DETECTING AFLATOXICOSIS IN BROILERS IN THE EVALUATION OF CLAY-BASED, TOXIN-BINDING FEED ADDITIVES

A Dissertation

by

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ABSTRACT

The objectives of this research were to evaluate common biological measures of aflatoxicosis in broilers (such as growth rate and relative organ weights) along with variables such as hepatic gene expression and aflatoxin residues in the liver, pursuant to identifying a more sensitive biological assay that will allow researchers to conduct three-week broiler trials at aflatoxin concentrations <1000 ppb, prior to significant changes in the growth rate or relative organ weights. This will help us both better understand how aflatoxicosis presents in broilers, as well as help us evaluate the efficacy of clay-based binders for their ability to ameliorate aflatoxicosis under experimental conditions.

In the first study, a recently mined calcium bentonite clay (TX4) was evaluated against Novasil™. Both clays appeared able to sequester aflatoxin, and overall TX4 appeared capable of ameliorating aflatoxicosis comparable to Novasil™.

In the second study, growth and relative organ weight data were compared with the gene expression of hepatic enzymes known to detoxify aflatoxin B_1 in broilers that had consumed a wide range of aflatoxin concentrations. When gene expression data from liver samples were analyzed, the genotypic effect of aflatoxin on the CYP1A1 and CYP2H1 isoforms simply mirrored the phenotypic effects seen in the growth and relative organ weights, suggesting that this variable was not any more sensitive than the more traditional ones.

The third study evaluated the TX4 clay when in diets containing <1000 ppb aflatoxin. Although weight gain was unaffected by aflatoxin at these lower levels (after three weeks on treatment diets, body weights between the 0 ppb treatment and the 700

ppb treatment only varied by 4%), there were negative effects on feed conversion and productivity index and there was an increase in the relative weights of the liver and kidney. The inclusion of TX4 to the treatment diets did not offer any amelioration from the main effects of aflatoxin.

Finally, a study was conducted to evaluate the effects of TX4 clay when using residues of aflatoxin B_1 in the liver as the primary variable of interest. Results after one week on treatment diets showed that TX4 was effective at reducing the accumulation of aflatoxin B_1 residues in liver. However, after the first week, liver residue data were not any more sensitive in evaluating aflatoxin or clay effects when compared to the "traditional" measures of growth performance and organ weights. Also, these results indicate that the clearance time required to remove aflatoxin residues from the liver is less than one week on a clean corn diet.

Based on these evaluations, attempts to characterize a more sensitive, sentinel-type response to aflatoxin exposure in broilers were not any more successful at evaluating aflatoxicosis than was the common bioassay measures such as growth rate and relative organ weights. These studies (by contaminating corn with aflatoxigenic species of *Aspergillus*) were able to find significant main effects for aflatoxin at lower concentrations (≤1000 ppb) than had been previously reported by the studies that included inoculated rice.

DEDICATION

For my companion and our fantastic four children.

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CHAPTER I

INTRODUCTION

Mycotoxins are a group of secondary metabolites that are produced by certain fungal species (e.g. *Aspergillus*, *Penicillium*, and *Fusarium*). Secondary metabolites are a category of compounds that are produced by an organism but that are not considered essential for their life-sustaining processes. Within the family of mycotoxins, there are compounds with varying chemical structures, biological properties, and levels of toxicity. Aflatoxins are a member of this mycotoxin family and are among the most potent natural carcinogens known to exist. Aflatoxins are detectable under ultraviolet light. Depending on the color of fluorescence, they are divided into aflatoxin B_1 and B_2 for blue (425 nm), and G_1 and G_2 for green (450 nm). In addition, there is aflatoxin M_1 and M_2, which are, respectively, detoxified metabolites of aflatoxin B_1 and B_2 found in milk or urine. Alternatively, the carbonyl oxygen on the furan ring of aflatoxin B_1 can be reduced to a hydroxyl group, forming a more water soluble metabolite, aflatoxicol. Of the categories of aflatoxin, aflatoxin B_1 has the most potent biological effects because of its ability to form an epoxide on the terminal furan ring, which easily binds to guanine base pairs and to proteins.

The fungal species that produce aflatoxin (e.g. *Aspergillus flavus* and *Aspergillus parasiticus*) are known to infect key feed stuffs that are used for animal production such as corn, sorghum, wheat, rice, cottonseed, and peanuts. Further, these species are ubiquitous and can contaminate grain or seed prior to harvest as well as during storage. Once produced, aflatoxins are relatively stable compounds and are not destroyed by feed

processing. In fact, they can be concentrated by processes such as distillation. Toxin production is most favorable under conditions of periods of high temperature, high humidity, drought, and insect damage. Aflatoxin is one of the most common mycotoxins found to contaminate feeds in warm and humid climates. No animal species is immune to its acute toxic effects, including humans.

Awareness of the problems associated with aflatoxin contamination in commercial poultry production began in 1960 when more than 100,000 turkeys in England died from what was initially characterized as "Turkey X Disease". It was later discovered that the Brazilian peanut meal used in that operation's diet formulation was contaminated with aflatoxin (Blount, 1961). Aflatoxin exerts its toxic effects upon absorption in the gastrointestinal tract and bioactivation in the liver. Aflatoxin B_1 undergoes epoxidation at the terminal furan ring (8,9 position) to form the carcinogenic metabolite aflatoxin B_1-8,9 epoxide (Figure 1-1). Alternatively, the carbonyl oxygen on the initial furan ring can be reduced to a hydroxyl group, forming aflatoxicol.

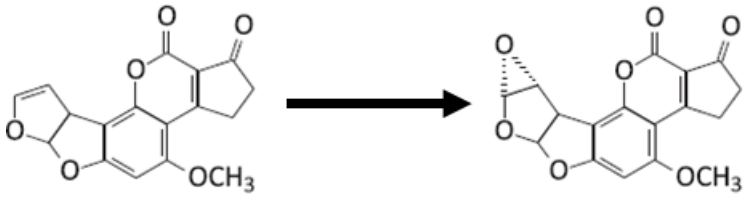


Figure 1-1: Biotransformation of aflatoxin B_1 into aflatoxin B_1-8,9 epoxide

Once activated into this highly toxic metabolite, the molecule is capable of forming adducts with DNA, RNA, and protein, inactivating tumor suppression and impairing protein synthesis and function (Campbell and Hayes, 1976; Phillips, 1999).

Aflatoxin exposure can impair many of the important production parameters in broilers: e.g. feed intake, weight gain, feed conversion, and processing yield. The inclusion of non-nutritive, clay-based adsorbents as aflatoxin binders in animal feeds has been shown through recent decades of research to have a significant protective effect against aflatoxicosis in a variety of species. Although these clay-based binders are generally recognized as safe (GRAS) to be used in diets for improved flowability, anti-caking, and pellet quality, no adsorbent has been approved by the Food and Drug Administration (FDA) for the prevention or treatment of aflatoxicosis.

The reported data on the effects of aflatoxin on the relative organ weights and serum biochemistry of broilers suggest that the liver can be considered the "target organ" for aflatoxicosis. Thus, the expression of specific genes in the liver tissue represent a likely "first stop" in the biological response to aflatoxin, as the contents absorbed by the gastrointestinal tract must all travel first to the liver via the portal blood system. The cytochrome p450 class of enzymes (CYP) are involved in the biotransformation of various xenobiotic compounds into metabolites that are more water soluble and available for excretion. Though the CYP-mediated reactions are essential for detoxification, they are responsible for the bioactivation of aflatoxin B_1 into the carcinogenic metabolite aflatoxin B_1-8,9 epoxide. Previous research has found that certain CYP-isoforms (CYP1A1 and CYP2H1) are upregulated while epoxide hydrolase, GST, and glutathione peroxidase genes are downregulated by 2000 ppb aflatoxin. This is a combination of factors that favors the formation of aflatoxin B1-8,9 epoxide, while providing less of a chance for any biological antioxidant response (Yarru, et al. 2009, 1). In Yarru, et al.

(2009, 2), there were significant increases in the expression rate of the CYP1A1 and CYP2H1 isoforms. It is likely that the liver will respond to dietary aflatoxin by upregulating specific CYP-isoforms prior to the overt tissue damage and lipid accumulation that characterizes aflatoxicosis at levels >1000 ppb.

Also, aflatoxins can accumulate in the milk of dairy cattle, as well as in the muscle, liver, and eggs of poultry (Neeff, et al. 2013 and Zaghini, et al. 2005) when detoxification cannot keep up with the intake from the diet. It is possible for there to be detectable levels of aflatoxin residues even when the animal's growth and performance are not noticeably affected. Since the liver is the first organ to receive any of the absorbed contents of the gastrointestinal tract via the hepatic portal system in poultry, it is the principle organ involved in the detoxification of compounds such as aflatoxin. In addition to that, the liver is also an edible tissue, which makes it of interest for human health as well.

The objectives of this research are to evaluate common biological assays for measuring aflatoxicosis in broilers (such as growth rate and relative organ weights) along with variables such as aflatoxin residues in the liver or hepatic gene expression, pursuant to identifying a more sensitive biological assay that will allow three-week broiler trials to be conducted at aflatoxin concentrations <1000 ppb, prior to significant changes in the growth rate or relative organ weights. This will help us both better understand how aflatoxicosis presents in broilers, as well as help us evaluate the efficacy of novel clay-based binders for their ability to ameliorate aflatoxicosis under experimental conditions.

CHAPTER II

LITERATURE REVIEW

Body weight

Smith and Hamilton (1970) obtained weekly body weights from male broilers reared for three weeks on treatment diets in which aflatoxin was introduced by inoculating *Aspergillus flavus* on rice as described by Shotwell et al. (1966). In the Shotwell et al. procedure, autoclaved rice is inoculated with spores of an aflatoxin-genic strain of *Aspergillus* and allowed to ferment in Erlenmeyer flasks at 28 °C for six days with continuous agitation. The moldy rice product can then be subjected to a chloroform/hexane extraction procedure to collect the aflatoxins. Alternatively, as was done in the Smith and Hamilton study, the rice can be dried and ground into a powder that can be added into diets to achieve the desired concentrations. Diets in Smith and Hamilton (1970) contained dried, ground rice powder so as to achieve the equivalent of 0, 625, 1250, 2500, 5000, and 10,000 ppb aflatoxin in the treatment diets. Aflatoxin concentrations of 625 and 1250 ppb did not reduce body weights during the first three weeks, while treatments at 2500 ppb and above did. In a second trial, Smith and Hamilton fed all broilers an aflatoxin-free control diet for 10 days, put them on the same treatment diets for one week, and then fed the control feed again until 67 days of age. With this feeding methodology, the body weights were not significantly reduced until the 10,000 ppb treatment. One week on the recovery diet of 0 ppb was sufficient to return the rate of growth in that treatment to the level of the control.

Hamilton, et al. (1974) conducted two separate three-week trials to evaluate the effect of aflatoxin in the presence of a vitamin deficiency. The first trial was arranged as a 2x2 factorial with aflatoxin at either 0 or 5000 ppb and the vitamin premix at either sufficient or four-times sufficient amount. Aflatoxin significantly reduced body weights, but the inclusion of four-times the normal amount of vitamin premix had no effect. The second trial fed either 0 or 625 ppb aflatoxin in the presence or absence of either a riboflavin or a vitamin D_3 deficiency to broilers for three weeks. In the non-deficient treatments, 625 ppb did not affect the body weights of three-week old broilers. However, when in the presence of either vitamin deficiency, the effect of aflatoxin on body weight became apparent.

Arafa, et al. (1981) found that broilers, when compared to turkeys and Bobwhite quail, were more robust against aflatoxicosis. Aflatoxin that had been produced by the inoculation of rice with *Aspergillus flavus* was added to diets to achieve 0, 700, 1400, or 2100 ppb and fed to 4 replicates of 4 strains or species of poultry: White Leghorn, broiler, Bobwhite quail, and gosling in a first experiment, and White Leghorn, broiler, Bobwhite quail, and turkey poults in a second experiment. Leghorns and broilers showed no toxic effects due to aflatoxin at any of the treatment levels at any point through the three-week trials. Goslings and turkey poults were the most sensitive, with goslings showing reductions in body weight and feed consumption at 1400 ppb and further reductions at 2100 ppb and turkey poults at 700 ppb. Quail appeared to be intermediate with respect to the response to aflatoxin. Body weight and feed intake were not affected

during the first week, but from the second week until the end of trial were reduced by 2100 ppb aflatoxin.

Doerr, et al. (1983) conducted two trials using male broiler chicks fed 0, 75, 225, or 675 ppb aflatoxin in the first experiment and 0, 300, 900, or 2700 ppb for the second for seven weeks. The aflatoxin for both trials was produced by the inoculation of rice with *Aspergillus parasiticus*. In the first experiment, all dose levels significantly reduced body weight relative to the control. However, carcass yield (the chilled, eviscerated weight as a percent of live weight) was not affected by aflatoxin. However, in the second experiment body weights were only reduced by 2700 ppb aflatoxin, including the percent carcass yield.

Giambrone, et al. (1985, 1) fed purified aflatoxin B_1 in gelatin tablets either alone or in combination with aflatoxin B_2 to two-week old broilers. The tablets were administered to achieve a final toxin exposure equivalent to consuming 0, 100, 200, 400, 500, or 1000 ppb of aflatoxin B_1 or 100, 200, or 400 ppb as aflatoxin B_1 and B_2 in the diet. Birds were fed their respective treatments for five weeks. Aflatoxin B_1 at 500 and 1000 ppb reduced weekly weight gain after two weeks of exposure (four-week old birds), while 100, 200, and 400 ppb only reduced weekly weight gains after the full five weeks (seven-week old birds). However, cumulative weight gains at the end of the seven-week trial were unaffected by any dose of aflatoxin B_1. The highest dose of aflatoxin fed as a combination of B_1 and B_2 (400 ppb) significantly reduced the cumulative five-week average for weight gain, suggesting an interaction effect when both toxins were present.

Miller and Wyatt (1985) fed male broiler chicks for three weeks on diets containing 0, 1250, 2500, and 5000 ppb aflatoxin, which was produced by the inoculation of rice with *Aspergillus parasiticus*. Three-week body weights were not reduced by either 1250 or 2500 ppb aflatoxin but were only decreased when fed the highest dose, 5000 ppb.

Giambrone, et al. (1985, 2) used field-contaminated corn to formulate broiler and turkey diets that contained 0, 100, 200, 400, or 800 ppb aflatoxin as the final concentration in the feed. After three weeks on the treatment diets, broilers showed no reductions in weight gain at any aflatoxin level, whereas the weight gain of turkeys was significantly reduced after one week by 800 ppb. At three weeks of age, both broilers and turkeys were subjected to an immune challenge and observed for two more weeks (a Newcastle disease virus challenge via the ocular route, a *Pasteurella multocida* oral swab, and a delayed hypersensitivity reaction assay against *Mycobacterium* antigen). At the five week time point, broilers still showed no reductions in weight gain (considered on a weekly or on a cumulative basis) at any of the dietary concentrations of aflatoxin. Turkeys, on the other hand, showed a reduction in cumulative 5-week weight gain at 400 ppb aflatoxin and a further reduction at 800 ppb. Upon termination, after five weeks on the aflatoxin, the turkeys on the 800 ppb treatment experienced 100% mortality, supporting the finding of Arafa, et al. (1981) that broilers are more robust against aflatoxin when compared to turkeys.

In Huff, et al. (1986) male broilers were fed treatment diets for three weeks that contained 0, 1250, 2500, or 5000 ppb aflatoxin that had been produced by the inoculation

of rice with cultures of *Aspergillus parasiticus*. To gain a perspective of the progression of aflatoxin exposure over time, body weight was measured at 3, 6, 9, 12, 15, 17, and 21 days of age. Doses as high as 5000 ppb had no effect on body weight at 3 days of age. By 17 days, 1250 ppb was sufficient to significantly decrease body weights, which were further depressed by both 2500 ppb and by 5000 ppb. At the end of three weeks however, only 2500 and 5000 ppb treatments were significantly lower than the control.

Huff, et al. (1988) reared male broilers for three weeks on treatments containing aflatoxin (0 or 2500 ppb) and T-2 toxin (0 or 4000 ppb) either alone or in combination arranged as a 2x2 factorial design. Aflatoxin at 2500 ppb alone reduced body weights beginning at two weeks of age. When aflatoxin was fed in combination with 4000 ppb T-2 toxin, reductions in body weights were significantly greater than when either toxin was fed alone.

Kubena, et al. (1990) fed 7500 ppb aflatoxin as purified aflatoxin B_1 to male broilers for three weeks. Aflatoxin significantly reduced weekly weight gain during all three weeks and reduced cumulative weight gain as well.

When feeding 3500 ppb aflatoxin that had been produced by the inoculation of rice with cultures of *Aspergillus parasiticus* to male broilers for three weeks, Huff, et al. (1992) found that aflatoxin significantly reduced body weights and that there was a greater reduction when aflatoxin was fed in combination with 2000 ppb ochratoxin-A. This, along with the results from Huff, et al. (1988), suggest that aflatoxin can have more pronounced effects when in combination with another mycotoxin (such as T-2 toxin or ochratoxin-A) to cause further pronounced effects.

Kubena, et al. (1993) fed 3500 ppb aflatoxin to male broilers for three weeks and found significant reductions in body weight beginning at two weeks. When aflatoxin was fed in combination with 5000 ppb zearalenone (another mycotoxin), body weights were reduced a week earlier (after one week), again demonstrating that a combination of aflatoxin with another type of mycotoxin can show increased toxicity compared to either toxin fed alone.

When feeding aflatoxin produced by inoculating rice with *Aspergillus parasiticus* at a dietary concentration of 5000 ppb for a three-week trial using male broilers, Kubena, et al. (1998) found that aflatoxin significantly reduced weekly weight gains after two weeks of exposure and that it reduced cumulative three-week weight gains.

Miazzo, et al. (2000) started feeding 2500 ppb aflatoxin to male broilers at three weeks of age for a three-week trial period. Upon termination at six weeks of age, broilers fed aflatoxin had significantly lower weight gains during the trial period.

Oguz, et al. (2000) reared straight-run broilers for six weeks on 0, 50, or 100 ppb aflatoxin produced by the fermentation of rice with cultures of *Aspergillus parasiticus*. Both weekly and cumulative weight gains were unaffected by 50 ppb. Aflatoxin at 100 ppb reduced weight gain during the sixth week, as well as on a cumulative basis.

Raju and Devegowda (2000) fed 300 ppb aflatoxin (either alone or in combination with ochratoxin-A and T-2 toxin) to treatment groups of male broilers for five weeks. Mycotoxins were produced by rice fermentation with fungal cultures of *Aspergillus parasiticus*, *Aspergillus ochraceus*, and *Fusarium sporotrichioides*. Aflatoxin

significantly reduced body weights and those weights were reduced further when aflatoxin was in combination with either other mycotoxin (or all three were fed together).

Treatment diets in Aravind, et al. (2003) were formulated using field contaminated corn to evaluate the efficacy of including esterified glucomannan to ameliorate the toxicity of mycotoxicosis. The final diets contained 168 pbb aflatoxin, as well as 8.4 ppb ochratoxin-A, 54 ppb zearalenone, and 32 ppb T-2 toxin. After five weeks on treatment diets, body weights were significantly reduced by this diverse combination of mycotoxins.

Del Bianchi, et al. (2005) reared male broilers under identical control conditions until three weeks of age, at which point the birds were moved onto their respective treatments. Aflatoxin that had been produced by the inoculation of yeast extract sucrose with *Aspergillus flavus* culture was included in treatment diets at 0, 350, or 2450 ppb either alone or in combination with 10,000 ppb fumonisin in the final feed. All birds were maintained on these treatment diets for three additional weeks (until six weeks of age). There was no effect of the toxins on weight gain for any of the treatments. It is likely that this effect is best explained by the age of the birds at the time the treatment diets were administered.

Raju, et al. (2005) fed 300 ppb aflatoxin to broilers for two separate six-week trials. Aflatoxin for both trials was produced by inoculating rice with cultures of *Aspergillus parasiticus* and then adding the contaminated rice as a dried, ground powder to achieve the desired final concentration. In both trials, body weights were significantly

reduced by 300 ppb aflatoxin. Though body weights were reduced, aflatoxin did not reduce the relative carcass yield.

In order to mirror industry conditions, Bailey, et al. (2006) fed straight-run boilers for six weeks on floor pens and fed them pelleted diets containing 0 or 3600 ppb aflatoxin. Aflatoxin was provided by a combination of field-contaminated corn and both rice and corn inoculated with cultures of *Aspergillus parasiticus*. Treatments were a 2x2 factorial of the two aflatoxin levels with and without 0.5% of the HSCAS Novasil™. The weight gained by birds consuming aflatoxin was significantly reduced at three, five, and six weeks into the study, and also on a cumulative basis. Including the clay-based additive significantly improved weight gains relative to the aflatoxin diet but the birds did not grow as well as the control birds fed no aflatoxin.

Bintvihok, et al. (2006) started three-day old, straight-run broilers on treatment diets containing 0, 50, and 100 ppb aflatoxin B_1. Aflatoxin was produced by the inoculation of corn with *Aspergillus flavus* culture. Birds were fed treatment diets for six weeks. Body weights were recorded every two weeks. After four weeks into the trial (when birds were 31 days old), aflatoxin at both dose levels significantly reduced the weekly weight gains relative to the control.

Gowda, et al. (2008) were successful in reducing cumulative weight gain in three-week old male broilers by feeding aflatoxin at 1000 ppb.

Kermanshahi, et al. (2009) fed 0, 500, or 1000 ppb aflatoxin that had been produced by inoculating rice with *Aspergillus parasiticus* to day-old male broilers for a six-week trial. Body weights were reduced equally by both 500 and 1000 ppb beginning

at week 3. Upon termination at week 6, 1000 ppb reduced body weight to a greater extent than 500 ppb. Aflatoxin also reduced relative carcass, thigh, and breast weights.

Zhao, et al. (2010) supplied 0, 1000, or 2000 ppb aflatoxin by purified *Aspergillus parasiticus* culture material containing 815,000 ppb of aflatoxin B_1. Treatment diets were fed to straight-run broilers for three weeks. Weight gain was not significantly reduced by 1000 ppb, but was reduced by 2000 ppb.

Magnoli, et al. (2011, 1) inoculated rice with *Aspergillus parasiticus* to produce aflatoxin that was added to treatment diets containing a concentration of 50 ppb. Day-old male broilers were reared under identical control conditions for 18 days, at which point treatment diets (containing either 0 or 50 ppb) were fed for four weeks. Under these conditions, aflatoxin had no effect on weight gain. Also, in Magnoli, et al. (2011, 2), birds reared under identical control conditions for five days old were started on treatment diets containing either 0 or 100 ppb aflatoxin and fed treatment diets for four weeks (until 33 days of age). Again aflatoxin had no significant effect on weight gain.

Instead of feeding known quantities of aflatoxin, Yang, et al. (2012) included field-contaminated corn in treatment diets in replacement of clean, control corn at 25% increments (0, 25, 50, 75, and 100%). The final treatment diets were analyzed and found to contain 0, 20, 43, 54, and 100 ppb of total aflatoxins during the first phase (weeks 1-3) and 0, 40, 81, 112, and 158 ppb during the final phase (weeks 4-6). During the first three weeks, body weights were significantly reduced by the 43 ppb diet and further reduced by 100 ppb. After six weeks on the treatment diets, body weights were no longer affected by aflatoxin, even though the dietary concentration increased during the final three

weeks. This suggests that the aflatoxicosis induced by such low doses was perhaps mild enough for the birds to experience some compensatory growth as they got older.

Reductions in weight gain are a "classic" effect that has been associated with aflatoxicosis since it was first characterized. There is a definite time component that factors into what given dose level of aflatoxin will cause significant reductions in weight gain. Dose levels that consistently have no significant effect during three-week trials become more likely to cause significant changes in five or six-week trials. Further, the age of broilers at the time that a given duration of aflatoxin is initiated affects the relative toxicity of a given dose as well. Day-old broilers fed aflatoxin for three weeks are more likely to have reductions in weight gain than older birds started on a three-week trial.

Feed conversion

Feed conversion, as a ratio of body weight and feed consumption, is often an inconsistent measure of toxicity across a range of poultry species and strains. In Arafa, et al. (1981), goslings fed 700 and 1400 ppb aflatoxin for three weeks showed decreased feed conversion ratios when compared to the control treatment, while broilers showed no change when fed aflatoxin as high as 2100 ppb.

When purified aflatoxin B_1 was administered in gelatin capsules to two-week old broilers for a five-week trial, concentrations equivalent to consuming 1000 ppb in the diet had no effect on feed conversion (Giambrone, et al., 1985, 1). However, when Giambrone, et al. (1985, 2) fed field-contaminated corn at a concentration of 800 ppb aflatoxin to day-old broilers through a five-week trial, the authors observed significant

increases in feed conversion ratios beginning at three weeks and continuing until termination.

Kubena, et al. (1990, 1993, 1998), in their evaluation of the efficacy of various absorptive feed additives, fed relatively high levels of aflatoxin (2500 – 7500 ppb) either alone or in combination with other mycotoxins (zearalenone, ochratoxin A, or T-2 toxin). In these trials, feed conversion was an inconsistent measure of toxicity. For example, feed conversion ratios were not affected by 7500 ppb aflatoxin fed for four weeks in one study (1990), but in a later study (1998) 5000 ppb was sufficient to significantly increase feed conversion after a three-week trial.

Oguz, et al. (2000) reported a significant increase in the weekly feed-to-gain ratio of straight-run broilers during the sixth week of exposure to 100 ppb aflatoxin. However, on a cumulative basis, feed conversion was unchanged.

Using field-contaminated corn to formulate treatment diets containing 168 pbb aflatoxin, as well as 8.4 ppb ochratoxin-A, 54 ppb zearalenone, and 32 ppb T-2 toxin, Aravind, et al. (2003) reported a significant reduction in feed consumption and a significant increase in feed conversion after a five-week trial.

Bailey, et al. (2006) reared broilers for six weeks on floor pens on diets containing either 0 or 3600 ppb aflatoxin provided by a combination of field-contaminated corn and both rice and corn inoculated with cultures of *Aspergillus parasiticus*. The feed-to-gain ratio was significantly increased by 3600 ppb aflatoxin at weeks three and five and also on a cumulative basis. Though diets containing 0.5% of the HSCAS Novasil™ were

significantly better than those fed aflatoxin without clay, they did not perform as well as the control treatment.

In Magnoli, et al. (2011, 1), 50 ppb aflatoxin had no effect on either feed conversion or feed consumption when fed for four weeks to 18-day old male broilers (day 18 – 46). Also, in Magnoli, et al. (2011, 2), when five-day old male broilers were fed treatment diets containing either 0 or 100 ppb aflatoxin for four weeks, aflatoxin had no effect on feed consumption or conversion parameters.

More often what happens is that feed consumption significantly decreases in conjunction with weight gain, which leaves the ratio of feed-to-gain unchanged. In Miazzo, et al. (2000), 2500 ppb aflatoxin was fed to male broilers for a three-week period beginning at three weeks of age. They observed a significant reduction in weight gain but no effect on feed conversion ratios. Raju and Devegowda (2000) fed 300 ppb aflatoxin (alone or in combination with ochratoxin-A and T-2 toxin) for five weeks and found aflatoxin significantly reduced feed consumption but found no difference in feed conversion ratios. In Raju, et al. (2005), two separate trials using 300 ppb aflatoxin fed to broilers for a six-week trial period were conducted. In both trials, they reported significant reductions in feed consumption and weight gain but no effect on the feed conversion ratio. Bintvihok, et al. (2006) started three-day old, straight-run broilers on treatment diets containing 0, 50, and 100 ppb aflatoxin B_1. At both four and six weeks into the trial, 50 and 100 ppb aflatoxin significantly reduced feed consumption relative to the control. However, when feed conversion was calculated, aflatoxin caused a significant improvement in the feed-to-gain ratio. Gowda, et al. (2008) fed 1000 ppb

aflatoxin to male broilers for three weeks and found a significant reduction in feed consumption, but no effect on feed conversion because weight gain was similarly reduced. In Kermanshahi, et al. (2009), aflatoxin at 500 ppb significantly reduced feed intake in broilers beginning at four weeks of exposure. However, since body weights were likewise reduced by aflatoxin beginning at three weeks, this led to no significant differences in feed conversion. Zhao, et al. (2010) reported no effect on feed conversion by 1000 or 2000 ppb aflatoxin fed to straight-run broilers for three weeks. However, feed consumption was reduced by 1000 ppb and further reduced by 2000 ppb.

Relative organ weight

Smith and Hamilton (1970) were able to observe linear increases in relative liver weights at aflatoxin doses 0, 625, 1250, and 2500 ppb, at which point increasing the dose to 5000 or 10,000 ppb plateaued and failed to elicit a further increase. An increase in the lipid content of the liver samples accounted for 60% of the increase in the relative liver weights, suggesting aflatoxin impairs lipid shuttling capacity. The relative spleen weight showed an increase beginning at 1250 ppb. The bursa of Fabricius decreased in relative weight beginning at 1250 ppb. The relative weight of the pancreas showed a similar threshold dose of 1250 ppb that had to be exceeded before an increase was observed. All organ weights measured by Smith and Hamilton showed a plateau effect, in which increasing the aflatoxin dose level above a certain point failed to illicit a further effect. Also the liver responded to aflatoxin at lower levels when compared to the other organs.

Doerr, et al. (1983) found that exposure to aflatoxin at only 75 ppb for a seven-week trial was sufficient to increase liver lipid content, while 225 and 675 ppb

significantly decreased lipids. In a second trial, liver lipids were significantly increased by 300 and 2700 ppb. The middle dose (900 ppb) also had elevated liver lipids; however the amount was not significantly different from the 0 ppb control. The supports the Smith and Hamilton (1970) finding that relative liver weights increase in response to aflatoxin impairing the liver's lipid shuttling capacity.

Miller and Wyatt (1985), reported that though weight gain was not significantly reduced by three-week exposure to 2500 ppb aflatoxin, both relative liver weights and crude lipid content were significantly increased by the 2500 ppb treatment and were further increased by the 5000 ppb. This suggests that the liver could be a potential "first site" of toxicity, showing effects in relative size prior to a change in the bird's growth rate.

To measure the progression of aflatoxin exposure over time, Huff, et al. (1986) fed male broilers treatment diets for three weeks that contained 0, 1250, 2500, or 5000 ppb aflatoxin that had been produced by the inoculation of rice with cultures of *Aspergillus parasiticus*. Relative organ weights were collected and measured at 3, 6, 9, 12, 15, 17, and 21 days of age. The relative proventriculus and ventriculus weights were only increased by 5000 ppb aflatoxin with the ventriculus increasing after six days, while the proventriculus increased only at 21 days. Aflatoxin at 5000 ppb increased relative spleen weights beginning at 12 days. Relative kidney weights were increased beginning at day-12 as well by both 2500 and 5000 ppb. Pancreas and bursa of Fabricius weights showed no consistent changes in response to treatment over time. While typically relative liver weights increase in response to aflatoxin, the organ weights for the 2500

and 5000 ppb treatments significantly decreased early on in this study (at 6 and 9 days). However, by 17 days of age, the relative liver weights from these treatments were significantly higher than the control. There may be a threshold of exposure necessary for toxicity to manifest itself, and the younger birds had not quite met that threshold. The 1250 ppb treatment never differed from control for relative liver weight throughout the trial. Liver lipid levels began to segregate in a dose-related manner beginning at 12 days of age, with the increased relative weight corresponding to an increase in lipid levels, supporting the observation by Smith and Hamilton (1970) that aflatoxin is impairing the lipid-shuttling ability of the liver and that the increased liver weights can be tied to an accumulation of fat.

Huff, et al. (1988) found that in male broilers reared for three weeks on treatment diets that 2500 ppb aflatoxin increased relative liver, kidney, spleen, pancreas, proventriculus, and heart weights but did not affect the ventriculus or the bursa of Fabricius. When 4000 ppb T-2 toxin was fed in combination with 2500 ppb aflatoxin, relative liver, proventriculus, ventriculus, and heart weights were increased to a greater extent, even though T-2 toxin alone had no effect on these organs. This suggests a level of interaction between aflatoxin and other mycotoxins present that can enhance the toxicity of any given dose level.

In evaluating the efficacy of various inorganic sorbents (especially hydrated sodium calcium aluminosilicates), Kubena, et al. (1990, 1993, 1998) found that feeding concentrations of aflatoxin ≥2500 ppb were consistently able to increase relative liver and kidney weights in three-week old broilers, either when fed alone or in combination with

other mycotoxins (zearalenone, ochratoxin A, or T-2 toxin). Relative ventriculus, spleen, and pancreas weights were also increased by aflatoxin but in a less consistent manner. In Kubena, et al. (1993), aflatoxin at 3500 ppb alone caused significant increases in the relative weights of the liver, kidney, heart, ventriculus, spleen, and pancreas, while zearalenone alone had no effect. In Kubena, et al. (1998), aflatoxin at 5000 ppb alone was sufficient to increase the relative liver, kidney, heart, spleen, pancreas, and proventriculus weights, but did not affect the ventriculus or the bursa of Fabricius. T-2 toxin had no effect on any of the relative organ weights in this study.

R.H. Bailey, et al. (1998) collected liver, kidney, heart, spleen, pancreas, proventriculus, ventriculus, and bursa of Fabricius from male broilers fed 5000 ppb aflatoxin for three weeks. They found significant increases in the relative organ weights for all the organs collected, except for the ventriculus and the bursa of Fabricius.

Miazzo, et al. (2000) fed 2500 ppb aflatoxin to three-week old male broilers for three weeks. Aflatoxin significantly increased relative liver weights under these conditions.

Raju and Devegowda (2000) fed 300 ppb aflatoxin (either alone or in combination with ochratoxin-A and T-2 toxin) for five weeks. Upon termination, the liver, kidneys, ventriculus, and adrenals were collected. Aflatoxin caused increases in the relative weight of the liver, kidney, and adrenals but had no effect on the relative ventriculus weight.

In Aravind, et al. (2003), straight-run broilers were fed treatment diets formulated using field-contaminated corn. Final diets contained 168 pbb aflatoxin, as well as 8.4

ppb ochratoxin-A, 54 ppb zearalenone, and 32 ppb T-2 toxin, and were fed to the birds for five weeks. Upon termination, the relative organ weights of the liver, kidney, and ventriculus were calculated. Relative liver and ventriculus weights were increased by the contaminated diets, but there was no effect on the relative kidney weights.

When broilers reared under identical control conditions until three-weeks of age were then fed treatment diets containing 0, 350, or 2450 ppb aflatoxin (either alone or in combination with 10,000 ppb fumonisin) for three weeks until six weeks of age, Del Bianchi, et al. (2005) found no treatment differences on the relative weights of the liver, kidney, heart, bursa of Fabricius, thymus, pancreas, or proventriculus from any of the treatment groups.

Ortatatli, et al. (2005) collected liver, kidney, spleen, thymus, and bursa of Fabricius from broilers fed 0, 50, and 100 ppb aflatoxin that was produced via the fermentation of rice with cultures of *Aspergillus parasiticus* for six weeks. Aflatoxin at either dose level failed to elicit any change in the relative organ weights for any of the organs collected. However, the authors failed to report any

During two separately performed six-week broiler trials, Raju, et al. (2005) found that 300 ppb aflatoxin was sufficient to increase relative liver weights. Relative kidney, bursa of Fabricius, spleen, thymus, and adrenal weights were not affected by treatment in their first trial. However, relative liver, adrenal, pancreas, kidney, and gall bladder weights were all significantly increased and the relative weight of the thymus was significantly reduced by 300 ppb in the second trial.

Bailey, et al. (2006) found that 3600 ppb aflatoxin significantly increased relative liver, kidney, and spleen weights. Though diets containing 0.5% of the HSCAS Novasil™ were significantly better than those fed aflatoxin without clay with respect to the relative liver and kidney weights, the feed additive only had complete protective efficacy on the relative spleen weights.

Gowda, et al. (2008) saw a significant increase in relative liver weights of three-week old male broilers by feeding aflatoxin at 1000 ppb. Lipid peroxides were also significantly higher, suggesting an effect on the lipid shuttling ability of the liver.

Yarru, et al. (2009, 1) significantly increased the relative liver weights of male broilers by feeding 2000 ppb for three weeks, but 1000 ppb had no effect. However, in Yarru, et al. (2009, 2) 1000 ppb aflatoxin was sufficient to increase relative liver weights. Similarly, relative liver weights were significantly increased by 1000 ppb and were further increased by 2000 ppb when fed for three weeks by Zhao, et al. (2010).

Magnoli, et al. (2011, 1) found 50 ppb aflatoxin to have no effect on relative liver weights when fed for four weeks to 18-day old male broilers (day 18 – 46). Magnoli, et al. (2011, 2) also fed five-day old male broilers treatment diets containing either 0 or 100 ppb aflatoxin for four weeks (day 5 – 33) and again failed to find significant changes in the relative liver weight with this dose.

Yang, et al. (2012) found no effect of low levels of aflatoxin (0, 20, 43, 54, and 100 ppb) on the relative liver weight when birds were fed for three weeks. At three weeks of age, birds were continued on diets that had higher levels of aflatoxin (0, 40, 81, 112, and 158 ppb) for three additional weeks. At six weeks of age, there was a

significant increase in relative liver weights beginning at the 81 ppb level, which continued for the higher two doses.

Along with reductions in growth, liver damage represents a "classic" effect of aflatoxin that has been associated with exposure since it was first characterized. Smith and Hamilton (1970) and Huff, et al. (1986) demonstrated that the increase in relative liver weights matches a similar increase in liver lipid content, suggesting aflatoxin-induced hepatitis is caused by the accumulation of lipids in the tissue.

Serum biochemistry

In addition to increasing in liver lipid content (and the relative liver weight), aflatoxin-induced liver damage results in changes to the levels of biomolecules and the activity of various liver enzymes in the serum. Serum total protein is principally comprised of the albumin and globulin components present in circulation. Albumin is made specifically in the liver and globulins are made by both the liver and the immune system. Decreased levels can indicate damage to the liver or kidney or impairment of immune function. Reductions in serum levels of cholesterol and triglycerides would also suggest an impairment of liver function with respect to its role in lipid shuttling. Reductions in serum calcium or phosphorous can be attributed to effects on renal function (Glahn, et al. 1991) and have the potential to impact bone development in broilers. Higher levels of particular liver enzymes (e.g., alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase, or alkaline phosphatase) in the serum represent the "leaking" of liver cell contents into general circulation. While enzymes that

do not arise primarily in the liver (e.g., lactate dehydrogenase or creatine kinase) can still be used to evaluate the incidence of general tissue damage.

Hamilton, et al. (1974) fed titrated amounts of aflatoxin (0, 625, 1250, 2500, 5000, and 10,000 ppb) to male broilers for three weeks. Plasma was collected and analyzed for the concentration of calcium. There was a significant reduction in serum calcium beginning at 1250 ppb, with higher doses not showing any greater effect.

Doerr, et al. (1983) measured plasma carotenoids after a seven-week trial on aflatoxin and found a dose of 2700 ppb was required to cause any significant reduction.

When feeding 2500 ppb aflatoxin for three weeks, Miller and Wyatt (1985) observed significant reductions in serum total protein, albumin, and globulin in male broilers.

In Huff, et al. (1986) male broilers were fed treatment diets for three weeks that contained 0, 1250, 2500, or 5000 ppb aflatoxin that had been produced by the inoculation of rice with cultures of *Aspergillus parasiticus*. Serum was collected and analyzed from birds at 3, 6, 9, 12, 15, 17, and 21 days of age to track the progression of the effect of aflatoxin over time. Reduction in serum globulin, albumin, and total protein responded in a dose-related fashion, becoming significantly apparent after 12 days. As early as 3 days of age, serum albumin was reduced by the highest dose (5000 ppb) of aflatoxin.

Huff, et al. (1988) found that aflatoxin at 2500 ppb reduced red blood cell counts in three-week old male broilers. Other hematological parameters (mean corpuscular volume, packed cell volume, and hemoglobin levels) were not affected by aflatoxin alone but were reduced when aflatoxin was fed in combination with 4000 ppb T-2 toxin.

Serum total protein, albumin, cholesterol, and glucose were significantly reduced by aflatoxin. Triglycerides and uric acid were unaffected. Total protein, albumin, cholesterol, triglycerides, and uric acid were reduced to a greater extent when T-2 was included with aflatoxin. Serum calcium was reduced by aflatoxin alone. Though T-2 toxin alone did not affect calcium levels, when the two mycotoxins were fed in combination, serum calcium was reduced to a greater extent than with aflatoxin alone. Serum activity of lactate dehydrogenase and alkaline phosphatase were reduced by aflatoxin. Feeding the combination mycotoxin treatment did not have a greater effect than either mycotoxin alone. The activity of creatine kinase was only significantly increased by aflatoxin and T-2 toxin in combination.

Ghosh, et al. (1990) formulated treatment diets using purified aflatoxin B_1 to contain 0, 300, or 1000 ppb in the final feed. Diets were fed to day-old broilers for a six-week rearing period. Weekly blood samples were obtained and used to determine the total albumin and globulin content of the plasma. For total plasma albumin, 300 ppb did not decrease levels until five weeks of exposure, while 1000 ppb decreased levels beginning at three weeks. Plasma globulin significantly decreased at days 14 and 28 only in the 300 ppb treatment group, and decreased only at days 7, 14, and 28 in the 1000 ppb group.

Kubena, et al. (1990, 1993, 1998) collected serum by cardiac puncture from three-week old broilers that had been exposed to a range of doses of aflatoxin (2500 – 7500 ppb) either fed alone or in combination with other mycotoxins (zearalenone, ochratoxin A, or T-2 toxin) and found significant reductions in serum total protein, albumin,

cholesterol, calcium, the activity of aspartate aminotransferase and alkaline phosphatase, and increased activity of creatine kinase attributed to aflatoxin.

R.H. Bailey, et al. (1998) reported that 5000 ppb aflatoxin significantly reduced serum total protein, albumin, calcium, cholesterol, uric acid, and activity of alkaline phosphatase as well as increased the activity of creatine kinase in serum collected from three-week old male broilers.

Raju and Devegowda (2000) fed 300 ppb aflatoxin (either alone or in combination with ochratoxin-A and T-2 toxin) for five weeks. Serum total protein, cholesterol, hemoglobin, urea nitrogen, and the activity of γ-glutamyltransferase, alanine aminotransferase, and aspartate aminotransferase were analyzed after three weeks and five weeks of exposure. Serum total protein and cholesterol were both reduced by aflatoxin as early as three weeks. For serum total protein, the treatment fed aflatoxin alone had significantly lower levels when compared to aflatoxin fed in combination with the other mycotoxins. Urea nitrogen was significantly reduced by aflatoxin after the full five-week trial. Aflatoxin did not affect serum activity of γ-glutamyltransferase at three weeks but caused significant increases after five weeks, which suggests damage of the liver tissue. Aspartate aminotransferase was not affected at three weeks, but there was a significant decrease in serum activity after five weeks. Alanine aminotransferase was not affected by aflatoxin throughout the trial.

Oguz, et al. (2002) fed day-old, straight-run broilers 0, 50, or 100 ppb aflatoxin for six weeks. Upon termination, serum was collected and analyzed for biochemical parameters and enzymatic activity. Aflatoxin at both 50 and 100 ppb had no effect on

serum total protein, albumin, cholesterol, uric acid, or potassium. With respect to the activity of liver enzymes, aflatoxin at 50 ppb significantly decreased the activity of aspartate aminotransferase and alanine aminotransferase, while 100 ppb significantly increased the activities of both. It may be that 50 ppb was enough to depress the synthesis of these enzymes but not enough to damage the liver, whereas the 100 ppb treatment began to cause the liver the leak these enzymes into circulation.

In Aravind, et al. (2003), straight-run broilers were fed treatment diets formulated using field-contaminated corn, containing 168 pbb aflatoxin, as well as 8.4 ppb ochratoxin-A, 54 ppb zearalenone, and 32 ppb T-2 toxin in the final diet. Serum total protein, cholesterol, urea nitrogen, and activity of γ-glutamyltransferase, alanine aminotransferase, and aspartate aminotransferase were analyzed at three and five weeks of age. Serum total protein and cholesterol were not affected by this combination of mycotoxins. Serum activity of γ-glutamyltransferase was significantly increased at both time-points. The serum activity of alanine aminotransferase and aspartate aminotransferase were reduced at three weeks but there was no difference after five weeks of age. Urea nitrogen was significantly reduced by the contaminated diet at both time-points.

When feeding three-week old male broilers 0, 350, or 2450 ppb aflatoxin either alone or in combination with 10,000 ppb fumonisin for a three-week trial period (from three to six weeks of age), Del Bianchi, et al. (2005) found aflatoxin alone had no effect on serum total protein, albumin, urea, creatinine, cholesterol, and enzymatic activity of aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase, and alanine

aminotransferase. There were also no treatment effects on red-blood cell and leukocyte counts or hemoglobin concentrations. Only serum albumin was affected by treatment, significantly lowered by the highest level of aflatoxin (2450 ppb) when it was fed in combination with 10,000 ppb of fumonisin.

After six weeks exposure to 300 ppb dietary aflatoxin, Raju, et al. (2005) collected serum and subjected samples to analysis for total protein, cholesterol, triglycerides, and activity of γ-glutamyltransferase. In both trials performed by the authors, serum γ-glutamyltransferase activity was increased by aflatoxin, and serum total protein, cholesterol, and triglycerides were significantly decreased.

Bintvihok, et al. (2006) started three-day old, straight-run broilers on treatment diets containing 0, 50, and 100 ppb aflatoxin B_1. After six weeks on the respective treatment diets, blood was collected and serum analyzed for activity of γ-glutamyltransferase, alanine aminotransferase, and aspartate aminotransferase. Aflatoxin at both levels increased the activity of all three enzymes in the serum relative to the control treatment, which suggests that tissue damage to the liver.

When Gowda, et al. (2008) fed male broilers 1000 ppb aflatoxin for three weeks, they reported significantly reduced serum total protein, albumin, cholesterol, and calcium. Values for uric acid, phosphorous, and activity of γ-glutamyltransferase were unaffected.

Kermanshahi, et al. (2009) found that aflatoxin at 500 ppb was sufficient to reduce the activity of aspartate aminotransferase and γ-glutamyltransferase in broilers

after three weeks of exposure. Alanine aminotransferase and lactate dehydrogenase activity were increased at three weeks by 1000 ppb, and by 500 ppb after six weeks.

Yarru, et al. (2009, 1) fed 0, 1000, and 2000 ppb aflatoxin to male broilers for three weeks. Serum total protein and serum calcium were both reduced by 1000 ppb and reduced further by 2000 ppb. Serum phosphorous showed no response at 1000 ppb but was significantly reduced by 2000 ppb.

Zhao, et al. (2010) saw deceases in serum total protein, albumin, and globulin when feeding 1000 ppb aflatoxin for three weeks, but saw no effect on serum glucose, sodium, potassium, chloride, calcium, phosphorous, uric acid, or the activities of aspartate aminotransferase, γ-glutamyl transferase, alkaline phosphatase, and creatine phosphokinase. In the treatment diet containing 2000 ppb, broilers showed significant decreases in serum total protein, albumin, globulin, calcium, phosphorous, glucose, and activity of alkaline phosphatase, but no effect was seen for serum sodium, potassium, chloride, uric acid, or the activities of the liver enzymes aspartate aminotransferase, γ-glutamyl transferase, and creatine kinase.

Magnoli, et al. (2011, 1) found 50 ppb aflatoxin had no effect on serum total protein, albumin, globulin, or the activity of aspartate aminotransferase and alanine aminotransferase when fed for four weeks to 18-day old male broilers (18-46 days of age). Magnoli, et al. (2011, 2) found that 100 ppb had no effect on serum total protein, albumin, or globulin when feeding five-day old male broilers treatment diets containing either 0 or 100 ppb aflatoxin for four weeks (5-33 days of age).

In the study conducted by Yang, et al. (2012), field contaminated corn was included in treatment diets as a replacement for clean, control corn at 25% increments. After the first three weeks, only the serum values of aspartate aminotransferase and γ-glutamyl transferase were increased by aflatoxin. Aspartate aminotransferase increased relative to the control beginning at 54 ppb, while, with γ-glutamyl transferase, no aflatoxin treatment was significantly different than the control. Instead, the levels of 54 and 100 ppb were increased relative to the 20 ppb treatment only. After six weeks on the treatment diets, there were no differences between treatments for any of the serum parameters measured (total protein, albumin, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transferase).

The significant effects from the above data are summarized in Table 2-1.

Table 2-1: Weeks of Exposure to Given Levels of Aflatoxin (ppb)

	1	2	3	4	5	6
50			↑ AST			↑ ALT, AST, GGT
100						↑ ALT, AST
200			↑ GGT			↑ GGT
300		↓ Glob	↓ TP, Chol		↓ TP, Alb, Chol ↑ GGT	↓ TP, Chol, Trig ↑ GGT
500			↓ AST, GGT			↑ ALT, L-DH
1000	↓ Glob		↓ TP, Alb, Glob, Chol, Ca ↑ ALT, L-DH			
2500			↓ TP, Alb, Glob, Ca, Glu, AST, L-DH, Alk Phos ↑ CK			

TP, total protein; Alb, Albumin; Glob, globulin; Chol, cholesterol; Trig, triglycerides; Ca, calcium; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; L-DH, lactate dehydrogenase; Alk Phos, alkaline phosphatase; CK, creatine kinase

The table illustrates the time-to-dose relationship of aflatoxin exposure in broilers. Dose levels that consistently elicit no significant effect during a three-week period become more likely to cause significant changes in five or six-week trials. Further, as the two trials by Magnoli, et al. demonstrate, the age of the birds at the initiation of their aflatoxin exposure can affect the relative toxicity of a given dose fed for a specific duration as well.

Changes in serum total protein, albumin, globulin, and activity of certain enzymes reflect the ability of the metabolite aflatoxin B_1-8,9 epoxide to bind DNA and impair the synthesis of RNA and protein. Significant increases of the serum activity of hepatic enzymes (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase) support the observation that the target organ for aflatoxin is the liver and that damage to the liver tissue has allowed "leaks" of the enzymes into circulation. On the other hand, Zhao, et al. (2010) noted a significant decrease in the activity of alkaline phosphatase in broilers fed 2000 ppb aflatoxin for three weeks. Instead of damage to the liver causing an increase in serum activity, it appears possible to completely impair or "crash" the liver's ability to synthesize these enzymes and cause a reduction in serum activity if aflatoxicosis is severe enough.

The data in Zhao, et al. (2010) support the use of serum biochemistry as a more sensitive bioassay for the toxicity of aflatoxin. The authors observed a significant main effect for the level of the adsorptive feed additive included (0.1% or 0.2%) in diets containing aflatoxin (0 or 1000 ppb) for the serum biochemical parameters. However this main effect for the additive level was not seen in the feed consumption or weight gain

data, suggesting serum biochemistry may be significantly affected prior to changes in growth parameters. Given that aflatoxin travels to the liver first and that it targets protein synthesis and function, serum total protein (and the albumin and globulin fractions) appear to be the more sensitive indicators. Activities of the liver enzymes are more variable, sometimes showing decreases under milder aflatoxicosis prior to increases when aflatoxin has been severe enough to damage liver tissue.

Immune response

In addition to reducing the globulin fraction of serum total protein (which is associated with immune function), aflatoxin impairs the efficiency of certain mechanisms in the immune system. Giambrone, et al. (1985, 1) fed purified aflatoxin B_1 in gelatin tablets either alone or in combination with aflatoxin B_2 to two-week old broilers. The tablets were administered to achieve a final toxin exposure equivalent to consuming 0, 100, 200, 400, 500, or 1000 ppb of aflatoxin B_1 or 100, 200, or 400 ppb as aflatoxin B_1 and B_2 in the diet. One week into the trial (when birds were three weeks of age), half were challenged with a Newcastle Disease Virus vaccine and half with a *Pasteurella multocida* vaccine. Immunological response data were obtained using hemagglutination inhibition, antibody titer via ELISA, delayed hypersensitivity reaction, graft-versus-host, and lymphocyte blastogenesis assays. There were no significant reductions in antibody titer, cell-mediated immune response, or resistance to specific challenge to either pathogen by aflatoxin B_1 fed alone at any dose. However, when B_1 was combined with B_2, there were reductions in cell-mediated immunity at 100 ppb, and at 400 ppb there was a significant reduction in three of the four cell-mediated immune response assays. This

suggests that, in addition to environmental stressors that may increase the toxicity of a given dose of aflatoxin (as seen in Doerr, et al. 1983), the presence of aflatoxin B_2 can increase the toxicity of B_1.

Giambrone, et al. (1985, 2) administered an immune challenge of either Newcastle Disease Virus or *Pasteurella multocida* to broilers and turkeys that had been reared on aflatoxin-contaminated diets containing 0, 100, 200, 400, or 800 ppb for three weeks. These birds were kept on their respective treatment diets and observed for two additional weeks post-challenge. Upon termination at five weeks, cell-mediated immunity was measured by two delayed hypersensitivity indices, a graft-to-host index, and a lymphocyte blastogenesis index. In turkeys, all four measures of cell-mediated immune response were depressed at exposure to 200 ppb. For broilers, only the two delayed hypersensitive indices (phytohemagglutin and mycobacterium) were depressed by aflatoxin (at 200 and 800 ppb respectively), supporting the conclusion that broilers are more robust against aflatoxin exposure when compared to turkeys.

Ghosh, et al. (1990) formulated treatment diets using purified aflatoxin B_1 to contain 0, 300, or 1000 ppb. Diets were fed to day-old broilers for a six-week rearing period. Weekly blood samples were obtained and used to determine the percentage of T-lymphocytes in the peripheral blood as measured by α–naphthyl acetate esterase (ANAE) staining. There was a significant increase in percent of ANAE-reacting lymphocytes at two and three weeks in the 300 ppb treatment group when compared with the control. The percent of positive lymphocytes then significantly decreased at five and six weeks. The 1000 ppb group showed a significant decrease in ANAE-reacting lymphocytes at all

weekly time-points when compared with control, except for three weeks, where the numeric decrease was not statistically significant. The data suggest that aflatoxin at 300 ppb caused a suppression of the cell-mediated immune response after five weeks of exposure and 1000 ppb after only one week.

When fed 300 ppb aflatoxin for six weeks, Raju, et al. (2005) found that broilers had a significantly depressed humoral immune response as measured by antibody response to sheep red blood cell inoculation.

Aflatoxin, especially once converted into aflatoxin B_1-8,9 epoxide by the liver, affects the synthesis of proteins (by binding to DNA and RNA) and protein function (by binding directly amino acid residues). The immunosuppressive effects of aflatoxin may, in part, result from the inhibition of antibody or complement control protein synthesis.

Hepatic gene expression

The reported data on relative organ weights and serum biochemistry suggest that the liver can be considered a target organ for aflatoxicosis. Thus, the expression of specific genes in the liver tissue represents a likely "first stop" in the biological response to aflatoxin, as the contents absorbed by the GI tract travel first to the liver via the portal blood system. Microarrays can be used to profile global gene expression pursuant to identifying candidate genes for diseases/disorders and to map growth, metabolic, and regulatory pathways that control chosen traits. Yarru, et al. (2009, 1) reported the measurement of gene expression in chicks fed aflatoxin using the microarray. Day-old male broilers were reared for three weeks on diets containing 0, 1000, or 2000 ppb aflatoxin that was supplied by purified *Aspergillus parasiticus* culture material. Upon

termination, liver tissue from one bird per treatment was collected, flash-frozen in liquid nitrogen, and stored at -80°C until the microarray and real-time PCR analyses. Microarray analysis identified 177 genes with significantly different expression between 0 ppb control and the 2000 ppb treatment. Quantitative real-time PCR was used to confirm the validity of the microarray results. Four randomly selected differentially expressed genes were evaluated and had a similar expression pattern as observed in microarray results, validating the microarray. In the liver tissue of broilers fed 2000 ppb aflatoxin for three weeks, gene expression for specific physiological pathways of:

- detoxification
- fatty-acid metabolism
- oxidative phosphorylation
- energy production
- cell proliferation
- immune response
- metabolism
- growth and development
- blood coagulation
- antioxidant activities

were affected. According to Yarru, et al., the genes specifically associated with electron transport and β-oxidation of fatty-acids were downregulated, which would cause decreased ATP production. The authors found a downregulation of genes involved in fatty-acid metabolism and transport (which could potentially explain the increases in

relative liver weights and liver lipid content other authors have reported when feeding aflatoxin). Genes associated with carbohydrate metabolism and gluconeogenesis were upregulated, which the authors suggest could be an effort to compensate for reduced feed intake and reduced energy production. Feeding 2000 ppb downregulated insulin-like growth factor-1, which could contribute to reductions in body weight. Genes associated with immune function were downregulated, while tumor necrosis factor-10 was upregulated, both suggesting aflatoxin at 2000 ppb causes immunotoxicity. Aflatoxin also upregulated genes associated with cell proliferation. The authors also observed a downregulation in genes responsible for the synthesis of coagulation factors and an upregulation in genes coding for anti-coagulative proteins.

The cytochrome p450 class of enzymes (CYP) are involved in the biotransformation of various xenobiotic compounds into metabolites that are more water soluble and available for excretion. CYP450 enzymes are classified into families identified by a number (1, 2, 3, 4), subfamilies identified by a letter (1A, 1B, 2A, 2B, etc.), and then specific members identified by a second number (CYP1A1, CYP2E1, CYP2H1, etc.). Though the CYP-mediated reactions are essential for detoxification, they can generate reactive oxygen species and are responsible for the bioactivation of aflatoxin B_1 into the carcinogenic metabolite aflatoxin B_1-8,9 epoxide. This epoxide can be detoxified by epoxide hydrolase and glutathione S-transferase (GST) enzymes. However, Yarru, et al. report that CYP-isoforms (CYP1A1 and CYP2H1) were upregulated and epoxide hydrolase, GST, and glutathione peroxidase genes were downregulated by 2000

ppb aflatoxin. This combination favors the formation of aflatoxin B1-8,9 epoxide and oxidative stress, while providing less of a chance for any biological antioxidant response.

In Yarru, et al. (2009, 2) day-old male broiler chicks were reared for three weeks on diets containing either 0 or 1000 ppb aflatoxin. Livers were collected from six birds per treatment to determine relative liver weight and for real-time PCR analysis. The expression patterns of hepatic genes involved in antioxidant function (catalase, superoxide dismutase, glutathione peroxidase, and GST), the biotransformation of aflatoxin (epoxide hydrolase, CYP1A1, and CYP2H1), and immune function (interleukin 6 and 2) were quantified. The gene expression of catalase, glutathione peroxidase, and interleukin-2 were unaffected by 1000 ppb aflatoxin. Aflatoxin caused a significant decrease in the expression of superoxide dismutase, epoxide hydrolase, and GST and caused significant increases in the expression of CYP1A1, CYP2H1, and interleukin-6. This is the combination the Yarru, et al. had highlighted previously as favoring the formation of aflatoxin B_1-8,9 epoxide while at the same time providing less of a chance for an antioxidant response.

Diaz, et al. (2010) showed that through the comparison of enzyme kinetics for aflatoxin B_1 epoxidation, quail liver tissue had a higher rate of aflatoxin B_1-8,9 epoxide production (with enzymes that had a higher affinity for aflatoxin B_1) when compared with broiler liver tissue. This effect in the liver expression of CYP450 genes was hypothesized as an explanation of the greater sensitivity of quail to dietary aflatoxins as compared to broilers, originally shown in Arafa, et al. (1981).

Organ residues

The liver and kidney are the main organs involved in detoxification. Detoxification sites can also be organs where residues of the toxins and their metabolites can accumulate. Residual amounts of aflatoxin B_1 can accumulate in edible tissues such as the liver and carry-over into human food. Fernández, et al. (1994) fed broiler chickens a diet containing 2500 and 5000 ppb aflatoxin and, after 32 days on the contaminated diets, residues of aflatoxin B_1 in the liver were only 0.16 and 0.15 ppb respectively. The relatively low numbers were attributed to a high activity of liver metabolization of aflatoxin B_1 into its detoxified forms for rapid excretion. In looking at the clearance time for residues once aflatoxin has been removed from the diet, birds from both doses had no detectable residues in their livers after only one day into the clearance period. These results show that the amount of residue accumulation in broilers can be quite low, and the clearance time once aflatoxin is removed from the diet can be short.

Bintvihok and Kositcharoenkul (2006) fed 50 and 100 ppb of aflatoxin B_1 to broilers for six weeks. The liver residues for aflatoxin B_1 were lower than those for aflatoxin M_1 (0.05 and 0.13 ppb for B_1 – 0.10 and 0.32 ppb for M_1) in both aflatoxin diets. However, the residues for both were well under a negligible amount (< 1 ppb).

Hussain et al. (2010) fed one, two, and four-week old broilers diets containing 0, 1600, 3200, and 6400 ppb aflatoxin. In the one-week old birds, they detected aflatoxin B_1 residues in the liver from all doses after three days of exposure (0.00, 0.83, 2.45, and 5.23 ppb in the 0, 1600, 3200, and 6400 ppb treatments, respectively). In two-week old birds, B_1 residues where also found in all doses after three days of exposure, and were

0.00, 2.67, 2.50, and 4.55 ppb in the respective treatments. In birds that were three weeks old at the start of aflatoxin exposure, residues where not found in any of the three doses until five days of exposure, at which point they were 0.00, 2.71, 2.91, and 2.77 ppb in the 0, 1600, 3200, and 6400 ppb treatments. Overall, older birds had lower tissue residues of aflatoxin B_1 when compared to younger birds (when they were fed aflatoxin for the same amount of time) and the elimination of residues following a withdrawal of contaminated diets in this study occurred earlier in the older birds, suggesting that as birds age, the liver's metabolizing mechanisms for toxins becomes more efficacious. The authors noted this could also have potentially been due to a dilution effect that occurred as birds (and their livers) got larger. In future work, this could be adjusted for by analyzing total residues as a function of body weight or organ weight.

In Neeff, et al. (2013), one sample from both the liver and kidney per replicate in the study were collected for analysis of aflatoxin residues [B_1, B_2, G_1, and G_2] and the detoxified metabolites aflatoxin M_1 and aflatoxicol. They found that 2500 ppb aflatoxin fed for three weeks significantly increased liver concentrations of aflatoxin B_1, B_2, and G_1 as well as M_1 and aflatoxicol [all of the ones analyzed except for aflatoxin G_2] relative to the control diet. Also, all forms of aflatoxin had significantly higher presence in the kidney. The amount of aflatoxin B_1 in the liver was 8.32 ppb, and total aflatoxins were 12.78 ppb. However, the variations in the aflatoxin residues between studies suggest that residues may be influenced by factors such as total ppb in the feed, length of time on feed, age of birds at the start of toxin exposure, or species/type of the birds.

Clay-based, toxin-binding additives

At present, the chief means of both effectively and feasibly protecting against the toxic effects of aflatoxin contamination in animal feeds is the inclusion of non-nutritive, clay-based binding agents included in formulated diets. According to Phillips (1999), the mechanism of protection for clay-based binders is a function of high-affinity binding of aflatoxin B_1 in the interlayers of the smectite clay inside the small intestine, thus preventing the molecule's absorption and transport to the liver. Evaluating the protective efficacy of various clay-based binders at ameliorating aflatoxicosis cannot be demonstrated in experimental trials where a relative low-level of aflatoxin fails to produce a significant response for the given parameter. However, feeding higher doses so as to obtain a greater degree of confidence that significant differences will be produced subjects the additive to a scenario less representative of "reality". Further, the concentration of aflatoxin present in a solution has been shown, *in vitro*, to affect the percent of the total toxin the clay will absorb (its "binding efficiency") by Kannewischer, et al. (2006).

It remains the common practice in the literature for studies to evaluate feed additives at aflatoxin levels anywhere from 50 to 250-times the amount allowed in broiler diets by regulation. The use of non-nutritive, clay-based adsorbents has proved effective at reducing the toxic effects of aflatoxin in various animal species, including broilers. These various aluminosilicate, bentonite, and zeolite clays have been shown *in vitro* to bind aflatoxin into the interlayers of the clay structure, as well as on the edge (Phillips, 1999; Desheng, 2005; and Kannewischer, 2006) with relatively high affinity. However,

to evaluate the efficacy of these clay-based additives as a viable strategy for detoxifying contaminated diets, studies typically use higher concentrations of aflatoxin than would ever be present in "real-world" scenarios. The FDA has established a set of specific "action levels" that constitute the acceptable levels of aflatoxin allowable in commerce. In Texas, these action levels are monitored by the Office of the Texas State Chemist. These regulatory levels of aflatoxin represent a "speed-limit" for the presence of the toxin in feed, meaning any feedstuff found to meet or exceed the set level at any point along the production cycle must be dealt with as directed. The legal action-level for the presence of aflatoxin in a commercial setting is ≤ 20 ppb for immature poultry and ≤ 100 ppb for mature poultry. However, the levels of aflatoxin that predominate in experimental trials range from 500 – 7500 ppb, with most data collected within the 1000 to 2500 ppb range. The reason for this is two-fold:

- Higher concentrations of aflatoxin have a higher likelihood of eliciting measurable aflatoxicosis, as well as doing so in a shorter experimental time-period (*e.g.*, a three-week battery trial).
- Multiple contributing factors leading to the toxicity of aflatoxin other than the concentration of toxin present in a well-controlled experimental setting.

There are toxin-related factors (other mycotoxins present, level and duration of intake), animal-related factors (species, sex, age, immune status), and environmental-related factors (farm management, nutritional status, temperature, disease control) that all interact with each other to cause the over-all toxic effects. The toxicity of aflatoxin is not solely dependent on the amount present in the feed. Rather, toxicity of a given level of

aflatoxin can vary according to different sets of other stressors present in a rearing environment.

Though feeding aflatoxin at >1000 ppb may make observing experimental effects with statistical confidence more likely, such trials subject the adsorptive capacity of the feed additive being evaluated to levels of aflatoxin 100x or more than what would be encountered in a real-world setting. The clay-based binders are most efficient at adsorbing aflatoxin in dose ranges where their absorbance curves are steepest. Kannewischer, et al. (2006) reported that the average sorption capacity of samples from 20 various smectite clays was 22.2% of the aflatoxin in solution at higher concentration (8000 ppb) compared to 47.7% of the aflatoxin in solution at the lowest concentration (400 ppb), with the best clay sample adsorbing 99.8% of the aflatoxin when its concentration in solution was lowest. Evaluating the efficacy of clay-based binders at ameliorating aflatoxicosis cannot be experimentally demonstrated in trials where a relative low level of aflatoxin does not produce a measurable response in the given parameter.

The efficacy of hydrated sodium calcium aluminosilicates (HSCAS) was evaluated extensively by Kubena, et al. during the 1990's. The clay-based additive was included in diets containing aflatoxin at concentrations exceeding 2000 ppb (either alone or in combination with other mycotoxins) and fed to broilers over a series of three-week trial periods. Ameliorative effects of the feed additive on weight gain, feed conversion, relative organ weights, and serum biochemical parameters were measured.

In Kubena, et al. (1990), 7500 ppb of purified aflatoxin B_1 and 0.5% of a HSCAS were fed to four treatments arranged as a 2 x 2 factorial for three weeks. Aflatoxin significantly reduced weekly weight gain during all three weeks, as well as cumulative weight gain. The HSCAS inclusion (0.5%) in the diet containing aflatoxin significantly improved the weight gain during the first week, to a level comparable to the control. Weekly weight gains during the second and third weeks and the cumulative weight gain were significantly higher in birds consuming aflatoxin when the HSCAS was included, but were still significantly lower than the control. The inclusion of HSCAS alone (without aflatoxin) showed no adverse effects on weight gain when compared with the control. This theme is common in acute aflatoxicosis trials (>1000 ppb for three weeks), where the absorptive feed additive offers a significant improvement compared to consuming aflatoxin without any additive but levels remain significantly lower compared to the control.

Huff, et al. (1992) fed 3500 ppb aflatoxin, 2000 ppb ochratoxin-A, and 0.5% of a HSCAS in a 2x2x2 complete factorial arrangement to male broilers for three weeks. Aflatoxin alone significantly reduced body weight, ochratoxin-A alone reduced body weight to a greater extent, and aflatoxin in combination with ochratoxin-A reduced weight still further. Including HSCAS at 0.5% with 3500 ppb aflatoxin significantly improved body weight, but as was seen in Kubena, et al. (1990), the treatment was still significantly lower than the control. However, including HSCAS had no effect on the toxicity of ochratoxin-A when fed alone or when in combination with aflatoxin. Aflatoxin alone significantly increased relative kidney, liver, proventriculus, and heart

weights. This effect was not any greater when ochratoxin-A was fed in combination. Including the HSCAS with aflatoxin significantly improved the adverse changes made to the relative organ weights (though weights were still significantly different when compared to the control). However, when aflatoxin and ochratoxin-A were fed in combination, HSCAS only improved the relative liver and kidney weights (not those of the proventriculus or heart). Aflatoxin and ochratoxin-A alone and in combination significantly reduced levels of serum total protein, albumin, and cholesterol to the same extent. HSCAS again offered significant protection against this effect in the presence of 3500 ppb aflatoxin, but had no effect when ochratoxin-A was present. Only aflatoxin alone significantly reduced the activity of aspartate aminotransferase, and including HSCAS restored the activity to levels similar to those seen in the control treatment. This study demonstrates that while HSCAS has high affinity binding with respect to aflatoxin, it shows less efficacy with a different mycotoxin (in this case ochratoxin-A). The authors suggest that, when ochratoxin-A is present, it both acts alone and in combination with free aflatoxin that was not adsorbed by the HSCAS to cause the further pronounced effects.

Kubena, et al. (1993) fed 3500 ppb aflatoxin, 5000 ppb diacetoxyscirpenol (a zearalenone mycotoxin), and 0.5% of a HSCAS in a 2x2x2 complete factorial arrangement to male broilers for three weeks. Aflatoxin alone did not significantly reduced body weights until the second week. When aflatoxin was fed with 0.5% HSCAS, body weights were not significantly different than the control. When aflatoxin was fed in combination with zearalenone, body weights were reduced earlier (after only

one week) and the 0.5% HSCAS inclusion offered no a significant protection. Aflatoxin alone caused significant increases in the relative weights of the liver, kidney, heart, ventriculus, spleen, and pancreas. These effects were not any different when zearalenone was fed in combination. Also, zearalenone alone did not affect any of the relative organ weights. HSCAS significantly improved the relative organ weights affected by aflatoxin alone, but failed to offer protection against the increased relative liver weight when both mycotoxins were fed. Aflatoxin alone significantly reduced the levels of serum total protein, albumin, triglycerides, cholesterol, calcium, and glucose. Activity of creatine kinase was significantly increased and activity of lactate dehydrogenase and aspartate aminotransferase significantly reduced by aflatoxin alone. HSCAS offered significant amelioration to the effects of aflatoxin alone, but was less efficacious when zearalenone was in combination with aflatoxin. This again demonstrates that HSCAS adsorbs aflatoxin with a specific affinity that is not present with other mycotoxins, and that a combination of aflatoxin with another type of mycotoxin can show increased toxicity compared to the effects of either toxin alone.

When feeding aflatoxin produced by inoculating rice with *Aspergillus parasiticus* at a dietary concentration of 5000 ppb for three weeks with or without a commercial HSCAS (T-Bind™) included at 0.25 or 0.375% as a 2x2 complete factorial, Kubena, et al. (1998) found that aflatoxin began to significantly reduce weekly weight gains after two weeks of exposure and that it reduced the cumulative three-week weight gains. The FCR was also significantly increased by aflatoxin after three weeks. Including the

HSCAS at both doses significantly improved weight gain and FCR relative to the birds fed 5000 ppb aflatoxin.

Miazzo, et al. (2000) fed 2500 ppb aflatoxin to three-week old male broilers for a three-week trial (until birds were six weeks of age) to evaluate the efficacy of another aluminosilicate material, a synthetic zeolite clay, when included in treatment diets at 1%. The zeolite used had been previously synthesized by Basaldella et al. (1997). Aflatoxin significantly reduced the cumulative weight gain during the study. When the synthetic zeolite was included at 1%, weight gains were not significantly different from either the aflatoxin treatment or the control, suggesting that there was some level of protection but not complete amelioration. Relative liver weights were significantly increased by 2500 ppb aflatoxin and were improved by the presence of the synthetic zeolite, but the livers were still significantly heavier than the control.

Oguz, et al. (2000 and 2002) evaluated clinoptilolite (a natural zeolite clay) included at 1.5% in treatment diets containing 50 or 100 ppb aflatoxin fed to broilers for a full six weeks. In Oguz, et al. (2000), aflatoxin at 100 ppb significantly reduced cumulative weight gains, but the inclusion of clinoptilolite offered no significant ameliorative effect. Aflatoxin at 100 ppb also significantly increased the weekly feed conversion ratio during the final week of the trial. However, there was no clay effect for the 1.5% clinoptilolite inclusion on the sixth week's FCR. In Oguz, et al. (2002), aflatoxin at either 50 or 100 ppb had no effect on serum total protein, albumin, cholesterol, uric acid, or potassium. Aflatoxin at 50 ppb significantly decreased the activity of aspartate amino transferase and alanine amino transferase, while 100 ppb

significantly increased these activities. Liver damage is typically associated with the increase of these particular liver enzymes. It is possible that the 50 ppb treatment was enough to impair general protein synthesis in the liver without being severe enough to actual damage the tissue and cause the enzymes to "leak" into general circulation. The inclusion of 1.5% clinoptilolite did not ameliorate either of these effects. The authors concluded that the ameliorative efficacy of clinoptilolite was not demonstrated in their trials because the relatively low level of aflatoxin used was not able to produce a significantly measurable level of aflatoxicosis that is seen when greater concentrations are included. This highlights the problem for researchers, which is that without a more sensitive measure of aflatoxicosis, the preventive efficacy of such additives cannot be demonstrated while using low levels (≤ 100 ppb).

In order to mirror industry rearing conditions, Bailey, et al. (2006) fed straight-run boilers for six weeks on floor pens and fed them pelleted diets containing 0 or 3600 ppb aflatoxin. Treatments were arranged as a 2x2 factorial of the two aflatoxin levels with and without 0.5% of the HSCAS Novasil™. The weight gained by birds consuming aflatoxin was significantly reduced at three, five, and six weeks into the study, and also on a cumulative basis. Including the clay-based additive significantly improved weight gains relative to the aflatoxin diet but the birds did not grow as well as the control birds fed no aflatoxin. The feed-to-gain ratio was significantly increased by 3600 ppb aflatoxin at weeks three and five and also on a cumulative basis. As seen with weight gain, while birds fed diets contaminated diets that included 0.5% of the HSCAS Novasil™ were significantly better than those fed aflatoxin without clay, they failed to perform as well as

the control treatment. Bailey, et al. also found that 3600 ppb aflatoxin significantly increased the relative liver, kidney, and spleen weights. Though diets containing 0.5% of the HSCAS Novasil™ were significantly better than those fed aflatoxin without clay with respect to the relative liver and kidney weights, the feed additive was able to offer complete protective efficacy for the relative spleen weights.

Gowda, et al. (2008) evaluated the efficacy of 0.5% HSCAS and 0.5% turmeric powder (which contained 74 mg/kg of antioxidant curcuminoids) in diets containing 1000 ppb aflatoxin fed to male broilers over a three-week period arranged as a complete 2x2x2 factorial. Aflatoxin significantly reduced feed intake and weight gain and significantly increased relative liver weights. The inclusion of 0.5% HSCAS fully ameliorated the effects of aflatoxin to levels comparable to that of the control. Turmeric powder alone had no effect on the reduction in feed intake and weight gain, and offered only a partial protection against the hepatitis. Supplementing both HSCAS and turmeric powder in combination provided no greater protection than the HSCAS alone. Aflatoxin significantly reduced serum total protein, albumin, cholesterol, and calcium. Values for uric acid, phosphorous, and activity of γ-glutamyl transferase were unaffected. Both HSCAS and turmeric powder alone significantly improved the affected serum parameters to a degree, but they remained significantly different from the control. However, when fed in combination in diets containing aflatoxin, the serum values were restored to levels comparable to the control. Aflatoxin caused a significant increase in lipid peroxides. When HSCAS and turmeric powder were included either alone or in combination, the lipid peroxides were reduced back to levels seen in the control treatment.

Zhao, et al. (2010), evaluated two additives: a mixture of different HSCAS clays and a combination of clay and yeast cell wall derivatives, at two concentrations (0.1 and 0.2%). These additives were included with either 1000 or 2000 ppb aflatoxin supplied by *Aspergillus parasiticus* culture material. Treatments were arranged as a complete 2x2x2 factorial arrangement and fed to straight-run broilers for three weeks. Results found no significant effect on weight gain with 1000 ppb. Reductions in weight gain were only evident with 2000 ppb aflatoxin. The authors also reported no effect on feed conversion by either level of aflatoxin; however feed consumption was reduced by 1000 ppb and further reduced by 2000 ppb. Both additives significantly improved performance when in the diets containing 1000 ppb. Only by including HSCAS at 0.2% were the effects of aflatoxin at 2000 ppb ameliorated. The clay/yeast cell wall combination product had no effect in the 2000 ppb treatment. Relative liver weights were significantly increased by 1000 ppb and further increased by 2000 ppb. Both levels of HSCAS offered partial amelioration of the effects of 1000 ppb aflatoxin, but only 0.2% had any significant effect on the 2000 ppb treatment. Again the clay and yeast cell wall product had no effect on the impact on the liver caused by aflatoxin. There were deceases in serum total protein, albumin, and globulin when feeding 1000 ppb aflatoxin for three weeks, but no effect on serum glucose, sodium, potassium, chloride, calcium, phosphorous, uric acid, or the activities of aspartate aminotransferase, γ-glutamyl transferase, alkaline phosphatase, and creatine phosphokinase. In the treatment diet containing 2000 ppb, broilers showed (in addition to the decreases seen with 1000 ppb) significant decreases in calcium, phosphorous, glucose, and the activity of alkaline phosphatase as well. Similar to the

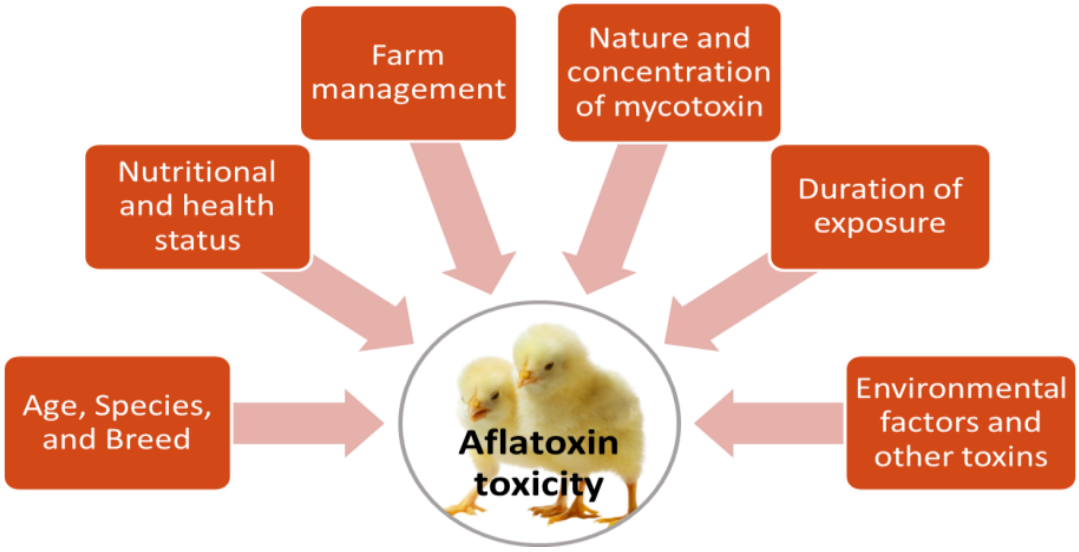
other data, HSCAS showed significant efficacy, whereas the clay and yeast cell wall product did not, and 0.2% HSCAS was required to significantly improve parameters in the 2000 ppb group. This meant that there was a significant main effect of adsorbent inclusion level observed for the serum biochemical parameters that was not seen in the performance data, suggesting serum biochemistry (specifically the total protein, albumin, and globulin) may be considered a more sensitive assay for toxicity in broilers, especially when evaluating an optimal inclusion level for feed additives.

In Neeff, et al. (2013), samples from both the liver and kidney were collected for analysis of aflatoxin residues [B_1, B_2, G_1, and G_2] and the detoxified metabolites AFM_1 and aflatoxicol from birds that had been fed either 0 or 2500 ppb aflatoxin in the presence or absence of a HSCAS included at 0.5%. They found that 2500 ppb aflatoxin fed for three weeks significantly increased liver concentrations of all forms of aflatoxin analyzed except for aflatoxin G_2 (all aflatoxin forms were increased in the kidney). The amount of aflatoxin B_1 in the liver was 8.32 ppb, and total aflatoxins were 12.78 ppb. Including HSCAS in the aflatoxin diet reduced liver residues of aflatoxin B_1, B_2, and G_1 and aflatoxicol (and kidney residues of B_1) to levels statistically similar to that of the 0 ppb diet (B_1 in the liver of the 2500 ppb + HSCAS treatment was 1.49 ppb, and total aflatoxins for that treatment were only 2.1 ppb). This was the only variable for which the HSCAS showed a full amelioration effect by returning the aflatoxin-contaminated diet to a level that was not significantly different than the aflatoxin-free control diet. This suggests that not only are liver residues of aflatoxin a sensitive measure of exposure, but that it may stand as a more sensitive indicator with respect to evaluating clay-based

adsorbents. Results also showed that HSCAS was effective at reducing aflatoxin residues, especially in liver (which is an edible tissue). The biotransformation of aflatoxin B_1 into aflatoxin M_1 and aflatoxicol in the liver also decreased in broilers receiving the HSCAS. Favoring the formation of a HSCAS-aflatoxin B_1 complex restricts the absorption of aflatoxin in the intestine, which prevents aflatoxin B_1 from being transported to the liver where it produces its toxic effects.

Evaluating the biological effects of aflatoxin on research animals can be complicated by the relatively diverse range of effects the toxin can have *in vivo*. There are toxin-related (the presence of other mycotoxins or the level and duration of intake), animal-related (species, age, breed, over-all health, immune status, or nutritional standing) and environmental (farm management, general hygiene, or temperature) factors that all contribute to the effect that a given level of aflatoxin may exert (Figure 2-1).

Figure 2-1: Combination of factors that contribute to the total biological effect of aflatoxin



For example, Doerr, et al. (1983) observed in two trials performed concurrently under the same rearing conditions that body weights were significantly reduced by 75 ppb aflatoxin in one experiment, while in the second body weights were not significantly affected until the dose was 2700 ppb. This demonstrates the unique challenge in both attempting to set a "safe level" of aflatoxin for regulation standards, as well as establishing consistent research methodology for generating aflatoxicosis for the evaluation of clay-based feed additives.

Giambrone, et al. (1985, 1) reported no significant reductions in antibody titer, cell-mediated immune response, or resistance to specific pathogens challenges by aflatoxin B_1 fed alone. However, when B_1 was fed in combination with B_2, reductions in cell-mediated immunity were significant at 100 ppb total aflatoxins, and at 400 ppb there was a significant reduction in three of the four cell-mediated immune response assays. This suggests that in addition to genetic variations in the birds and any environmental stressors that may increase the toxicity of a given dose of aflatoxin, the presence of aflatoxin B_2 can interact with B_1, increasing the toxicity of a given dose level of total aflatoxins.

When straight-run broilers were fed 50 ppb aflatoxin, Oguz, et al. (2000) reported no significant differences in weight gain, feed consumption, or feed conversion during a six-week trial, when compared to the control treatment. However, the authors considered this a function of a well-controlled experimental environment, rather than suggesting that 50 ppb aflatoxin is non-toxic or inert.

Huff, et al. (1992), Kubena, et al. (1993), and Raju and Devegowda (2000) reported additive effects whenever two mycotoxins were included in combination with aflatoxin, though the authors found that simultaneous feeding of three common mycotoxins (aflatoxin, ochratoxin-A, and T-2 toxin) fails to cause an increased effect relative to feeding any two.

Nutritional status may also affect the susceptibility of broilers to aflatoxin. Hamilton, et al. (1974) found a significant interaction between the presence of 625 ppb aflatoxin and the absence of both riboflavin and vitamin D_3. Male broilers had no change in body weights in the presence of aflatoxin alone. However, when aflatoxin was in combination with either a riboflavin or a D_3 deficient treatment diet, that dose was sufficient to reduce body weight.

The nature of how grain is contaminated in the field with the *Aspergillus* species that may go on to produce aflatoxin contributes to a heterogeneous distribution of the toxin within the feedstuff. A sample can mostly contain corn particles with less than 20 ppb aflatoxin but be punctuated by a relatively small amount of aflatoxin "hot-spots" that contain much higher amounts (in the 1000's of ppb). The "hot-spots" are the areas where *Aspergillus* species were able to gain entrance and establish a presence in the corn grain, and where they went on to produce aflatoxin. This inherent heterogeneity of aflatoxin distribution within contaminated feedstuffs can contribute variability into analytical quantifications of aflatoxin concentration in a specific lot and imparts variability into experimental bioassays. Should one particular bird happen to eat more feed that came from one of these contaminated "hot-spots" than its pen mates, the variability of the

response within that pen would increase. Greater variability within experimental treatments contributes to less observed amelioration *in vivo* than would be expected from feed additives that show a high-binding affinity for aflatoxin in a pure solution *in vitro*.

The objectives of this research will be to evaluate common biological assessments for measuring aflatoxicosis in broilers (such as growth rate and relative organ weights) along with variables such as aflatoxin-residues in the liver or hepatic gene expression, pursuant to identifying a more sensitive biological assay that will allow three-week broiler trials to be conducted at aflatoxin concentrations <1000 ppb, prior to significant changes in the growth rate or relative organ weights. This will help us both better understand how aflatoxicosis presents in broilers, as well as help us evaluate the efficacy of novel clay-based binders for their ability to ameliorate aflatoxicosis under experimental conditions. In terms of the evaluation of clay-based additives, this may also be a variable that will allow us to more accurately reflect what happens in a "real world" scenario.

CHAPTER III

EFFECTS OF HYDRATED SODIUM CALCIUM ALUMINOSILICATE AND CALCIUM BENTONITE CLAYS WHEN INCLUDED IN DIETS CONTAINING AFLATOXIN-CONTAMINATED CORN

Introduction

Mycotoxins are a group of secondary metabolites produced by certain fungal species (e.g. *Aspergillus*, *Penicillium*, or *Fusarium*). Within the family of mycotoxins, there are a variety of compounds with varying chemical structures, biological properties, and levels of toxicity. Aflatoxins are a member of this mycotoxin family and are among the most potent natural carcinogens. The use of clay-based adsorbents has proved effective at reducing the toxic effects of aflatoxin-contamination in animal feeds. These various aluminosilicate and bentonite clays have been shown *in vitro* to bind aflatoxin with relatively high affinity (Phillips, 1999; Desheng, 2005; and Kannewischer, 2006). This binding within clay smectite layers allows the aflatoxin to pass through the gastrointestinal tract unabsorbed, reducing its toxic effects.

The efficacy of hydrated sodium calcium aluminosilicates (HSCAS) has been evaluated extensively by Kubena, et al. during the 1990's (Kubena et al, 1990, 1993, and 1998). In those studies, the clay-based additive Novasil™ was included in diets containing aflatoxin at concentrations exceeding 2000 ppb (either alone or in combination with other mycotoxins) and fed to male broilers over three-week trial periods. Significant ameliorative effects on weight gain, feed conversion, relative organ weights, and serum biochemical parameters were observed. Bentonite clays are also

adsorbent aluminum silicates that have been produced from volcanic ash deposited in marine environments. There are differences among bentonites with respect to the exchangeable cations in the clay structure (e.g., calcium or sodium). Calcium bentonites are considered better adsorbents of aflatoxin due to their greater smectite layer separation when compared with sodium bentonites.

This current study was designed to evaluate a calcium bentonite clay recently mined from the Gonzalez region of Texas with respect to its ability to ameliorate aflatoxicosis when compared to the commercially-available hydrated sodium-calcium aluminosilicate (Novasil™) as a positive control.

Materials and methods

Aflatoxin for this trial was produced by inoculating yellow dent corn with live fungal cultures of *Aspergillus parasiticus*. Inoculated grains were incubated for five days at 37°C and then kept in a portable cement mixer under green-house conditions for one week with daily mixing. After being dried and ground, the contaminated corn was then assayed by the Office of the Texas State Chemist for total aflatoxin to estimate how much should be included to achieve the desired concentrations in the final diets. Four mash basal diets were prepared by blending increasing concentrations of the aflatoxin-contaminated corn mixed with non-contaminated corn into a basal soybean meal concentrate such that there would be 0, 500, 1000, and 2000 ppb of total aflatoxins in the final diets. Each diet was then sub-divided and blended with either 0.5% of the HSCAS Novail™ (NOVA), a calcium bentonite (TX4), or a non-aflatoxin binding kaolinite clay (NBC).

A total of 240 straight-run, Ross 308 broiler chicks obtained on the day of hatch were housed in a Petersime battery brooder unit. Five birds were randomly selected for placement into one of the 24 pens. Treatments were assigned such that each had equal representation throughout the rearing room. Also, initial weight per pen was controlled so as to be within ±10 grams of the total average flock weight of five chicks (30% of all birds were sampled to obtain an average flock weight per chick). Birds were allowed *ad libitum* access to feed and water for 21 days. Feed consumption and body weight per pen were recorded weekly. Mortality was checked for and monitored daily. Upon termination, the organ weights for the liver and the kidney were collected and relative organ weights were calculated on a per bird basis. All personnel handling birds, feed, or fecal material from this study were required to wear N95 particulate respirators (3M™, No. 9211), latex gloves, and spun-bonded polypropylene coveralls (VWR®, Basic Protection SPP Coveralls). All methods were approved by the Texas A&M Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Data were analyzed as a two-way ANOVA for main effects of clay, aflatoxin, and their interaction using the General Linear Model procedure of SPSS. Significant means ($p \leq 0.05$) were separated using Duncan's Multiple Range Tests.

Results

Aflatoxin had no effect on any of the growth parameters during the first week (Table 3-1). The TX4 clay had a higher FCR when compared to the NBC at the first week, but this effect was not present throughout the rest of the study. After two weeks consuming the treatment diets, there was a significant main effect for aflatoxin

concentration, which showed that the highest dose (2000 ppb) reduced body weights. There was no significant clay effect for body weight that may have suggested amelioration. The results for both performance and organ weights upon completion of the study at three weeks of age are presented in Table 3-2. Aflatoxin had significant effects on body weight, productivity index, and the relative weights of the liver and kidney. Body weights were reduced by 1000 ppb and were reduced further by 2000 ppb. There was no difference between 500 ppb and the 0 ppb control for any of the variables. The clay additive had a significant main effect for both body weight and productivity index. Both aflatoxin-binding clay types (NOVA and TX4) had higher body weights when compared to NBC. However, with respect to the broiler productivity index, only NOVA had a higher value than the NBC treatment. An interaction between aflatoxin and clay was seen in the relative liver weights. The nature of the interaction was that in the NBC treatment, 500 ppb caused a significant increase in the relative liver weights, whereas for both of the aflatoxin-binding clays, 0 and 500 ppb liver weights were not considered different from each other.

Table 3-1: Performance effects during the first two weeks on aflatoxin-contaminated diets

	Day 7		Day 14	
	BW	FCR	BW	FCR
0 ppb	130±3	1.28±0.02	354±7 [a]	1.47±0.02
500 ppb	131±6	1.28±0.02	344±11 [a]	1.41±0.04
1000 ppb	132±5	1.29±0.03	322±16 [a]	1.47±0.05
2000 ppb	129±3	1.26±0.03	267±10 [b]	1.50±0.04
NBC	129±4	1.24±0.02 [a]	309±17	1.47±0.03
NOVA	133±4	1.27±0.02 [ab]	331±16	1.43±0.02
TX4	130±4	1.32±0.02 [b]	326±13	1.48±0.05
		ANOVA		
Aflatoxin	0.973	0.766	<0.001	0.380
Clay	0.790	0.037	0.236	0.573
*Aflatoxin*Clay*	0.900	0.350	0.287	0.273

Data are presented as means ± SEM
[a,b] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
n for clay type = 8
n for aflatoxin level = 6

Table 3-2: Performance variables and relative organ weights after three weeks on aflatoxin-contaminated diets

	BW	FCR	Mort	PI	Relative Liver (%)	Relative Kidney (%)
0 ppb	706±15 [a]	1.60±0.03	0.0±0.0	196±6 [ab]	3.37±0.10 [a]	0.76±0.03 [a]
500 ppb	694±26 [a]	1.54±0.04	3.3±3.3	204±13 [a]	3.54±0.14 [a]	0.87±0.04 [a]
1000 ppb	619±26 [b]	1.61±0.06	3.3±3.3	173±14 [b]	4.04±0.11 [b]	1.23±0.05 [b]
2000 ppb	492±16 [c]	1.61±0.04	6.7±6.7	139±8 [c]	4.71±0.19 [c]	1.61±0.09 [c]
NBC	590±38 [b]	1.62±0.04	0.0±0.0	160±13 [b]	4.09±0.17	1.17±0.08
NOVA	651±36 [a]	1.54±0.02	10.0±5.4	193±10 [a]	3.77±0.12	1.08±0.08
TX4	642±32 [a]	1.60±0.05	0.0±0.0	180±12 [ab]	3.84±0.13	1.08±0.07
				ANOVA		
Aflatoxin	<0.001	0.651	0.726	0.001	<0.001	<0.001
Clay	0.026	0.324	0.110	0.037	0.140	0.374
*Aflatoxin*Clay*	0.220	0.752	0.835	0.533	0.023	0.338

Data are presented as means ± SEM
[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
Mort = Cumulative mortality (%)
PI = Broiler productivity index (Livability [%] x Live weight [kg] / age [d] / FCR x 100)
n for clay type = 24
n for aflatoxin level = 12

Discussion

During the first week, aflatoxin as high as 2000 ppb did not affect either body weight or feed conversion. The 2000 ppb dose began to reduce growth after two weeks, and it took the full three weeks before it was reduced by 1000 ppb. There is a definite time-factor involved in determining what a given dose of aflatoxin will do in terms of production parameters like growth rate or feed conversion. In this study, it was not until the second week that the effects of aflatoxin were becoming apparent. This suggests that very young broilers can be robust against acute doses if they are only fed for a very short period of time.

The body weights and feed conversion ratios between 500 ppb and the control were not significantly different at any point throughout this study, however doses of 500 ppb and lower have reduced weight gain in other trials when fed for 4-6 weeks (Giambrone, et al. 1985; Raju, et al. 2005; Kermanshahi, et al. 2009). Though aflatoxin did not affect the body weights or the relative organ weights until the dose was 1000 ppb, this would not necessarily mean that 500 ppb can be considered "negligible" or "safe" (an observation also noted by Oguz, et al. 2000). This finding simply highlights the difficulty in "scaling-up" findings from well-controlled experimental trials, as well as the need to identify a more sensitive biological assay that allows for broiler trials to be conducted at aflatoxin concentrations less than 1000 ppb, prior to the significant impacts in variables like growth and relative liver weight.

In terms of the effects of the calcium bentonite (TX4) compared with NOVA, both clays appeared able to sequester aflatoxin, as both had significantly higher body

weights when compared to the NBC control at the end of the three-week study. In terms of the broiler productivity index (which includes mortality, body weight, and feed conversion into one value measuring over-all bird performance), only the NOVA clay was significantly better than NBC. Both aflatoxin-binding clays appeared able to manage 500 ppb, as relative liver weights from that dose were not different from the 0 ppb control (and they were different in the NBC treatment). Since there was no main effect for the clay-type in either relative organ weights, it is possible that higher doses exceeded the capacity of NOVA and TX4 to bind all of the aflatoxin present.

In evaluating this particular calcium bentonite alongside Novasil™ as a positive control (both included at 0.5%), there may be some concerns with the TX4 clay having increased the one-week FCR and not having altered the three-week productivity index, but overall it appears capable of ameliorating aflatoxicosis when compared to NOVA.

CHAPTER IV

EVALUATION OF HEPATIC CYP1A1 AND CYP2H1 GENE EXPRESSION IN THE LIVER TISSUE OF BROILERS FED A RANGE OF DIETARY AFLATOXIN CONCENTRATIONS

Introduction

Aflatoxins are secondary metabolites produced by certain *Aspergillus* species of mold that, once produced, are extremely carcinogenic to a wide-range of animal species. The mold species that produce aflatoxin are ubiquitous and considered unavoidable contaminants of major cereal grains, even where good manufacturing practices have been followed. The Food and Drug Administration has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed by establishing action levels that allow for the removal of violative feed lots from commerce. The action level for most poultry feeds is 20 ppb.

The reported data on the effects of aflatoxin on the relative organ weights and serum biochemistry of broilers suggest that the liver can be considered the "target organ" for aflatoxicosis. Thus, the expression of specific genes in the liver tissue represent a likely "first stop" in the biological response to aflatoxin, as the contents absorbed by the gastrointestinal tract travel first to the liver via the portal blood system. The cytochrome p450 class of enzymes (CYP) are involved in the biotransformation of various xenobiotic compounds into metabolites that are more water soluble and available for excretion. Though the CYP-mediated reactions are essential for detoxification, they can generate reactive oxygen species and are responsible for the bioactivation of aflatoxin B_1 into the

carcinogenic metabolite aflatoxin B_1-8,9 epoxide. Previous research by Yarru et al. (2009) has found that certain CYP-isoforms (specifically CYP1A1 and CYP2H1) are upregulated while epoxide hydrolase, GST, and glutathione peroxidase genes are downregulated by 2000 ppb aflatoxin. This combination favors the formation of aflatoxin B1-8,9 epoxide, while providing less of a chance for any biological antioxidant response (Yarru, et al. 2009, 1). In Yarru, et al. (2009, 2) day-old male broiler chicks were reared for three weeks on diets containing either 0 or 1000 ppb aflatoxin. The expression patterns of hepatic genes involved in antioxidant function, the biotransformation of aflatoxin, and immune function were quantified via real-time PCR. Real-Time PCR detects the accumulation of the amplified material through each cycle of the reaction. This is in contrast to "basic" PCR, which detects the product at the end-point, after the reaction has plateaued. Detection has greater sensitivity when it is done during the exponential portion of the reaction, when product is doubling at every cycle. Through using this more sensitive detection method, Yarru, et al. found that aflatoxin at 1000 ppb caused significant increases in the expression rate of the CYP1A1 and CYP2H1 isoforms.

This current study was designed to evaluate the expression of the two CYP isoforms (CYP1A1 and CYP2H1) highlighted in the work by Yarru, et al. under a wider range of aflatoxin concentrations to determine the lowest level of toxin at which the CYP genes would be differentially expressed. The hypothesis of the study was that hepatic gene expression serves as a more sensitive bioassay when compared to the dose response of variables such as weight gain or relative organ weight.

Materials and methods

Bird performance

Aflatoxin for this trial was produced by inoculating yellow dent corn with live fungal cultures of *Aspergillus parasiticus*. Inoculated grain was then incubated for five days at 37°C and then kept in a portable cement mixer under green-house conditions for one week with daily mixing. A total of 60 straight-run, Ross-308 broilers reared under identical conditions on a control corn-soy diet for three days and were started on treatment diets containing 0, 300, 500, 1000, 2000 and 4000 ppb in the final feed. Body weights and the relative organ weights of the liver and kidney were measured for 2 or 3 birds per treatment after 7, 10, and 13 days of age. Upon termination at 16 days of age, body weights, liver tissues, and liver and kidney weights from two birds per treatment were collected. The liver samples were flash-frozen in liquid nitrogen and stored at -80°C prior to an evaluation of the expression of the hepatic CYP1A1 and CYP2H1 genes using quantitative real-time PCR (qRT-PCR).

The body weight and relative liver and kidney weights from the two birds per treatment were averaged to determine the dose response to aflatoxin. All personnel handling birds, feed, or fecal material from this study were required to wear N95 particulate respirators (3M™, No. 9211), latex gloves, and spun-bonded polypropylene coveralls (VWR®, Basic Protection SPP Coveralls). All methods were approved by the Texas A&M Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

RNA extraction

RNA was extracted from each of the liver samples collected from each treatment. Tissue samples (100 mg) were homogenized in 1 ml TRIzol® reagent and allowed to incubate for 5 minutes at room temperature. Chloroform was added (0.2 ml) and the samples vortexed and then centrifuged (12,000 x g) for 15 minutes at 4°C. The upper aqueous phase (containing the RNA) was collected and 0.5 ml of RNase-free isopropyl alcohol was added. RNA isolates were then incubated at RT for 10 minutes and centrifuged (12,000 x g) for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol prior to vortexing followed by centrifuging (7500 x g) for 5 minutes at 4°C. The ethanol wash was discarded and the pellet was air-dried for 5 minutes at RT. Finally, the RNA pellet was resuspended in DEPC-treated water.

A 1 µl sample of the RNA suspension was diluted with 49 µl of DEPC-treated water and placed in a cuvette to analyze the absorption ratio of 260 nm vs. 280 nm. Ratios were verified to be approximately 2.0 because that is generally accepted as "pure" RNA prior to cDNA synthesis. Lower ratios would suggest contamination by DNA, protein, or phenols from the TRIzol®.

Gene expression

Reverse transcription of cDNA from the RNA isolates was conducted using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Total RNA used for each reaction was 2 µg per 20 µl reaction volume. Samples were placed in a thermal cycler for 10 minutes at 25°C, then 120 minutes at 37°C, followed by 5 minutes

at 85°C to terminate the reaction. Samples were stored at -20°C prior to qRT-PCR analysis. Specific primers designed for the two CYP genes (CYP1A1 and CYP2H1) and for GAPDH (to be used as an endogenous control) were used (Table 4-1).

Table 4-1: Primers used for quantitative real-time PCR analysis of liver tissues

Gene	Forward	Reverse
GAPDH	GGAGTCCACTGGRGTCTTCAC	CTTAGCACCACCCTTCAGATG
CYP1A1	AAGCGCTACGACCACCACGAC	AAACTCATCGACCACGTTCA
CYP2H1	CCCAGAGACTTCACCATCA	GCACAGCTTTAGCGCTTCTC

Relative changes in the gene expression between treatments for the two CYP isoforms were determined via qRT-PCR using Applied Biosystems® 7900HT Fast Real-Time PCR machine. The double-stranded DNA was visualized using *Power* SYBR® Green Master Mix (Applied Biosystems®). The data were analyzed using the comparative $\Delta\Delta C_t$ method, where $\Delta\Delta C_t = \Delta C_{t,\ sample} - \Delta C_{t,\ reference}$. Here, $\Delta C_{t,\ sample}$ is the C_t value of CYP1A1 and CYP2H1 for each of the aflatoxin doses normalized to the endogenous control gene (GAPDH) and $\Delta C_{t,\ reference}$ is the C_t value for the 0 ppb treatment also normalized to the expression of GAPDH. Data were expressed as fold-change in expression across treatments.

Results

The results for body weight and relative liver and kidney weights represent the average (± SEM) of the two broilers selected from each treatment group for tissue collection at 16 days of age after birds had consumed their respective treatment diets for 13 days (Figures 4-1, 4-2, and 4-3). All three variables showed a consistent dose response that demonstrated a sharp change beginning at the 1000 ppb dose that then plateaued as aflatoxin increased up to 4000 ppb.

The impact of aflatoxin on the expression of CYP1A1 and CYP2H1 are shown in Figures 4-4 and 4-5. The dose response of CYP1A1 was relatively unchanged until 1000 ppb, at which point the gene showed an expression rate approximately 6-fold that of the 0 ppb control diet. That same increase in expression rate continued as aflatoxin increased up to 4000 ppb. The CYP2H1 isoform showed no changes in response to the entire range of dietary aflatoxin.

Figure 4-1. Body weight per bird (g) after 13 days on aflatoxin treatments (16 days of age).

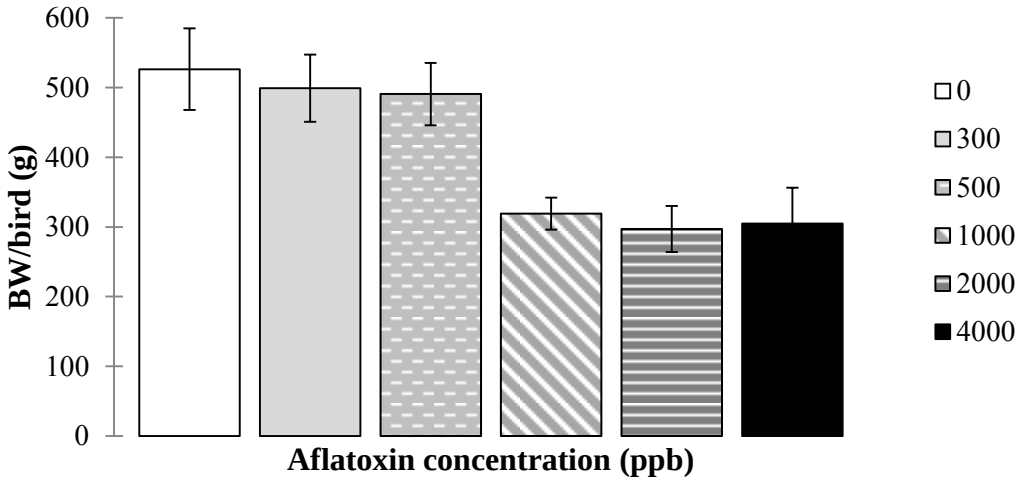


Figure 4-2. Relative liver weights after 13 days on aflatoxin treatments (16 days of age).

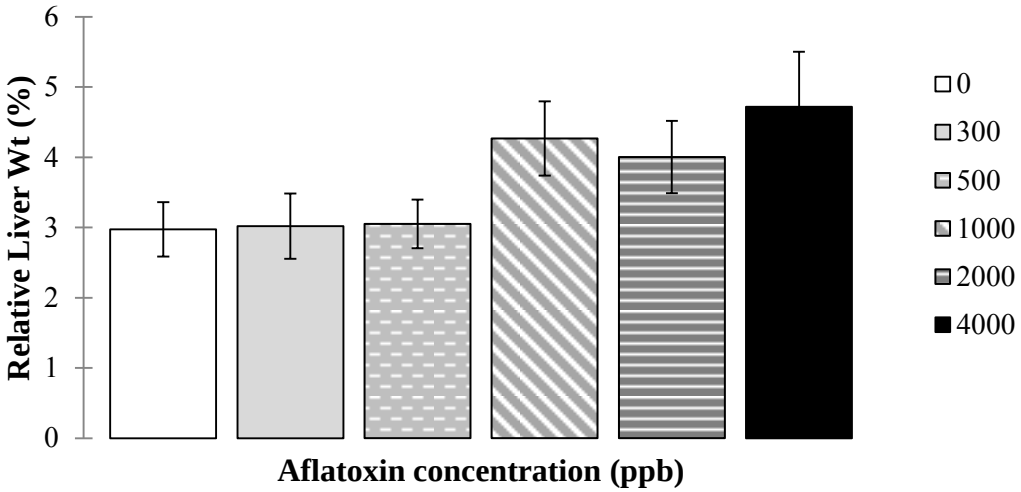


Figure 4-3. Relative kidney weights after 13 days on aflatoxin treatments (16 days of age).

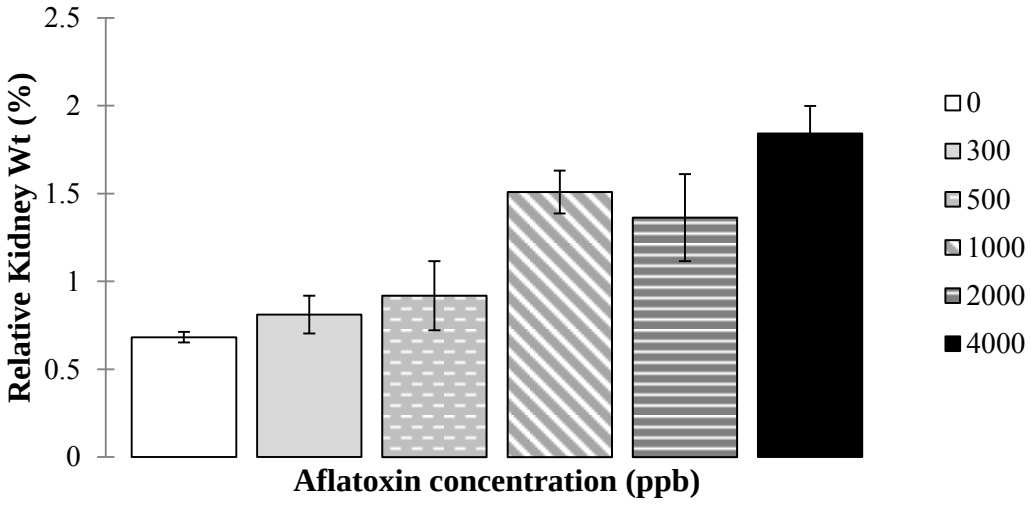


Figure 4-4. CYP1A1 gene expression.

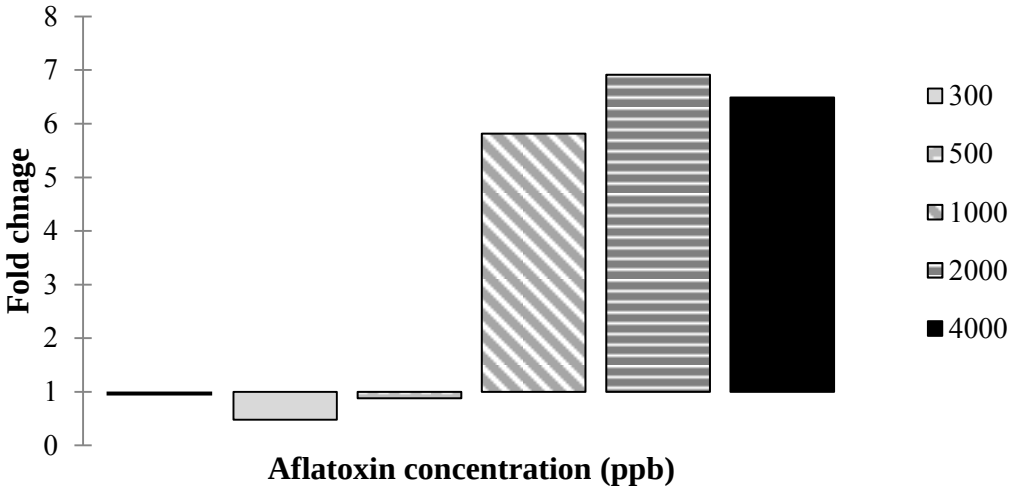
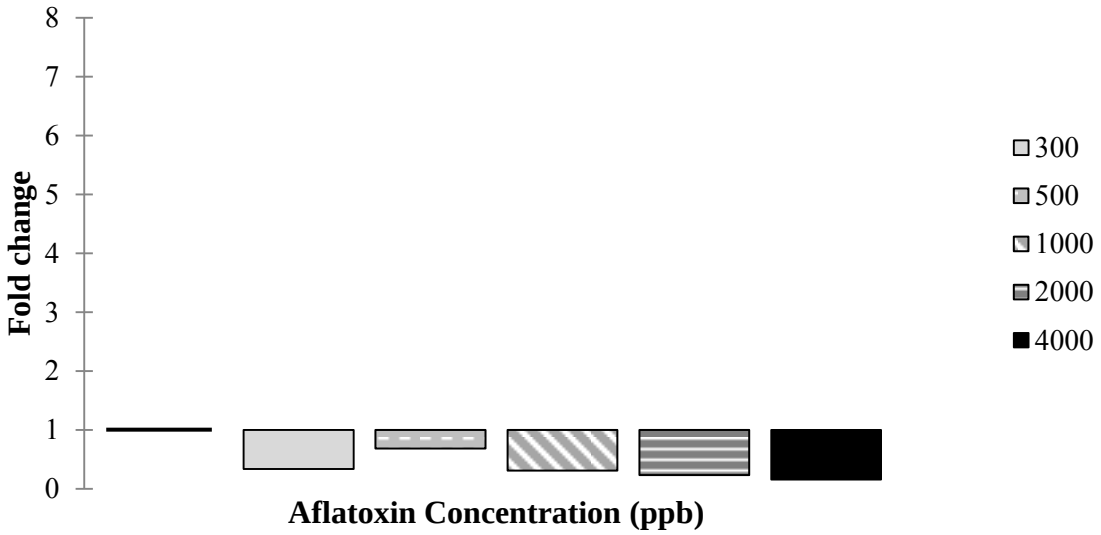


Figure 4-5. CYP2H1 gene expression.



Discussion

Aflatoxin B_1 is biotransformed in the liver by the cytochrome P-450 class of enzymes into the carcinogenic metabolite aflatoxin B_1-8,9 epoxide. In Yarru, et al. (2009), two CYP isoforms (CYP1A1 and CYP2H1) were highlighted as being upregulated in response to levels of aflatoxin ≥1000 ppb. Also, in Diaz, et al. (2010) it was reported that CYP1A1 was an isoform that could be considered responsible for the bioactivation of aflatoxin B_1 into aflatoxin B_1-8,9 epoxide in chicken livers. However, in attempting to identify a sentinel for aflatoxin exposure in the hepatic gene regulation, the two CYP isoforms evaluated in this study failed to differentiate aflatoxin exposure in a manner different than what could have been done by looking at the body weight or relative organ weights alone.

The growth and relative organ weight data were consistent with previous experience in our laboratory, meaning we saw no significant impact of aflatoxin at doses less than 1000 ppb. When the gene expression data from the liver samples were analyzed, the genotypic effect of aflatoxin on the CYP1A1 and CYP2H1 isoforms simply mirrored the phenotypic effects on growth and relative organ weight. While the upregulation of CYP1A1 seen in this study mirrored the reduction in body weight and the increases in relative liver and kidney weights seen at the same concentration (1000 ppb), CYP2H1 showed no upregulation in response to aflatoxin. Though the upregulation of CYP1A1 at 1000 ppb that was seen in this study was in agreement with the findings in Yarru, et al. (2009), it failed to confirm our hypothesis that aflatoxin would alter the gene expression prior to measurable phenotypic changes.

CHAPTER V

UTILIZATION OF A SPRAY-APPLIED CALCIUM BENTONITE CLAY TO AMELIORATE THE EFFECTS OF LOW-LEVELS OF AFLATOXIN IN BROILER DIETS CONTAINING DDGS

Introduction

With ethanol receiving considerable attention as a potential source for renewable fuel in recent years, corn prices have increased and the use of fermentation co-products, such as distiller's dried grains and solubles (DDGS), have become more common in animal feeds. Any mycotoxins contained in grain bound for fermentation will still appear in the co-products that end up in animal feeds (Lillehoi, et al. 1979). In fact, fermentation and distillation can increase mycotoxin concentrations up to three-fold in corn-derived co-products (Wu and Munkvold, 2008).

Mycotoxins are secondary metabolites produced by certain fungal species (e.g. *Aspergillus*, *Penicillium*, and *Fusarium*). Within the family of mycotoxins, there are a variety chemical structures, biological properties, and levels of toxicity. Aflatoxins are a member of this mycotoxin family and are considered one of the most potent natural carcinogens. Of the categories of aflatoxin, aflatoxin B_1 has the most potent biological effects. No animal species is immune to the effects of aflatoxin contamination in the diet. The *Aspergillus* species that produce aflatoxins are ubiquitous in the soil and are a common contaminant of feed crops in warm, humid climates. In poultry, aflatoxin exposure has been shown to have effects ranging from reduced feed intake and weight gain, decreased egg production, immunosuppression, increased relative liver and kidney

weights, impaired serum biochemistry, and ultimately mortality (Raju et al., 2005; Gowda et al., 2008; Zhao et al. 2010; Lee et al. 2012). It has been demonstrated that aflatoxin exerts its toxic effects upon absorption in the gastrointestinal tract and bioactivation of the molecule in the liver. Aflatoxin B_1 undergoes epoxidation at the terminal furan ring (8,9 position) to form the carcinogenic metabolite aflatoxin B_1–8,9 epoxide. Once activated into the epoxide, aflatoxin B_1 can form adducts with DNA, RNA, and protein, inactivating tumor suppression and impairing protein synthesis and function (Phillips, 1999).

Once produced, aflatoxins are relatively stable compounds that are not destroyed by conventional feed processing techniques. If improperly handled during harvest, transport, or storage, *Aspergillus* spp. can continue to produce more aflatoxin. Preventing the growth of aflatoxin-producing mold species on crops in the field would be an advisable strategy for reducing the incidence of aflatoxin contamination in feedstuffs. For example, the commercial product afla-guard™ is designed to deliver spores of a nontoxic strain of *A. flavus* to offer protection in the field via competitive exclusion. However, in cases where such preventative management either cannot or has not occurred, poultry producers require cost-effective methods of detoxifying contaminated seed or grain. The use of non-nutritive, clay-based adsorbents has proven effective at reducing the toxic effects of aflatoxins in various animal species. These various aluminosilicate and bentonite clays have been shown *in vitro* to bind aflatoxins in the interlayers of the clay structure as well as on the edge with relatively high affinity (Phillips, 1999; Desheng et al., 2005; Kannewischer et al., 2006). Once bound, the

aflatoxins can pass through the gastrointestinal tract unabsorbed, thereby reducing the toxic effects. The inclusion of such clay-based binders has been shown *in vivo* to have a significant protective effect against aflatoxicosis in a variety of species (Kubena et al., 1990; Huff et al., 1992; Kubena et al., 1993; Kubena et al., 1998).

The objective of the present study is to determine the protective efficacy of including a particular calcium bentonite clay as a wet, spray-application in the presence of dietary aflatoxin on the growth and performance of starting broilers fed diets containing 20% DDGS.

Materials and methods

Diet formulation

Aflatoxin for this trial was produced by inoculating yellow dent corn and DDGS with live fungal cultures of *Aspergillus parasiticus*. Inoculated grains were incubated for five days at 37°C and then kept in a portable cement mixer under green-house conditions for one week with daily mixing. The contaminated grains were then assayed by the Office of the Texas State Chemist for total aflatoxin concentration and were determined to contain 6000 and 1400 ppb for the corn and DDGS, respectively.

Dietary treatments were arranged as a complete 2 x 4 factorial design, with two clay sources at four concentrations of aflatoxin. Four mash basal diets (calculated to be isocaloric and isonitrogenous and to contain 20% DDGS) were prepared by blending various concentrations of the non-contaminated corn and DDGS with the aflatoxin-contaminated corn and DDGS with a basal soybean meal concentrate such that there would be a 0, low, medium, and high aflatoxin treatment diet (by doubling the amount of

aflatoxin-contaminated grain). Each diet was then sub-divided and blended with either 0.5% of a non-aflatoxin binding kaolinite clay (NBC) or a high-affinity binding calcium bentonite clay (TX4). Both clays were applied as a water-suspension that was spray-applied to the treatment diets with a commercial paint sprayer during mixing.

Analysis of samples of the NBC treatments diets by the Office of the Texas State Chemist found there to be 16, 228, 366, and 681 ppb in our 0, low, medium, and high aflatoxin groups, respectively. The previous work conducted using NBC and TX4 clays were at levels closer to 0, 500, 1000, and 2000 ppb. So this study presented an opportunity to evaluate the efficacy of the TX4 calcium bentonite clay at ameliorating aflatoxicosis at relatively lower levels of aflatoxin.

Growth and performance

A total of 288 straight-run, Ross 308 broiler chicks obtained on the day of hatch were housed in two Petersime battery brooder units. Six birds were randomly selected for placement into one of the 48 pens. The 8 treatments (n=6) were assigned such that each treatment had equal representation throughout the rearing room. Also, initial weight per pen was controlled so as to be within ±10 grams of the total average flock weight of five chicks (30% of all birds were sampled to obtain an average flock weight per chick). Birds were allowed *ad libitum* access to feed and water for 21 days. Feed consumption and body weight per pen were recorded weekly. Mortality was checked for and monitored daily. Upon termination, the organ weights for the liver, kidney, spleen, and bursa of Fabricius were collected and relative organ weights were calculated on a per bird basis. All personnel handling birds, feed, or fecal material from this study were required

to wear N95 particulate respirators (3M™, No. 9211), latex gloves, and spun-bonded polypropylene coveralls (VWR®, Basic Protection SPP Coveralls). All methods were approved by the Texas A&M Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Data analysis

All data were analyzed as a 2x4 full-factorial using the General Linear Model procedure of SPSS. Means for significant main effects were separated using Duncan's Multiple Range Tests at a significance defined at $p \leq 0.05$.

Results

The results for the growth parameters are presented in Table 5-1. Aflatoxin-inclusion had no effect on BW after 7 days. However, there was a significant main effect for the FCR beginning at the 250 ppb level, which was significantly higher than 0 ppb. The same effect was not seen for the cumulative FCR at 14 days, nor was there an effect on BW. Upon termination at 21 days of age, there was still no main effect for aflatoxin-inclusion on BW. The cumulative FCR was significantly increased equally by all three aflatoxin levels relative to 0 ppb. Further, a broiler productivity index was calculated (Livability [%] x Live weight [kg] / age [d] / FCR x 100), which was significantly reduced by the inclusion of aflatoxin at any of the three levels. There was no significant difference between clay-types for any variable at any of the three weeks. There was also no aflatoxin*clay interaction at any point.

Table 5-1: ANOVA and main effect means for growth parameters

	Day 7		Day 14		Day 21		
	BW	FCR	BW	FCR	BW	FCR	PI
0 ppb	186 ± 2.9	1.02 ± 0.01[a]	478 ± 6.6	1.28 ± 0.04	856 ± 15.5	1.47 ± 0.01[a]	274 ± 7.0 [a]
250 ppb	183 ± 2.0	1.08 ± 0.01[c]	455 ± 14.7	1.29 ± 0.01	819 ± 21.6	1.57 ± 0.02[b]	245 ± 8.8 [b]
400 ppb	184 ± 1.7	1.04 ± 0.01[ab]	456 ± 6.5	1.31 ± 0.01	814 ± 11.6	1.61 ± 0.04[b]	229 ± 9.7 [b]
700 ppb	186 ± 2.1	1.05 ± 0.01[b]	458 ± 4.8	1.31 ± 0.01	822 ± 12.3	1.58 ± 0.02[b]	239 ± 7.4 [b]
NBC	186 ± 1.4	1.04 ± 0.01	460 ± 8.2	1.30 ± 0.01	834 ± 12.4	1.55 ± 0.02	252 ± 7.2
TX4	184 ± 1.7	1.05 ± 0.01	464 ± 4.2	1.30 ± 0.02	822 ± 10.1	1.57 ± 0.02	242 ± 6.0
			ANOVA				
Aflatoxin	0.800	<0.001	0.238	0.623	0.258	0.001	0.004
Clay	0.342	0.340	0.695	0.804	0.446	0.339	0.245
*Aflatoxin*Clay*	0.969	0.518	0.446	0.462	0.543	0.882	0.853

Data are presented as means ± SEM
[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
PI = Broiler productivity index (Livability [%] x Live weight [kg] / age [d] / FCR x 100)
n for clay type = 24
n for aflatoxin level = 12

The relative organ weights taken at 21 days of age are presented in Table 5-2. The presence of aflatoxin significantly increased relative liver weights beginning at the 250 ppb level, and again at 400 ppb. The 700 ppb level was not significantly different from the two lower levels. Relative kidney weights were significantly increased at 400 ppb, and kidney weights from the 700 ppb level were significantly higher than the 250 ppb. The spleen and bursa of Fabricius were unaffected. The inclusion of the spray-applied TX4 clay had no significant effect on the weights of any of the organs when compared to NBC. There was also no aflatoxin*clay interaction.

Table 5-2: ANOVA and main effect means for organ collection (21 days of age)

	Liver Wt (%)	Kidney Wt (%)	Spleen Wt (%)	Bursa Wt (%)
0 ppb	2.63 ± 0.05 [a]	0.77 ± 0.02 [a]	0.09 ± 0.004	0.29 ± 0.01
250 ppb	2.78 ± 0.05 [b]	0.80 ± 0.02 [ab]	0.09 ± 0.003	0.27 ± 0.01
400 ppb	2.94 ± 0.06 [c]	0.83 ± 0.02 [bc]	0.09 ± 0.003	0.28 ± 0.01
700 ppb	2.82 ± 0.06 [bc]	0.87 ± 0.02 [c]	0.08 ± 0.003	0.28 ± 0.01
NBC	2.79 ± 0.04	0.81 ± 0.01	0.09 ± 0.002	0.28 ± 0.01
TX4	2.78 ± 0.04	0.82 ± 0.01	0.09 ± 0.002	0.28 ± 0.01
		ANOVA		
Aflatoxin	0.001	0.001	0.219	0.126
Clay	0.862	0.665	0.633	0.528
*Aflatoxin*Clay*	0.882	0.939	0.429	0.771

Data are presented as means ± SEM
[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
Organ weights are presented as a percent of the body weight
n for clay type = 24
n for aflatoxin level = 12

Discussion

This study evaluated the effects of relatively lower levels of aflatoxin (< 1000 ppb) on starter broiler performance and relative organ weights. Although weight gain was unaffected by aflatoxin, there were negative effects on feed conversion and productivity index and there was an increase in the relative weights of the liver and kidney. In this study, after 21 days on treatment diets, body weights between the 0 ppb treatment and the 700 ppb treatment only varied by 4%. In most cases in the literature where reductions in BW are reported, levels of aflatoxin in the feed are greater than 1000 ppb, whereas in this study all assayed levels were less than 1000 ppb.

Feed conversion can often present an inconsistent measure of aflatoxicosis. Most often, it is found that feed consumption significantly decreases in conjunction with weight gain, leaving the FCR unchanged. Miazzo et al. (2000) fed 2500 ppb aflatoxin to male broilers for 3 weeks beginning when the birds were 3 weeks of age. The researchers observed a significant reduction in weight gain but saw no effect on FCR. In two separate trials, Raju et al. (2005) fed 300 ppb aflatoxin to broilers for 6 weeks. In both trials, there were significant reductions in feed consumption and weight gain but no effect on FCR. Zhao et al. (2010) reported no effect on feed conversion by 1000 or 2000 ppb aflatoxin fed to straight-run broilers for 3 weeks. However, feed consumption was reduced by 1000 ppb and was further reduced by 2000 ppb. In this study, FCR was increased by 200 ppb aflatoxin after 3 weeks on treatment diets, while body weight was unaffected. This supports the conclusion that FCR should not be considered in isolation from the other production parameters with respect to aflatoxin exposure.

Along with reductions in growth rate, liver damage is a common effect that has been associated with aflatoxicosis since it was first characterized (Smith and Hamilton, 1970 and Huff et al., 1986). In this study, both the relative liver weights and relative kidney weights were significantly increased by aflatoxin. Organ weights appear to be more sensitive to aflatoxin exposure when compared with body weight or feed consumption.

The inclusion of TX4 as a clay amendment to treatment diets did not offer any amelioration for the main effects of aflatoxin dose when compared to treatment diets containing the NBC. Previous reports that included the same concentration of a clay-based binder found that the additives offer significant protection against the effects of aflatoxin. In Gowda, et al. (2008), broilers fed 0.5% of a hydrated sodium-calcium aluminosilicate in diets containing 1000 ppb aflatoxin showed significant improvements in the weight gain and relative liver weights at three weeks of age. Kermanshahi, et al. (2009) found that 0.5% of a sodium bentonite offered significant amelioration against the effects 1000 ppb aflatoxin had on weight gain for three-week old broilers. However, since the 500 ppb aflatoxin treatment had no effect on weight gain, the efficacy of the sodium bentonite at that level could not be determined. Aflatoxin levels in this trial were all less than 1000 ppb, which may explain the lack of main effects of the clay inclusion.

The use clay-based adsorbents have proven effective at binding aflatoxin with relatively high affinity *in vitro* and at reducing the toxic effects of aflatoxin in various animal species *in vivo*. Evaluating the efficacy of clay-based binders as possible interventions for ameliorating aflatoxicosis in animal feeds cannot be experimentally

demonstrated in trials where relatively low-levels of aflatoxin do not produce significant main effects. However, feeding larger doses of aflatoxin to obtain a higher level of confidence will subject the additive to a scenario that is less representative of a "real world" situation. This particular spray-applied calcium bentonite clay included at 0.5% of the diet did not ameliorate the toxicity caused by aflatoxin levels less than 1000 ppb.

CHAPTER VI

EFFECTS OF A CALCIUM BENTONITE CLAY IN DIETS CONTAINING LOW-LEVELS OF AFLATOXIN WHEN USING LIVER RESIDUES OF AFLATOXIN B_1 AS A RESPONSE VARIABLE

Introduction

Aflatoxins, which are secondary metabolites produced by the *Aspergillus flavus* and *parasiticus* species of mold, are among the most potent natural carcinogens. No animal species is immune to the toxic effects of consuming food contaminated with aflatoxin, and *Aspergillus* species are known to infect important agricultural crops. Therefore, in cases where preventative management strategies in the field have failed to prevent *Aspergillus* species from producing aflatoxin in a crop, poultry producers require cost-effective methods of detoxifying potentially contaminated grain. The use of clay-based adsorbents has proved effective at reducing the toxic effects of aflatoxin-contamination in animal feeds (Kubena, et al., 1990, 1993, 1998). Specifically, bentonite clays are adsorbent aluminum silicates that are capable of adsorbing aflatoxin within the clay smectite layers, which allows any adhering aflatoxin to pass through the gastrointestinal tract unabsorbed. Calcium bentonite is a better absorbent of aflatoxin when compared to sodium bentonites because the calcium ions provide a better separation of the clay layers when compared to sodium ions (McClure, et al. 2013).

The inclusion of non-nutritive, clay-based adsorbents as aflatoxin binders in animal feeds has been shown through recent decades of research to have a significant protective effect against aflatoxicosis in a variety of species. Although these clay-based

binders are generally recognized as safe (GRAS) to be used in diets for improved flowability, anti-caking, and pellet quality, no adsorbent has been approved by the Food and Drug Administration (FDA) for the prevention or treatment of aflatoxicosis. Concerns over the deposition of aflatoxin residues in edible tissues represent a part of the reason why. Aflatoxins can accumulate in the milk of dairy cattle, as well as in the muscle, liver, and eggs of poultry. It is also possible for there to be detectable levels of aflatoxin residues without the animal's growth and performance affected noticeably.

In this study we will perform a residue analysis for aflatoxin B_1 in samples of liver tissue collected at weekly intervals from broiler chickens consuming a range of dietary aflatoxin with and without a calcium bentonite clay additive. These residue data will be compared to the more "traditional" measures of aflatoxicosis (growth rate, feed consumption, and the relative weights of the liver, kidney, and immune organs). This will help us both better understand how aflatoxin affects broilers, as well as evaluate the efficacy of clay-based binders for their ability to prevent or treat aflatoxicosis.

Materials and methods

Feed formulation

Aflatoxin for this trial was produced by inoculating yellow dent corn with live fungal cultures of *Aspergillus parasiticus*. Inoculated grain was then incubated for five days at 37°C and then kept in a portable cement mixer under green-house conditions for one week with daily mixing. Four mash basal diets were prepared by blending increasing concentrations of the aflatoxin-contaminated corn with non-contaminated corn (0, 25, 50, and 75% of the contaminated corn, respectively) into a basal soybean meal concentrate.

Each diet was then sub-divided and one was blended with 0.2% of a calcium bentonite (TX4) and the other received no clay (as a Control). These eight diets were analyzed for total aflatoxins by the Office of the Texas State Chemist and were fed to the birds for three weeks. A single corn-soy finisher diet was formulated using only non-contaminated corn to be fed to all birds during the next three weeks as a "clearance" feed.

Bird performance and tissue collection

A total of 336 straight-run, Ross 308 broiler chicks obtained on the day of hatch were housed in three Petersime battery brooder units. Six birds were randomly selected for placement into one of the 56 pens. The 8 treatments (n=7) were assigned such that each treatment had equal representation throughout the rearing room. Also, initial weight per pen was controlled so as to be within ±10 grams of the total average flock weight of six chicks (30% of all birds were sampled to obtain an average flock weight per chick). All birds were allowed *ad libitum* access to feed and water throughout the study.

At weekly intervals, feed intake and body weight per pen were recorded and organs were collected and weighed. For the first two weeks, the liver and kidney were removed from one bird per pen for the determination of the relative organ weight. For the final four weeks, the relative organ weight was determined for the liver, kidney, spleen, and bursa of Fabricius. At each weekly point, the livers collected were retained for an analysis of aflatoxin B_1 residues in the tissue. All personnel handling birds, feed, or fecal material from this study were required to wear N95 particulate respirators (3M™, No. 9211), latex gloves, and spun-bonded polypropylene coveralls (VWR®, Basic

Protection SPP Coveralls). All methods were approved by the Texas A&M Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Liver residues

A 7.5 g sample of defrosted liver tissue (from 5 birds per treatment) was homogenized in 30 ml of 80% methanol. These samples were centrifuged (1500 x g) for 10 minutes and a 15 ml aliquot of the supernatant was collected and diluted in a 1:1 ratio with 20% methanol. An internal standard of aflatoxin B_1 (5 ng B_1 per ml) was added to each sample followed by vortexing. Samples were filtered through a 0.2 μm Whatman filter and subjected to tandem LC-MS analysis to detect aflatoxin B_1.

Data analysis

Data were analyzed as a two-way ANOVA for main effects of clay, aflatoxin, and their interaction using the General Linear Model procedure of SPSS. Significant means ($p \leq 0.05$) were separated using Duncan's Multiple Range Tests.

Results

The results from data collected during the first two weeks are presented in Tables 6-1 and 6-2. Aflatoxin had a significant effect on the broiler productivity index (which is a cumulative measure taking into account mortality, body weight, and feed conversion), suggesting that 1800 ppb reduced overall performance. By the time birds had eaten treatment diets for two weeks, aflatoxin significantly reduced body weights as well as productivity index beginning at 600 ppb. Also, cumulative mortality was significantly higher in the birds fed 1800 ppb when compared to those fed the 0 ppb control. The relative kidney weight began to be affected by aflatoxin during the second week, showing

a significant increase at the 600 ppb level. During week three (which was the final week on aflatoxin-contaminated diets), aflatoxin had a significant main effect on all variables expect for the relative bursa of Fabricius weight (Table 6-3). For body weight, cumulative feed-to-gain ratio, and productivity index, the 600 ppb treatment impaired performance relative to 0 ppb. Cumulative mortality was increased by the inclusion of 1800 ppb aflatoxin. The relative liver weights showed a significant increase in response to aflatoxin at 1200 ppb. Kidney and spleen weights were slightly more sensitive, showing changes at the 600 ppb level. The only variable to show a main effect for 0.2% of the TX4 clay was cumulative mortality. Diets that included the calcium bentonite clay had significantly lower mortality than the control diets that had no clay added.

Table 6-1: The effect of calcium bentonite (TX4) included in the diet at 0.2% at ameliorating the effects of aflatoxin contamination on the performance of broilers during the first week on treatment diets.

	Week 1					
	BW	FCR	PI	Mort	Rel Liver	Rel Kidney
0 ppb	168 ± 5	1.14 ± 0.02	212 ± 7 [a]	0.0 ± 0.0	4.54 ± 0.21	0.83 ± 0.06
600 ppb	165 ± 3	1.11 ± 0.01	211 ± 6 [a]	1.2 ± 1.2	4.61 ± 0.18	0.84 ± 0.06
1200 ppb	157 ± 7	1.12 ± 0.02	199 ± 10 [ab]	1.2 ± 1.2	4.74 ± 0.17	1.01 ± 0.07
1800 ppb	151 ± 5	1.16 ± 0.02	182 ± 8 [b]	2.6 ± 1.7	4.73 ± 0.16	0.89 ± 0.07
Control	163 ± 3	1.12 ± 0.02	204 ± 5	1.9 ± 1.0	4.66 ± 0.13	0.90 ± 0.05
TX4	159 ± 4	1.14 ± 0.01	198 ± 6	0.6 ± 0.6	4.65 ± 0.12	0.88 ± 0.04
			ANOVA			
Aflatoxin	0.088	0.254	0.044	0.533	0.860	0.172
Clay	0.447	0.322	0.489	0.292	0.938	0.588
*Aflatoxin*Clay*	0.372	0.190	0.752	0.842	0.269	0.099

Data are presented as means ± SEM
[a,b] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
PI = Broiler productivity index (Livability [%] x Live weight [kg] / age [d] / FCR x 100)
Mort = Cumulative mortality (%)
Rel Liver = Liver weight as a % of BW
Rel Kidney = Kidney weight as a % of BW
n for clay type = 28
n for aflatoxin level = 14

Table 6-2: The effect of calcium bentonite (TX4) included in the diet at 0.2% at ameliorating the effects of aflatoxin contamination on the performance of broilers during the second week on treatment diets.

	Week 2					
	BW	FCR	PI	Mort	Rel Liver	Rel Kidney
0 ppb	388 ± 9 [a]	1.49 ± 0.02	185 ± 5 [a]	1.2 ± 1.2 [a]	3.74 ± 0.12	0.76 ± 0.04 [a]
600 ppb	354 ± 9 [b]	1.57 ± 0.05	162 ± 8 [b]	1.2 ± 1.2 [a]	3.66 ± 0.07	1.03 ± 0.07 [b]
1200 ppb	326 ± 14 [b]	1.54 ± 0.04	149 ± 9 [b]	3.6 ± 2.0 [ab]	4.14 ± 0.23	1.06 ± 0.08 [b]
1800 ppb	293 ± 7 [c]	1.62 ± 0.06	121 ± 7 [c]	7.7 ± 2.4 [b]	4.02 ± 0.22	1.10 ± 0.10 [b]
Control	343 ± 10	1.56 ± 0.03	154 ± 7	3.7 ± 1.4	3.79 ± 0.11	1.00 ± 0.06
TX4	339 ± 10	1.54 ± 0.03	156 ± 7	3.0 ± 1.2	3.99 ± 0.14	0.97 ± 0.06
			ANOVA			
Aflatoxin	<0.001	0.190	<0.001	0.047	0.154	0.002
Clay	0.805	0.533	0.733	0.651	0.276	0.409
*Aflatoxin*Clay*	0.256	0.251	0.799	0.502	0.233	0.001

Data are presented as means ± SEM
[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
PI = Broiler productivity index (Livability [%] x Live weight [kg] / age [d] / FCR x 100)
Mort = Cumulative mortality (%)
Rel Liver = Liver weight as a % of BW
Rel Kidney = Kidney weight as a % of BW
n for clay type = 28
n for aflatoxin level = 14

Table 6-3: The effect of calcium bentonite (TX4) included in the diet at 0.2% at ameliorating the effects of aflatoxin contamination on the performance of broilers during the third week on treatment diets.

	Week 3							
	BW	FCR	PI	Mort	Rel Liver	Rel Kidney	Rel Spleen	Rel Bursa
0 ppb	764±23 [a]	1.72±0.03 [a]	210±8 [a]	1.2±1.2 [a]	3.23±0.12 [a]	0.69±0.03 [a]	0.11±0.01 [a]	0.21±0.02
600 ppb	645±17 [b]	2.00±0.12 [bc]	156±13 [b]	4.8±2.1 [a]	3.85±0.16 [a]	0.98±0.09 [b]	0.16±0.01 [b]	0.20±0.02
1200 ppb	581±21 [b]	1.83±0.06 [ab]	141±7 [b]	7.1±2.9 [ab]	4.87±0.23 [b]	1.30±0.11 [c]	0.19±0.02 [bc]	0.22±0.01
1800 ppb	448±40 [c]	2.12±0.10 [c]	94±11 [c]	15.4±4.8 [b]	4.93±0.32 [b]	1.27±0.12 [c]	0.20±0.02 [c]	0.22±0.01
Control	602±34	1.95±0.07	144±12	9.9±2.9 [b]	4.04±0.18	1.02±0.08	0.16±0.01	0.21±0.01
TX4	623±22	1.88±0.06	158±9	4.2±1.4 [a]	4.31±0.22	1.07±0.08	0.17±0.01	0.21±0.01
				ANOVA				
Aflatoxin	<0.001	0.010	<0.001	0.008	<0.001	<0.001	<0.001	0.617
Clay	0.278	0.421	0.117	0.040	0.395	0.944	0.972	0.529
*Aflatoxin*Clay*	0.220	0.565	0.224	0.631	0.993	0.738	0.332	0.011

[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
BW = Average body weight per bird (g)
FCR = Cumulative feed-to-gain ratio
PI = Broiler productivity index (Livability [%] x Live weight [kg] / age [d] / FCR x 100)
Mort = Cumulative mortality (%)
Rel Liver = Liver weight as a % of BW
Rel Kidney = Kidney weight as a % of BW
Rel Spleen = Spleen weight as a % of BW
Rel Bursa = Bursa of Fabricius weight as a % of BW
n for clay type = 28
n for aflatoxin level = 14

The accumulation of aflatoxin B_1 residues in the liver are presented in Table 6-4. After one week on treatment diets, aflatoxin at 1200 ppb caused a significant increase in liver residues when compared to both the 0 and 600 ppb treatments. A further increase was seen in the 1800 ppb treatment. There was a significant main effect for the inclusion of 0.2% TX4 clay, with clay providing an approximate 43.5% reduction in the accumulation of aflatoxin B_1 residues in liver tissue. After birds had been consuming contaminated diets for two weeks, the main effect for aflatoxin remained the same, with 1200 ppb causing a significant increase in liver residues when compared to the 0 ppb control. Though the trend in dose response remained the same, after two weeks, the 600 and 1200 ppb treatments were no longer significantly different from each other. During the final week on aflatoxin (week 3), only birds fed the diet containing the highest level of aflatoxin (1800 ppb) without clay had detectable levels of aflatoxin B_1 in the liver. One week on the 0 ppb control diet was sufficient to remove any detectable residues from all of the treatments. The livers collected from the second and third week on the 0 ppb control diet (weeks 5 and 6 of age) were not analyzed due to the non-detectable levels found at the completion of the first week of the "clearance" period.

Table 6-4: Liver residues of aflatoxin B_1 (ppb), analyzed on a weekly basis. Three weeks were spent on aflatoxin-contaminated feed, and during the fourth week, all birds consumed a non-contaminated finisher diet.

	Week 1	Week 2	Week 3	Week 4
0	ND [a]	ND [a]	ND	ND
600	0.53 ± 0.27 [a]	1.51 ± 0.47 [ab]	ND	ND
1200	1.24 ± 0.36 [b]	2.21 ± 0.67 [b]	ND	ND
1800	2.05 ± 0.29 [c]	3.84 ± 0.63 [c]	0.27 ± 0.27	ND
Control	1.15 ± 0.26 [b]	2.18 ± 0.48	0.14 ± 0.14	ND
TX4	0.65 ± 0.23 [a]	1.60 ± 0.43	ND	ND
		ANOVA		
Aflatoxin	<0.001	<0.001	0.403	.
Clay	0.034	0.277	0.323	.
Aflatoxin*Clay	0.010	0.884	0.403	.

Data are presented as means ± SEM
[a,b,c] Means for main effects within a column lacking a common superscript differ ($p \leq 0.05$)
n for clay type = 20
n for aflatoxin level = 10

Discussion

This study evaluated the effects of aflatoxin on broiler performance, relative organ weights, and residues of aflatoxin B_1 in the liver. Also, the "clearance" time required to remove aflatoxin residues from the liver once birds had been placed on a diet containing non-contaminated corn was evaluated. The relationship between a given dose of aflatoxin and the time spent on treatment diets was evident in terms of the production parameters. After one week on aflatoxin diets, only the cumulative measure of the broiler productivity index was significantly affected by the highest concentration of dietary aflatoxin (1800 ppb). However, once birds had consumed their treatment diets for two weeks, cumulative mortality was also increased by the 1800 ppb treatment, and body weights were reduced by the 600 ppb treatment. Also, at week two, there began to be a significant effect of aflatoxin on the relative organ weights, with the kidney weights showing an increase when aflatoxin was present in the diet. Finally, during the third and final week on aflatoxin-contaminated diets, body weights, cumulative feed conversion, and the productivity index were all affected by the 600 ppb treatment.

For the liver residues of aflatoxin B_1, when Fernández, et al. (1994) fed 2500 and 5000 ppb aflatoxin to broilers for 32 days, liver residues of aflatoxin B_1 were only 0.16 and 0.15 ppb. The authors attributed the low values to rapid liver metabolization of aflatoxin B_1 into the other more water soluble forms destined for excretion from the body. In this study, only the highest level of aflatoxin (1800 ppb) had detectable levels of aflatoxin B_1 in the liver after three weeks on the contaminated diets. In Hussain et al. (2010) it was found that, overall, older birds have lower tissue residues of aflatoxin B_1

when compared to younger birds, even when they were fed aflatoxin for the same amount of time. Also, the authors found that the elimination of residues occurred earlier in older birds. This supports the finding in these data, which suggest that as birds age, the liver's metabolizing mechanisms for dealing with incoming aflatoxin B_1 becomes more efficient and residues no longer accumulate.

Results from the first week showed that 0.2% TX4 was effective at reducing the accumulation of aflatoxin B_1 residues in liver. Neeff, et al. (2013), who fed 2500 ppb aflatoxin to broilers for three weeks, found significant increases liver concentrations of aflatoxin B_1, as well as B_2, G_1, M_1, and aflatoxicol (all of the forms of aflatoxin analyzed except for aflatoxin G_2) relative to the 0 ppb control diet. The amount of aflatoxin B_1 in the liver, after the three weeks, was 8.32 ppb (total aflatoxins were 12.78 ppb). In that study, the liver residue analysis was the only variable for which the clay effect showed complete amelioration by returning the aflatoxin-contaminated diet to a level that was not significantly different than the aflatoxin-free control diet. The results from this study (during the first week) agree with this, in that a significant clay effect (for 0.2% of TX4) was seen only in the liver residues. However, after the first week, liver residue data were not any more sensitive in evaluating aflatoxin or clay effects when compared to the "traditional" measures of growth performance and organ weights. Also, these results indicate that the clearance time required to remove aflatoxin residues from the liver is less than one week on a clean corn diet.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Mycotoxins are a group of secondary metabolites that are produced by certain fungal species (e.g. *Aspergillus*, *Penicillium*, and *Fusarium*). Within the family of mycotoxins, there are compounds with varying chemical structures, biological properties, and levels of toxicity. Aflatoxins are a member of this mycotoxin family and are among the most potent natural carcinogens known to exist. Aflatoxin exerts its toxic effects once absorbed by the gastrointestinal tract and bioactivation in the liver (into the more water-soluble metabolite, aflatoxin B_1-8,9 epoxide). Once activated into the epoxide, the molecule is capable of forming adducts with DNA, RNA, and protein, inactivating tumor suppression and impairing protein synthesis and function.

The initial work undertaken to characterize aflatoxicosis experimentally was done via the inoculation of rice with cultures of aflatoxigenic *Aspergillus* species. Rice, as a relatively singular source of starch, provided a highly pure and high concentration sample of aflatoxins. Those studies were conducted at final dietary concentrations ≥2000 ppb (100-times greater than the legal action level for poultry diets) because, at lower concentrations, there were not significant main effects for aflatoxin. This research was conducted using a more natural matrix, by inoculating corn with *Aspergillus parasiticus*. In the present studies, we began to see significant main effects for aflatoxin at lower concentrations (≤1000 ppb). This was beneficial because, at present, the chief means of both effectively and feasibly protecting against aflatoxin contamination in feeds is the inclusion of clay-based binding products, and evaluating the protective efficacy of the

various clay-based binders at ameliorating aflatoxicosis cannot be demonstrated in experimental trials where relative low-levels of aflatoxin fail to produce significant responses in a given parameter. On the other hand, by feeding higher doses of aflatoxin (so as to obtain a greater degree of confidence that significant differences will be produced) subjects the additive to a scenario less representative of "reality". Further, the concentration of aflatoxin present in a solution has been shown, *in vitro*, to affect the binding efficiency of a given clay.

The inclusion of non-nutritive, clay-based adsorbents as aflatoxin binders in animal feeds has been shown through recent decades of research to have significant protective effects against aflatoxicosis in a variety of species. The objectives of this research have been to evaluate common biological assays for measuring aflatoxicosis in broilers (such as production characteristics and relative organ weights) along with variables such as hepatic gene expression or aflatoxin residues in the liver, pursuant to identifying a more sensitive biological assay that will allow for three-week broiler trials to be conducted under experimental conditions at aflatoxin concentrations <1000 ppb (prior to significant changes in the growth rate or the relative organ weights).

In the first study, a calcium bentonite that was mined from the Gonzales region of Texas (TX4) was evaluated against Novasil™, which is a commercially-available hydrated sodium calcium aluminosilicate clay that has been consistently shown through research by Kubena, et al. (1990, 1993, 1998) to significantly bind with aflatoxin and make it unavailable for absorption (used as a "positive control" clay-based adsorbent). Both clays were included in diets containing total aflatoxins of 0, 500, 1000, and 2000

ppb and each appeared able to sequester aflatoxin, especially at the 500 ppb level. Overall the TX4 clay appeared as capable of ameliorating aflatoxicosis when compared to Novasil™. During the first week, aflatoxin as high as 2000 ppb did not affect either body weight or feed conversion. The 2000 ppb dose began to reduce growth after two weeks, and it took the full three weeks before it was reduced by 1000 ppb. This study highlighted the time-factor associated with determining what a given dose of aflatoxin will do in terms of production parameters. This suggested that very young broilers can be considered quite robust against acute doses if they are only fed for a very short period of time.

The reported data on the effects of aflatoxin on relative organ weights and serum biochemistry suggest that the liver can be considered a "target organ" for aflatoxicosis. Thus, it is reasonable that the expression of specific detoxification genes in the liver tissue represent a likely "first stop" in the biological response to aflatoxin, as the contents absorbed by the gastrointestinal tract travel first to the liver via the portal blood system. Once in the liver, the cytochrome p450 class of enzymes (CYP) are involved in the biotransformation of various xenobiotic compounds into metabolites that are more water soluble and available for excretion by the kidneys. Though the CYP-mediated reactions are essential for detoxification, they can generate reactive oxygen species and are responsible for the bioactivation of aflatoxin B_1 into the carcinogenic metabolite aflatoxin B_1-8,9 epoxide. Previous research by Yarru, et al. (2009) has found that certain CYP-isoforms (specifically CYP1A1 and CYP2H1) are upregulated while epoxide hydrolase, GST, and glutathione peroxidase genes are downregulated by 2000 ppb aflatoxin. This

combination favors the formation of aflatoxin B1-8,9 epoxide, while providing less of a chance for any biological antioxidant response.

In the second study, growth and relative organ weight data were compared with the gene expression of hepatic enzymes known to detoxify aflatoxin B_1 in birds fed a wide range of total aflatoxin concentration (0, 300, 500, 1000, 2000 and 4000 ppb). When gene expression data from liver samples were analyzed, the genotypic effect of aflatoxin on the CYP1A1 and CYP2H1 isoforms simply mirrored the phenotypic effects seen in the growth and relative organ weights, suggesting that this variable was not more sensitive than the more traditional ones and thus would not be considered a better alternative when it comes to accessing aflatoxicosis in broilers.

The third study evaluated the TX4 clay (0.5%) when in diets containing <1000 ppb aflatoxin (0, 250, 400, and 700 ppb) as provided by both *Aspergillus*-contaminated corn and contaminated distiller's dried grains and solubles (DDGS were included in the diets at 20%). With ethanol receiving considerable attention as a potential source for renewable fuel in recent years, corn prices have increased and the use of fermentation co-products such as DDGS have become more common in animal feeds. Any mycotoxins contained in grain bound for fermentation will still appear in the co-products that end up in animal feeds. In fact, fermentation and distillation can increase mycotoxin concentrations up to three-fold in corn-derived co-products. Although the growth rate between treatments was unaffected by aflatoxin at these lower levels (after three weeks on treatment diets, body weights between the 0 ppb treatment and the 700 ppb treatment only varied by 4%), there were negative effects associated with feed conversion and

productivity index and there was an increase in the relative weights of the liver and kidney. The inclusion of TX4 to the treatment diets failed to offer any amelioration for the main effects of aflatoxin, though it may be because aflatoxin levels in this trial were all less than 1000 ppb (levels that failed to significantly reduce body weights).

Although clay-based binders are generally recognized as safe (GRAS) to be used in diets for improved flowability, anti-caking, and pellet quality, no adsorbent has been approved by the Food and Drug Administration (FDA) for the prevention or treatment of aflatoxicosis. Concerns over the deposition of aflatoxin residues in edible tissues represent a part of the reason why. Aflatoxins can accumulate in the milk of dairy cattle, as well as in the muscle, liver, and eggs of poultry. It is also possible for there to be detectable levels of aflatoxin residues without the animal's growth and performance affected noticeably.

The final study was conducted to evaluate the effects of the TX4 calcium bentonite clay when residues of aflatoxin B_1 in the liver were used as the major variable of interest. Results after one week on treatment diets (containing 0, 600, 1200, or 1800 ppb total aflatoxins) showed that TX4 (included at 0.2%) was effective at reducing the accumulation of aflatoxin B_1 residues in liver. However, after the first week, liver residue data were not any more sensitive in evaluating aflatoxin or clay effects when compared to the "traditional" measures of growth performance, mortality, or relative organ weights. Once birds had been consuming aflatoxin for three weeks, residues of aflatoxin B_1 were not detectable in the liver (the only treatment with detectable levels for the control treatment containing 1800 ppb aflatoxin). Also, these results indicated that

the clearance time required to remove aflatoxin residues from the liver is less than one week on a clean corn diet.

A global conclusion based on both the literature review and the research conducted herein suggest that an alternative solution to aflatoxin contamination in feedstuffs such as corn may not necessarily be dilution throughout the lifetime of a chicken, but rather it may be to give short, punctuated exposure (of approximately one week) of higher levels of aflatoxin (of potentially 100-500 ppb), so long as that exposure was followed by at least week of a withdrawal period on a clean diet free of aflatoxin. Also, as birds age, it appears that the liver's capacity to metabolize aflatoxin B_1 becomes more efficient. Though the liver is well-known as the target organ for aflatoxicosis, this research has shown consistent effects on relative kidney weights as well. The goal of the liver is to convert the relatively lipophilic aflatoxin B_1 molecule into something more water-soluble so that it can be excreted from the body via the kidneys. It may be that future studies could evaluate the accumulation of the aflatoxin B_1-8,9 epoxide (and the other metabolites) in the kidneys as the birds age and aflatoxin is being more efficiently "cleaned-out" by the liver.

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