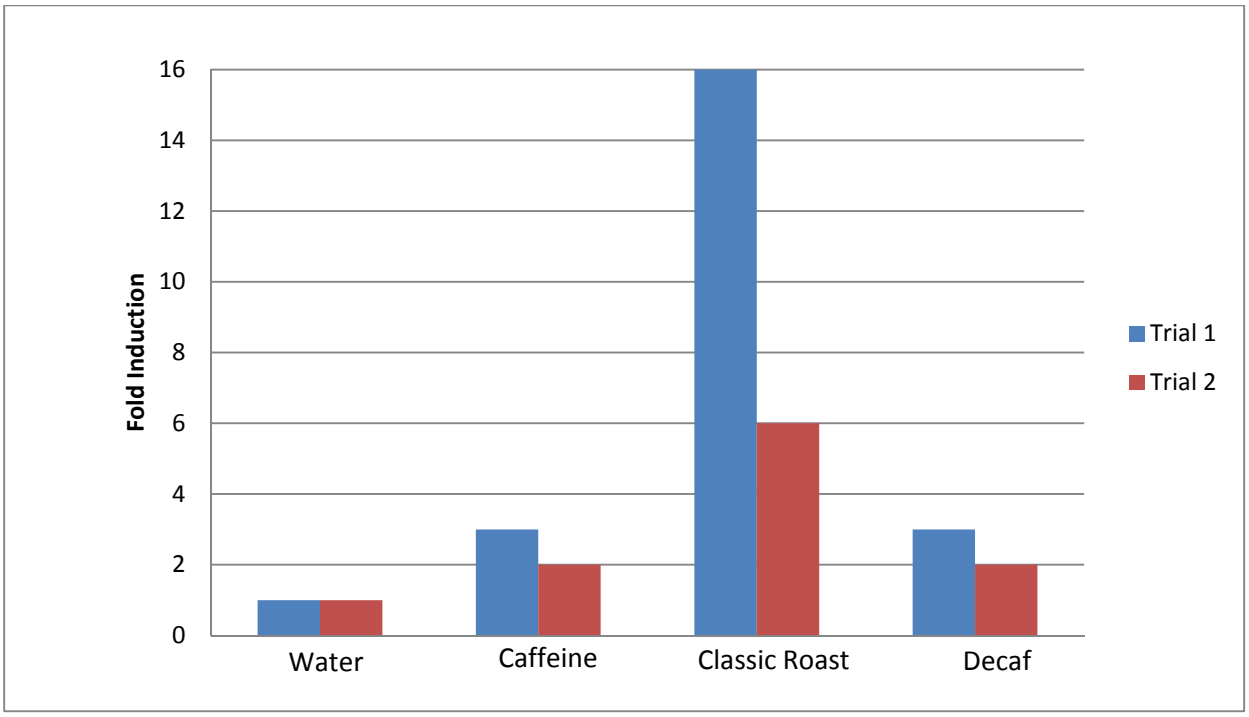

Figure 2. Induction of CYP6a8 gene promoter in flies treated with Kroger coffee. Fold induction was measured compared to water.