12-2021

Comparative Metabolism of Aflatoxin B1 in Two Quail Genera Coturnix japonica and Callipepla californica

Sean Moody
Utah State University

COMPARATIVE METABOLISM OF AFLATOXIN B_1 IN TWO QUAIL GENERA *Coturnix japonica* AND *Callipepla californica*

by

Sean L. Moody

A research paper submitted in partial fulfillment

of the requirements for the degree

of

MASTER OF SCIENCE

In

Animal Management

Approved:

Roger A. Coulombe, Jr., Ph.D.
Major Professor

Jeffery O. Hall, D.V.M., Ph.D.
Committee Member

Kerry Rood, D.V.M., M.S., M.P.H.
Committee Member

UTAH STATE UNIVERSITY
Logan, Utah

2021

Abstract

Avian species are highly susceptible to the hepatotoxic mycotoxin aflatoxin B_1 (AFB_1). Domesticated turkeys are exquisitely sensitive, due to a combination of highly-efficient hepatic cytochrome P450 (CYP)-mediated bioactivation, and to dysfunctional alpha-class glutathione S-transferases (GSTAs) which typically detoxify the bioactivated electrophilic metabolite exo-AFB_1-8,9-epoxide (AFBO). Wild turkeys are relatively resistant to AFB_1 in large part due to expression of functional GSTAs. Quail, a related Galliforme, are slightly less sensitive *in vivo* to AFB_1, but whether this is related to the hepatic metabolic profiles of these two critical enzymes has not been rigorously evaluated. The purpose of this study was to compare hepatic CYP-mediated bioactivation and GST-mediated detoxification activities toward AFB_1 in *Callipepla californica* (HQ) and *Coturnix japonica* (JQ) against those from domesticated Broad-breasted White (BB) or Eastern Wild (EW) turkeys. Although *Callipepla californica* is commonly known as the California quail, the flock investigated in this study originated in Hawaii, hence use of the identifier "HQ" (noted above) that will be used throughout this report. Hepatic expression of CYP1A5 was significantly greater in both quail species, whereas there was no difference in CYP3A37 expression between species. Likewise, livers from both quail species expressed significantly greater GSTA3 than that from both turkey types, whereas turkeys expressed greater hepatic GSTA4 than those from both quail species. Kinetic analysis confirmed that liver microsomes from turkeys bioactivated AFB_1 more efficiently (high V_{max}, K_{cat}; low K_m) than those from quail, whereas hepatic cytosols from quail were significantly more efficient in detoxifying AFBO than those from turkeys. Conversely, turkey hepatic cytosols were more efficient than quail at detoxifying GST indicator substrates 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic

acid (ECA), and cumene hydroperoxide (CHP), but not 1,2-dichloro-4-nitrobenzene (DCNB), indicating greater presence of GST isoforms not relevant to AFB_1 detoxification. In total, our data shows that the relative resistance of quail compared to turkeys is reflected in the relatively lower *in vitro* bioactivation and higher detoxification activities.

Acknowledgements

I would like to thank my Major Professor, Dr. Roger Coulombe, Jr. for his support and guidance on this project. I would like to thank my committee members, Dr. Kerry Rood and Dr. Jeffery Hall for their help and support. I would additionally like to acknowledge and thank Dr. Kevin Welch for the use of his laboratory at the USDA-ARS Poisonous Plant Research Laboratory, and Dr. Casey Simmons for his help and expertise in optimizing my HPLC protocols. I would like to thank my fellow students and lab mates for their support and help as I have worked through this project; Andy Nguyen, Dr. Aaron Duffy, Nick Grooms, Schyler Odum, and Austin Sears. Lastly, I want to thank my wife, Natalie, and my family for the constant encouragement and support in my schooling.

Table of Contents

List of Tables

List of Figures

Literature Review

Aflatoxins: Overview

Aflatoxins are one of the most important mycotoxins in terms of occurrence and potency (Pitt, 2013). Aflatoxin B_1 (AFB_1; Figure 1) was discovered when it was identified as the etiological agent of "Turkey X Disease" in 1960, which caused extensive and widespread deaths of turkeys and other poultry species across Europe. The source of AFB_1 that caused the disease outbreak was traced to contaminated Brazilian peanut meal a widely used feed ingredient for poultry (Blount, 1961; Stevens *et al.*, 1960). This event demonstrated the extreme sensitivity of turkeys to this mycotoxin, and it is now clear that domesticated turkeys (*Meleagris gallopavo*) are one of the most sensitive animals known (Kim *et al.*, 2013; Monson *et al.*, 2015).

Aflatoxins are naturally occurring metabolites produced by the fungus *Aspergillus flavus*, *A. parasiticus*, and others. Derivatives of difuranocoumarin, individual aflatoxins (AFs) are named according to their blue (B) or green (G) fluorescence under UV light and elution position on a thin-layer chromatograph, hence AFB_1, AFB_2, AFG_1, and AFG_2 (Dalvi, 1986; Yu *et al.*, 2004); of these, AFB_1 is the most potently hepatotoxic, mutagenic, and carcinogenic (Coulombe, 1993; Monson, et al., 2015; Rawal, et al., 2010a). Aflatoxin B_1 is hepatocarcinogenic in livestock, laboratory animals, and humans (Kim, et al., 2013; Wogan, 1992). Aflatoxin B_1 is also considered a "pro-carcinogen", because it requires enzymatic bioactivation for its carcinogenic and toxic effects to occur (Diaz *et al.*, 2010a; Diaz *et al.*, 2010b;

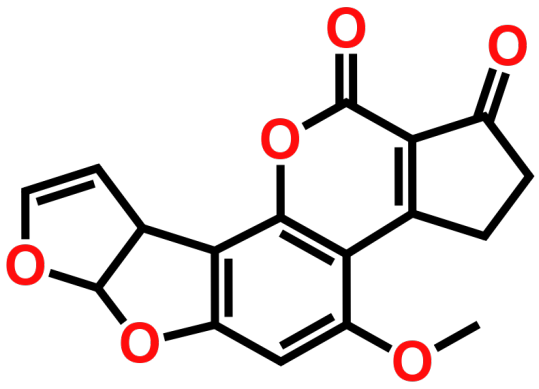

Figure 1: Chemical structure of Aflatoxin B_1

Rawal and Coulombe, 2011; Rawal, et al., 2010a). Aflatoxin B_1 also acts as a "force multiplier" synergizing the adverse effects of microbial and viral pathogens, and other agents (Monson, et al., 2015). While AFB_1 is toxic to every animal species examined, the primary focus of this review is on the effects of this mycotoxin in poultry, specifically turkey and quail. When relevant, molecular mechanisms of toxicity in other species that may also aid in our understanding of a response in poultry will be briefly presented.

Aflatoxins: Toxicity and Carcinogenicity

The acute and chronic diseases caused by AFB_1 consumption are termed aflatoxicoses (Bennett and Klich, 2003). Aflatoxin B_1 affects the liver as the primary target organ, where it causes hemorrhagic necrosis, fatty infiltration, and bile duct proliferation (Coulombe, 1993). Chronic AFB_1 exposure results in immunosuppression, as well as reductions in growth rates, productivity, fertility, feed utilization, milk production, and egg production (Diekman and Green, 1992; Galvano *et al.*, 1996; Monson, et al., 2015; Pandey and Chauhan, 2007; Polychronaki *et al.*, 2006).

Species susceptibility to the acute toxic effects of AFB_1 varies considerably. Mice are one of the most resistant species to both the acute and chronic toxic effects of AFB_1 whereas rats, fish, and poultry are extremely sensitive to both (Bailey *et al.*, 1988; Bedard *et al.*, 2005; Gold *et al.*, 1984; Klein *et al.*, 2002a; Monson, et al., 2015; Rawal, et al., 2010a; Wogan, 1992). Chronic aflatoxicosis inflicts an estimated US$143 million yearly loss to the turkey industry in the United States from hepatotoxicity, reduced performance, and secondary infections (CAST, 1989, 2003).

Acute AFB_1 exposure leads to structural and functional damage to the liver (Busby and Wogan, 1984). The clinical signs and pathological changes associated with acute AFB_1 exposure in humans include vomiting, abdominal pain, pulmonary edema, and fatty acid infiltration into the liver (Shank *et al.*, 1972); and in animals there are parenchymal cell necrosis, bile duct proliferation, and hepatic lesions (Newberne and Butler, 1969). Additionally, acute toxicosis may be associated with an increased future risk of cancer, through the formation of DNA adducts, especially in transcriptionally active genes (Irvin and Wogan, 1984; Yu, 1983). Acute toxicosis in turkeys is evident by enlarged mottled livers that have periportal necrosis, hemorrhage, and accumulation of fat (Wannop, 1960).

Chronic exposure more readily leads to cancer than does acute exposure (Edds, 1973). In rats chronic treatment of AFB_1 resulted in failure to gain body weight, loss of liver weight, and loss of hepatic DNA content (Liu *et al.*, 1988). The clinical signs of chronic AFB_1 exposure are anemia; jaundice; anorexia; hemorrhage; embryotoxicity; increased susceptibility to environmental and microbial stressors; hepatic necrosis; hepatobiliary hyperplasia; and it can alter the gut microbiota allowing colonization of gut pathogens (CAST, 2003; Klein *et al.*, 2002b; Reed *et al.*, 2019).

Aflatoxin B_1: Metabolism

Once ingested, AFB_1 is initially converted by oxidative phase I hepatic enzymes to a number of metabolites, many of which are detoxified and excreted *per se*, whereas others are of greater bioactivity. Many phase I metabolites are further metabolized through one or more secondary (phase II) conjugations with endogenous endocons such as glucuronic acid and

glutathione (GSH) (Deng *et al.*, 2018; Lozano and Diaz, 2006). Aflatoxin B_1 requires prior bioactivation by cytochrome P450s to become toxic, as shown in Figure 2 (Ball and Coulombe, 1991; Coulombe, 1993). The main bioactive metabolite is the electrophilic and highly reactive *exo*-AFB_1-8,9-epoxide (AFBO), which reacts with cellular macromolecules, most significantly DNA to form the AFB_1- N^7-guanine adduct that is the principal mutagenic and carcinogenic cellular lesion (Ball *et al.*, 1990; Iyer *et al.*, 1994). Although AFBO is the most toxic, other metabolites of AFB_1 include aflatoxin M_1 (AFM_1; first named due to its prevalence in cow's milk), aflatoxin Q_1 (AFQ_1), and aflatoxicol (AFL) (Coulombe, 1993; Monson, et al., 2015) (Figure 2).

Aflatoxin M_1 (4-hydroxy AFB_1; AFM_1) represents the primary hydroxylated metabolite of AFB_1. Aflatoxin M_1 is classified as a group 2B carcinogen, possibly carcinogenic to humans. In the United States, the maximum acceptable limit of AFM_1 in fluid milk products is 0.5 ppb. Aflatoxin Q_1 is the 3-hydroxy metabolite of AFB_1 and is considerably less toxic than AFB_1 (IARC, 2012; Raney *et al.*, 1992).

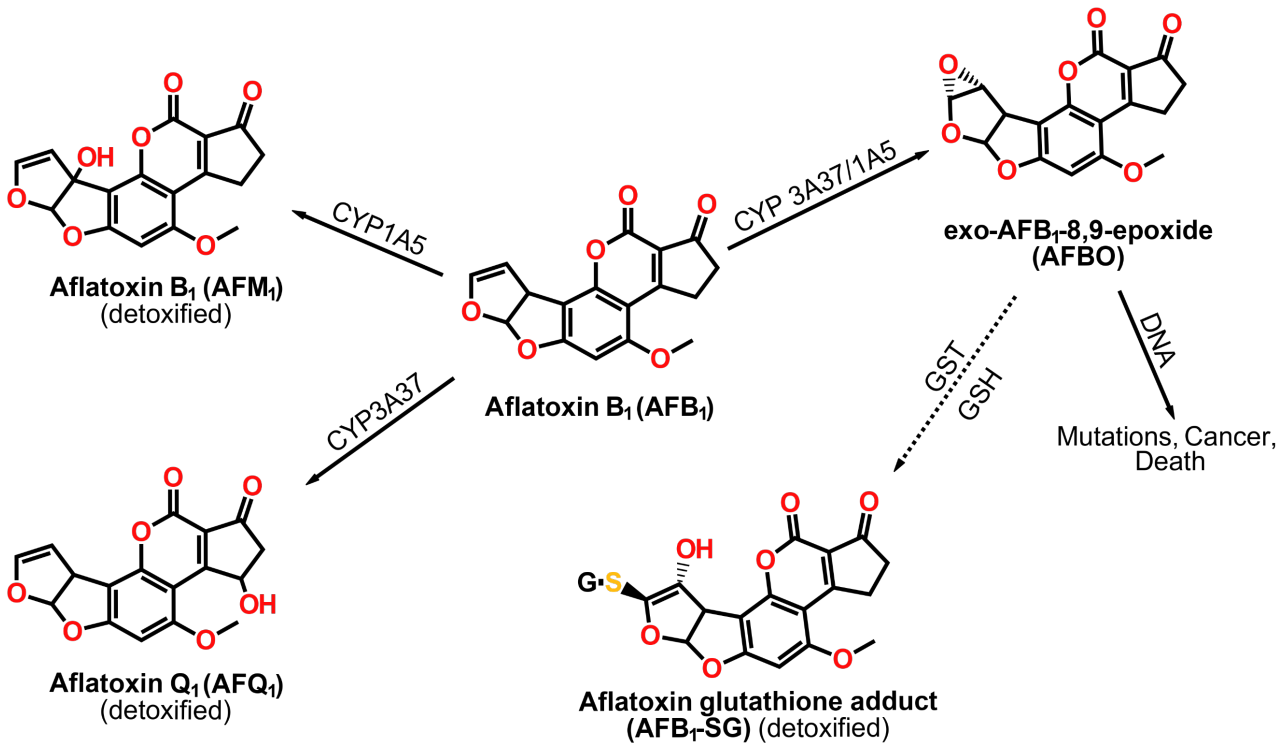

Figure 2: The extreme sensitivity of turkeys to AFB_1 is associated with efficient AFB_1 epoxidation catalyzed by CYP1A5 and CYP3A37, coupled with deficient GST detoxification. The hydroxylated metabolites, AFM_1, and AFQ_1 are formed by CYP1A5 and CYP3A37, respectively. Dashed line indicates this critical detoxification pathway is deficient in domesticated turkeys. Adapted from (Rawal et al., 2010a).

Role of Cytochrome P450 in AFB_1 Metabolism

The principal phase I metabolic enzymes that metabolize AFB_1 are the cytochrome P450 proteins (CYPs), hemeproteins principally located in the smooth endoplasmic reticulum (Nebert and Russell, 2002). An important endogenous role of CYPs is to catalyze the synthesis of steroid hormones (hCYP17A1, 19A1, 21A2), cholesterol (hCYP11A1, 27A1, 39A1) and bile acids (hCYP7A1, 27A1) (Guengerich, 2017; Nebert and Russell, 2002; Staels and Fonseca, 2009). Cytochrome P450s are named and classified according to nucleotide and amino acid sequence homology by a family number (e.g., CYP1, CYP3) and a subfamily letter (e.g., CYP1A, CYP3A)

then by isoform number (e.g., CYP1A5, CYP3A37) (McDonnell and Dang, 2013). Additionally, a lowercase letter can precede the cytochrome designation to indicate species of the enzyme, for example, mouse (mCYP1A2) or turkey (tCYP1A5).

In turkeys, AFB_1 is metabolized primarily by two hepatic P450s, CYP1A5 and CYP3A37 (Rawal and Coulombe, 2011). For example, AFQ_1 is the principal product of tCYP3A37 metabolism, and AFM_1 is produced primarily by tCYP1A5 (Rawal and Coulombe, 2011). Both isoforms bioactivate AFB_1 to AFBO, but show significant substrate affinities in that tCYP1A5 predominates at low, pharmacologically-relevant concentrations (~0.1 µM), whereas CYP3A37 catalysis is significant only at much greater (> 50 µM) concentrations of AFB_1 that are unlikely to be achieved *in vivo* in most if not all "real world" exposures (Rawal and Coulombe, 2011).

The relative contribution of both microsomal CYP1A5 and CYP3A37 AFB_1 bioactivation is approximately 98% and 2%, respectively, at sub-micromolar (0.1 µM) concentrations of AFB_1 (Rawal and Coulombe, 2011). At 0.1 µM AFB_1, CYP1A5 produces a higher ratio (50:1) of bioactivation to detoxification product (AFBO:AFM_1) compared to CYP3A37 (AFBO:AFQ_1 = 0.17:1) (Rawal and Coulombe, 2011). Using custom anti-peptide antibodies to perform a series of immunoinhibition experiments to independently characterize CYP 1A5 and 3A37 metabolism in hepatic microsomes, two kinetic models were observed (Rawal and Coulombe, 2011; Yip and Coulombe, 2006). At sub-micromolar AFB_1 concentrations CYP1A5 is characterized by hyperbolic Michaelis kinetics producing only AFBO and AFM_1 (Rawal and Coulombe, 2011). Identical kinetics have been observed from *E. coli* that expressed tCYP1A5 (Yip and Coulombe, 2006) and hCYP1A2 (Gallagher *et al.*, 1996). The inhibitory effect of BHT on CYP1A5 exhibited Michaelis competitive inhibitory kinetics (K_i = 0.81 µM) (Guarisco *et al.*, 2008). Conversely,

when CYP1A5 was immunoinhibited, isolating CYP3A37 catalysis, turkey liver microsomes produced AFBO and AFQ_1, following sigmoidal Hill kinetics (Rawal and Coulombe, 2011). The sigmoidal relationship observed between the substrate concentrations and the rates of product formation suggests cooperativity between CYP3A37 and AFB_1 (Rawal and Coulombe, 2011). This relationship was explored previously in our laboratory where *E. coli* expressed CYP3A37 exhibited a sigmoidal relationship suggesting that both AFBO and AFQ_1 formation is driven by an allosteric interaction between AFB_1 and CYP3A37, showing positive cooperativity (Rawal *et al.*, 2010b).

The epoxidation of AFB_1 to form AFBO is the most critical step in the toxic and carcinogenic effects of this mycotoxin (Rawal, et al., 2010b). Turkey hepatic P450s are among the most efficient at bioactivating AFB_1 to AFBO compared to other poultry species, specifically ducks, quail, chickens, and pheasants (Monson, et al., 2015; Rawal and Coulombe, 2011). Cytochrome P450s of turkey poults are more active toward AFB_1 than those of mature birds (Klein, et al., 2002a). The dominant catalyst of AFB_1 epoxidation at low, environmentally-relevant concentrations found in poultry liver is CYP1A5 (Rawal and Coulombe, 2011). The critical role of CYP1A5 was further illustrated by the use of butylated hydroxytoluene (BHT) an hCYP1A5 inhibitor to alleviate the symptoms of aflatoxicosis in AFB_1 exposed poultry (Guarisco, et al., 2008).

Aflatoxin B_1: Carcinogenicity

Aflatoxin B_1 is one of the most potent hepatocarcinogens known, and its potency has been established in numerous avian, mammalian, aquatic, and non-human primate species

(Bailey *et al.*, 1984; Eaton and Schaupp, 2014; Monson, et al., 2015; Muller *et al.*, 1970). Numerous epidemiological and clinical studies have led the International Agency for Research on Cancer (IARC) to classify AFB_1 as a class 1 or recognized human carcinogen (IARC, 1993). Essential to AFB_1 toxicity and carcinogenesis is the formation of the N^7-guanine adduct (Figure 3), the principal mutagenic and carcinogenic cellular lesion in animals (Ball, et al., 1990; Iyer, et al., 1994). Bailey *et al.* (1988) demonstrated that rainbow trout, when exposed to AFB_1, exhibited an 62% tumor incidence after 12 months compared to Coho salmon that had no incidence of tumors when they were treated identically. Additionally, that study demonstrated that rainbow trout AFB-DNA binding is 18 times greater than salmon AFB-DNA binding after a three-week dietary exposure to AFB_1 (Bailey, et al., 1988). Studies working with rats have shown that through the use of a detoxifying enzyme inducer, Oltipaz, N^7-guanine adducts could be reduced by 97% with no incidence of AFB_1-induced hepatocellular neoplasms (Kensler *et al.*, 1985; Roebuck *et al.*, 1991). The AFBO metabolite has also been found to cause genetic damage in bacteria, as well as in human and animal cells (IARC, 2012). Cancer results from the introduction of a G→T transversion in hepatic DNA in human hepatocytes (Mace *et al.*, 1997); this is the result of AFB_1 adducts predisposing DNA synthesis machinery to make G→T transversions (Smela *et al.*, 2002). The G→T transversion is the predominant mutation induced by AFB_1 exposure (Foster *et al.*, 1983). The specific mutation is made to codon 249 of the p53 tumor suppressor gene (IARC, 2012; Kensler *et al.*, 2011; Mace, et al., 1997), which occurs in more than half of the patients with hepatocellular carcinoma (HCC)(Bressac *et al.*, 1991; Hsu *et al.*, 1991).

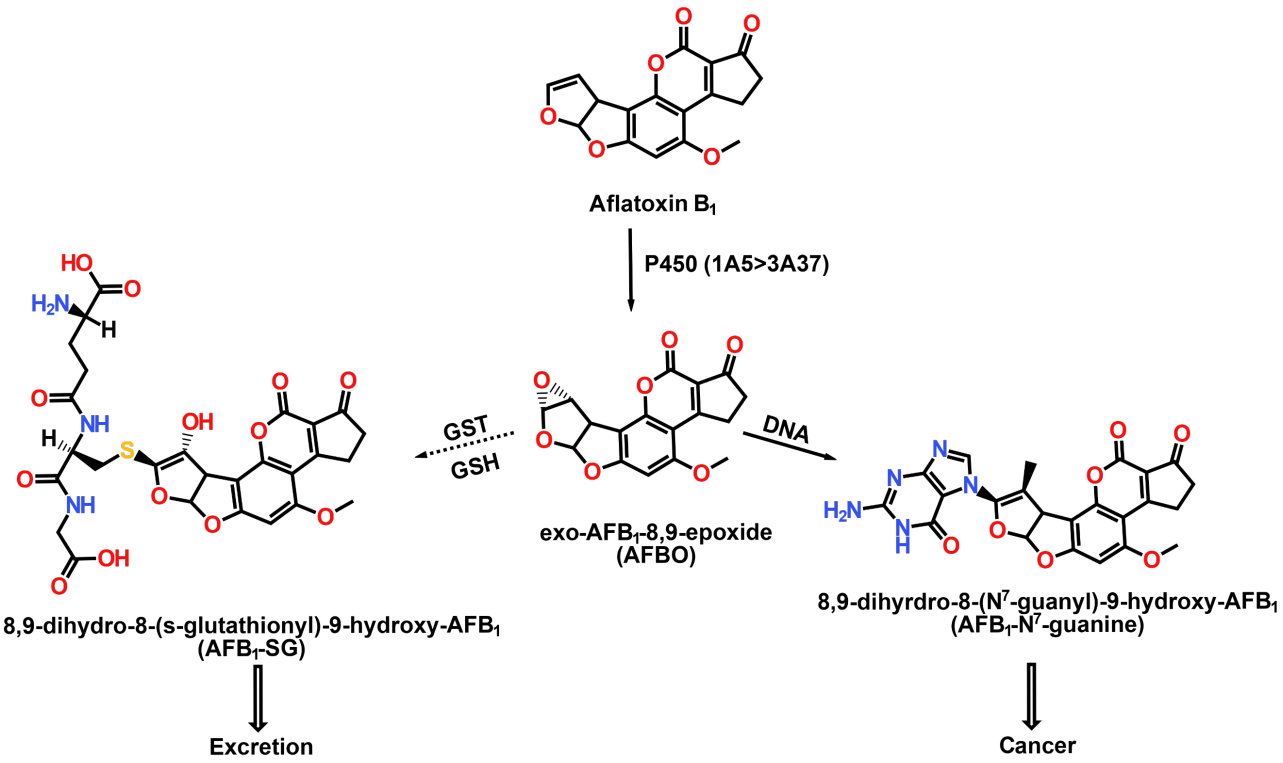

Figure 3: Metabolites and enzymes involved in aflatoxin B_1 (AFB_1) metabolism in turkey liver microsomes. Hepatic glutathione S-transferases (GSTs) from domesticated turkeys lack exo-AFB_1-8,9-epoxide (AFBO) conjugating activity (dashed line), while those from wild turkeys are fully functional. [Adapted from Monson, et. al., 2015]

Role of Glutathione S-transferases in AFB_1 Detoxication

Phase II metabolism is an enzymatic process whereby a phase I metabolite is conjugated with an endogenous cofactor, or "endocon" such as glucuronic acid, sulfate acetic acid, or glutathione with the resultant metabolite being safely excreted(Zamek-Gliszczynski *et al.*, 2006) . In nearly all animals studied, the primary route of AFB_1 detoxification is through hepatic glutathione S-transferases (GSTs) which are critical phase II detoxification enzymes that specifically recognize, react with, and detoxify electrophilic intermediates, such as AFBO, by conjugating this reactive intermediate with the cofactor glutathione (GSH)(Coles and Kadlubar,

2003). Glutathione S-transferases are dimeric cytosolic enzymes that are organized, based upon their amino acid homology, into six classes- zeta (ζ), pi (π), alpha(α), omega (Ω), mu (μ), and theta (Θ). Glutathione is a tri-peptide consisting of glutamate and cysteine through gamma linkage and glycine through peptide bond (Eaton and Bammler, 1999; Kim, et al., 2013; Ziglari and Allameh, 2013).

In the case of AFB_1, GSTs catalyze the reaction between the nucleophilic sulfur atom in the cysteine residue of GSH with the electrophilic substrate, with the resultant AFB-GSH adduct safely eliminated in the urine and feces (Wang *et al.*, 1999; Ziglari and Allameh, 2013) (Figure 3). Glutathione S-transferase activity is routinely quantified using prototype assays involving bioactivated substrates such as 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ECA), and cumene hydroperoxide (CHP) (Awasthi, 2007; Habig and Jakoby, 1981; Habig *et al.*, 1974b). Previous studies in our laboratory identified and cloned six turkey alpha class GSTs, tGSTA 1.1, 1.2, 1.3, 2, 3, and 4 (Kim *et al.*, 2010; Kim *et al.*, 2011). Cloned and expressed tGSTAs possessed detectable activities toward prototypical substrates (Kim, et al., 2011). The cloned tGSTA1.2 and A3 had the highest activities toward CDNB, ECA, and CHP, whereas tGSTA1.1 possessed the highest activity toward DCNB (Kim, et al., 2011). Even though tGSTA1.1 is missing a signature motif, detectable catalytic activity was measured for all substrates (Kim, et al., 2011). Cytosolic tGSTAs possessed activities toward all GST prototype substrates, however these activities were significantly lower than those observed for mouse cytosol except for ECA (Kim, et al., 2011). All cloned and expressed tGSTAs possessed measurable AFBO-conjugating activity compared to non-cloned hepatic tGSTAs (Kim, et al., 2011; Kim, et al., 2013). Domesticated hepatic turkey GSTs lack detoxifying activity, while

wild varieties have fully functional GSTs (Kim, et al., 2011; Kim, et al., 2013). These six turkey GST genes are homologs of AFB_1-protective GSTAs in mice and other species (Kim, et al., 2010). The catalytic activity of hepatic turkey GSTAs toward AFBO are substantially lower or not detected compared to that of mGSTA3, the "gold standard" for AFBO detoxification (Kim, et al., 2013). The importance of GSTA3 in other species was confirmed with the demonstration that GSTA3 knockout mice are substantially more AFB_1-susceptible than wild-type mice (Ilic *et al.*, 2010). Studies in our laboratory have consistently demonstrated that hepatic GSTs from domesticated turkeys lack AFBO detoxifying activity (Kim, et al., 2013), indicating that the rate-limiting step in species sensitivity toward AFB_1 is GST mediated detoxification of the AFBO metabolite (Eaton and Bammler, 1999; Kim, et al., 2011). Additionally, the phylogeny of poultry GSTs was constructed to illustrate the relatedness of these GSTAs between chickens, heritage, wild, and domesticated turkeys (Kim, et al., 2013). This study concluded that turkey and chicken GSTAs are orthologous, and that the GSTA loci was established before speciation (Kim, et al., 2013).

Quail

Our laboratory has previously determined the extreme sensitivity of poultry species to AFB_1 that was observed during "Turkey X Disease" is most likely due to a deficiency of protective hepatic glutathione S-transferases (GSTs) (Kim, et al., 2011; Klein *et al.*, 2000). Importantly, studies from our laboratory showed that unlike the hepatic forms, recombinant GSTAs from domesticated turkeys have the ability to conjugate and detoxify AFBO (Kim, et al., 2013), leading to the conclusion that GST silencing is not due to sequence changes within the

GST coding region, but more plausibly due to regulatory gene abnormalities in domestic turkeys not present in their wild counterparts (Coulombe *et al.*, 2005; Kim, et al., 2011). The use of purified metabolic proteins has provided many insights and answers to how these enzymes interact with AFB_1.

While turkeys are among the most susceptible species studied, quail, a related Galliforme, appeared in one *in vivo* feeding study to be less susceptible AFB_1 when considering gross hepatic lesions and lethality (Arafa *et al.*, 1981). Another study demonstrated that quail microsomal preparations produced significantly lower bioactivation metabolites (as measured by both production of metabolites AFB_1-dihydrodiol, AFB-GSH, and AFL) than that from turkey (Lozano and Diaz, 2007). To our knowledge, there have been no studies focusing specifically on the functional characteristics of CYP- and GST-mediated bioactivation and detoxification in quail liver.

Given the centrality of these two opposing metabolizing pathways in AFB_1 toxicology in turkeys, the purpose of this study was to characterize the kinetic and functional properties of microsomal CYP and cytosolic GST enzymatic activities of *Coturnix japonica* (JQ) and *Callipepla californica* (HQ). These properties were compared against those of domesticated and wild turkeys with a view to determine whether these reflect differences in *in vivo* susceptibility between quail and turkeys.

Research Goals

The research goals were to characterize microsomal and cytosolic enzyme activities from Japanese (JQ) and Hawaiian (HQ) quail with regards to AFB_1, CDNB, DCNB, CHP, and ECA. Comparing the results to Broad-breasted white (BB) and Eastern Wild turkeys (EW). Doing so would allow the assessment of the susceptibility of quail with respect to other poultry species and it would generate further insight about the mechanisms of AFB_1 bioactivation and detoxification.

Materials and Methods

Supplies and Reagents

1-chloro-2,4-dinitrobenzene (CDNB), 1,2-Dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ECA), cumene hydroperoxide (CHP), GSH, glutathione reductase, nicotinamide adenine dinucleotide (NADPH), AFB_1, aflatoxin G_1 (AFG_1), butylated hydroxytoluene (BHT), phenylmethylsulphonyl fluoride (PMSF), sucrose, 85% H_3PO_4, tetrahydrofuran (THF), and isopropyl-b-D-thiogalactopyranoside (IPTG) were obtained from Sigma–Aldrich (St Louis, MO). Bradford Protein Assay Kit was from Bio-Rad (Philadelphia, PA). The PTFE membranes were purchased from Millex Samplicity Filters (Tullagreen, Carringtonhill, Co Cork IRL). Turkey starter mash obtained from IFA Country Stores (Salt Lake City, UT). Custom anti-peptide antibodies for P450 1A5 (Rabbit anti-serum BLD#1, Lot#33964, Rabbit#G5415), P450 3A37 (Rabbit anti-serum BLD#1, Lot#33965, Rabbit#G5424), GSTA3 (Rabbit anti-serum #1 [A3R2], Lot#35788-35789, Rabbit#G7496), and GSAT4 (Rabbit anti-serum #2 [A4R2-2], Lot#35790-35791, Rabbit# G7510) were prepared by Genemed Synthesis Inc. (San Antonio, TX). The anti- β-actin antibody

(ab8224) was purchased from Abcam (Cambridge, UK). The remaining immunoblot reagents (streptavidin-HRP, anti-rabbit secondary, and the chemiluminescent substrate) were from ProteinSimple (San Jose, CA).

Animal Tissues

The liver tissues used were from two varieties of Quail, *Callipepla californica* (HQ) and *Coturnix japonica* (JQ) obtained from the Deschambault Research Station Quebec, Canada. The livers were flash-frozen in liquid N_2, shipped overnight on dry ice, and stored at -80 °C until use. Turkeys were purchased from Strombergs hatchery (Hackensack, MN) and raised at Utah State University South Farm. Female poults were identified by PCR and culled (Kalina *et al.*, 2012). Male poults were fed starter mash and livers harvested at approximately three weeks of age. Livers were flash frozen in liquid N_2, transported on dry ice, and stored at -80 °C. Swiss-Webster mice were obtained from Charles River Laboratories (Wilmington, MA) through Laboratory Animal Research Center, Utah State University and were given a diet containing 0.75% butylated hydroxyanisole (BHA) using corn oil for 14 days. Animals were housed in an AAALAC-accredited facility, and all procedures involving animal care and tissue collection were approved by Utah State University's Animal Care and Use Committee (approvals #2668 and 2670).

Isolation of Hepatic and Cytosolic Proteins

Microsomes and cytosols were isolated by pooling approximately 2 g of liver tissue from two birds in each group, resulting in a total of 4 HQ and 3 JQ samples. Proteins were purified through differential centrifugation as described previously (Klein, et al., 2000). All steps were

performed at 4 °C. Frozen livers were homogenized using a Polytron (Brinkman, Westbury, NY) in a 1:3 w/v cold homogenizing buffer (50 mM Tris, 1 mM EDTA, 0.25 M sucrose, 150 mM KCl, 20 *m*M BHT, 200 *m*M PMSF, pH 7.4). Homogenate was centrifuged at 10,000 x g for 10 min then supernatant was centrifuged at 16,000 x g for 10 min then at 105,000 x g for 60 min. Supernatants (consisting of liver cytosols) were collected, aliquoted and stored at -80 °C. The microsomal pellet was homogenized and centrifuged at 105,000 x g (L7-55 ultracentrifuge Beckman Coulter, Indianapolis, IN), for 60 min and the pellet collected, aliquoted, and stored at -80 °C until assayed. Proteins were quantified using the Bradford assay (Kim, et al., 2011).

Quantifying AFB_1 Bioactivation and Detoxification

Bioactivation of AFB_1 to AFBO and detoxification activities of AFBO to *exo*-AFB_1-GSH (AFB-GSH) were performed according to published methods(Kim, et al., 2011; Rawal and Coulombe, 2011) . Turkey liver microsomes (~400 μg total protein for control sample) and quail microsomes (~50 μg total protein) were used as the P450 source for AFB_1 bioactivation which were reacted in 0.1 mM Tris buffer (pH 7.6) containing 10-1024 μM AFB_1 in HPLC grade dimethyl sulfoxide, 2 mM NADPH, 5 mM GSH, and BHA-Induced mouse cytosol (~800 μg total protein for each sample and ~3 μg total protein for control sample) as the GST source to "trap" the short-lived AFBO (Guarisco, et al., 2008; Klein, et al., 2000). The mixture was incubated in epoxide trapping buffer (5 mM $MgCl_2$, 25 mM KCl, 0.25 mM sucrose, and 80 mM K_2HPO_4, pH 7.6) to give a final volume of 250 µl. The mixture was incubated for 10 min with the cofactor mix at 37 °C, then incubated an additional 20 min with the AFB_1. The reaction was stopped by adding 250 uL of cold MeOH spiked with 24 μM AFG_1 as an internal standard. The samples were

left overnight at -20 °C to facilitate precipitation and then centrifuged for 11 min at 17,000 G. Supernatants were filtered through a 0.2 μM hydrophilic PTFE membrane and 160 μL was injected into an HPLC. Metabolites were separated on a Shimadzu LC system (Pleasanton, CA), equipped with a model LC-20AD pump, a model SPD-20AV UV/vis detector, and an Allsphere ODS-2.5 *u*M (250 × 4.6 mm) column (Grace Davison Discovery Sciences, Columbia, MD) kept at 40 °C. The elution of the peaks was monitored by UV absorbance (λ = 365 nm). Mobile phases and elution programs used were as previously reported (Kim, et al., 2011). Metabolite formation was quantified using a calibration curve generated using authentic *exo*-AFB_1-GSH standard. Bioactivation of AFB_1 from turkey samples was performed as described (Rawal and Coulombe, 2011).

Quail hepatic cytosolic GST-mediated detoxification of AFBO to AFB-GSH was measured by a modification of the above protocol. Quail liver cytosol (~100 μg total protein) was substituted for the BHA-induced mouse cytosol. The AFBO metabolite was enzymatically generated by using turkey liver microsomes (~400 μg total protein). Detoxification of AFBO from turkey samples was performed as described (Kim, et al., 2011).

GST Prototype assays

Specific enzyme activities were assayed using previously established protocols for activity toward prototype substrates ethacrynic acid (ECA), 1,2-dichloro-4-nitrobenzene (DCNB), and 1-chloro-2,4-dinitrobenzene (CDNB) (Habig and Jakoby, 1981; Habig *et al.*, 1974a), and cumene hydroperoxide (CHP) (Lawrence and Burk, 1976). The assays were optimized for a final volume of 200 µL of 100 mM phosphate buffer at room temp (25°C) using a Synergy H1

microplate reader (Biotek, Winooski, VT), with the exception of ECA which was performed in a 1 mL volume using a Genesys 6 spectrophotometer (Thermo Spectronic, Madison, WI): (1) 0.2 mM ECA, 0.25 mM GSH, ΔA270 nm (Extinction coefficient: 5/mM/cm), and buffer pH 6.5, (2) 1 mM DCNB, 5mM GSH, ΔA345 nm (8.5/mM/cm), and buffer pH7.5, (3) 1 mM CDNB, 1 mM GSH, ΔA340 nm (9.6/mM/cm), and buffer pH 6.5, and (4) CHP glutathione peroxidase activity was determined using 1.2 mM CHP, 2mM GSH, 1 U Glutathione reductase, 0.2 mM NADPH, ΔA340 nm (6.22/mM/cm), and buffer pH 7.0.

Expression of CYPs and GSTs

Expression of CYPs and GSTs were quantified by immunoblots using ProteinSimple Wes™ system utilizing the 12-230 kDa 25 capillary separation module, performed according to the manufacturer's instructions, except the antibody incubation time was increased from 30 min to 60 min per the manufacturers recommendation. The proteins were diluted to 0.50 mg/mL for both quail varieties and for EW; while BB was diluted to 2.0 mg/mL for labeling with both isoforms of P450 (1A5 and 3A37) and GSTA (A3 and A4). The anti-peptide dilution used was 1:50 for both P450 1A5 and 1:10 for P450 3A37 for all samples. Proteins were identified using the following antipeptide concentrations 1:50 tGSTA3 and 1:50 tGSTA4 for turkey, 1:50 tGSTA3 and 1:250 tGSTA4 for quail. 1:10 (29 kDa) System loading control and 1:100 (90 kDa) System loading control were used for P450 and GSTA immunoblots, respectively.

Statistical Analysis

Kinetic data and statistics analyzed Prism by nonlinear regression for Michaelis-Menten and K_{cat} equations using (GraphPad Software, San Diego, CA).

Results and Discussion

Although quail are generally regarded to be of intermediate susceptibility among poultry, with turkeys the highest (Arafa et al., 1981), a direct comparison has been complicated by a lack of direct *in vivo* AFB_1 feeding trials between these two species of equivalent age, gender, AFB_1 intake, and toxicity endpoints. In the absence of such definitive studies, quantifying activities and metabolic profiles of specific phase I and phase II enzymes can accurately predict *in vivo* comparative toxicity (Kim, et al., 2013; Klein, et al., 2000).

In turkeys and most other animals studied, metabolic bioactivation of AFB_1 is catalyzed by orthologs of turkey CYPs 1A5 and 3A37 (Rawal, et al., 2010b; Yip and Coulombe, 2006). Hepatic expression of these CYPs was determined from immunoblots using custom anti-peptide antibodies prepared against the turkey forms of these enzymes previously cloned, expressed and functionally characterized in our laboratory (Rawal, et al., 2010b; Yip and Coulombe, 2006). For all liver samples, CYPs 1A5 and 3A37 appeared at the expected molecular weights of 60 and 58kD, respectively (Rawal, et al., 2010b; Yip and Coulombe, 2006). As shown in Figure 4, quail liver, like that from turkey, constitutively express both of these CYP forms. Hepatic expression of CYP1A5 was not significantly different between quail, but was significantly greater than that

from turkey. Conversely, there were no significant differences between and among species with respect to expression of CYP3A37 (Figure 4).

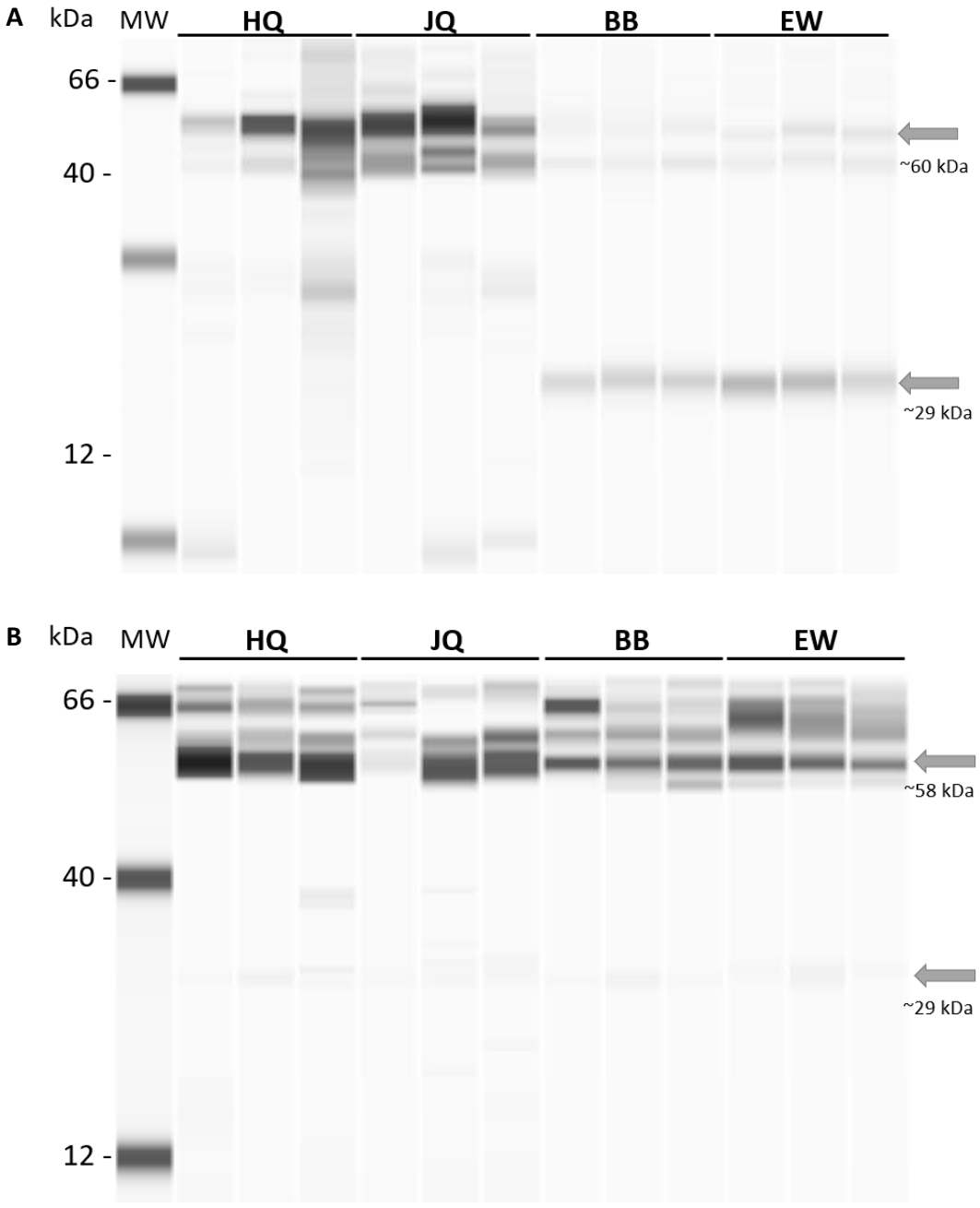

Figure 4: ***Immunoblots showing expression of CYP1A5 (A) and 3A37 (B) from liver microsomes from Japanese (JQ) and Hawaiian Quail (HQ), and Broad-breasted White (BB) and Eastern Wild (EW) Turkeys. A] CYP1A5 B] CYP3A37.*** *Custom Turkey anti-P450 peptides and HRP-conjugated anti-Rabbit secondary antibody were used and bands were detected by chemiluminescence analysis. Each sample represents two pooled liver samples corresponding to three unique pooled microsome samples. Arrows indicate the protein band of interest and the associated molecular weight.*

Cytochrome P450-mediated bioactivation to AFBO, the metabolite most responsible for the toxic and carcinogenic effects of this mycotoxin, is accurately quantified as the trapped AFB-GSH adduct (Klein, et al., 2000) using exogenous GST in the microsomal incubations due to the transient nature of AFBO ($t_{0.5}$ ~ 0.5 sec; (Eaton *et al.*, 1994)). Bioactivation from livers prepared from both quail and turkeys exhibited traditional Michaelis saturation kinetics (Figure 5). At a substrate concentration of 128 μM, CYP-mediated formation of AFBO in microsomes

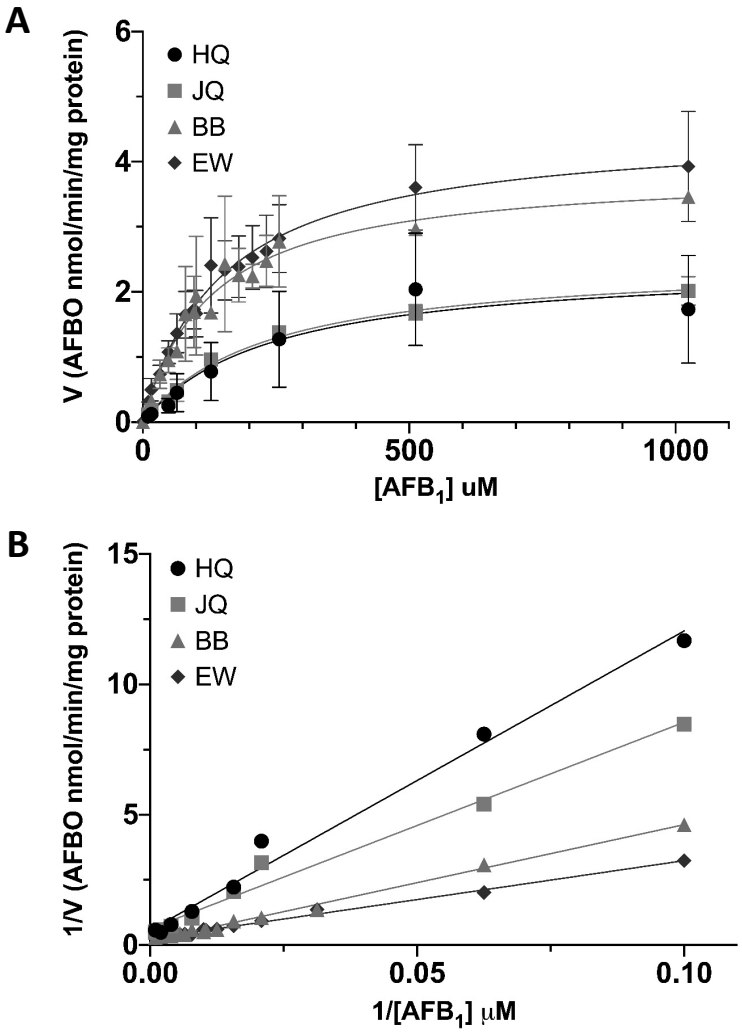

Figure 5: ***Michaelis-Menten (A) and Lineweaver-Burke (B) plots for AFB_1 bioactivation by Japanese and Hawaiian Quail (JQ, HQ) and Domesticated and Wild Turkey (BB, EW) Liver Microsomes.*** Enzyme kinetics of AFB_1 epoxidation activity in liver microsomal proteins (A and B). Panel A shows SE for each data point, with an N of 3. R^2 values were 0.97, 0.84, 0.84, and 0.89 respectively.

prepared from HQ and JQ were not significantly different from each other, but were significantly ($p < 0.05$) lower than those from domesticated and wild turkeys, respectively (HQ: 2.14 ± 1.80; JQ: 1.89 ± 0.69; BB: 69.06 ± 6.28; EW: 104.90 ± 3.72 nmol/min/mg protein). In all cases, quail microsomes exhibited significantly lower maximum rates (V_{max}), lower catalytic affinity of enzyme for substrate (K_m), and lower specificity (K_{cat}) (Table 1).

Table 1 **Kinetics of Hepatic Microsomal P450 Bioactivation of AFB_1 to AFBO for Hawaiian (HQ) and Japanese Quail (JQ) and, Broad-breasted White (BB) and Eastern Wild (EW) Turkeys.**

	V_{max} (nmol/min/mg protein)	K_m (μM AFB_1)	K_{cat} (s^{-1})
HQ	2.28 ± 0.54 [a]	214.92 ± 13.43 [a]	$8.61X10^{-04}$ ± $1.79X10^{-04}$ [a]
JQ	2.49 ± 0.12 [a]	231.50 ± 18.96 [a]	$1.08X10^{-03}$ ± $5.03X10^{-05}$ [a]
BB	3.87 ± 0.27 [b]	178.25 ± 2.17 [b]	$1.73X10^{-03}$ ± $9.29X10^{-06}$ [b]
EW	4.52 ± 0.28 [b]	129.10 ± 18.91 [c]	$1.82X10^{-03}$ ± $1.09X10^{-04}$ [b]

Data are mean (± SE; n=3); different superscript letters represent significant difference ($P < 0.05$). The AFBO column represents the bioactivation of Aflatoxin B_1 (AFB_1) to the AFB_1-8,9-epoxide (AFBO) intermediate in microsomes, calculated for the 128 μM AFB_1 treatment.
[1] AFB_1-8,9-epoxide (AFBO); GSH (5mM)

In humans and in most animals studied, the principal route of detoxification and single most important determinant for species resistance regardless of efficiency of AFB_1 bioactivation is via hepatic GSTs (Eaton and Bammler, 1999). As shown in Figure 6, liver cytosolic fractions prepared from all species expressed both GSTA3 and GSTA4, subunits that putatively catalyze conjugation of AFBO with GSH (Kim, et al., 2013). Bands were observed for GSTA3 and GSTA4 at the expected a MW of 29 kDa (Figure 6 A and B) (Kim, et al., 2011). Apparent expression shows significant difference in expression of GSTA3 between turkey and quail (Figure 6).

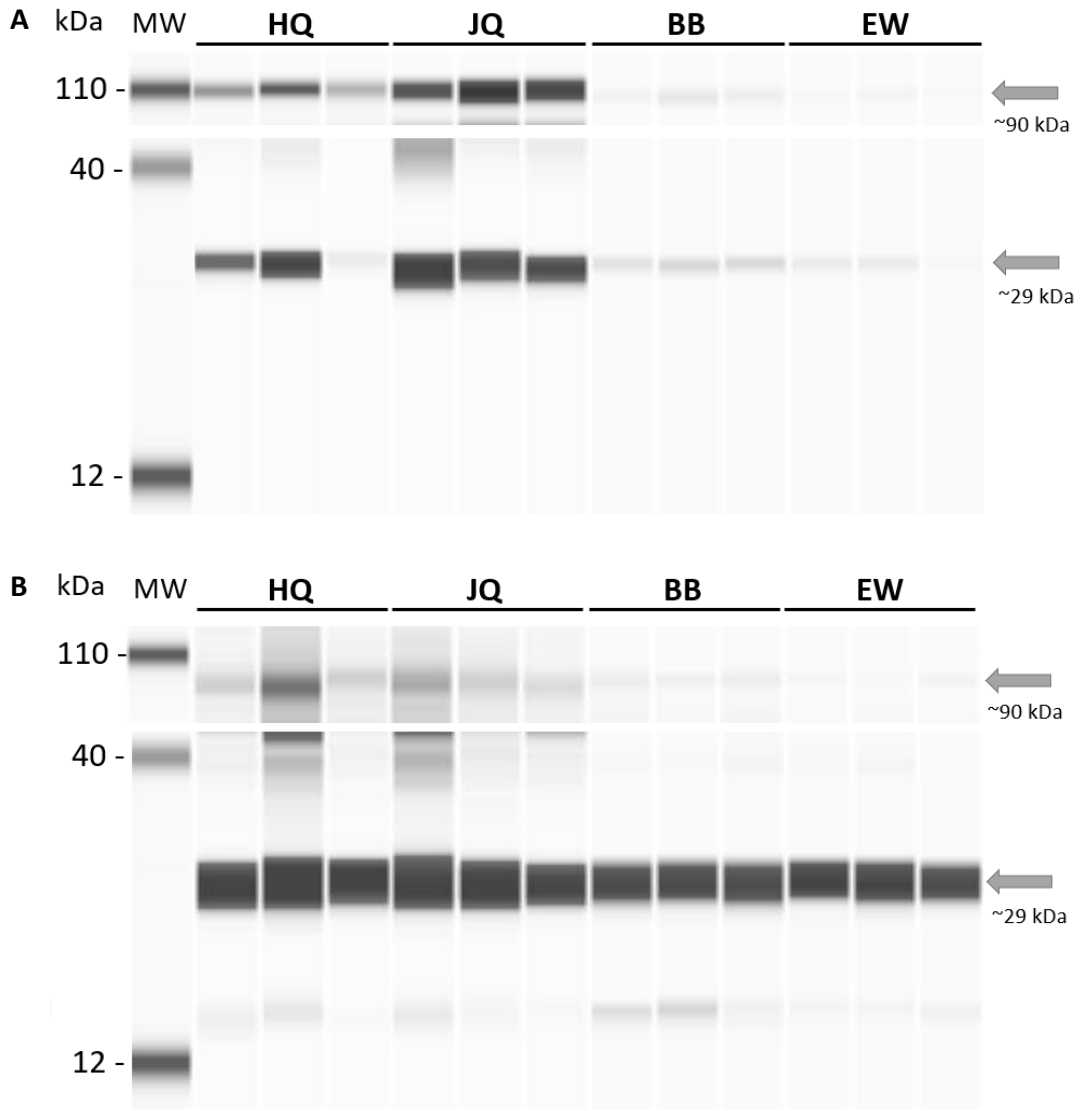

Figure 6: **Immunoblots of Japanese (JQ) and Hawaiian Quail (HQ), and Broad-breasted White (BB) and Eastern Wild (EW) Turkey Hepatic Protein Expression for GSTA3 (A) and GSTA4 (B)**. **A] GSTA3 B] GSTA4.** Custom Turkey anti-GSTA peptides and HRP-conjugated anti-Rabbit secondary antibody were used and bands were detected by chemiluminescence analysis. Each sample represents two pooled liver samples corresponding to three unique pooled cytosol samples. Arrows indicate the protein band of interest and the associated molecular weight. Blots were truncated between ~50-110kd.

The most specific and state-of-the-art method of measuring GST-mediated AFB_1 detoxification in liver cytosolic fractions is by an HPLC-based method that employs liver microsomes to generate AFBO that is then trapped by GSTs in the cytosolic fraction (Eaton, et

al., 1994). Cytosolic AFBO detoxification in livers prepared from both quail and turkeys exhibited traditional Michaelis saturation kinetics (Figure 7). At a substrate concentration of 128 μM, hepatic cytosols from quail were significantly more active in AFB-GSH formation than that in turkeys (HQ: 188.69 ± 0.13; JQ: 504.17 ± 0.24; BB: n.d.; EW: 18.40 ± 0.40 pmol/min/mg protein). Among quail, conjugation activity in JQ was significantly greater than in HQ (Table 2).

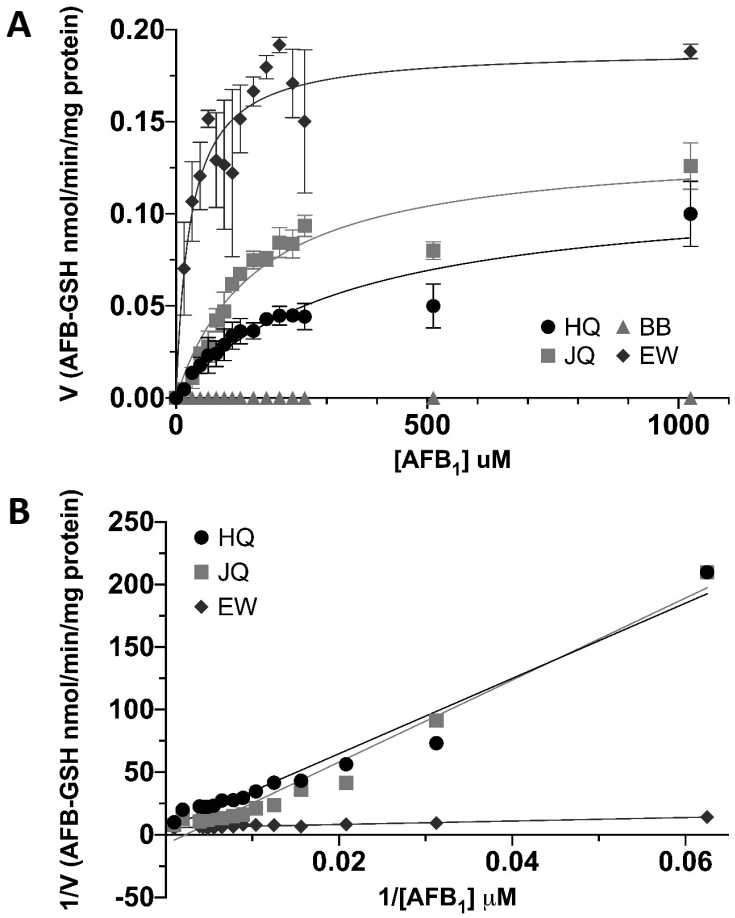

Figure 7: ***Michaelis-Menten (A) and Lineweaver-Burke (B) plots showing kinetics of GST-mediated AFBO detoxification by Japanese and Hawaiian Quail (JQ, HQ) and Domesticated and Eastern Wild Turkey (BB, EW) Liver Cytosols.*** *Enzyme kinetics of AFB_1 detoxification in liver cytosolic proteins (A and B). Saturation curve was determined using AFB_1 concentrations of 10 to 1024 μM. Panel A shows SE for each data point with an N of 3. R^2 values were 0.92, 0.81, 1.00, 0.79 respectively.*

The AFB-GSH formation, presented as V_{max} rates, is significantly different between HQ and JQ. However, both quail species presented rates slightly lower than that of EW. Cytosolic AFBO detoxification in livers from HQ and JQ exhibited significantly lower catalytic affinity of enzyme for the AFBO substrate (K_m), and lower specificity (K_{cat}) than that from EW. (Table 2).

Detoxification of the prototype substrates CDNB, DCNB, ECA and CHP provide further information on hepatic GST activity, though these activities are not relevant to AFB_1 *per se* but can be used in larger cross-species comparisons. Unlike BB and EW, neither HQ nor JQ possessed the ability to metabolize DCNB, while quail had significantly lower metabolic activity toward CDNB than turkeys (Table 3). Cytosols from both quail species had lower metabolic activities toward ECA and CHP compared to those from BB and EW.

Table 2 **Kinetics of Hepatic Cytosolic GST mediated Detoxification of AFBO to AFB-GSH in Hawaiian (HQ) and Japanese Quail (JQ) and, Broad-breasted White (BB) and Eastern Wild (EW) Turkeys.**

	V_{max} (nmol/min/mg protein)	K_m (μM AFB_1)	K_{cat} (s^{-1})
HQ	0.12 ± 0.02 [a]	169.82 ± 32.71 [a]	$1.75X10^{-05}$ ± $8.76X10^{-06}$ [a]
JQ	0.15 ± 0.01 [b]	184.34 ± 25.09 [b]	$3.25X10^{-05}$ ± $2.03X10^{-06}$ [b]
BB	n.d.	n.d.	n.d.
EW	0.18 ± 0.01 [c]	27.03 ± 9.69 [c]	$4.21X10^{-05}$ ± $4.21X10^{-06}$ [c]

Data are mean (± SE; n=3); different superscript letters represent significant difference ($P < 0.05$). The AFB-GSH column represents the formation of the trapped epoxide in cytosol, calculated for the 128 μM AFB_1 treatment.
Abbreviations: n.d. is not detected.

Table 3 Cytosolic Detoxification of GST Pseudosubstrates in Hepatic Cytosols from Hawaiian (HQ) and Japanese Quail (JQ) and, Broad-breasted White (BB) and Eastern Wild (EW) Turkeys.

	Specific enzyme activity (nmol/min/mg protein)			
	CDNB[1]	**DCNB[2]**	**ECA[3]**	**CHP[4]**
HQ	19.10 ± 1.67 [a]	n.d.	25.38 ± 0.10 [a]	141.33 ± 4.94 [a]
JQ	25.64 ± 1.77 [a]	n.d.	27.45 ± 1.44 [a]	172.86 ± 8.37 [a]
BB	380.40 ± 40.07 [b]	0.80 ± 0.08 [a]	78.12 ± 2.67 [b]	56.70 ± 1.14 [b]
EW	306.40 ± 24.99 [b]	0.76 ± 0.10 [a]	47.65 ± 7.43 [c]	46.40 ± 1.98 [b]

Data are mean (± SE; n=3); different superscript letters represent significant difference ($P < 0.05$). The AFBO column represents the bioactivation of Aflatoxin B_1 (AFB_1) to the AFB_1-8,9-epoxide (AFBO) intermediate in microsomes. The AFB-GSH column represents the formation of the trapped epoxide in cytosol.

[1]1-Chloro-2,4-dinitrobenzene (CDNB): GSH (1mM);

[2]1,2-Dichloro-4-nitrobenzene (DCNB): GSH (5mM);

[3]Ethacrynic acid (ECA); GSH (2.5mM);

[4] Cumene Hydroperoxide (CHP); GSH (2mM);

Abbreviations: n.d. is not detected.

Quail are like other avian species regarding metabolism of exogenous substrates by liver enzymes. This study examined two genera of quail, *Gallipepla californica* (HQ) and *Coturnix japonica* (JQ), to rank quail susceptibility to AFB_1 toxicity as a species against turkey of which we have known susceptible (BB) and resistant (EW) varieties (Kim, et al., 2011). Species susceptibility to AFB_1 has been described as a balance between P450-mediated AFBO production, and the efficiency with which this reactive intermediate is detoxified through GST conjugation (Eaton and Gallagher, 1994). Mice efficiently bioactivate AFB_1 to produce AFBO through high-affinity P450s and are substantially resistant to AFB_1 owing to the expression of the A3 GST subunit (mGSTA3), which has a high catalytic activity toward AFBO (Kim, et al., 2011). The current knowledge indicates that the efficiency of GST conjugation is a principal "rate-limiting" determinant for AFB_1 action in individuals and species (Ilic, et al., 2010). Investigations from our laboratory have previously demonstrated the extreme sensitivity of turkeys to the effects of AFB_1, due to the presence of high affinity and high epoxidation activity of P450s toward AFB_1 (Rawal, et al., 2010a; Rawal *et al.*, 2009; Rawal, et al., 2010b; Yip and

Coulombe, 2006). The toxic effects of the bioactivated AFBO metabolite in quail was studied by the Oliveira group who observed typical aflatoxicosis symptoms such as, hepatic cell vacuolation with fatty infiltration in all treatment groups (Oliveira *et al.*, 2002). Additionally, bile duct proliferation and trabecular disorder in treatment groups that received 0.1 ppm AFB_1 (Oliveira, et al., 2002). These studies provided important information on the acute and chronic effects of AFB_1 for quail production, giving insight to the relevant toxic doses and confirming that quail suffer from the same toxic effect as other poultry species.

Only a few research groups have done metabolic analysis of quail enzymatic bioactivation and detoxification toward AFB_1 and AFBO. As was observed in turkey hepatic microsomes (Rawal, et al., 2010b), cytosols (Kim, et al., 2011), and expressed tGSTAs, quail microsomal and cytosolic proteins possess catalytic activity for both bioactivating AFB_1 to AFBO and detoxifying AFBO to AFB-GSH. There have not been any studies, to date, that address the relative susceptibility of quail versus other Galliformes to AFB_1 insult. Those previous studies focused more on the transportation and movement of AFB_1 and related metabolites within a given species of bird. These studies cannot accurately be analyzed and compared to the current study. The current study utilized the same techniques previously performed on turkey species to determine relative resistance and susceptibility toward AFB_1. Compared to previous studies utilizing the same techniques and end points it is clear that quail microsomes are not as efficient at bioactivating AFB_1 to AFBO (Rawal, et al., 2010b). However, quail cytosols possess similar catalytic detoxifying activity toward AFBO compared to turkeys (Kim, et al., 2011).

As previously observed in turkey hepatic cytosols (Kim, et al., 2011), quail cytosols possessed catalytic activity toward the GST substrates CDNB, ECA, and CHP, however, DCNB

activity was not detected. Similar observations have been previously made (Dai *et al.*, 1996), however, the GSTs isolated were fractioned and tested individually, as opposed to using whole cytosolic proteins. The Dai group observed similar trends in the conjugating activity of quail GSTs toward prototype substrates. Notably, DCNB is not a suitable substrate for quail Alpha class GSTs as either no or only marginal activity was measured, a conclusion that is similar to current study. However, the specific activity of the enzymes is different, most likely an effect of the difference in and the purity of the protein preparations. Therefore, direct comparisons cannot be made between the two studies as the protein preparations are quite different; however, activity was detected for CDNB, CHP, and ECA in both studies. The GST enzyme activities for the prototypical substrates were determined using established protocols (Habig and Jakoby, 1981; Lawrence and Burk, 1976).

The molecular weights (MW) of P450 CYP1A5 and CYP3A37 along with GSTA3 and GSTA4 were consistent with the expected molecular weights of analogous proteins in turkey. The MW of quail P450s and GSTs observed in this study correlate with the MW observed previously for CYP1A5 (Diaz, et al., 2010a) and CYP3A37 (Dai, et al., 1996). Where the Diaz group only observed quail P450 enzymes analogous to the hCYP2A6 and hCYP3A4 families the current study observed the presence of P450 enzymes analogous to tCYP1A5 and tCYP3A37, homologs of human CYP1A2 and 3A4, using turkey antipeptide serum to label the proteins (Diaz, et al., 2010a). The Dai group (Dai, et al., 1996) observed molecular weights between 26 – 27 kDa as opposed to the 29 kDa observed in this study for the GSTA enzymes. This observation confirms that quail hepatic proteins possess P450 and GSTA enzymes homologous to human and turkey enzymes (Dai, et al., 1996).

The current study characterized the kinetics of quail P450s and GSTs to understand the susceptibility of quail to the toxic effects of AFB_1 in comparison to other Galliformes. The data indicates there is as much variation between quail genera as there is between poultry species, which suggests that quail susceptibility to AFB_1 varies between genera. Quail P450s bioactivate AFB_1 to AFBO at a significantly slower rate (V_{max}), a greater substrate concentration (K_m), and at a slower turnover rate (K_{cat}) than those of turkeys. Concluding that quail P450s are less efficient at bioactivating AFB_1 to AFBO. The detoxifying activity of quail GSTs toward AFBO is significantly different from both mouse and turkey cytosolic proteins. The EW turkey species possessed greater detoxifying ability, while the two genera of quail are statistically different from one another with JQ having greater detoxifying activity than that of HQ. The expression of each metabolizing enzyme isoform and the correlated kinetic rates indicate the observed difference in susceptibility. Quail livers expressed more CYP1A5 than turkey livers did, however, turkey liver CYP1A5 proteins have an overall lower K_m of bioactivation. Leading to the conclusion that the order of susceptibility based on bioactivation is not different than what has previously been published, ducklings >>> turkey (EW > BB) > quail (JQ > HQ) > mouse. Conversely, quail livers expressed more GSTA3 enzyme with a significantly greater cytosolic protein detoxifying K_m compared to the BB turkey species, with JQ being more efficient than HQ. Interestingly the detoxifying ability of EW turkey species is more efficient than both of the quail species and the BB turkey species. In terms of detoxification the data concludes that the order of susceptibility is ducklings >>> turkey (BB) > quail (HQ > JQ)> turkey (EW) > mouse. Additional studies are needed to further clarify our understanding of quail phase I and II metabolic enzymes in relation to other Galliformes. Specifically, additional studies isolating and identifying the

specific contributions of the identified individual P450s and GSTA enzymes involved in the metabolism of AFB_1 with a greater number of individual animals and variety of poultry species. The study should prioritize characterizing the above poultry species utilizing the AFB-GSH detoxified metabolite as the endpoint. The AFB-GSH detoxified metabolite is the best metric to determine susceptibility of poultry species to the toxic effects of AFB_1. As such, a study in which duck, chicken, turkey, quail, pheasant, mice, and rats are all characterized at the same time will provide the most comprehensive data on the matter.

Bibliography

Arafa, A. S., Bloomer, R. J., Wilson, H. R., Simpson, C. F., and Harms, R. H. (1981). Susceptibility of various poultry species to dietary aflatoxin. *Br Poult Sci* **22**(5), 431-6.

Awasthi, Y. C. (2007). *Toxicology of glutathione transferases*. CRC Taylor & Francis, Boca Raton, FL.

Bailey, G. S., Taylor, M. J., Loveland, P. M., Wilcox, J. S., Sinnhuber, R. O., and Selivonchick, D. P. (1984). Dietary modification of aflatoxin B1 carcinogenesis: mechanism studies with isolated hepatocytes from rainbow trout. *Natl Cancer Inst Monogr* **65**, 379-85.

Bailey, G. S., Williams, D. E., Wilcox, J. S., Loveland, P. M., Coulombe, R. A., and Hendricks, J. D. (1988). Aflatoxin B1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. *Carcinogenesis* **9**(11), 1919-26.

Ball, R. W., and Coulombe, R. A., Jr. (1991). Comparative biotransformation of aflatoxin B1 in mammalian airway epithelium. *Carcinogenesis* **12**(2), 305-10.

Ball, R. W., Wilson, D. W., and Coulombe, R. A., Jr. (1990). Comparative formation and removal of aflatoxin B1-DNA adducts in cultured mammalian tracheal epithelium. *Cancer Res* **50**(16), 4918-22.

Bedard, L. L., Alessi, M., Davey, S., and Massey, T. E. (2005). Susceptibility to aflatoxin B1-induced carcinogenesis correlates with tissue-specific differences in DNA repair activity in mouse and in rat. *Cancer Res* **65**(4), 1265-70.

Bennett, J. W., and Klich, M. (2003). Mycotoxins. *Clin Microbiol Rev* **16**(3), 497-516.

Blount, W. P. (1961). Turkey "X" disease. *Turkeys* **9**(7), 52-77.

Bressac, B., Kew, M., Wands, J., and Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* **350**(6317), 429-31.

Busby, W. F., and Wogan, G. (1984). Aflatoxins. In "Chemical Carcinogenesis". *American Chemical Society*, 945-1136.

CAST (1989). Council for Agricultural Science and Technology. Mycotoxins: Economic and Health Risks,. *CAST, Ames, IA,* **116**, 52.

CAST (2003). Council for Agricultural Science and Technology. Mycotoxins: risks in plant, animal, and human systems. *CAST, Ames, IA,* **139**, 63-4, 140.

Coles, B. F., and Kadlubar, F. F. (2003). Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *Biofactors* **17**(1-4), 115-30.

Coulombe, R. A., Guarisco, J. A., Klein, P. J., and Hall, J. O. (2005). Chemoprevention of aflatoxicosis in poultry by dietary butylated hydroxytoluene. *Anim Feed Sci Tech* **121**(1-2), 217-25.

Coulombe, R. A., Jr. (1993). Biological action of mycotoxins. *J Dairy Sci* **76**(3), 880-91.

Dai, H., Edens, F. W., and Roe, R. M. (1996). Glutathione S-transferases in the Japanese quail: tissue distribution and purification of the liver isozymes. *J Biochem Toxicol* **11**(2), 85-96.

Dalvi, R. R. (1986). An overview of aflatoxicosis of poultry: Its characteristics, prevention and reduction. *Vet. Res. Commun.* **10**(6), 429-43.

Deng, J., Zhao, L., Zhang, N. Y., Karrow, N. A., Krumm, C. S., Qi, D. S., and Sun, L. H. (2018). Aflatoxin B1 metabolism: Regulation by phase I and II metabolizing enzymes and chemoprotective agents. *Mutat Res* **778**, 79-89.

Diaz, G. J., Murcia, H. W., and Cepeda, S. M. (2010a). Cytochrome P450 enzymes involved in the metabolism of aflatoxin B1 in chickens and quail. *Poultry science* **89**(11), 2461-9.

Diaz, G. J., Murcia, H. W., Cepeda, S. M., and Boermans, H. J. (2010b). The role of selected cytochrome P450 enzymes on the bioactivation of aflatoxin B1 by duck liver microsomes. *Avian Pathol* **39**(4), 279-85.

Diekman, M. A., and Green, M. L. (1992). Mycotoxins and reproduction in domestic livestock. *J Anim Sci* **70**(5), 1615-27.

Eaton, D. L., and Bammler, T. K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci* **49**(2), 156-64.

Eaton, D. L., and Gallagher, E. P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol* **34**, 135-72.

Eaton, D. L., Ramsdell, H. S., and Neal, G. E. (1994). Biotransformation of Aflatoxins. *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Signigicance*, 45-72.

Eaton, D. L., and Schaupp, C. M. (2014). Of mice, rats, and men: Could Nrf2 activation protect against aflatoxin heptocarcinogenesis in humans? doi: 10.1158/1940-6207.CAPR-14-0119.

Edds, G. T. (1973). Acute aflatoxicosis: A review. *J Am Vet Med Assoc* **162**(4), 304-9.

Foster, P. L., Eisenstadt, E., and Miller, J. H. (1983). Base substitution mutations induced by metabolically activated aflatoxin B1. *Proc Natl Acad Sci U S A* **80**(9), 2695-8.

Gallagher, E. P., Kunze, K. L., Stapleton, P. L., and Eaton, D. L. (1996). The kinetics of aflatoxin B1 oxidation by human cDNA-expressed and human liver microsomal cytochromes P450 1A2 and 3A4. *Toxicol Appl Pharmacol* **141**(2), 595-606.

Galvano, F., Galofaro, V., and Galvano, G. (1996). Occurrence and stability of aflatoxin M1 in milk and milk products: A worldwide review. *J Food Prot* **59**(10), 1079-90.

Gold, L. S., Sawyer, C. B., Magaw, R., Backman, G. M., de Veciana, M., Levinson, R., Hooper, N. K., Havender, W. R., Bernstein, L., Peto, R*., et al.* (1984). A carcinogenic potency database of the standardized results of animal bioassays. *Environ Health Perspect* **58**, 9-319.

Guarisco, J. A., Hall, J. O., and Coulombe, R. A., Jr. (2008). Mechanisms of butylated hydroxytoluene chemoprevention of aflatoxicosis--inhibition of aflatoxin B1 metabolism. *Toxicol Appl Pharmacol* **227**(3), 339-46.

Guengerich, F. P. (2017). Intersection of roles of cytochrome P450 enzymes with xenobiotic and endogenous substrates. Relevance to toxicity and drug Interactions. *Chem Res Toxicol* **30**(1), 2-12.

Habig, W. H., and Jakoby, W. B. (1981). Assays for differentiation of glutathione S-transferases. *Methods Enzymol* **77**, 398-405.

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974a). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**(22), 7130-9.

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974b). Glutatione S-Transferase. *The Journal of Biological Chemistry* **249**(22), 7130-9.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* **350**(6317), 427-8.

IARC (1993). Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 56.
IARC Working Group on the Evaluation of Carcinogenic Risk to Humans.
Lyon (FR): International Agency for Research on Cancer; 1993.

IARC (2012). Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 100F.
IARC Working Group on the Evaluation of Carcinogenic Risk to Humans.
Lyon (FR): International Agency for Research on Cancer; 2012.

Ilic, Z., Crawford, D., Vakharia, D., Egner, P. A., and Sell, S. (2010). Glutathione-S-transferase A3 knockout mice are sensitive to acute cytotoxic and genotoxic effects of aflatoxin B1. *Toxicol Appl Pharmacol* **242**(3), 241-6.

Irvin, T. R., and Wogan, G. N. (1984). Quantitation of aflatoxin B1 adduction within the ribosomal RNA gene sequences of rat liver DNA. *Proc Natl Acad Sci U S A* **81**(3), 664-8.

Iyer, R. S., Coles, B. F., Raney, K. D., Thier, R., Guengerich, F. P., and Harris, T. M. (1994). DNA adduction by the potent carcinogen aflatoxin B1: Mechanistic studies. *Journal of the American Chemical Society* **116**(5), 1603-9.

Kalina, J., Mucksova, J., Yan, H., and Trefil, P. (2012). Rapid sexing of selected Galliformes by polymerase chain reaction. *Czech J. Anim. Sci.* **57**(14), 187-92.

Kensler, T. W., Egner, P. A., Trush, M. A., Bueding, E., and Groopman, J. D. (1985). Modification of aflatoxin B1 binding to DNA in vivo in rats fed phenolic antioxidants, ethoxyquin and a dithiothione. *Carcinogenesis* **6**(5), 759-63.

Kensler, T. W., Roebuck, B. D., Wogan, G. N., and Groopman, J. D. (2011). Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci* **120**(Suppl 1), S28-48.

Kim, J. E., Bauer, M. M., Mendoza, K. M., Reed, K. M., and Coulombe, R. A., Jr. (2010). Comparative genomics identifies new alpha class genes within the avian glutathione S-transferase gene cluster. *Gene* **452**(2), 45-53.

Kim, J. E., Bunderson, B. R., Croasdell, A., and Coulombe, R. A., Jr. (2011). Functional characterization of alpha-class glutathione s-transferases from the Turkey (meleagris gallopavo). *Toxicol Sci* **124**(1), 45-53.

Kim, J. E., Bunderson, B. R., Croasdell, A., Reed, K. M., and Coulombe, R. A., Jr. (2013). Alpha-class glutathione S-transferases in wild turkeys (Meleagris gallopavo): Characterization and role in resistance to the carcinogenic mycotoxin aflatoxin B1. *PLoS One* **8**(4), e60662.

Klein, P. J., Buckner, R., Kelly, J., and Coulombe, R. A., Jr. (2000). Biochemical basis for the extreme sensitivity of turkeys to aflatoxin B(1). *Toxicol Appl Pharmacol* **165**(1), 45-52.

Klein, P. J., Van Vleet, T. R., Hall, J. O., and Coulombe, R. A., Jr. (2002a). Biochemical factors underlying the age-related sensitivity of turkeys to aflatoxin B1. *Comp Biochem Physiol C Toxicol Pharmacol* **132**(2), 193-201.

Klein, P. J., Van Vleet, T. R., Hall, J. O., and Coulombe, R. A., Jr. (2002b). Dietary butylated hydroxytoluene protects against aflatoxicosis in Turkeys. *Toxicol Appl Pharmacol* **182**(1), 11-9.

Lawrence, R. A., and Burk, R. F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and biophysical research communications* **71**(4), 952-8 (Research Support, U.S. Gov't, P.H.S.).

Liu, Y. L., Roebuck, B. D., Yager, J. D., Groopman, J. D., and Kensler, T. W. (1988). Protection by 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) against the hepatotoxicity of aflatoxin B1 in the rat. *Toxicol Appl Pharmacol* **93**(3), 442-51.

Lozano, M. C., and Diaz, G. J. (2006). Microsomal and cytosolic biotransformation of aflatoxin B1 in four poultry species. *Br Poult Sci* **47**(6), 734-41.

Mace, K., Aguilar, F., Wang, J. S., Vautravers, P., Gomez-Lechon, M., Gonzalez, F. J., Groopman, J., Harris, C. C., and Pfeifer, A. M. (1997). Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* **18**(7), 1291-7.

McDonnell, A. M., and Dang, C. H. (2013). Basic review of the cytochrome P450 system. *J Adv Pract Oncol* **4**(4), 263-8.

Monson, M., Coulombe, R., and Reed, K. (2015). Aflatoxicosis: Lessons from toxicity and responses to aflatoxin B1 in poultry. *Agriculture* **5**(3), 742-77.

Muller, R. D., Carlson, C. W., Semeniuk, G., and Harshfield, G. S. (1970). The response of chicks, ducklings, goslings, pheasants and poults to graded levels of aflatoxins. *Poult Sci* **49**(5), 1346-50.

Nebert, D. W., and Russell, D. W. (2002). Clinical importance of the cytochromes P450. *Lancet* **360**(9340), 1155-62.

Newberne, P. M., and Butler, W. H. (1969). Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Res* **29**(1), 236-50.

Oliveira, C., Rosmaninho, J., Butkeraitis, P., Correa, B., Reis, T., Guerra, J., Albuquerque, R., and Moro, M. (2002). Effect of low levels of dietary aflatoxin B1 on laying japanese quail. *Poultry Science* **81**(7), 976-80.

Pandey, I., and Chauhan, S. S. (2007). Studies on production performance and toxin residues in tissues and eggs of layer chickens fed on diets with various concentrations of aflatoxin AFB1. *Br Poult Sci* **48**(6), 713-23.

Pitt, J. I. (2013). Mycotoxins. In *Foodborne Infections and intoxications*, Vol. Fourth Volume, pp. 409-18. Academis Press.

Polychronaki, N., P, C. T., Mykkanen, H., Gong, Y., Amra, H., Abdel-Wahhab, M., and El-Nezami, H. (2006). Determinants of aflatoxin M1 in breast milk in a selected group of Egyptian mothers. *Food Addit Contam* **23**(7), 700-8.

Raney, K. D., Shimada, T., Kim, D. H., Groopman, J. D., Harris, T. M., and Guengerich, F. P. (1992). Oxidation of aflatoxins and sterigmatocystin by human liver microsomes: significance of aflatoxin Q1 as a detoxication product of aflatoxin B1. *Chem Res Toxicol* **5**(2), 202-10.

Rawal, S., and Coulombe, R. A., Jr. (2011). Metabolism of aflatoxin B(1) in turkey liver microsomes: The relative roles of cytochromes P450 1A5 and 3A37. *Toxicology and Applied Pharmacology* **254**(3), 349-54.

Rawal, S., Kim, J. E., and Coulombe, R., Jr. (2010a). Aflatoxin B1 in poultry: toxicology, metabolism and prevention. *Res Vet Sci* **89**(3), 325-31.

Rawal, S., Mendoza, K. M., Reed, K. M., and Coulombe, R. A., Jr. (2009). Structure, genetic mapping, and function of the cytochrome P450 3A37 gene in the turkey (Meleagris gallopavo). *Cytogenet Genome Res* **125**(1), 67-73.

Rawal, S., Yip, S. S., and Coulombe, R. A., Jr. (2010b). Cloning, expression and functional characterization of cytochrome P450 3A37 from turkey liver with high aflatoxin B1 epoxidation activity. *Chem Res Toxicol* **23**(8), 1322-9.

Reed, K. M., Mendoza, K. M., and Coulombe, R. A., Jr. (2019). Differential transcriptome responses to aflatoxin B_1 in the cecal tonsil of susceptible and resistant turkeys. *Toxins* **11**(1), 55.

Roebuck, B. D., Liu, Y. L., Rogers, A. E., Groopman, J. D., and Kensler, T. W. (1991). Protection against aflatoxin B1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz): predictive role for short-term molecular dosimetry. *Cancer Res* **51**(20), 5501-6.

Shank, R. C., Siddhichai, P., Subhamani, B., Bhamarapravati, N., Gordon, J. E., and Wogan, G. N. (1972). Dietary aflatoxins and human liver cancer. V. Duration of primary liver cancer and prevalence of hepatomegaly in Thailand. *Food Cosmet Toxicol* **10**(2), 181-91.

Smela, M. E., Hamm, M. L., Henderson, P. T., Harris, C. M., Harris, T. M., and Essigmann, J. M. (2002). The aflatoxin B1 formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. In *Proc Natl Acad Sci U S A*, Vol. 99, pp. 6655-60.

Staels, B., and Fonseca, V. A. (2009). Bile Acids and Metabolic Regulation: Mechanisms and clinical responses to bile acid sequestration. In *Diabetes Care*, Vol. 32, pp. S237-45.

Stevens, A. J., C. N. Saunders, Spence, J. B., and Newnham, A. G. (1960). Investigations into Disease of Turkey Poults. *Vet. Rec.* **72**(31), 627-28.

Wang, J. S., Shen, X., He, X., Zhu, Y. R., Zhang, B. C., Wang, J. B., Qian, G. S., Kuang, S. Y., Zarba, A., Egner, P. A*., et al.* (1999). Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. *J Natl Cancer Inst* **91**(4), 347-54.

Wannop, C. C. (1960). Disease of turkey poults. *Vet. Rec.* **72**(33), 671-72.

Wogan, G. N. (1992). Aflatoxins as risk-factors for hepatocellular-carcinoma in humans. *Cancer Research* **52**(7), S2114-S8.

Yip, S. S., and Coulombe, R. A., Jr. (2006). Molecular cloning and expression of a novel cytochrome p450 from turkey liver with aflatoxin b1 oxidizing activity. *Chem Res Toxicol* **19**(1), 30-7.

Yu, F.-L. (1983). Preferential binding of aflatoxin B1 to the transcriptionally active regions of rat liver nucleolar chromatin *in vivo* or *in vitro*. *Carcinogenesis* **4**(7), 889-93.

Yu, J., Chang, P. K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, G. A., Linz, J. E., Woloshuk, C. P., and Bennett, J. W. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl Environ Microbiol* **70**(3), 1253-62.

Zamek-Gliszczynski, M. J., Hoffmaster, K. A., Nezasa, K.-I., Tallman, M. N., and Brouwer, K. L. R. (2006). Integration of hepatic drug transporters and phase II metabolizing enzymes: Mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *European Journal of Pharmaceutical Sciences* **27**(5), 447-86.

Ziglari, T., and Allameh, A. (2013). The significance of glutathione conjugation in aflatoxin metabolism. In *Aflatoxins - Recent Advances and Future Prospects* (M. Razzaghi-Abyaneh, Ed.), Vol. 2, pp. 129-35. InTech.