



HAL
open science

Cytochrome P450-dependent metabolism of caffeine in [i]*Drosophila melanogaster*[/i]

Alexandra Coelho, Stéphane Fraichard, Gaëlle Le Goff, Philippe Faure, Yves Artur, Jean-François Ferveur, Jean-Marie Heydel

► **To cite this version:**

Alexandra Coelho, Stéphane Fraichard, Gaëlle Le Goff, Philippe Faure, Yves Artur, et al.. Cytochrome P450-dependent metabolism of caffeine in [i]*Drosophila melanogaster*[/i]. PLoS ONE, 2015, 10 (2), 10.1371/journal.pone.0117328 . hal-01212004

HAL Id: hal-01212004

<https://hal.science/hal-01212004>

Submitted on 5 Oct 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

RESEARCH ARTICLE

Cytochrome P450-Dependent Metabolism of Caffeine in *Drosophila melanogaster*

Alexandra Coelho¹, Stephane Fraichard¹, Gaëlle Le Goff², Philippe Faure¹, Yves Artur¹, Jean-François Ferveur^{1*}, Jean-Marie Heydel^{1*}

1 CNRS 6265, INRA 1324, Université de Bourgogne, Centre des Sciences du Goût et de l'Alimentation, F-21000, Dijon, France, **2** INRA, CNRS, UNSA, UMR 1355, Institut Sophia Agrobiotech, F-06903, Sophia Antipolis, France

* jean-marie.heydel@u-bourgogne.fr (JMH); jean-francois.ferveur@u-bourgogne.fr (JFF)



OPEN ACCESS

Citation: Coelho A, Fraichard S, Le Goff G, Faure P, Artur Y, Ferveur J-F, et al. (2015) Cytochrome P450-Dependent Metabolism of Caffeine in *Drosophila melanogaster*. PLoS ONE 10(2): e0117328. doi:10.1371/journal.pone.0117328

Academic Editor: Kun Yan Zhu, Kansas State University, UNITED STATES

Received: August 19, 2014

Accepted: December 21, 2014

Published: February 11, 2015

Copyright: © 2015 Coelho et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Microarray data obtained from this study can be accessed at NCBI GEO (GSE59084).

Funding: This work was funded in part by the Regional Council of Burgundy France (PARI), the European Funding for Regional Economical Development (FEDER) the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Recherche Agronomique (INRA) and the French Ministry of Research and Higher Education. The funders had no role in study design, data

Abstract

Caffeine (1, 3, 7-trimethylxanthine), an alkaloid produced by plants, has antioxidant and insecticide properties that can affect metabolism and cognition. In vertebrates, the metabolites derived from caffeine have been identified, and their functions have been characterized. However, the metabolites of caffeine in insects remain unknown. Thus, using radiolabelled caffeine, we have identified some of the primary caffeine metabolites produced in the body of *Drosophila melanogaster* males, including theobromine, paraxanthine and theophylline. In contrast to mammals, theobromine was the predominant metabolite (paraxanthine in humans; theophylline in monkeys; 1, 3, 7-trimethyluric acid in rodents). A transcriptomic screen of *Drosophila* flies exposed to caffeine revealed the coordinated variation of a large set of genes that encode xenobiotic-metabolizing proteins, including several cytochromes P450s (CYPs) that were highly overexpressed. Flies treated with metyrapone—an inhibitor of CYP enzymes—showed dramatically decreased caffeine metabolism, indicating that CYPs are involved in this process. Using interference RNA genetic silencing, we measured the metabolic and transcriptomic effect of three candidate CYPs. Silencing of *CYP6d5* completely abolished theobromine synthesis, whereas *CYP6a8* and *CYP12d1* silencing induced different consequences on metabolism and gene expression. Therefore, we characterized several metabolic products and some enzymes potentially involved in the degradation of caffeine. In conclusion, this pioneer approach to caffeine metabolism in insects opens novel perspectives for the investigation of the physiological effects of caffeine metabolites. It also indicates that caffeine could be used as a biomarker to evaluate CYP phenotypes in *Drosophila* and other insects.

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is a typical purine alkaloid that is produced in a variety of plants, including coffee (*Coffea arabica*) and tea (*Camellia sinensis*) [1]. Caffeine is involved in plant chemical defense, acting as a repellent, pesticide and allelopathic agent [2–4]. With its

collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

psychostimulant, cardiac and diuretic effects, caffeine is one of the most widely used plant secondary metabolites, primarily as a food additive or an ingredient in drugs [5].

Various insect studies have revealed that caffeine can induce similar effects than in vertebrates: inhibition of feeding and sleep [6–8], increased heart rate [9], and mutagenic and mitotic action [10,11]. In insects in particular, caffeine can affect olfactory and visual associative learning, as demonstrated by studies conducted in honeybees [12]. Caffeine can also affect the reproductive (egg laying ability) [3,13,14] and dopaminergic systems, calcium exchange and potassium currents in the central nervous system of insects [15–17]. In *Drosophila*, the effect of caffeine was measured in regard to bitter taste-induced aversive behavior [18]. Caffeine induces similar repulsive effects to a male sex pheromone, as shown by both the male courtship behavior and the feeding response [19,20]. Several gustatory receptors sensitive to caffeine, such as Gr66a, Gr33a and Gr93a, were also characterized [19,21].

All of these effects may involve metabolites derived from caffeine, which could have, as in humans, their own biological activities [22–24]. Caffeine derivatives in the human liver, metabolized by the cytochrome P450 oxidase enzyme system (in particular by the CYP1A2 isoenzyme), include three major dimethylxanthine metabolites (paraxanthine, theobromine and theophylline) and one hydroxylated metabolite (1, 3, 7-trimethyluric acid) [25]. Paraxanthine is the predominant caffeine-derived metabolite in humans, but 1, 3, 7-trimethyluric acid and theophylline are the major metabolites found in rodents and monkeys, respectively [26–28].

Among the compounds known to induce toxic effects in *Drosophila melanogaster*, caffeine has been studied primarily in regard to its regulatory effect on detoxification enzymes. Exposure to caffeine enabled the identification of CYP enzymes involved in insecticidal or toxic host plant resistance and metabolism [29,30]. These studies led to a better understanding of insecticide resistance [31] and the regulation of CYP expression [32–34]. CYPs have also been proposed to interact in the metabolism of odorant compounds [35,36]. Cytochrome P450s make up a diverse and important gene super-family in all organisms. In insects, CYPs are known to catalyze a diverse range of chemical reactions important for both developmental processes and the detoxification of exogenous compounds. We focused on the *CYP12d1*, *CYP6a8* and *CYP6d5* genes, which showed the largest amplitude of variation in our initial tests. These enzymes were also selected because they belong to two of the four large clades of insect P450 genes: the CYP2 clade, the CYP3 clade (*CYP6a8*, *CYP6d5*), the CYP4 clade and the mitochondrial P450 clade (*CYP12d1*).

Interestingly, there have been few insect reports providing a global picture of the CYP-related metabolism of a xenobiotic compound. Our study takes into account the activity of the genes induced by xenobiotic compounds as well as the catalytic function of the coded enzymes leading to compound degradation. More specifically, we identified the metabolites of caffeine in *Drosophila melanogaster* and screened the “xenobiotic-metabolizing-enzyme genes” affected by caffeine exposure. Among the genes strongly impacted by caffeine exposure, three CYP candidates were silenced, the effects of which were measured on caffeine metabolism together with the coordinated variation of expression between the three CYPs.

Materials and Methods

Chemicals

Caffeine (58–08–2), Theobromine (83–67–0), Theophylline (58–55–9), Paraxanthine (611–59–6) and Metyrapone (54–36–4) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 1, 3, 7 trimethyluric acid (5415–44–1) from ChemCruz (Santa Cruz, CA, USA).

Drosophila strains and rearing conditions

Flies were reared on a standard yeast/cornmeal/agar medium at 25°C on a 12L:12 D cycle. w^{1118} ($y1 w+$) and P{Act5C-GAL4}25FO1/CyO strains, identified as w^{1118} and *Actin-GAL4*, respectively, were obtained from the Bloomington stock center. All of the UAS-RNAi strains (*dsCYP12d1* (109256); *dsCYP6a8* (4884); *dsCYP6d5* (12139)) were purchased from the Vienna Drosophila Resource Center (stock ID indicated in parentheses). The UAS RNAiCYP/*Actin-GAL4* system was used to target all tissues (with an *Actin-GAL4* driver) potentially involved in the metabolism of caffeine and to inhibit (with UAS-RNAiCYP) the expression of the selected CYPs in the targeted tissues.

Caffeine treatment and microarray analysis

Four-day-old adult males were starved for 12 hours in a vial containing a filter paper impregnated with water before being transferred to caffeine-rich medium (18 mM; Sigma-Aldrich) for 12 hours. Food mixed with water served as a control. Taking into account the studies reported by Le Goff G (2006), and Willoughby L, (2006) [29–52], we decided to expose adult male flies for 12 hours on an 18 mM caffeine-rich medium to obtain a trade-off between the optimal induction of CYP genes and the lower mortality for the flies. For each food treatment (caffeine and water), RNA was isolated from 10 fly bodies (thorax and abdomen) using Isol RNA Lysis reagent (5Prime). All samples were prepared in triplicate to permit statistical analysis. Probe labeling, hybridization to single color Agilent 4x44k arrays, scanning and statistical analysis were performed by the IMAXIO company. Genes showing a 2-fold change and a significant p-value < 0.05 were considered to be differentially expressed. Microarray data obtained from this study can be accessed at NCBI GEO (GSE59084).

RNA extraction and RT-qPCR

Total RNA was extracted from 10 fly bodies using Isol RNA Lysis reagent (5Prime) and treated with RNase-free DNase (Euromedex) to avoid genomic DNA contamination. Total RNA (1 µg) was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad). The qPCR reactions were carried out using a MyIQ (BioRad) and the IQ SYBR Green SuperMix (BioRad). Each reaction was performed in triplicate and all results were normalized to the tubulin and *rp-49* mRNA levels and calculated using the $\Delta\Delta C_t$ method.

The following forward and reverse primers were used: *tubulin* TGTCGCGTGTGAAACACTTC and AGCAGTAGAGCTCCCAGCAG; *rp-49* CCCAAGGGTATCGACAACAG and GTTCGATCCGTAACCGATGT; *cyp12d1p* TTAGCTTGTTTCATGTGCC and ATTTACGTGGTCCCCTTC; *cyp6a8* GGCTGAGGTGGAGGAGGT and CGATGACGAAGTTTGGATGA; *cyp6d5* AAGCAACTGCCTGCGAAC and CAATAATGTCGATGGCGTATGT. For each gene, primer efficiency was calculated: *tubulin* (1.90), *rp-49* (2.00), *cyp12d1p* (1.98), *cyp6a8* (1.92); *cyp6d5* (2.00) [37].

Western-blot

Protein extracts were prepared from male adult flies. Equal amounts of protein were separated on a 4–15% SDS-polyacrylamide gel (Bio-Rad) and blotted using standard procedures. The membrane was incubated with a polyclonal anti-CYP6d5 (1/5000, Proteogenix) and a specific antibody against Actin (1/2000, Abcam) was used for loading controls.

Caffeine exposure

Four-day-old male adult flies, starved for 12 hours under humid conditions and pre-exposed for 12 hours to a caffeine-rich medium (18 mM), were placed under humid conditions for 3 hours and transferred, without anesthesia, into a MultiCAFE device [38]. Briefly, pre-exposed individuals were transferred into a box containing 4 capillaries filled with a red dye used for further quantification (0.2 mg/mL sulforhodamine, Sigma-Aldrich), 100 mM sucrose (Euro-medex) and 0.04 μ M radiolabeled caffeine ($8\text{-}^{14}\text{C}$, Bio trend, $3.7\cdot 10^3$ Bq/ μ L). Flies were fed in the dark for 2 hours at 27°C under high humidity (>60%).

For the metyrapone experiment (Fig. 1B), the 4 capillaries were filled with 100 mM sucrose and 0.04 μ M radiolabeled caffeine, as well as 25 mM metyrapone (Sigma-Aldrich).

Radioactive caffeine and thin layer chromatography (TLC)

We initially performed mass spectrometry analysis to identify caffeine metabolites but faced technical problems, including low metabolism recording and matrix effect on mass detection, which led to a lack of reproducibility (with four attempts). If a similar experiment is never reported in insects, it may be partly because xenobiotic metabolic activity can be inhibited by endogenous compounds present in homogenates [40,41]. This was the reason for the development of a radiolabeled TLC method.

Exposed flies with red-dye-colored abdomens were pooled into groups of 50, homogenized with 150 μ L of lysis buffer (Tris 0.1 M, 0.1% SDS, 20% glycerol, 10% protease inhibitor), and centrifuged for 5 min at 16000 g. An aliquot of the supernatant (5 μ L) was mixed with scintillation liquid, and the radioactivity was counted with a Tri-Carb 3110TR scintillation counter (PerkinElmer). A similar amount of each sample, or radiolabeled caffeine alone (positive control), was directly applied to a thin layer chromatography plate (Nano-SIL NH2/UV, Macherey-Nagel). The mobile phase used for the separation was composed of chloroform, dichloromethane and isopropanol (v/v/v 4:2:1). After migration, the plate was autoradiographed. The total amount of radiolabeled caffeine-derived metabolites was quantified by densitometry and normalized to non-metabolized caffeine using ImageJ (Software, NIH, Bethesda, MD, USA). Each experiment was conducted in triplicate for appropriate statistical analysis.

Statistical analyses

For qPCR, transcript level ratios were compared between strains using the Relative Expression Software Tool (REST, REST-MCS beta software version 2) with 2000 iterations [39]. For the radioactivity assay, the amount of caffeine metabolites was compared between control strains and transgenic or treated flies. Normal distribution and homoscedasticity of the variances were checked. In two-group comparisons, Student's t tests were performed for parametrically distributed data. When more than two groups were compared, ANOVA was performed for parametrically distributed data using R software.

Results

Caffeine metabolism

Drosophila melanogaster adult males fed with radiolabeled caffeine for 2 hours produced eight caffeine-derived metabolites. These metabolites were distinctly separated by thin layer chromatography (Fig. 1A-lane 2) and additionally identified based on their physical characteristics (retention factors = RFs) compared to commercially available compounds (Fig. 1A-lane 1). The pattern of *Drosophila* caffeine-derived metabolites was compared with that observed in mammals, in which caffeine degradation generally yields four primary metabolites:

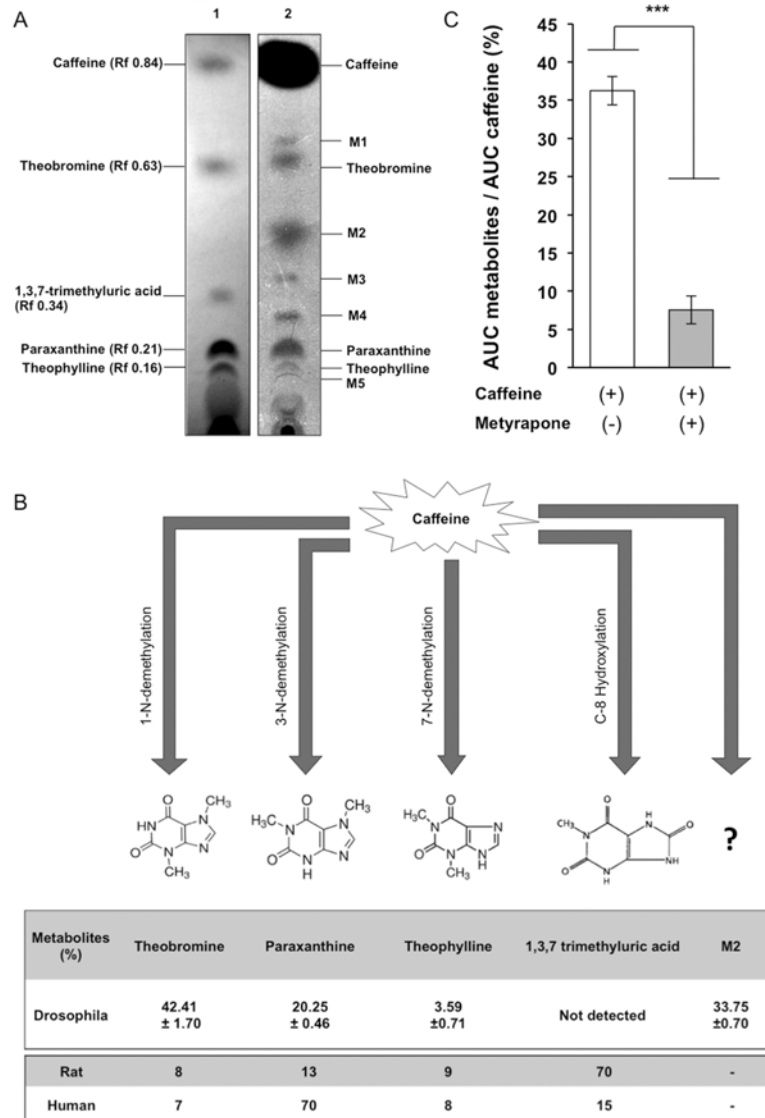


Fig 1. Caffeine metabolism in *Drosophila melanogaster* and the influence of cytochrome P450 inhibitor. A- Separation of non-radiolabeled standards of caffeine and metabolites (lane 1) with a homogenate of wild-type four-days-old *Drosophila* male flies (w^{1118}) exposed with radiolabeled caffeine for 2 hours (lane 2) on thin layer chromatography. B- The total amount of caffeine derived metabolites was strongly decreased in the body of male flies after exposure to caffeine mixed with metyrapone (+), as compared to control food (-). The amounts were evaluated by densitometry which is based on the comparison of the areas under the curve (AUC) for both metabolites and caffeine. Bars indicate the mean values (\pm s.e.m.). Significant differences are indicated by asterisks (Student's t-test *** $p < 0.001$; $n = 3$). C- Interspecific variation for the level of major caffeine metabolites. The proportions of theobromine, paraxanthine and theophylline and of the unidentified M2 metabolite detected in fly bodies after caffeine exposure (18mM) are indicated as the mean (\pm s.e.m.; $n = 4$). In *Drosophila melanogaster*, these amounts were evaluated by measuring the relative intensity of the radiolabelled signal associated with each metabolite after thin layer chromatography separation. The proportion of these compounds detected in *Drosophila melanogaster* was compared with that reported for *Rattus norvegicus* and *Homo sapiens* [25–28].

doi:10.1371/journal.pone.0117328.g001

theobromine, 1, 3, 7-trimethyluric acid, paraxanthine and theophylline (RFs: 0.63, 0.34, 0.21, 0.16, respectively) (Fig. 1A-lane 1).

The pattern of caffeine-derived metabolites in mammals partially overlapped with that obtained in *Drosophila melanogaster*. Notably, theobromine, paraxanthine and theophylline were found in both human and flies. Five additional metabolites detected remain unidentified (M1–M5; Fig. 1A-lane 2). However, the relative amount of caffeine metabolites diverged between flies, rat and human (Fig. 1B). While the caffeine ingested by *Drosophila* was metabolized into 42% theobromine, 20% paraxanthine, and 4% theophylline, mammals produced much less theobromine and slightly more theophylline. Humans also produced more paraxanthine than the two other species. The presence of the M2 metabolite (34%) in *Drosophila* did not match the mammalian standard metabolite tested. Reciprocally, 1, 3, 7-trimethyluric acid was detected in mammals, but apparently not in *Drosophila*.

Genes induced by caffeine exposure

Next, we screened for xenobiotic-metabolizing-enzyme genes (XMEs) induced by caffeine exposure. We carried out a microarray experiment (with a *Drosophila* pangenomic array on flies exposed or not exposed to caffeine). Interestingly, several genes belonging to all major classes of detoxification enzymes, including XMEs, were among those that were the most highly induced (Tables 1 and 2). Among XME genes, several CYPs were up-regulated (including *CYP12d1*, *CYP6a8* and *CYP6d5*), whereas others were down regulated (Table 1). Remarkably, all induced CYPs were already known to be inducible by a broad range of compounds, such as phenobarbital or the herbicide atrazine (Table 1). In a similar manner, several CYPs known to be repressed by paraquat, a compound known to induce stress by producing reactive oxygen species, were also down-regulated by caffeine [42] (Table 1). Our screen also found several other major detoxification enzymes known to be regulated by different xenobiotics (Table 2).

Pharmacological and genetic silencing of CYP: effect on caffeine metabolism

Given that the expression of several CYP genes was significantly modulated after caffeine exposure, we tested the effect of metyrapone, a general CYP inhibitor [35]. Male flies fed for 2 hours with the “caffeine + metyrapone” mixture produced much less caffeine metabolites in their body than controls (Fig. 1C). Furthermore, we manipulated the expression of several CYP candidate genes by using the GAL4/UAS-RNAi binary system. In particular, a ubiquitously expressed driver (*Actin-GAL4*), allowed us to separately target the RNAi of three CYP candidates (*dsCYP12d1*, *dsCYP6a8* and *dsCYP6d5*) that were highly up-regulated after caffeine exposure (Table 1). Given the simultaneous up-regulation of these three CYP genes by caffeine, we also measured their potential interaction and coordinated regulation.

First, ubiquitous silencing of *CYP6d5* strongly altered the production of two caffeine-derived metabolites in caffeine-exposed male flies: the production of theobromine was dramatically decreased, whereas M2 was significantly increased (in *dsCYP6d5/Actin-GAL4*). This effect was specifically due to *CYP6d5* silencing because no such variation was detected in the two transgenic controls (*Actin-GAL4/+* and *dsCYP6d5/+*). Fig. 2A shows that the paraxanthine and theophylline levels did not differ between the RNAi-targeted and control strains. The silencing efficiency of *dsCYP6d5*, measured by RT-qPCR in the body of male adult flies, was measured not only on the expression of *CYP6d5* but also of *CYP12d1* and *CYP6a8* genes (Fig. 2B). The mRNA level of *CYP6d5* was strongly decreased (-95%) in *dsCYP6d5/Actin-GAL4* strain, whereas the expression level of two other CYPs remained unaffected. Moreover,

Table 1. Expression of CYP genes in *Drosophila melanogaster* after caffeine exposure.

Gene name	Microarray	P value	Quantitative PCR	P value	Regulation by other compounds ^a
	Up or down (-) regulation		Up or down (-) regulation		(+) up-expression or (-) down-expression
CYP12d1-p	21.41	1.7E-3	15	1.0E-3	+ (phenobarbital, atrazine, piper nigrum, piperonyl butoxide, pyrethrum, ethanol, DDT, chlorpromazine)
CYP12d1-d	19.55	1.5E-3	15	1.0E-3	
CYP6a8	13.48	3.2E-3	20.5	1.0E-3	+ (phenobarbital, piper nigrum, ethanol, DDT, chlorpromazine)
CYP6d5	3.46	2.6E-3	11.8	1.0E-3	+ (phenobarbital, atrazine, piper nigrum, piperonyl butoxide, paraquat, cadmium, ethanol, zinc, rotenone)
CYP4p1	2.95	1.9E-3	2.1	1.0E-2	+(paraquat, tunicamycin, piperonyl butoxide, ethanol, cadmium, rotenone)
CYP304a1	2.85	2.9E-2	2.1	5.0E-2	+(atrazine)
CYP28a5	2.45	3.0E-3	Nd	nd	+(paraquat, tunicamycin, ethanol, rotenone)
CYP12a5	2.42	5.0E-3	3.5	1.0E-2	+(cadmium, ethanol, rotenone)
CYP6a9	2.39	6.3E-3	3.9	1.0E-2	+(ethanol, rotenone)
CYP6a20	2.30	5.0E-3	2.0	1.0E-2	+(cadmium, copper, paraquat)
CYP6w1	2.06	4.2E-3	Nd	nd	+(phenobarbital, atrazine, piper nigrum, piperonyl butoxide, cadmium, zinc, ethanol, paraquat)
CYP313a3	-2.05	4.4E-2	Nd	nd	-
CYP6a18	-2.39	3.8E-3	Nd	nd	-(paraquat)
CYP4d8	-2.55	3.3E-3	Nd	nd	-
CYP316a1	-2.81	3.9E-3	Nd	nd	-
CYP4e1	-3.55	2.2E-3	Nd	nd	-(paraquat), +(tunicamycin)
CYP4d20	-4.20	2.6E-3	-2.3	1.0E-3	-(paraquat, phenobarbital)
CYP313a1	-4.29	3.6E-3	-5.3	1.0E-3	-(paraquat, phenobarbital), + (tunicamycin)
CYP4ac1	-4.95	3.5E-3	-3.5	1.0E-3	+(endosulfan)
CYP4ac2	-9.61	3.5E-3	-3.0	1.0E-3	-(paraquat)
CYP4d21	-15.43	3.5E-3	-9.8	1.0E-3	-(paraquat, phenobarbital)

Adult males were exposed to 18mM caffeine during 12 hours.

^a Data extracted from the following references [42], [32], [55], [56], [57], [52], [34], [58], [59], [60] and from RNAseq experiments referenced in modENCODE treatment expression data in Flybase (<http://www.flybase.org>).

doi:10.1371/journal.pone.0117328.t001

the level of the CYP6D5 protein was strongly decreased in dsCYP6d5/Actin-GAL4 males compared to transgenic control males as demonstrated by western blot (Fig. 2C).

The ubiquitous silencing effects of CYP12d1 and CYP6a8 on metabolism and transcription were also similarly assessed.

Caffeine-exposed dsCYP6a8/Actin-GAL4 males produced much more theobromine, M2 and theophylline compared to transgenic controls (Actin-GAL4/+, dsCYP6a8/+; Fig. 3A). In dsCYP6a8/Actin-GAL4 flies, the mRNA level of CYP6a8 was significantly decreased (-90%), whereas the transcription of CYP12d1 and CYP6d5 was not affected (Fig. 3B).

dsCYP12d1/Actin-GAL4 males fed with caffeine only showed a significant decrease of the M2 metabolite compared to controls (Actin-GAL4/+, dsCYP12d1/+; Fig. 3C). Moreover, if the mRNA level of CYP12d1 was significantly decreased in dsCYP12d1/Actin-GAL4 males (-70%), the expression level of CYP6a8 was strongly increased (+400%), whereas CYP6d5 expression remained affected (Fig. 3D).

Table 2. Expression of major detoxification genes in *Drosophila melanogaster* after caffeine exposure.

Gene name	Up (+) or down (-) regulation	P value	Regulation by other compounds ^(a)
Esterase			
Alpha-Est7	-2.74	2.2E-3	Rotenone, ethanol, cadmium
Est-6	-3.00	3.1E-3	Paraquat, ethanol, heat shock, zinc
Alpha-Est2	-4.06	2.9E-3	-
Glutathione-S-transferase			
GSTD6	4.05	8.4E-3	Rotenone, ethanol
GSTE1	4.04	3.3E-4	Paraquat, ethanol, heat shock, phenobarbital, cadmium
GSTD5	3.08	6.7E-3	Ethanol, cadmium
GstE12	-2.09	1.1E-4	Heat shock, cadmium, ethanol, rotenone, copper, paraquat
GSTD10	-2.40	2.3E-2	Heat shock, cadmium, ethanol, rotenone, atrazine
GSTE9	-2.46	1.8E-3	paraquat
GSTD8	-2.84	6.8E-3	-
GstZ1	-3.22	1.9E-4	copper
GSTE10	-3.74	2.2E-3	-
GstD11	-4.04	2.5E-3	-
UDP-glycosyltransferase			
CG6475	2.58	1.9E-02	-
CG4302	-2.28	5.5E-04	ethanol
CG6850	-2.59	3.6E-05	-
Ugt35b	-2.86	2.5E-05	-
CG17322	-2.87	1.6E-03	cadmium
CG30438	-3.34	2.4E-04	-
Ugt37b1	-3.43	2.3E-05	-
ATP-binding cassette transporter			
CG8908	3.36	1.7 E-3	-
CG4562	-2.04	8.6 E-3	-
CG31792	-2.09	3.5 E-3	-
CG9664	-2.77	3.4 E-3	-
CG33970	-3.05	1.7 E-3	-

Adult males were exposed to 18mM caffeine during 12 hours.

^(a) according to data available from RNAseq experiments referenced in modENCODE treatment expression data in Flybase (<http://www.flybase.org>), [57], [52].

doi:10.1371/journal.pone.0117328.t002

Discussion

While the metabolism of caffeine has been extensively investigated in vertebrates, this is not the case in invertebrates. Medical and pharmacological applications based on human caffeine metabolism are widely available. For example, the measure of caffeine metabolites in urine provides an accurate assessment of an individual's ability to metabolize drugs [43]. This measure is often based on the activity of CYP1A2, one of the main human enzymes involved in caffeine metabolism. In cases of combined ingestion of caffeine with pharmaceutical compounds, the measurement of CYP1A2 activity allows for precise adjustment of the optimal drug amount required for each person [44,45].

Despite the multiple effects induced by caffeine in insects (genome defect, growth, metamorphosis, sleep, pesticide adaptation, gene regulation), CYP-related metabolism of caffeine was previously unknown in invertebrates. We detected (on TLC) theobromine, paraxanthine

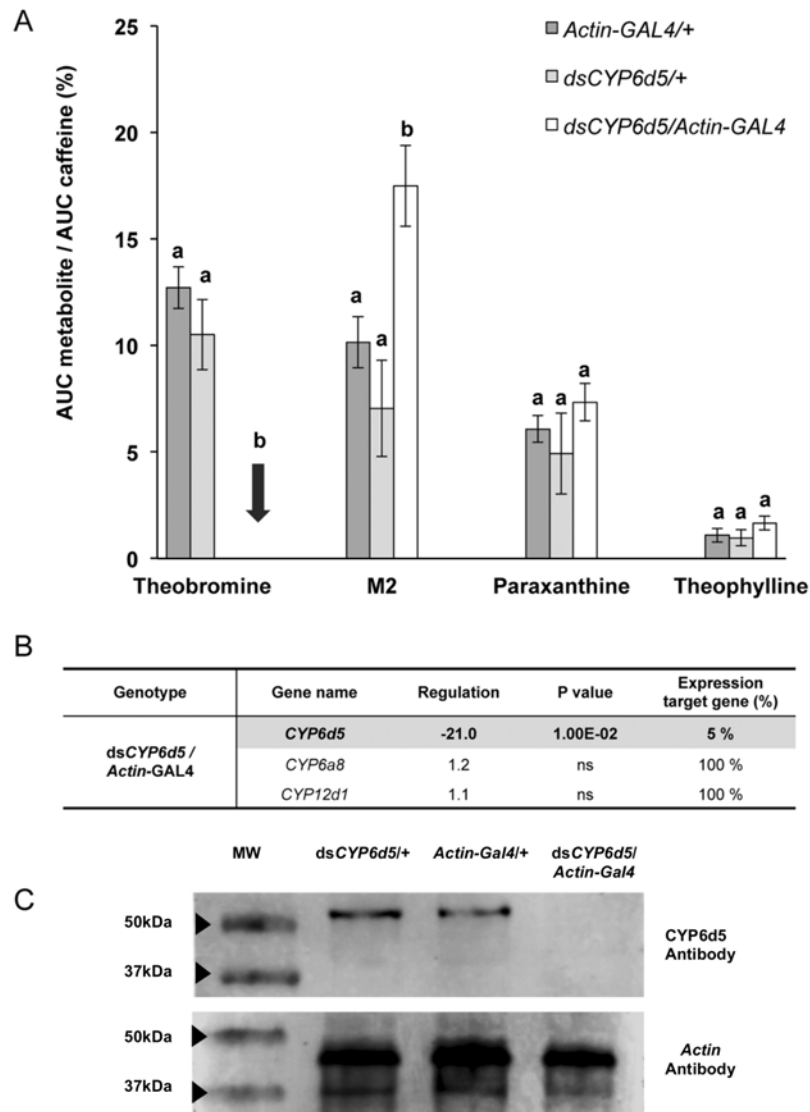


Fig 2. Effect of CYP6d5 knockdown in Drosophila caffeine metabolism. A- The comparison of the normalized quantities of caffeine metabolites reveals a dramatic decrease of theobromine combined with a substantial increase of M2 in CYP6d5 silenced flies (dsCYP6d5/Actin-GAL4) compared to the two transgenic parental controls (Actin-GAL4/+, dsCYP6d5/+). This analysis was performed by measuring the relative intensity of the radiolabelled signal detected in the bodies of male flies of these three genotypes. Bars represent mean values (\pm s.e.m). For each metabolite, the statistical differences are indicated by different letters (ANOVA, n = 4). B- CYP genetic targeting induces specific effect on transcript levels. The comparison of fold change expression between CYP12d1, CYP6a8 and CYP6d5 (measured with RT-qPCR) reveals that only CYP6d5 level was affected in silenced CYP6d5 males (dsCYP6d5/Actin-GAL4) (Statistical analysis by REST, p < 0.01; n = 3). Data are given relatively to normalized expression fold variation compared to controls. C- Comparison of expression level of CYP6D5 protein in experimental and control genotypes by western blotting with a CYP6D5 antibody. The Actin antibody was used to provide a control measurement.

doi:10.1371/journal.pone.0117328.g002

and theophylline, three *Drosophila* caffeine-derived metabolites that are also present in mammals. This indicates that the substances resulting from caffeine metabolism have been partially conserved across evolution. We were not able to relate the 1, 3, 7-trimethyluric acid (TMUA) standard to any of the five unknown metabolites detected. Alternatively, the absence of 1, 3, 7-TMUA in flies can also be due to a slight change of the migratory properties (on TLC) of some

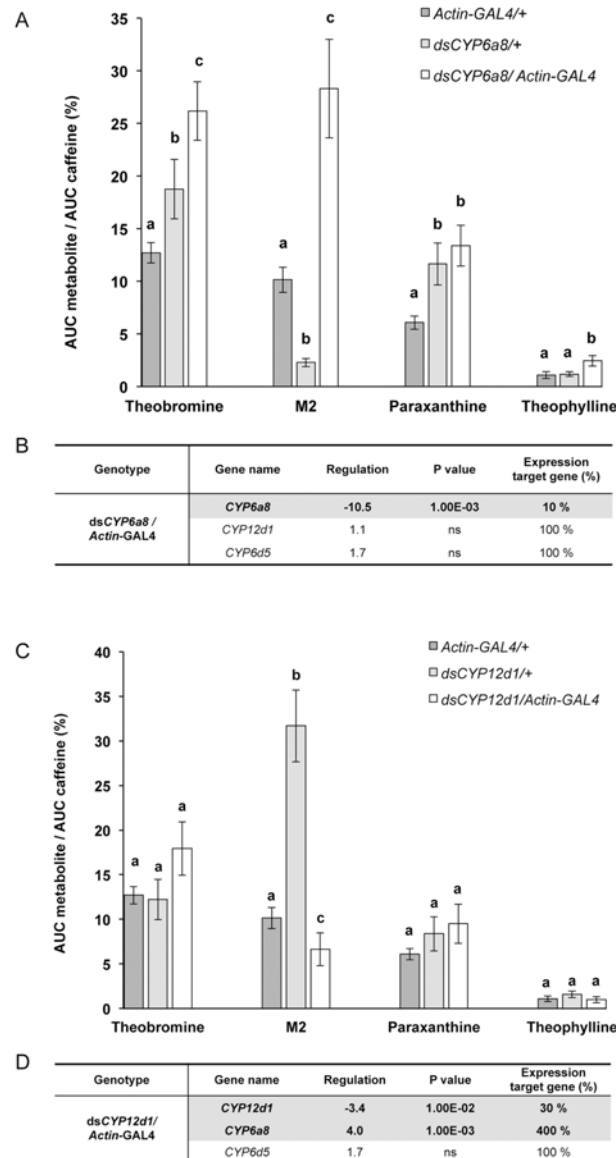


Fig 3. Influence of *CYP6a8* and *CYP12d1* genetic silencing in the metabolism of caffeine and gene expression. A and C: Comparison of normalized quantity of caffeine metabolites in males with respective silencing of *CYP6a8* and *CYP12d1* genes. The relative amount of metabolites was evaluated based on the intensity of the radiolabelled signal detected in the male bodies of experimental genotypes (dsCYP6a8/+ and dsCYP12d1/+ combined with *Actin-GAL4*+) and transgenic controls. Bars represent mean values (\pm s.e.m.). For each metabolite, the statistical differences are indicated by different letters (ANOVA, $n = 3$). B and D: Comparison of fold change expression of *CYP12d1*, *CYP6a8* and *CYP6d5* in dsCYP6a8/*Actin-GAL4* and dsCYP12d1/*Actin-GAL4*, respectively, and their transgenic controls. The quantitative variation of transcript level was measured with RT-qPCR analysis. Data are shown as normalized expression fold variation compared to controls. Highlighted data indicate statistical differences ($p < 0.01$; $n = 4$). For detailed methods and statistics, please refer to legend of Fig. 2.

doi:10.1371/journal.pone.0117328.g003

caffeine metabolites induced by radiolabelling [46]. This “human vs *Drosophila*” variation for caffeine metabolites is not surprising, given the known inter-mammal differences. The origin of species-specific differences may be related to the main oxidation pathways that diverges between human (3-N demethylation), monkeys (7-N demethylation) and rat, mouse and rabbit

(C-8 hydroxylation; [26]). Our data suggest that 1-N demethylation is the main *Drosophila* pathway leading to the production of 42% theobromine, whereas the secondary “3-N and 7-N demethylation” pathways lead to 20% paraxanthine and 4% theophylline, respectively.

Our pharmacological and genetic manipulation of *Drosophila* caffeine metabolism strongly supports the involvement of CYPs in this process. First, caffeine metabolism was drastically reduced in flies treated with metyrapone, a potent pharmacological inhibitor of CYP activity known to affect insect CYP in relation to hormone synthesis [47] and pheromone catabolism [35,48].

Second, genetic down-regulation of the expression of three CYP candidates revealed (i) CYP-specific effect on metabolism and (ii) CYP-CYP interactive regulation.

More precisely, genetic silencing of *CYP6d5* pinpoints its key-role in the synthesis of theobromine, but not in the two other identified metabolites. In contrast, *CYP6a8* or *CYP12d1* silencing induced a reciprocal effect on the level of theobromine that increased along with other metabolites. This indicates that *CYP6d5* and the two latter enzymes act in distinct steps of the caffeine metabolic pathway. Based on these results, we propose a hypothetical caffeine metabolism pathway in *Drosophila* (Fig. 4). Currently, we do not know whether another CYP acts with *CYP6d5*, as in humans with CYP1A2 [25]. Our genetic manipulation of the three CYPs indicates a possible regulatory interaction between these genes. If the manipulation of either *CYP6d5* or *CYP6a8* did not affect the expression of the two other tested enzymes, that of *CYP12d1* induced a 4-fold transcriptional increase in of *CYP6a8*, suggesting a potential mechanism of transcriptional compensation between these two CYPs (and possibly with other CYPs not studied here). The non-optimal efficiency of ds*CYP12d1* and the transcriptional compensation between *CYP12d1* and *CYP6a8* do not allow us to conclude that *CYP12d1* is directly implicated in the metabolic transformation of caffeine into M2 metabolite.

The regulation of genes expression by xenobiotic treatments or exposure is a universal feature of animals. Toxicity can result from genetic deregulation, either induced by the down-regulation of detoxification enzymes and/or by the up-regulation of enzymes involved in the bioactivation of xenobiotic compounds. Our transcriptomic screen allowed us to detect a large set of proteins potentially involved in caffeine catabolism. This includes phase I enzymes (CYPs), which often exhibited the highest response to the treatment, as well as phase II (GST and UGT) and phase III (ABC transporter) proteins. Because these proteins control xenobiotic detoxification, regulatory mechanisms may strongly enhance the kinetics of xenobiotic catabolism. Moreover, *Drosophila* xenobiotic response can be regulated by CncC (Cap n' collar isoform C), a transcription factor ortholog to Nrf2 (NF-E2-related factor 2), which is activated by coffee [49] in humans [34]. Nine of the eleven CYP genes induced by caffeine in our study (*CYP12d1-p* and *-d*, *CYP6a8*, *CYP6d5*, *CYP4p1*, *CYP28a5*, *CYP12a5*, *CYP6a20*, *CYP6w1*) were also up-regulated in transgenic flies over-expressing CncC [34]. Similarly, *CYP6a18*, *CYP313a1* and *CYP4d21* were down-regulated in CncC transgenic caffeine-fed flies. Furthermore, caffeine-induced effects depend on one binding site of CncC located in the promoter region of *CYP6a8* [34]. This strongly suggests that CncC could be a major player in the coordinated response to caffeine.

In addition to the fundamental relevance of our study, the response of CYPs to environmental stress may also represent a reliable marker with regard to the acquisition of resistance in insects repeatedly exposed to xenobiotics or to toxic compounds. One way to assess insect adaptation (and resistance) to caffeine and to other environmentally stressful molecules may be provided by the measure of theobromine amount (or other metabolites) after exposure to these toxic substances. Additionally, as previously successfully demonstrated with other CYPs, *CYP6d5* RNAi silencing could be used to screen for xenobiotic resistance [31]. Furthermore, given that CYPs are also involved in drug metabolism of vertebrates, the measurement of

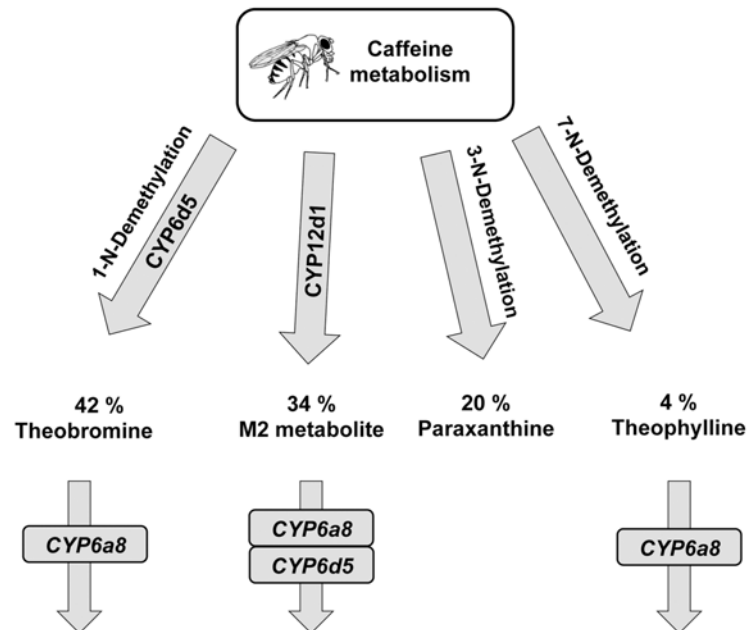


Fig 4. Hypothetic model for *CYP6d5*, *CYP6a8* and *CYP12d1* involvement in caffeine metabolism pathway in *Drosophila melanogaster*. In male adult fly bodies, caffeine is degraded into 4 major metabolites: theobromine, M2, paraxanthine and theophylline. *CYP6d5* and *CYP12d1* seem to metabolize caffeine in theobromine and M2 metabolite, respectively. The metabolism of theobromine and theophylline into unknown secondary metabolites may be controlled by *CYP6a8* whereas the M2 degradation could involve cooperation between *CYP6a8* and *CYP6d5*.

doi:10.1371/journal.pone.0117328.g004

theobromine in *Drosophila* could offer a suitable tool for therapeutic drug discovery [50] and provide a simple but robust marker of *CYP6D5* activity. For example, *CYP6d5* expression is up-regulated by the drug phenobarbital (barbituric compound), but the involvement of this enzyme in drug metabolism is not completely understood [51,52]. In parallel to drug response, CYP activity could explain the variation in response of *Drosophila* between mutants or natural populations, hence the potential adaptive response of their metabolism after caffeine exposure.

We have not studied the potential effect of metabolites—in particular of theobromine—in *Drosophila* because this metabolite was shown to induce a weaker effect than caffeine on mortality, female fecundity, and male mating preference [54]. Similar to humans, caffeine and theobromine were shown to differentially affect mood, psychomotor performance and blood pressure [53].

In summary, using *Drosophila*, we (i) discovered several products derived from caffeine metabolism, (ii) partly unveiled the potential genetic network underlying this process, (iii) showed similarities between insects and mammals and (iv) demonstrated a high specificity of *CYP6D5* with regard to caffeine degradation. With the current possibility to link the metabolic transformation of a natural compound with the transcriptomic identity of each animal, our study provides a useful base to design accurate bioassays for the evaluation of metabolic ability among individuals or between populations.

Acknowledgments

We thank Jean-Philippe Charles for pertinent comments and useful advice, Marina Dobreva for proofreading and Serge Loquin and José Solonot for their technical help.

Author Contributions

Conceived and designed the experiments: AC SF JFF JMH. Performed the experiments: AC SF. Analyzed the data: AC SF JFF JMH PF GLG. Wrote the paper: AC SF YA GLG JFF JMH.

References

1. Ashihara H, Suzuki T (2004) Distribution and biosynthesis of caffeine in plants. *Front Biosci* 9: 1864–1876. PMID: [14977593](#)
2. Nathanson JA (1984) Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science* 226: 184–187. PMID: [6207592](#)
3. Mathavan S, Premalatha Y, Christopher MS (1985) Effects of caffeine and theophylline on the fecundity of four lepidopteran species. *Exp Biol* 44: 133–138. PMID: [3850026](#)
4. Hollingsworth RG, Armstrong JW, Campbell E (2002) Caffeine as a repellent for slugs and snails. *Nature* 417: 915–916. PMID: [12087394](#)
5. Chou TM, Benowitz NL (1994) Caffeine and coffee: effects on health and cardiovascular disease. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 109: 173–189. PMID: [7881818](#)
6. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G (2000) Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 287: 1834–1837. PMID: [10710313](#)
7. Wu MN, Ho K, Crocker A, Yue Z, Koh K, et al. (2009) The effects of caffeine on sleep in *Drosophila* require PKA activity, but not the adenosine receptor. *J Neurosci* 29: 11029–11037. doi: [10.1523/JNEUROSCI.1653-09.2009](#) PMID: [19726661](#)
8. Thimgan MS, Duntley SP, Shaw PJ (2011) Changes in gene expression with sleep. *J Clin Sleep Med* 7: S26–27. doi: [10.5664/JCSM.1352](#) PMID: [22003326](#)
9. Zornik E, Paisley K, Nichols R (1999) Neural transmitters and a peptide modulate *Drosophila* heart rate. *Peptides* 20: 45–51. PMID: [10098623](#)
10. Kuhlmann W, Fromme H-G, Heege E-M, Ostertag W (1968) The mutagenic action of caffeine in higher organisms. *Cancer Res* 28: 2375–2389. PMID: [4881507](#)
11. Itoyama MM, Bicudo HEMdC, Cordeiro JA (1997) Effects of caffeine on mitotic index in *Drosophila prosaltans* (Diptera). *Brazilian Journal of Genetics* 20.
12. Si A, Zhang SW, Maleszka R (2005) Effects of caffeine on olfactory and visual learning in the honey bee (*Apis mellifera*). *Pharmacol Biochem Behav* 82: 664–672. PMID: [16375953](#)
13. Laranja AT, Manzato AJ, Bicudo HEMdC (2006) Caffeine effect on mortality and oviposition in successive generations of *Aedes aegypti*. *Revista de Saúde Pública* 40: 1112–1117. PMID: [23559050](#)
14. Itoyama MM, de Campos Bicudo HEM (1992) Effects of caffeine on fecundity, egg laying capacity, development time and longevity in *Drosophila prosaltans*. *Brazilian Journal of Genetics* 15: 303–321.
15. Alshuaib WB, Mathew MV (2006) Caffeine modulates potassium currents in *Drosophila* neurons. *Int J Dev Neurosci* 24: 249–253. PMID: [16621420](#)
16. Nakayama S, Sasaki K, Matsumura K, Lewis Z, Miyatake T (2012) Dopaminergic system as the mechanism underlying personality in a beetle. *J Insect Physiol* 58: 750–755. doi: [10.1016/j.jinsphys.2012.02.011](#) PMID: [22414537](#)
17. Kucharski R, Maleszka R (2005) Microarray and real-time PCR analyses of gene expression in the honeybee brain following caffeine treatment. *J Mol Neurosci* 27: 269–276. PMID: [16280596](#)
18. Ebbs ML, Amrein H (2007) Taste and pheromone perception in the fruit fly *Drosophila melanogaster*. *Pflugers Arch* 454: 735–747. PMID: [17473934](#)
19. Moon SJ, Lee Y, Jiao Y, Montell C (2009) A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr Biol* 19: 1623–1627. doi: [10.1016/j.cub.2009.07.061](#) PMID: [19765987](#)
20. Lacaille F, Hiroi M, Twele R, Inoshita T, Umemoto D, et al. (2007) An inhibitory sex pheromone tastes bitter for *Drosophila* males. *PLoS One* 2: e661. PMID: [17710124](#)
21. Lee Y, Moon SJ, Montell C (2009) Multiple gustatory receptors required for the caffeine response in *Drosophila*. *Proc Natl Acad Sci U S A* 106: 4495–4500. doi: [10.1073/pnas.0811744106](#) PMID: [19246397](#)
22. Franco R, Onatibia-Astibia A, Martinez-Pinilla E (2013) Health benefits of methylxanthines in cacao and chocolate. *Nutrients* 5: 4159–4173. doi: [10.3390/nu5104159](#) PMID: [24145871](#)
23. Hetzler RK, Knowlton RG, Somani SM, Brown DD, Perkins RM 3rd (1990) Effect of paraxanthine on FFA mobilization after intravenous caffeine administration in humans. *J Appl Physiol* (1985) 68: 44–47.

24. Welsh EJ, Bara A, Barley E, Cates CJ (2010) Caffeine for asthma. *Cochrane Database Syst Rev*: CD001112. doi: [10.1002/14651858.CD001112.pub2](https://doi.org/10.1002/14651858.CD001112.pub2) PMID: [20091514](https://pubmed.ncbi.nlm.nih.gov/20091514/)
25. Kot M, Daniel WA (2008) The relative contribution of human cytochrome P450 isoforms to the four caffeine oxidation pathways: an in vitro comparative study with cDNA-expressed P450s including CYP2C isoforms. *Biochem Pharmacol* 76: 543–551. doi: [10.1016/j.bcp.2008.05.025](https://doi.org/10.1016/j.bcp.2008.05.025) PMID: [18619574](https://pubmed.ncbi.nlm.nih.gov/18619574/)
26. Berthou F, Guillois B, Riche C, Dreano Y, Jacqz-Aigrain E, et al. (1992) Interspecies variations in caffeine metabolism related to cytochrome P4501A enzymes. *Xenobiotica* 22: 671–680. PMID: [1441590](https://pubmed.ncbi.nlm.nih.gov/1441590/)
27. Bonati M, Latini R, Tognoni G, Young JF, Garattini S (1984) Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. *Drug Metab Rev* 15: 1355–1383. PMID: [6543526](https://pubmed.ncbi.nlm.nih.gov/6543526/)
28. Kot M, Daniel WA (2008) Relative contribution of rat cytochrome P450 isoforms to the metabolism of caffeine: the pathway and concentration dependence. *Biochem Pharmacol* 75: 1538–1549. doi: [10.1016/j.bcp.2007.12.017](https://doi.org/10.1016/j.bcp.2007.12.017) PMID: [18279840](https://pubmed.ncbi.nlm.nih.gov/18279840/)
29. Willoughby L, Chung H, Lumb C, Robin C, Batterham P, et al. (2006) A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and phenobarbital. *Insect Biochem Mol Biol* 36: 934–942. PMID: [17098168](https://pubmed.ncbi.nlm.nih.gov/17098168/)
30. Mahapatra CT, Bond J, Rand DM, Rand MD (2010) Identification of methylmercury tolerance gene candidates in *Drosophila*. *Toxicological sciences* 116: 225–238. doi: [10.1093/toxsci/ktq097](https://doi.org/10.1093/toxsci/ktq097) PMID: [20375079](https://pubmed.ncbi.nlm.nih.gov/20375079/)
31. Shah S, Yarrow C, Dunning R, Cheek B, Vass S, et al. (2012) Insecticide detoxification indicator strains as tools for enhancing chemical discovery screens. *Pest Manag Sci* 68: 38–48. doi: [10.1002/ps.2218](https://doi.org/10.1002/ps.2218) PMID: [21681918](https://pubmed.ncbi.nlm.nih.gov/21681918/)
32. Giraudo M, Unnithan GC, Goff GL, Feyereisen R (2010) Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights. *Pestic Biochem Physiol* 97: 115–122. PMID: [20582327](https://pubmed.ncbi.nlm.nih.gov/20582327/)
33. Morra R, Kuruganti S, Lam V, Lucchesi JC, Ganguly R (2010) Functional analysis of the cis-acting elements responsible for the induction of the *Cyp6a8* and *Cyp6g1* genes of *Drosophila melanogaster* by DDT, phenobarbital and caffeine. *Insect Mol Biol* 19: 121–130. doi: [10.1111/j.1365-2583.2009.00979.x](https://doi.org/10.1111/j.1365-2583.2009.00979.x) PMID: [20167023](https://pubmed.ncbi.nlm.nih.gov/20167023/)
34. Misra JR, Horner MA, Lam G, Thummel CS (2011) Transcriptional regulation of xenobiotic detoxification in *Drosophila*. *Genes Dev* 25: 1796–1806. doi: [10.1101/gad.172809.11](https://doi.org/10.1101/gad.172809.11) PMID: [21896655](https://pubmed.ncbi.nlm.nih.gov/21896655/)
35. Maibeche-Coisne M, Nikonov AA, Ishida Y, Jacquin-Joly E, Leal WS (2004) Pheromone anosmia in a scarab beetle induced by in vivo inhibition of a pheromone-degrading enzyme. *Proc Natl Acad Sci U S A* 101: 11459–11464. PMID: [15277687](https://pubmed.ncbi.nlm.nih.gov/15277687/)
36. Pottier MA, Bozzolan F, Cheretemps T, Jacquin-Joly E, Lalouette L, et al. (2012) Cytochrome P450s and cytochrome P450 reductase in the olfactory organ of the cotton leafworm *Spodoptera littoralis*. *Insect Mol Biol* 21: 568–580. doi: [10.1111/j.1365-2583.2012.01160.x](https://doi.org/10.1111/j.1365-2583.2012.01160.x) PMID: [22984814](https://pubmed.ncbi.nlm.nih.gov/22984814/)
37. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55: 611–622. doi: [10.1373/clinchem.2008.112797](https://doi.org/10.1373/clinchem.2008.112797) PMID: [19246619](https://pubmed.ncbi.nlm.nih.gov/19246619/)
38. Sellier MJ, Reeb P, Marion-Poll F (2011) Consumption of bitter alkaloids in *Drosophila melanogaster* in multiple-choice test conditions. *Chem Senses* 36: 323–334. doi: [10.1093/chemse/bjq133](https://doi.org/10.1093/chemse/bjq133) PMID: [21173029](https://pubmed.ncbi.nlm.nih.gov/21173029/)
39. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30: e36. PMID: [11972351](https://pubmed.ncbi.nlm.nih.gov/11972351/)
40. Kulkarni AP, Motoyama N, Dauterman WC, Hodgson E (1978) Inhibition of housefly glutathione S-transferase by catecholamines and quinones. *Bull Environ Contam Toxicol* 20: 227–232. PMID: [698432](https://pubmed.ncbi.nlm.nih.gov/698432/)
41. Visetson S (1991) Insecticide Resistance Mechanism in the Red Rust Flour Beetle (*Tribolium castaneum* Burst). PhD Thesis The University of Sydney, Australia.
42. Girardot F, Monnier V, Tricoire H (2004) Genome wide analysis of common and specific stress responses in adult *drosophila melanogaster*. *BMC Genomics* 5: 74. PMID: [15458575](https://pubmed.ncbi.nlm.nih.gov/15458575/)
43. Raj MA, John SA (2013) Simultaneous determination of uric acid, xanthine, hypoxanthine and caffeine in human blood serum and urine samples using electrochemically reduced graphene oxide modified electrode. *Anal Chim Acta* 771: 14–20. doi: [10.1016/j.aca.2013.02.017](https://doi.org/10.1016/j.aca.2013.02.017) PMID: [23522107](https://pubmed.ncbi.nlm.nih.gov/23522107/)
44. Tang BK, Zhou Y, Kadar D, Kalow W (1994) Caffeine as a probe for CYP1A2 activity: potential influence of renal factors on urinary phenotypic trait measurements. *Pharmacogenetics* 4: 117–124. PMID: [7920691](https://pubmed.ncbi.nlm.nih.gov/7920691/)

45. Carrillo JA, Christensen M, Ramos SI, Alm C, Dahl ML, et al. (2000) Evaluation of caffeine as an in vivo probe for CYP1A2 using measurements in plasma, saliva, and urine. *Ther Drug Monit* 22: 409–417. PMID: [10942180](#)
46. Benchekroun Y, Dautraix S, Desage M, Brazier JL (1997) Isotopic effects on retention times of caffeine and its metabolites 1, 3, 7-trimethyluric acid, theophylline, theobromine and paraxanthine. *J Chromatogr B Biomed Sci Appl* 688: 245–254. PMID: [9061462](#)
47. Bannenberg G, Martin HJ, Belai I, Maser E (2003) 11beta-Hydroxysteroid dehydrogenase type 1: tissue-specific expression and reductive metabolism of some anti-insect agent azole analogues of metyrapone. *Chem Biol Interact* 143–144: 449–457. PMID: [12604248](#)
48. Wojtasek H, Leal WS (1999) Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllorhiza diversus* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450. *FEBS Lett* 458: 333–336. PMID: [10570935](#)
49. Trinh K, Andrews L, Krause J, Hanak T, Lee D, et al. (2010) Decaffeinated coffee and nicotine-free tobacco provide neuroprotection in *Drosophila* models of Parkinson's disease through an NRF2-dependent mechanism. *J Neurosci* 30: 5525–5532. doi: [10.1523/JNEUROSCI.4777-09.2010](#) PMID: [20410106](#)
50. Pandey UB, Nichols CD (2011) Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Pharmacol Rev* 63: 411–436. doi: [10.1124/pr.110.003293](#) PMID: [21415126](#)
51. Sun W, Margam VM, Sun L, Buczkowski G, Bennett GW, et al. (2006) Genome-wide analysis of phenobarbital-inducible genes in *Drosophila melanogaster*. *Insect Mol Biol* 15: 455–464. PMID: [16907832](#)
52. Goff GL, Hilliou F, Siegfried BD, Boundy S, Wajnberg E, et al. (2006) Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. *Insect Biochem Mol Biol* 36: 674–682. PMID: [16876710](#)
53. Mitchell ES, Slettenhaar M, vd Meer N, Transler C, Jans L, et al. (2011) Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure. *Physiol Behav* 104: 816–822. doi: [10.1016/j.physbeh.2011.07.027](#) PMID: [21839757](#)
54. Matsagas K, Lim DB, Horwitz M, Rizza CL, Mueller LD, et al. (2009) Long-term functional side-effects of stimulants and sedatives in *Drosophila melanogaster*. *PLoS One* 4: e6578. doi: [10.1371/journal.pone.0006578](#) PMID: [19668379](#)
55. Jensen HR, Scott IM, Sims S, Trudeau VL, Amason JT (2006) Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper nigrum*. *J Agric Food Chem* 54: 1289–1295. PMID: [16478250](#)
56. Jensen HR, Scott IM, Sims SR, Trudeau VL, Amason JT (2006) The effect of a synergistic concentration of a *Piper nigrum* extract used in conjunction with pyrethrum upon gene expression in *Drosophila melanogaster*. *Insect Mol Biol* 15: 329–339. PMID: [16756552](#)
57. King-Jones K, Horner MA, Lam G, Thummel CS (2006) The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. *Cell Metab* 4: 37–48. PMID: [16814731](#)
58. Morozova TV, Anholt RR, Mackay TF (2006) Transcriptional response to alcohol exposure in *Drosophila melanogaster*. *Genome Biol* 7: R95. PMID: [17054780](#)
59. Sharma A, Mishra M, Ram KR, Kumar R, Abdin MZ, et al. (2011) Transcriptome analysis provides insights for understanding the adverse effects of endosulfan in *Drosophila melanogaster*. *Chemosphere* 82: 370–376. doi: [10.1016/j.chemosphere.2010.10.002](#) PMID: [21036383](#)
60. Willoughby L, Batterham P, Daborn PJ (2007) Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Manag Sci* 63: 803–808. PMID: [17514638](#)