Virginia Journal of Science
Volume 42, Number 3
Fall 1991

Utilization of Response Surface Modeling to Evaluate the Interaction between Aflatoxin B_1 and Caffeine on Egg-Adult Viability in *Drosophila melanogaster.*

David A. Bettinger and **Joseph P. Chinnici**
Department of Biology, Box 2012
Virginia Commonwealth University
Richmond, VA 23284-2012

ABSTRACT

Utilizing five different concentrations of aflatoxin B_1 (AFB_1) and caffeine, the effect of continuous larval exposure to AFB_1 and/or caffeine at all possible concentration combinations on egg-adult viability was determined for several wildtype strains of *Drosophila melanogaster.* The data were analyzed by ANOVA and Response Surface Methodology (RSM) using a beta (ß) binomial model. RSM analyses were used to generate 3-dimensional plots visually predicting the interactions of the tested compounds at concentration combinations not actually tested. Analyses indicate that slightly to moderately toxic levels of caffeine dramatically reduce the toxic effect of AFB_1, and that the compounds interact synergistically to reduce lethality.

INTRODUCTION

Aflatoxins are oxygenated, heterocyclic secondary metabolites of some species of the fungus *Aspergillus*, often contaminating agricultural products such as peanuts, corn, cottonseed, tree nuts, and other crops not only in the field but also during harvesting, in storage, and during processing (Wood, 1989). Aflatoxins, particularly aflatoxin B_1 (AFB_1) are powerful mutagens and teratogens, as well as vertebrate hepatocarcinogens and hepatotoxins (Ong 1975, Wogan and Busby 1980). Human health effects apparently include liver cancer, though the association between aflatoxin in the diet and liver cancer is confounded by the incidence of hepatitis B virus (Yeh et al. 1989, Stoloff 1989). Aflatoxins are also toxic to invertebrates, including *Drosophila* (Kirk, et.al. 1971), with larval growth in media contaminated with AFB_1 leading to decreased viability, smaller pupal case and adult body lengths, and increased developmental times (Lalor, et.al. 1976).

Caffeine (1,3,7-trimethylxanthine), a natural metabolite of *Coffea* and other plant species, is neurogenic, mutagenic, and toxic to a number of species under certain conditions (Kihlman 1977, Tanzarella et.al. 1984). However, under other conditions, caffeine has the ability to protect cells and organisms to some extent from the adverse effects of other toxigenic agents (Kakunaga 1975, Nomura 1980, Nigsch et.al. 1977), including aflatoxin (Cramer and Painter 1981, Chinnici and Bettinger 1984.)

This study was undertaken to further characterize the interaction between AFB_1 and caffeine affecting egg-adult viability in *Drosophila melanogaster* by applying an analytical technique called Response Surface Methodology (RSM) to the viability data.

RSM is a multivariate method widely used in engineering, agronomy, and product development (Mead and Pike 1975); recently, the utility of RSM in toxicological studies has been demonstrated (Carter et.al. 1985). RSM involves development of mathematical models and equations that relate the biological response to the concentrations of the agents used. The models/equations indicate the relative importance of each agent in producing the biological response and permit the interpretation of the type (inhibitory, additive, or synergistic) and relative strengths of each agent involved in the interaction. In addition, RSM can predict the response that would occur if combinations of concentrations different from those investigated experimentally were used. In this study, RSM was used to determine the effects of AFB_1 and caffeine applied singly and in combination on egg-adult viability in *Drosophila*.

METHODS AND MATERIALS

Insects. Three wildtype strains of *D. melanogaster* were used: Lausanne-S (A-11), a standard laboratory strain obtained from the Mid-America *Drosophila* Center, Bowling Green OH, and strains 3B and 34, originally captured from Virginia localities in 1980 (Delawder and Chinnici 1983) and maintained by mass culture.

Chemicals and culture medium. The culture medium consisted of yeast, dextrose, agar and several inorganic salts, with tegosept added as a mold inhibitor. Control (no AFB_1 or caffeine) and stock solutions containing either 0.00, 0.16, 0.32, 0.48, or 0.64 X 10^{-5} M AFB_1 (Grade A, Calbiochem-Behring, LaJolla CA) alone, 0.0, 0.5, 1.0, 1.5, or 2.0 X 10^{-2}M caffeine alone, and mixture of all pairwise combinations of AFB_1 and caffeine were produced. These were poured into a series of 8-dram shell vials (8 ml medium per vial), stoppered with foam plugs and refrigerated until used. All experiments were performed at 25 ± 1°C.

Experimental procedures. Flies from the three strains were allowed to lay eggs for 12 hr in half pint culture bottles containing control medium. The eggs were then collected, and groups of 25 were placed on small squares of moistened blotting paper. Each shell vial containing medium received one group of 25 eggs, and each of the 25 treatments was replicated six times. As the cultures developed, data were collected daily on egg-pupal and egg-adult viabilities and development times and adult body lengths (tip of head to tip of abdomen). Only the egg-adult viability data are reported here.

RSM analysis. The data were analyzed by Drs. Hans Carter and Vernon Chinchilli of the Department of Biostatistics at the Medical College of Virginia of Virginia Commonwealth University to determine a mathematical model that best approximates the observed relationships between levels of treatments and effects on viability. A beta (ß) binomial model with the following equation provided the closest approximation to the observed results:

$$\text{Expected } (Y) = 1/[\exp\{-\beta_0 - \beta_1 X_1 - \beta_2 X_2 - \beta_{11}(X_1)^2 - \beta_{22}(X_2)^2 - \beta_{12}(X_1X_2)\}]$$

where

Y is the dependent variable: proportion of eggs producing larvae that develop into adults

X_1 is one independent variable: concentration of AFB_1

X_2 is the other independent variable: concentration of caffeine

β_0 is the population parameter denoting the Y-intercept (i.e., percentage of egg-adult viability in the control treatment)
β_1 is the population parameter denoting the slope of the linear response curve associated with AFB_1 concentration
β_2 is the population parameter denoting the slope of the linear response curve associated with caffeine concentration
β_{11} is the population parameter describing curvature in the dose-response relationship associated with AFB_1 concentration
β_{22} is the population parameter describing curvature in the dose-response relationship associated with caffeine concentration
β_{12} is the population parameter describing the interaction between the independent variables (AFB_1 and caffeine)

The ß coefficients are determined by the method of maximum likelihood (Williams 1975). Once the ß coefficients are determined from a set of actual data, any values for X_1 and X_2 (within the ranges of concentrations used in the experiment) can be used to calculate expected Y values (egg-adult viabilities). The full array of Y values may then be plotted as a three-dimensional "response curve".

RESULTS

For strains A-11, 3B, and 34, respectively, Figures 1, 2, and 3 graphically represent the egg-adult viability data for the 25 treatments, replicated six times each, of adding various concentrations of AFB_1 and/or caffeine to the culture medium in which larvae were grown. Inspection of the graphs indicates the following general trends:

(1) Viability decreases with increasing concentration of AFB_1 alone in the medium (compare the solid bars in each graph);
(2) Viability decreases with increasing concentration of caffeine alone in the culture medium (compare the first set of bars in each graph);
(3) Caffeine, at moderate concentrations ($0.5\text{-}1.5 \times 10^{-2}$ M), lessens the toxic effect of AFB_1 on viability (compare viabilities at various concentrations of caffeine for 0.00 and 0.48×10^{-5} M AFB_1 for strain A-11, or for 0.00 and 0.32×10^{-5} M AFB_1 for strain 34);
(4) To some extent, AFB_1 lessens the toxic effect of caffeine on viability (for strain A-11, compare viabilities at 1.0 or 1.5×10^{-2} M caffeine at increasing concentrations of AFB_1).

The best fit ß coefficients for the ß binomial formulas were calculated from the raw data for each strain of *Drosophila*. The RSM ß coefficients and statistics are presented in Tables 1, 2, and 3. For strain A-11 for instance, the ß binomial formula is: Expected (Y) $= 1 / [\exp \{- (0.407) - (-3.217)X_1 - (0.674)X_2 - (14.288)(X_1)^2 - (-2.787)(X_2)^2 - (7.839)(X_1X_2)\}]$. Substituting all values for X_1 and X_2 within the actual concentration ranges used in the experiments, response surfaces were generated for each strain predicting the probabilities of survival (Y) for each combination of AFB_1 and caffeine concentrations. These response surfaces for strains A-11, 3B, and 34, respectively, are illustrated in Figures 4, 5, and 6.

DISCUSSION

The data from this study, showing that AFB_1 and caffeine each have increasingly deleterious effects on egg-adult viability as concentrations are increased, and that

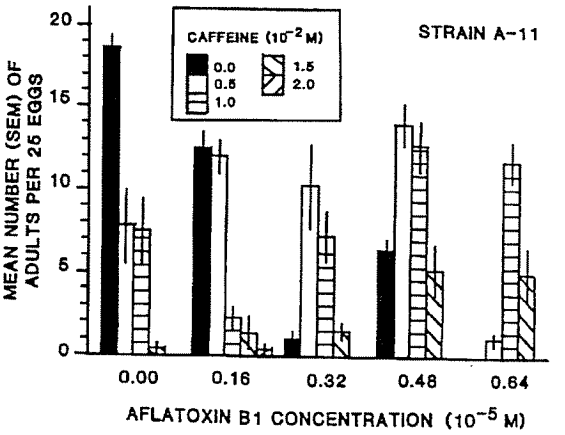

FIGURE 1. For strain A-11, numbers of adults (± standard error of the mean for six replications) that developed in vials initially containing 25 eggs and various concentrations of aflatoxin B1 and/or caffeine in the culture medium.

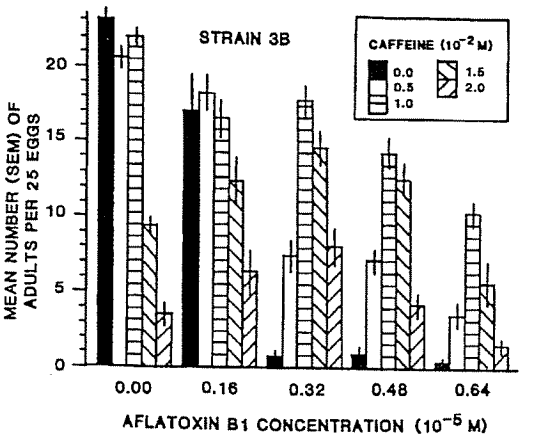

FIGURE 2. For strain 3B, numbers of adults (± standard error of the mean for six replications) that developed in vials initially containing 25 eggs and various concentrations of aflatoxin B1 and/or caffeine in the culture medium.

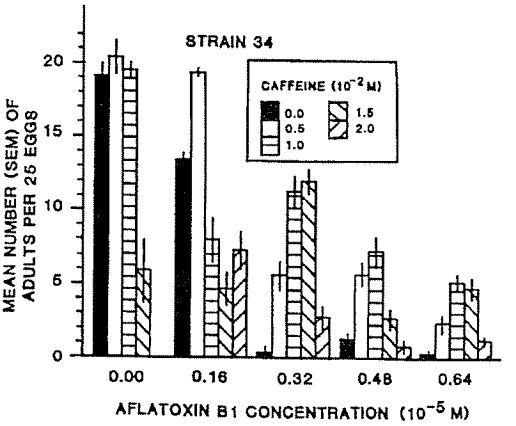

FIGURE 3. For strain 34, numbers of adults (± standard error of the mean for six replications) that developed in vials initially containing 25 eggs and various concentrations of aflatoxin B1 and/or caffeine in the culture medium.

TABLE 1. Beta coefficients and standard errors, as determined by least squares analysis, that best define the beta binomial formula for strain A-11. Results of chi square analyses, testing each beta coefficient against the null hypothesis that that the beta value is zero.

VARIABLE	BETA COEFFICIENT	STANDARD ERROR	CHI-SQUARE	P
Intercept	0.407	0.123	10.89	0.001
X_1	-3.217	0.702	20.99	0.0001
X_2	0.674	0.250	7.26	0.007
$(X_1)^2$	-4.288	1.130	14.39	0.0001
$(X_2)^2$	-2.787	0.184	229.04	<0.0001
X_1X_2	7.739	0.539	211.09	<0.0001

TABLE 2. Beta coefficients and standard errors, as determined by least squares analysis, that best define the beta binomial formula for strain 3-B. Results of chi square analyses, testing each beta coefficient against the null hypothesis that that the beta value is zero.

VARIABLE	BETA COEFFICIENT	STANDARD ERROR	CHI-SQUARE	P
Intercept	1.330	0.135	96.54	<0.0001
X_1	-7.155	0.673	113.05	<0.0001
X_2	2.039	0.209	95.23	<0.0001
$(X_1)^2$	-3.154	0.963	10.74	0.001
$(X_2)^2$	-1.871	0.103	327.54	<0.0001
X_1X_2	5.145	0.323	254.28	<0.0001

TABLE 3. Beta coefficients and standard errors, as determined by least squares analysis, that best define the beta binomial formula for strain 34. Results of chi square analyses, testing each beta coefficient against the null hypothesis that that the beta value is zero.

VARIABLE	BETA COEFFICIENT	STANDARD ERROR	CHI-SQUARE	P
Intercept	1.127	0.131	73.44	<0.0001
X_1	-8.182	0.697	137.86	<0.0001
X_2	1.263	0.222	32.30	<0.0001
$(X_1)^2$	-0.943	1.060	0.79	0.373
$(X_2)^2$	-1.619	0.117	192.02	<0.0001
X_1X_2	4.873	0.370	173.51	<0.0001

the toxic effect is somewhat ameliorated when both agents are jointly administered collaborates previous work (Chinnici and Bettinger 1984). ANOVA of the previous data indicated that caffeine at moderate concentrations significantly reduced the toxic effects of AFB_1 on viability and developmental parameters. RSM analysis in this study has allowed the nature of the interaction to be more precisely defined.

The response surfaces depicted in Figures 4, 5, and 6 may be described by using an analogy. The three dimensions (X_1, X_2, and Y) describe a box. The vertical "walls" (Y axis) indicate the probability of survival. The "floor" has length (X_1 axis) and depth (X_2 axis). A sheet of paper, placed within the box on the floor, may be raised to various heights along the Y axis to indicate degree of viability associated with particular combinations of test agents. The front and left edges of the paper indicate viability levels due to AFB_1 alone (front edge) or due to caffeine alone (left side edge).

The ß binomial equation provides a reasonably good approximation of the interactions of AFB_1 and caffeine as they affect egg-adult viability in *Drosophila melanogaster*. The ß coefficients themselves provide information about the nature of the actions and interactions of the toxins. A positive ß term for the cross-products (X_1X_2) variable indicates a synergistic (greater than additive) interaction between the test agents while a negative ß term for the (X_1X_2) variable indicates a inhibitory interaction between the agents. For all strains studied, B_{12} is positive, indicating a synergistic interaction between AFB_1 and caffeine on viability (flies survive better when both are present than when either one alone is present).

The ß coefficients of the X_1 and X_2 variables may be compared to determine the relative strengths of the X toxic agents regarding the response Y: the greater the ß coefficient of an X variable, the more the X variable contributes to the response. For each strain tested, X_1 describes a negative slope and X_2 describes a positive slope. This indicates that caffeine has a much greater effect in protecting the larvae from the harmful effects of AFB_1 on viability than does AFB_1 in protecting larvae from harm due to caffeine.

Little is known about the biochemical effects of AFB_1 or caffeine ingestion in insects (Al-Adil et.al. 1973, Watson 1975, Foerster et.al. 1984), or about the interaction of these agents (Cramer and Painter 1981). In vertebrate systems, it is known that AFB_1 is activated by a type I, P-450 mixed function oxidase (MFO) to the toxic and reactive epoxide that binds to DNA, RNA, and protein macromolecules (Shepherd et.al. 1984, Kitada et.al. 1989, Shimada et.al. 1989). Detoxification mechanisms include conversion of the epoxide by an MFO into less toxic aflatoxin M, hydrolyzing the epoxide by an epoxide hydrolase, or conjugating the epoxide to glutathione S-transferase (Lotikar et.al. 1980, Shelton et.al. 1984). Elucidation of the mechanisms by which caffeine may inhibit the production of the AFB_1-epoxide and/or its binding to macromolecules, or may enhance epoxide metabolism awaits further investigation.

ACKNOWLEDGMENTS

This work was supported by the Grants-in-Aid Program for faculty at Virginia Commonwealth University. Dr. Vernon Chinchilli of the Department of Biostatistics at MCV/VCU provided helpful criticism of an earlier version of this paper.

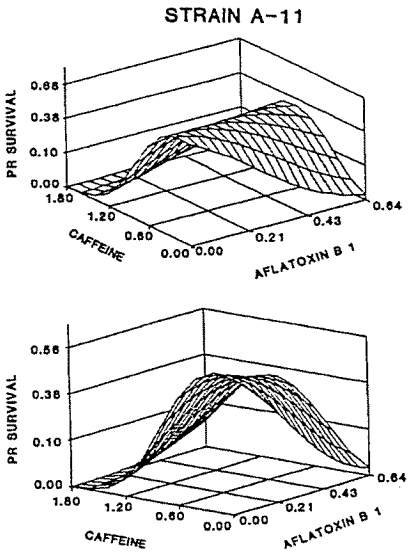

FIGURE 4. For strain A-11, the response surface generated by RSM for egg-adult viability at various concentrations of aflatoxin B1 and/or caffeine. Two views of the surface are shown. X1 axis = AFB1 concentrations, X2 axis = cafffeine concentrations, Y axis = probabilities of eggs surviving to the adult stage.

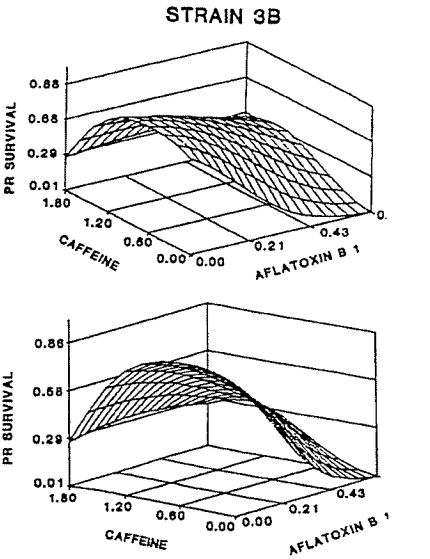

FIGURE 5. For strain 3B, the response surface generated by RSM for egg-adult viability at various concentrations of aflatoxin B1 and/or caffeine. Two views of the surface are shown. X1 axis = AFB1 concentrations, X2 axis = cafffeine concentrations, Y axis = probabilities of eggs surviving to the adult stage

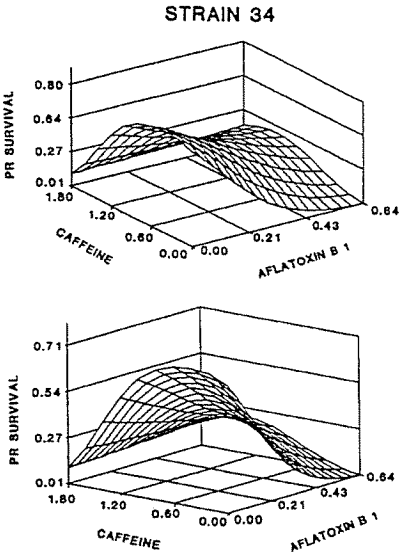

FIGURE 6. For strain 34, the response surface generated by RSM for egg-adult viability at various concentrations of aflatoxin B1 and/or caffeine. Two views of the surface are shown. X1 axis = AFB1 concentrations, X2 axis = cafffeine concentrations, Y axis = probabilities of eggs surviving to the adult stage.

This paper is based on research performed by DAB as partial fulfillment of the MS in Biology requirements. JPC directed the project and prepared the manuscript.

REFERENCES

Al-Adil, K. M., W. W. Kilgore and R. R. Painter. 1973. Effects of orally ingested aflatoxin B_1 on nucleic acids and ribosomes of housefly ovaries. Toxicol. Appl. Pharmacol. 26:130-136.

Carter, W. H., D. E. Jones and R. A. Carchman. 1985. Application of response surface methods for evaluating the interactions of soman, atropine, and pralidoxime chloride. Fund. Appl. Toxicol. 5:S232-S241.

Chinnici, J. P., and D. A. Bettinger. 1984. Effects of aflatoxin B_1 and caffeine on viability in natural strains of *Drosophila melanogaster*. J. Invert. Pathol. 44:263-266.

Cramer, P., and R. B. Painter. 1981. Effects of activated aflatoxin B_1 and caffeine on DNA replicon initiation in HeLa cells. Carcinogenesis 2:379-384.

Delawder, S., and J. P. Chinnici. 1983. Degree of aflatoxin B_1 sensitivity in Virginia natural populations of *Drosophila melanogaster*. VA. J. Sci. 34:48-57.

Foerster, R. E., and F. E. Wurgler. 1984. In vitro studies on the metabolism of aflatoxin B_1 and aldrin in testes of genetically different strains of *Drosophila melanogaster*. Arch. Toxicol. 56:12-17.

Kakunaga, T. 1975. Caffeine inhibits cell transformation by 4-nitroquinoline-1-oxide. Nature 258:248-250.

Kihlman, B. A. 1977. *Caffeine and Chromosomes*. Elsevier Scien-tific Publ. Co., Amsterdam, New York, and Oxford. 504 pages.

Kirk, H. D., A. B. Ewen, H. E. Emson and D. G. R. Blair. 1971. Effect of aflatoxin B_1 on development of *Drosophila melanogaster* (Diptera). J. Invert. Path. 18:313-315.

Kitada, M., M. Taneda, H. Ohi, M. Komori, K. Itahashi, M. Nagao and T. Kamataki. 1989. Mutagenic activation of aflatoxin B_1 by P-450 HFLa in human fetal livers. Mutat. Res. 227:53-58.

Lalor, J. H., J. P. Chinnici and G. C. Llewellyn. 1976. Effects of a fungal metabolite, aflatoxin B_1, on larval viability and gross morphology in *Drosophila melanogaster*. Dev. Ind. Microbiol. 17:443-449.

Lotikar, P. D., S.M. Insetta, P.R. Lyons and E. Jhee. 1980. Inhibition of microsome-mediated binding of aflatoxin B_1 to DNA by glutathione S-transferase. Cancer Lett. 9:143-149.

Mead, R., and D. J. Pike. 1975. A review of response surface methodology from a biometric viewpoint. Biometrics 31:803-851.

Nigsch, J., V. Graf and F. E. Wurgler. 1977. Caffeine toxicity in *Drosophila* strains having different MMS sensitivities. Mutat. Res. 43:57-64.

Nomura, T. 1980. Timing of chemically induced neoplasia in mice revealed by the antineoplasmic action of caffeine. Cancer Res. 40:1332-1340.

Ong, T. M. 1975. Aflatoxin mutagenesis. Mutat. Res. 32:35-53.

Shelton, D. W., J. D. Hendricks, R. A. Coulombe and G. S. Bailey. 1984. Effect of dose on the inhibition of carcinogenesis/mutagenesis by aroclor 1254 in rainbow trout fed aflatoxin B_1. J. Tox. Envt. Health 13:649-657.

Shepherd, E. C., T. D. Phillips and T. R. Irvin. 1984. Aflatoxin B_1 metabolism in the rat: Polyhalogenated biphenyl conversion to aflatoxin M_1. Xenobiotics 14:714-750.

Shimada, T., and F. P. Guengerich. 1989. Evidence for cytochrome P-450_{NF}, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. Proc. Natl. Acad. Sci. USA 86:462-465.

Stoloff, L. 1989. Aflatoxin is not a probable human carcinogen: The published evidence is sufficient. Regulatory Toxicol. Pharmacol. 10:272-283.

Tanzarella, C., S. Astolfi and F. Palitti. 1984. Effect of caffeine on induced damage in cells of *Xenopus laevis*. Mutat. Res. 128:25-29.

Watson, W. A. G. 1975. Lack of an effect of caffeine on repair systems in oocytes of *Drosophila melanogaster* following treatment of mature sperm with alkylating agents. Mutat. Res. 33:395-398.

Williams, D. A. 1975. The analysis of binary responses from toxicological experiments involving reproduction and teratogenicity. Biometrics, 31:949-952.

Wogan, G. N., and W. F. Busby. 1980. Naturally occurring carcinogens. In: *Toxic Constituents in Plant Foodstuffs*, Liener, I. E., ed., Academic Press, NY. 502 pps.

Wood, G. E. 1989. Aflatoxins in domestic and imported foods and feeds. J. Assoc. Off. Anal. Chem. 72:543-548.

Yeh, F-S., M. C. Yu, C-C. Mo, S. Luo, M. J. Tong and B. E. Henderson. 1989. Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in southern Guangxi, China. Cancer Res. 49:2506-2509.