RESEARCH REPORT
ANIMAL SCIENCE

Title: Effects of Dietary Aflatoxin on Hepatic Gene Expression in Swine - **NBP** #08-075

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Industry Summary

Aflatoxins, especially aflatoxin B1 (AFB1), can be high in dried distillers grains with solubles (DDGS) when concentrated during the ethanol production process. Increased use of DDGS in swine diets could potentially lead to an increased incidence of aflatoxicosis, a disease associated with decreased feed intake, reduced BW gain, and impaired liver function. The objectives of this study were to determine the effects of AFB1 on 1) the health, performance, and serum profiles and 2) the hepatic gene expression of growing barrows. Ninety Duroc x Yorkshire crossbred barrows (age = 35 ± 5 d; initial BW = 14.2 ± 3.0 kg) were randomly assigned to receive 0 (CON), 250 ppb AFB1 (LOW), or 500 ppb AFB1 (HIGH) for 7, 28, or 70 d. Feed intake was measured daily, and pigs were weighed and blood samples collected weekly. Serum was analyzed for concentrations of liver specific enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (BILI), and blood urea nitrogen (BUN). Average feed intake was less in HIGH barrows than in CON barrows from wk 5 to 10, and was less in LOW barrows than in CON barrows in wk 5 and again from wk 8 to 10. Also, ADFI was lower in HIGH barrows than LOW barrows in wk 10. Lower ADG was observed in HIGH barrows than in CON barrows in wk 8 and 10; no differences in ADG were noted between CON and LOW barrows. There was no effect of AFB1 treatment on ALT or BILI concentrations. However, both AST and BUN were affected ($P < 0.05$) by AFB1 treatment. Serum AST was greater in LOW barrows than CON barrows in wk 5, and serum BUN was greater in CON barrows than LOW barrows in wk 3. Both ADFI and ADG were negatively affected by AFB1 treatment. Liver samples were fixed and stained for detection of cellular damage and inflammation. No statistical differences in liver health as assessed by histological grading were observed among the 70 d treatment groups. These results demonstrate that performance and blood parameters in young growing barrows are affected by consumption of an aflatoxin-contaminated diet, especially when the concentration of aflatoxin is high (≥ 500 ppb); however, even lower concentrations (250 ppb) are detrimental to performance when administered for a more chronic period. Liver samples from d 70 barrows were used for RNA-Sequencing (RNA-Seq). Genomic analysis indicates considerable variation in the number of transcripts, with most probed sequences having low levels of transcription. Changes in the expression of genes involved in a variety of functions related to cellular stress and toxicity responses, such as apoptosis, regulation of cell growth and proliferation, and mRNA processing, are differentially regulated in response to AFB1. Of specific interest, 15 genes with apoptotic roles were differentially expressed after long-term aflatoxin

exposure (70 d). Of those 15 genes, 4 (*CDKN1A*, *ZMAT3*, *YWHAZ*, and *AEN*) were also differentially expressed after only a short-term exposure (7 d), indicating those genes may be early indicators of an apoptotic response to aflatoxins. Further study of these differentially expressed genes may lead to prevention strategies and treatments for aflatoxicosis. Additionally, early response genes may prove to be useful for early diagnosis of aflatoxicosis, or even indicate differences in tolerance to dietary aflatoxins.
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Scientific Abstract

Aflatoxins, especially aflatoxin B1 (AFB1), can be high in dried distillers grains with solubles (DDGS) when concentrated during the ethanol production process. Increased use of DDGS in swine diets could potentially lead to an increased incidence of aflatoxicosis, a disease associated with decreased feed intake, reduced BW gain, and impaired liver function. The objectives of this study were to determine the effects of AFB1 on 1) the health, performance, and serum profiles and 2) the hepatic gene expression of growing barrows. Duroc x Yorkshire crossbred barrows (n = 90; age = 35 ± 5 d; initial BW = 14.2 ± 3.0 kg) were randomly assigned in a 3 x 3 factorial design to receive 0 (CON), 250 ppb AFB1 (LOW), or 500 ppb AFB1 (HIGH) for 7, 28, or 70 d. Feed intake was measured daily, and pigs were weighed and blood samples collected weekly. Serum was analyzed for concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (BILI), and blood urea nitrogen (BUN). Average feed intake was lower ($P < 0.01$) in HIGH and LOW barrows than in CON barrows in wk 8 and 9, and was lower ($P < 0.05$) in HIGH barrows only in wk 10. Lower ADG ($P < 0.05$) was observed in HIGH barrows than in CON barrows in wk 8 and 10; no differences in ADG were noted between CON and LOW barrows. There was no effect of AFB1 treatment on ALT or BILI concentrations. However, both AST and BUN were affected ($P < 0.05$) by AFB1 treatment. Concentrations of AST were greater in both HIGH ($P = 0.067$) and LOW ($P = 0.073$) barrows than CON barrows, while BUN concentrations were lower in both HIGH ($P = 0.073$) and LOW ($P = 0.012$) barrows.

RNA-Seq was performed on liver samples from the d 70 groups. Forty-three functional groups were identified using DAVID, and genes within the apoptosis regulation functional group were selected for 1) validation of d 70 gene expression differences using real-time RT-PCR (n = 4 genes), and 2) investigation of d 7 expression to identify early response to aflatoxins (n = 15 genes). Expression levels of the four apoptosis genes selected for validation, *CDKN1A*, *ZMAT3*, *KNG1*, and *PIM1*, were confirmed with real-time RT-PCR. Of the 15 genes tested in the d 7 liver samples, 4 were differentially expressed: *CDKN1A*, *ZMAT3*, *YWHAZ*, and *AEN*. Results from this study demonstrate that administration of an AFB1-contaminated diet to growing barrows reduces performance, compromises health, and alters hepatic gene expression.

Introduction

Mycotoxins are secondary metabolites produced by molds on crops grown in stressful or poor conditions (Dharmarha, 2009). Aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are some of the most potent mycotoxins, of which aflatoxin B1 (AFB1) is the most toxic (Devegowda and Murthy, 2005). Feeds contaminated with AFB1 can cause aflatoxicosis, which is manifested through liver damage and subsequently poor health and performance. Like other nonruminant species, swine are unable to efficiently metabolize aflatoxins, making them especially susceptible to aflatoxicosis. Susceptibility also varies with age, aflatoxin concentration, and duration of exposure (CAST, 2003). It was estimated that swine producers in 1980 lost $100 million of potential income due to use of aflatoxin-contaminated feeds (Lindemann et al., 1993). Regulatory limits for AFB1 in swine feed are < 20 ppb AFB1 for young, immature pigs, < 100 ppb AFB1 for breeding pigs, and < 200 ppb AFB1 for finishing pigs (USDA, 2005; FDA, 2009). As producers are looking to increase the use of distillers dried grains with solubles (DDGS) as an energy source in swine diets, AFB1 can become problematic. Aflatoxins produced on corn are not destroyed during ethanol production, but are instead concentrated approximately 3- to 4-fold (FDA, 2006; Wilkinson and Abbas, 2008). No treatment for aflatoxicosis currently exists, and while AFB1 levels in DDGS are typically low (Zhang et al., 2009),

mycotoxin sampling and testing procedures can be difficult and inconsistent, limiting the reliability of detection. Administration of high AFB1 concentrations (≥ 420 ppb) for short durations can be detrimental to swine health and performance (Harvey et al., 1988; Lindemann et al., 1993). However, the effects of administering lower concentrations of AFB1 to growing pigs for an extended period are relatively unknown. Identification of genes and pathways altered due to dietary aflatoxin consumption may lead to enhanced diagnostics and treatment and prevention strategies for aflatoxicosis.

Objectives

Objective 1: Swine producers are considering use of DDGS to reduce feed costs and improve economic viability of their operations. However, an increased risk of aflatoxicosis is associated with these byproducts as aflatoxins are concentrated during ethanol production. The liver is the primary target organ for aflatoxins. Therefore, our first objective is to gain a better understanding of genomic alterations in the liver due to consumption of an aflatoxin-contaminated diet.

Objective 2: Differences in gene expression associated with performance may be used to determine more effective treatments and/or prevention strategies for aflatoxicosis. Our second objective is to determine correlations between expression levels of differentially expressed genes and performance traits.

Materials & Methods

Objective 1

All animal procedures used were approved by the University of Wyoming Institutional Animal Care and Use Committee.

Animal Procedures and Treatments

Ninety Duroc x Yorkshire crossbred barrows were purchased from a commercial farm (average age = 35 ± 5 d at time of purchase). Barrows were allocated to 9 pens with 10 pigs per pen, and each pen was randomly assigned to 1 of 9 dietary treatments: 0 ppb AFB1 (CON), 250 ppb AFB1 (LOW), or 500 ppb AFB1 (HIGH) for 7, 28, or 70 d in a factorial arrangement of treatments. Similar concentrations of AFB1 have been shown to elicit a response in swine (Southern and Clawson, 1979; Lindemann et al., 1993), and evenly spaced increments of AFB1 administration were used to determine if responses were linear, quadratic, or both.

After pen and treatment assignment, barrows were allowed a 6-d adjustment period (d -6 to -1) before starting their respective dietary treatments. Barrows were housed in the nursery unit until wk 4, after which all pigs were transferred to the grower unit for the remainder of the trial period; pen mate assignments, or contemporary groups, remained the same throughout the trial. Barrows were allowed ad libitum access to feed and water throughout the study. The non-medicated starter diet (Table 1) was formulated (Swine Premix Catalog; Custom Ag Products, Inc., Fairview, KS) to be fed from d 0 to 20, at which time mean BW was expected to exceed 22.7 kg. The grower diet (Table 1; Swine Premix Catalog) was fed from d 21 until the end of the trial period. Ground corn containing AFB1 culture material was added to the LOW and HIGH starter and grower diets to reach dietary concentrations of 250 and 500 ppb AFB1, respectively. Barrows were monitored daily for signs of aflatoxicosis, including decreased feed intake, icterus, rough coat, and lethargy (Coppock et al., 1989; Harvey et al., 1990).

Pen feed intake was measured daily beginning on d -2. Adequate feed for ad libitum feeding was provided each morning. All remaining feed was removed the following morning and weighed. Barrows were weighed and bled on d -1, and then weekly throughout the duration of the trial starting on d 7. Blood samples were collected via jugular venipuncture into 16 x 100 mm blood collection tubes (Tyco Healthcare Group LP, Mansfield, MA). Samples were allowed to clot for ≥ 20 min and then centrifuged for 20 min at 1,200 × *g* at 4˚C. Serum was collected and stored at -20˚C until analyzed. All serum analyses were conducted at the Wyoming State Veterinary Laboratory (Laramie, WY). Analyses of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (BILI), and blood urea nitrogen (BUN) were performed (VetEx Serum Chemistry Analyzer; Alfa Wassermann Diagnostic Technologies, West Caldwell, NJ) according to the manufacturer's instructions. Because AST measurements are subject to variability due to muscle stress, AST measurements were closely examined for extreme values that were likely due to stress associated with

movement and blood collection, not aflatoxin consumption. Normal range for AST in pigs is 15.3 to 55.3 U/L. For this study, AST values > 2 SD greater than the upper limit of the normal range were eliminated if the other serum measurements (ALT, BILI, and BUN) were not atypical, as this would indicate that the increased AST was due to the movement or handling stress instead of AFB1 exposure. Alternately, if the other serum measurements were also atypical, this would indicate liver damage associated with AFB1 consumption, and those AST values were retained.

Pigs were euthanized on the last day of their respective treatment [1 pen of pigs or 10 pigs per treatment on d 7, 28, and 70; 1 mL of Beuthanasia-D (Schering-Plough Animal Health Corp., Union, NJ)/22 kg BW]. Liver, kidneys, and pancreas were collected and weighed. Each tissue was subsampled and preserved in 4% paraformaldehyde fixative for histological analyses. All carcasses and remaining AFB1-contaminated feed were incinerated at the Wyoming State Veterinary Laboratory.

Aflatoxin Production and Analysis

The AFB1 was produced on rice using Aspergillus *parasiticus* (NRRL2999, USDA Midwest Area-National Center for Agricultural Utilization Research, Peoria, IL) at the University of Missouri (Columbia, MO) using methods adapted from Shotwell et al. (1966). Aflatoxin B1 culture material containing 800 mg AFB1/kg was added to the LOW and HIGH diets to achieve targeted concentrations of 250 and 500 ppb, respectively. Samples from each diet were analyzed for final AFB1 concentration using methods previously described (Gomez-Catalan et al., 2005; Gowda et al., 2009). The final AFB1 concentrations within the starter diets of CON, LOW, and HIGH treatment groups were 0, 185, and 510 ppb, respectively. For the grower diets, the final AFB1 concentrations were 0, 170, and 455 ppb for CON, LOW, and HIGH treatment groups, respectively. The CON diet was analyzed for mycotoxins, using methods similar to those previously described; there was no detection of aflatoxin, fumonisin, vomitoxin, zearalenone, or ochratoxin contamination.

Histology

At time of euthanasia, cross sections of liver from the d 70 groups were excised and fixed in 4% buffered paraformaldehyde for 24 h followed by two 70% ethanol washes. Tissues were dehydrated through a series of graded ethanol baths followed by xylene before being infiltrated and embedded in paraffin. Liver cross sections (6 µm) were placed on Superfrost Plus slides were deparaffinized and rehydrated in distilled water. For rehydration, slides were placed sequentially into three 10 min xylene baths, two 5 min 100% ethanol baths, two 5 min 95% ethanol baths, one 5 min 70% ethanol bath, and finally, briefly into 1 water bath. Hematoxylin/eosin staining was performed according to standard laboratory protocol [Bancroft and Gamble, 2002; Mayer Hematoxylin/Eosin Staining (Sigma-Aldrich, St. Louis, MO)]. Liver tissue sections were evaluated at the University of Missouri (Columbia). Sections were individually graded from 0 to 5, similar to that described by Miller et al. (1981). A grade of 0 indicated no detectable changes in the liver tissue, and a grade of 1 indicated that portal tracts were infiltrated by very small numbers of lymphocytes, macrophages, and eosinophils. Grades 2 through 4 represented increasing degrees of liver damage as evidenced by the presence of bile duct proliferation in the portal areas. A grade of 5 indicated severe inflammation involving portal tracts and parenchyma.

Statistical Analyses

There were 10 pigs per treatment combination (CON, LOW, or HIGH for 7, 28, or 70 d). This resulted in 3 pens each with 10 pigs per pen for CON, LOW, and HIGH treatments from d 0 to 7 of the trial; 2 pens each with 10 pigs per pen for CON, LOW, and HIGH treatments from d 8 to 28; and 1 pen each with 10 pigs per pen for CON, LOW, and HIGH treatments from d 29 to 70. Treatments (CON, LOW, or HIGH) were allocated by pen, thus pen was the experimental unit for treatment and pen(treatment) was used as the error term for all analyses to test treatment effects (Kaps and Lamberson, 2009). Feed intake was measured daily for each pen, and analyzed as ADFI. Average daily feed intake was analyzed as repeated measures using the compound symmetry covariance structure in the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) for effects of treatment, week, and their interaction. Weekly measures of ADG and serum measurements (AST, ALT, BILI, and BUN) were also analyzed using MIXED procedure of SAS for effects of treatment, week, and their interaction. For ADG, initial BW (measured on d -1) was also used as a covariate. Organ weights were analyzed for to determine effects of treatment, duration of treatment (i.e., 7, 28, or 70 d), and their interaction

using an analysis of variance in the GLM procedure of SAS. Finally, histology grades of d 70 liver tissues were tested for the effect of treatment also using an analysis of variance. For all analyses, separation of least-squares treatment × week means was performed when a significant interaction was detected in the analysis of variance, using LSD with a Tukey's adjustment and assuming an alpha level of 0.05. Linear and quadratic contrasts were additionally performed using the MIXED procedure when the main effect of treatment was significant.

Objective 2

RNA Isolation

Pigs were euthanized on their last day on trial for tissue collections. Liver samples from d 70 animals were snap-frozen after collection. Liver tissue (0.05 to 0.1 g) from d 70 and d7 barrows was homogenized in 1 mL of TRI-Reagent (Sigma-Aldrich, St. Louis, MO). The RNA was isolated and the resulting RNA pellet was purified using the RNeasy clean-up kit protocol (Qiagen, Valencia, CA), which included on-column DNase digest. All purified RNA was measured for quality and quantity using a Nanodrop spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE). Each sample was aliquotted into 2 µg concentrations for real-time RT-PCR analysis, and the d 70 samples were also aliquotted into 10 µg concentrations for RNA-Seq analysis.

RNA- Seq

Quality analysis was performed on each d 70 sample before library preparation using the Experion RNA Analysis Kit (Bio-Rad, Hercules, CA) at the DNA Core at the University of Missouri, Columbia. A total of 9 CON, 6 LOW, and 9 HIGH samples were selected for RNA-Seq based on their high quality scores. Library preparations to synthesize double stranded cDNA and purify samples were conducted according to protocol (Illumina) at the University of Missouri, Columbia. DNA samples were diluted with a 99.9% EB and 0.1% Tween20 mixture to make working stocks of 10 nM per sample.

RNA-Seq Analysis

RNA-Seq was conducted at the University of Missouri DNA Core (Columbia, MO) using the Illumina Genome Analyzer (Illumina). Flowcells contain 8 lanes with fields of oligos to bind the samples for bridge amplification and sequencing. Barcoded adaptors are a 4 base sequence that are bound to every sequence from an individual sample, which allows results of each individual animal to be identified. By using barcoded adaptors, 3 samples (10 nM working stocks described above) with different barcodes but like treatment (CON, LOW, or HIGH) were combined, diluted to 4-12 pM single stranded sequences, and added to each lane, for a total of 24 samples across the 8 lanes. In the cluster station, the samples were applied to the flowcell where clusters of each oligo were replicated through bridge amplification, with the amount of initial RNA corresponding with the number of clusters for each transcript. After replication, the flowcell was placed in the sequencing machine, where fluorescently labeled bases (unique to each A, C, G, and T) were attached to the complementary bases of the RNA sequences. Laser excitation was used to activate the fluorescently labeled bases. The Illumina Genome Analyzer (Illumina) then recorded the first 42 bases of each labeled sequence, with the first 4 bases being the barcoded adapters to identify each sample. The sequence information was recorded and compiled for each sample, and stored at the University of Missouri. For each unique transcript of a sample, the number of copies was quantified. This transcript copy number is relative to transcript abundance (and hence mRNA abundance).

RNA-Seq Statistical Analysis

Treatments were assigned relative levels, with CON = 0, LOW = 1, and HIGH = 2. Correlation coefficients between transcript copy number and treatment level were estimated for each sample and unique transcript using MicroSoft Excel (MicroSoft Corp., Redmond, WA). For those transcripts with a significant correlation coefficient ($r \geq |0.80|$; $P < 0.01$), linear regression of transcript copy number of treatment level was also performed. Genes associated with transcripts with significant correlation coefficients were identified by blasting the transcript sequence against the Human Genome (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml; Appendix 1).

Gene Annotation

Genes detected as different from RNA-Seq by correlation of transcript copy number to level of treatment ($r > |0.8|$; $P < 0.01$) were clustered using the highest stringency option (less functional groups with

more tightly associated genes per group) according to functional groups by the Database for Annotation, Visualization, and Integrated Discovery, or DAVID (Dennis et al., 2003) using the Expression Analysis Systemic Explorer (EASE) program. A modified Fisher exact test was used within EASE to identify overrepresented functional groups. Genes within functional group(s) of interest were tested for treatment effects using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Least squares means were estimated, and tested for pair-wise treatment differences using the Tukey adjustment, assuming an alpha level of 0.05.

Real-time RT-PCR

Fifteen genes were clustered within the regulation of apoptosis function by DAVID. Of those 15 genes, 2 up-regulated and 2 down-regulated genes were chosen for validation with real-time RT-PCR. In addition, real-time RT-PCR was used to determine expression levels of all 15 of those genes in randomly selected d 7 liver samples, with 8 samples per treatment group. For real-time RT-PCR, 2 µg of purified RNA (described previously) were lyophilized, then converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Primers (Table 9) were designed to have a melting temperatures of 60°C and to yield 150 bp amplicons using Primer3 Software v. 0.4.0 (Rozen and Skaletsky, 2000), and were confirmed by blasting the forward and reverse primers in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/). The primers were synthesized by Invitrogen (Carlsbad, CA), and were diluted to a 5 µM working stock. Fifteen µL of master mix consisting of 12.5 µL of SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of forward and reverse primers, and 0.5 µL of H_2O were added to 10 µL diluted cDNA in a 96-well plate in duplicate. For the PCR reaction, the IQ5 (BioRad, Hercules, CA) was programmed to run 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 10 s and 60°C for 30 s. Melting curve analysis was performed using 1 cycle of 95°C for 1 min, and then 1 cycle of 55°C for 1 min followed by a 0.5°C increase in temperature with each cycle for 81 cycles to ensure quality and amplification. For each gene, the number of threshold cycles was obtained for each sample in duplicate. Relative gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and were expressed relative to GAPDH as the housekeeping gene.

Real-Time RT-PCR Statistical Analysis

Relative gene expression values obtained from real-time RT-PCR were analyzed using the GLM procedure in SAS, with LSMEANS for mean separation using a Tukey adjustment, and assuming an alpha of 0.05.

Results

Objective 1

Weekly ADFI of barrows administered dietary treatments is presented in Table 2. Weekly ADFI was affected by treatment ($P < 0.001$), week ($P < 0.001$), and a week × treatment interaction ($P < 0.001$). Average daily feed intake was decreased ($P < 0.05$) in HIGH barrows compared to CON barrows from wk 5 to 10, and was decreased ($P < 0.05$) in LOW barrows compared to CON barrows in wk 5 and again from wk 8 to 10. Additionally, ADFI was lower ($P = 0.022$) in HIGH barrows than LOW barrows in wk 10. Both an overall linear ($P < 0.001$) and quadratic ($P = 0.030$) response were observed for ADFI.

Weekly ADG of barrows fed dietary treatments is summarized in Table 3. Average daily gain was affected by treatment ($P = 0.001$), week ($P < 0.001$), and the week × treatment interaction ($P = 0.002$). Average daily gain was greater ($P < 0.05$) in CON barrows than HIGH barrows in wk 8 and 10; no differences in ADG were detected between CON and LOW barrows, or between HIGH and LOW barrows. There was an overall linear response ($P < 0.001$) in ADG, but no quadratic response was detected.

Serum AST, ALT, BILI, and BUN measures are summarized in Table 4. Serum AST was affected by treatment ($P = 0.050$), week ($P < 0.001$), and the interaction of treatment × week ($P = 0.020$). Specifically, serum AST was greater ($P = 0.010$) in LOW barrows than CON barrows in wk 5; no other differences were observed. An overall linear response ($P = 0.030$), but no quadratic response, was observed for AST. There was no overall effect of treatment or interaction of treatment × week on ALT concentrations; however, serum ALT was affected ($P < 0.001$) by week. There was no effect of treatment ($P = 0.080$) on BILI concentrations, but effects ($P < 0.001$) of week and the treatment × week interaction were observed. The BILI concentration was

greater ($P < 0.001$) in LOW barrows than both CON and HIGH barrows in wk 5. Upon further investigation of BILI measurements during that week, 1 LOW barrow exhibited a BILI concentration reading outside the range considered typical for swine, and that observation likely contributed to the differences observed in wk 5. However, the other serum measures (AST, ALT, and BUN) were also outside their respective typical ranges, indicating that this high BILI reading was not due to laboratory error, but, instead, may be indicating a response by that particular barrow to aflatoxin consumption. Concentrations of BUN were affected by treatment ($P = 0.032$), week ($P = 0.003$), and the interaction of treatment × week ($P = 0.002$). Concentration of BUN was greater ($P = 0.004$) in CON barrows than LOW barrows in wk 3, but no other differences were observed. There was an overall quadratic response ($P = 0.028$), but not linear, response observed for BUN.

Organ weights of barrows fed dietary treatments, measured as a percentage of BW, are summarized in Table 5. Liver weight was affected by the main effects of AFB1 concentration ($P < 0.001$) and duration of feeding ($P < 0.05$), but not their interaction. Liver weight was greater ($P < 0.05$) in HIGH barrows than CON barrows on d 70 only. Weights of d 70 livers increased linearly ($P < 0.001$) with increasing AFB1 concentrations. It should also be noted that 7 of 10 barrows in the d 70, HIGH treatment group exhibited liver discolorations, including paleness and yellowing. Table 6 summarizes results from the histological analysis of liver tissues collected from d 70 barrows. No differences in liver health as assessed by histological grading were observed among the 70 d treatment groups. However, there were 2 CON barrows with a grade of 1 (limited portal tract infiltration), and 3 CON barrows displayed mild extramedullary hematopoiesis (EMH). In the LOW treatment group, 5 barrows were graded 1, and 5 also showed mild EMH. In addition, 1 barrow in the LOW group had moderate EMH, and 3 barrows had mild nuclear pleomorphisms. The HIGH treatment group had 2 barrows that graded 1, and 1 barrow that graded 2 (bile duct proliferation). There were 9 barrows in the HIGH group with mild EMH, 2 with nuclear pleomorphisms, and 1 with mid-zonal vacuolation. Bile duct proliferation was more prominent in the HIGH barrows than the CON barrows. Vacuolation was seen in 1 HIGH animal.

Objective 2:

Hepatic Gene Expression

All d 70 RNA samples used for RNA-Seq exceeded minimum quality requirements based on Experion results, with a RNA Quality Indicator (RQI) > 8.0 on a scale of 1.0 (fully degraded) to 10.0 (intact). Of 82,744 sequences probed using RNA-Seq, 179 had transcript numbers that were highly correlated ($r \geq |0.8|$; $P < 0.01$) with treatment level. There were 46 sequences with a negative relationship between transcript copy number and treatment level, and 133 sequences with a positive relationship. Of these 179 genes, 150 were recognized by DAVID, and subsequently clustered into 43 functional groups (Table 7). The functional groups that contained ≥ 5 genes included catabolic processes, vesicle membrane, apoptosis, positive apoptosis regulation, negative apoptosis regulation, nucleotide binding, ATP binding, ion binding, zinc finger, protein kinase activity, actin binding, GTP binding, metabolism regulation, and reproduction. The functional groups of apoptosis regulation were selected for further genetic investigation because of the role of apoptosis in aflatoxicosis. The RNA-Seq results for the apoptosis regulating genes are reported in Table 8, along with treatment differences found using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). Four genes in the apoptosis regulation functional category, the 2 most up-regulated and the 2 most down-regulated from RNA-Seq, were selected for validation and compared to results from real-time RT-PCR (Table 10, Table 11); all 15 apoptosis regulation genes were tested for expression differences in d 7 liver samples (Table 12). The genes chosen for validation in d 70 samples included cyclin-dependent kinase inhibitor 1A (*CDKN1A*), kininogen 1 (*KNG1*), zinc finger matrin type 3 (*ZMAT3*), and pim-1 oncogene (*PIM1*).

The apoptosis genes chosen for further analysis in the d 7 tissues included *CDKN1A*, *KNG1*, *ZMAT3*, *PIM1*, signal transducer and activator of transcription 1, 91 kDa (*STAT1*), protein phosphatase 2 (catalytic subunit, beta isoform) (*PP2CB*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), B-cell lymphoma-2 (*BCL2*)/adenovirus E1B 19kDa interacting protein 2 (*BNIP2*), fem-1 homolog b (*FEM1B*), cullin 2 (*CUL2*), insulin-like growth factor 2 (somatomedin A) (insulin; INS-IGF2 read-through transcript) (*IGF2*), NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa (NADH-coenzyme

Q reductase) (*NDUFS3*), collagen (type II, alpha 1) (*COL2A1*), apoptosis enhancing nuclease (*AEN*), and NEDD8 activating enzyme E1 subunit 1 (*NAE1*).

Apoptosis Functional Group

Cyclin-dependent kinase inhibitor 1A (CDKN1A). RNA-Seq showed *CDKN1A* expression was up-regulated in HIGH and LOW barrows ($P < 0.001$) compared to CON barrows, and additionally up-regulated in HIGH barrows ($P = 0.023$) compared to the LOW barrows. Real-time RT-PCR results mostly validated these results in the d 70 tissues; *CDKN1A* expression was up-regulated ($P < 0.001$) in the HIGH and LOW barrows compared to the CON barrows, but not different ($P = 0.692$) between LOW and HIGH barrows. Real-time RT-PCR results of d 7 liver tissues also showed up-regulation of *CDKN1A* ($P < 0.001$) in LOW and HIGH barrows compared to CON, and no difference ($P = 0.057$) in HIGH compared to LOW barrows.

Zinc finger matrin type 3 (ZMAT3). RNA-Seq of d 70 samples identified *ZMAT3* expression as greater ($P < 0.001$) in LOW and HIGH than CON barrows, and greater ($P = 0.008$) in HIGH than LOW barrows. Real-time RT-PCR results from the d 70 tissues supported RNA-Seq; *ZMAT3* expression was up-regulated in LOW ($P = 0.009$) and HIGH ($P < 0.001$) barrows compared to CON barrows, with no expression differences ($P = 0.236$) between LOW and HIGH barrows observed. Real-time RT-PCR of d 7 samples also revealed greater expression of *ZMAT3* in LOW and HIGH ($P < 0.001$) than CON barrows, and greater expression ($P = 0.006$) in HIGH than LOW barrows.

Kininogen 1 (KNG1). RNA-Seq of d 70 samples showed a down-regulation ($P < 0.001$) of *KNG1* in LOW and HIGH barrows compared to CON barrows, with no differences ($P = 0.968$) observed between HIGH and LOW barrows. Real-time RT-PCR of d 70 samples confirmed the RNA-Seq results, showing a down-regulation ($P < 0.001$) in LOW and HIGH barrows compared to CON barrows, with no differences ($P = 0.861$) between LOW and HIGH barrows. No differences ($P > 0.203$) in *KNG1* expression were observed in d 7 samples using real-time RT-PCR.

Pim-1 oncogene (PIM1). A down-regulation of *PIM1* was detected in d 70 samples using RNA-Seq, with LOW and HIGH barrows having lower expression ($P < 0.001$) than CON barrows; no expression differences ($P > 0.392$) between LOW and HIGH barrows were detected. Real-time RT-PCR of d 70 samples confirmed the RNA-Seq results, showing a down-regulation of *PIM1* in LOW ($P = 0.020$) and HIGH ($P = 0.003$) compared to CON barrows, and no differences ($P = 0.866$) between LOW and HIGH barrows. No differences ($P > 0.226$) in *PIM1* expression were detected in d 7 samples using real-time RT-PCR.

Signal transducer and activator of transcription 1 (STAT1). RNA-Seq showed that *STAT1* was up-regulated in LOW ($P = 0.001$) and HIGH ($P < 0.001$) barrows on d 70 compared to CON barrows, with no differences ($P = 0.123$) between LOW and HIGH barrows. Real-time RT-PCR of d 7 samples showed no differences ($P > 0.439$) in *STAT1* expression between treatment groups.

Protein phosphatase 2 (catalytic subunit, beta isoform) (PP2CB). RNA-Seq showed higher *PP2CB* expression in LOW ($P = 0.003$) and HIGH ($P < 0.001$) barrows than CON barrows on d 70, and no differences ($P = 0.085$) between LOW and HIGH barrows. No differences ($P > 0.744$) in PP2CB expression between treatment groups were observed in d 7 samples.

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). RNA-Seq of d 70 samples showed an up-regulation of *YWHAZ* in LOW ($P = 0.004$) and HIGH barrows ($P < 0.001$) compared to CON barrows, but no expression differences ($P = 0.164$) between LOW and HIGH barrows. Real-time RT-PCR of d 7 samples revealed a down-regulation ($P = 0.038$) of *YWHAZ* in HIGH compared to CON barrows, with no differences ($P > 0.282$) between CON and LOW barrows, or between HIGH and LOW barrows.

B-cell Lymphoma 2 (BCL2)/adenovirus E1B 19kDa interacting protein 2 (BNIP2). Using RNA-Seq, *BNIP2* expression of d 70 liver samples was greater in the LOW ($P = 0.001$) and HIGH barrows ($P < 0.001$) than the CON barrows, and *BNIP2* was additionally greater ($P = 0.003$) in HIGH barrows than LOW barrows. No differences ($P > 0.130$) in *BNIP2* d 7 expression were detected using real-time RT-PCR.

Fem-1 homolog b (FEM1B). RNA-Seq identified an up-regulation of *FEM1B* in LOW ($P = 0.030$) and HIGH ($P < 0.001$) barrows on d 70 compared to CON barrows, and also an up-regulation ($P = 0.039$) in HIGH

barrows compared to LOW barrows. Real-time RT-PCR detected no differences ($P > 0.364$) in d 7 *FEM1B* expression between treatments.

Cullin 2 (CUL2). RNA-Seq showed d 70 *CUL2* was up-regulated in LOW ($P = 0.001$) and HIGH ($P < 0.001$) barrows compared to CON barrows; no differences ($P = 0.216$) in *CUL2* expression were observed between LOW and HIGH barrows. Real-time RT-PCR of d 7 tissues revealed no differences ($P > 0.133$) in *CUL2* expression between treatment groups.

Insulin-like growth factor 2 (IGF2). RNA-Seq showed a down-regulation of *IGF2* in LOW and HIGH barrows ($P < 0.001$) compared to CON barrows on d 70, but no expression differences ($P = 0.422$) between LOW and HIGH barrows. Real-time RT-PCR analysis of the d 7 samples revealed no differences ($P > 0.091$) between treatment groups.

NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3). RNA-Seq showed d 70 expression of *NDUFS3* was up-regulated in LOW ($P = 0.008$) and HIGH ($P < 0.001$) barrows compared to CON barrows. No differences ($P = 0.086$) in NDUFS3 expression were observed between LOW and HIGH barrows. No differences ($P > 0.167$) were found in d 7 samples by real-time RT-PCR analysis.

Collagen (type II, alpha 1) (COL2A1). According to RNA-Seq, *COL2A1*expression was up-regulated in LOW ($P = 0.003$) and HIGH ($P < 0.001$) barrows compared to CON barrows, with no expression differences ($P = 0.146$) between LOW and HIGH barrows observed. Real-time RT-PCR revealed no differences ($P > 0.284$) between treatment groups in the d 7 barrows.

Apoptosis enhancing nuclease (AEN). Using RNA-Seq, an up-regulation ($P < 0.001$) of *AEN* gene expression in LOW and HIGH compared to CON barrows was observed; no expression differences ($P = 0.125$) between LOW and HIGH barrows were detected. Apoptosis enhancing nuclease expression in d 7 samples was up-regulated ($P < 0.001$) in LOW and HIGH barrows compared with CON, and higher ($P = 0.026$) in HIGH barrows than LOW.

Neural precursor cell expressed developmentally down-regulated 8 (NEDD8) activating enzyme E1 subunit 1 (NAE1). In d 70 samples, *NAE1* was up-regulated ($P < 0.001$) in LOW and HIGH compared to CON barrows, with no expression differences ($P = 0.354$) between LOW and HIGH barrows detected using RNA-Seq. No differences ($P > 0.195$) between treatments were detected in d 7 samples using real-time RT-PCR.

Discussion

Objective 1

Similar to our results, Southern and Clawson (1979) reported linear decreases in feed intake over a 66-d feeding period with increasing levels of AFB1 (0, 20, 385, 750, and 1,480 ppb). Lindemann et al. (1993) also observed a linear reduction in feed intake in growing swine administered feeds contaminated with 0, 420, or 840 ppb AFB1 for 49 d. In this study, feed intake increased in all treatment groups over the trial period, but to a lesser extent in the LOW and HIGH treatment groups. Schell et al. (1993) reported decreased ADG in pigs (barrows and gilts) fed a diet contaminated with 800 ppb AFB1. Linear decreases in ADG have been previously reported in barrows fed increasing concentrations of 0 ppb to 840 ppb (Lindemann et al., 1993) and 20 ppb to 1480 ppb (Southern and Clawson, 1979) of AFB1 in their diets. Similar to feed intake, ADG in this study increased in all treatments throughout the trial period, but this increase was less in the aflatoxin-treated barrows.

Swine administered aflatoxin-contaminated feed have increased activity of liver-specific enzymes (Harvey et al., 1990; Lindemann et al., 1993; Schell et al., 1993). Lindemann et al. (1993) reported linear increases in AST in swine fed diets with 0 ppb to 840 ppb AFB1. Schell et al. (1993) found serum AST levels were elevated in pigs fed 800 ppb AFB1. Both AST and ALT have been proposed as indicators of depressed liver function due to hepatocellular injury leading to changes in plasma membrane permeability (Miller et al., 1982). Because AFB1 induces liver damage, it would be expected that both AST and ALT concentrations would be increased in AFB1 treated pigs. However, the levels of AFB1 used in this study (250 and 500 ppb) may not have been great enough to elicit substantial changes in ALT concentrations. Additionally, the variability associated with laboratory analysis of AST may have contributed to the limited differences in AST observed in this study. Bilirubin, a byproduct of hemoglobin degradation, is conjugated with glucuronic acid

and secreted with the bile in the liver. Increased bilirubin, resulting from decreased liver function, is another potential indicator of aflatoxicosis (Sisk et al., 1968). Lindemann et al. (1993) reported linear increases of total bilirubin with increasing AFB1 concentrations of 0, 420, and 840 ppb. Measurements of BUN reflect the concentration of urea in blood. Urea is excreted by the kidneys, and high urea concentration is indicative of impaired kidney function. Lower BUN concentrations have been observed in barrows with severe liver impairment, indicating decreased protein synthesis (Hussein and Brasel, 2001). Schell et al. (1993) found serum urea nitrogen decreased with 500 ppb AFB1 in the feed. Harvey et al. (1990) reported decreased urea nitrogen in pigs fed 2,500 ppb AFB1. Lindemann et al. (1993) found no differences in 2 trials, and decreased BUN in 1 trial with 800 ppb AFB1.

Similarly to our study, Sisk et al. (1968) and Miller et al. (1982) found that animals treated with aflatoxins had livers that were pale and yellow in color. There were no differences ($P > 0.05$) in kidney and pancreas weights (each measured as a percentage of BW). Southern and Clawson (1979) found that aflatoxin ingestion in finishing swine caused a linear increase in liver weights (expressed as a percent of BW) with increasing levels from 20 ppb to 1480 ppb of AFB1. Harvey et al. (1990) reported higher liver and kidney weights in barrows fed 2,500 ppb AFB1 compared with barrows fed a non-contaminated feed. Increased kidney weights but no differences in pancreas weights have been reported in barrows and gilts fed AFB1 concentrations increasing from 83.4 µg/kg BW to 333.6 µg/kg BW (Sisk et al., 1968).

The lack of differences in histological analysis in our study could be attributed to the lower AF concentration (250 and 500 ppb) than those used in previous research. Miller et al. (1982) found that pigs fed 1.2 mg AFB1/kg BW had varying degrees of mild karyomegaly, hepatocellular degeneration, bile ductile proliferation, and mild fibrosis. Sisk et al. (1968) reported vacuolation of cytoplasm of centrilobular cells, and bile ductile cell proliferation in the liver of pigs given AFB1 orally at concentrations from 83.4 µg/kg BW to 333.6 µg/kg BW. Performance results from this study demonstrated that feeding an AFB1-contaminated diet, especially for an extended period, is detrimental to the health and performance of growing barrows.

Objective 2

Apoptosis Functional Group

Apoptosis is a complex process that is necessary for regulating cell survival through removal of diseased or damaged cells. Because of the liver damage, especially DNA damage, caused by aflatoxins in swine, increased activity of genes involved in the apoptosis process would be expected.

Cyclin-dependent kinase inhibitor 1A (CDKN1A). Upon DNA damage, *CDKN1A* regulates cell cycle arrest in the *p53* checkpoint pathway (Bendjennat et al., 2003). The *p53* tumor suppressor is regulated by many apoptosis genes. This transcription factor triggers cell cycle arrest, senescence, and apoptosis; too much can lead to cell death and too little can lead to tumor development (Vilborg et al., 2009). Cyclin-dependent kinase inhibitor 1A binds and inhibits cyclin-dependent kinase activity, preventing phosphorylation of substrates and blocking cell cycle progression. Tumor suppressor *p53* can activate *CDKN1A*, and *CDKN1A* can be a negative regulator of *p53* stability (Broude et al., 2007). Over-expression of *CDKN1A* results in cell cycle arrest. Under conditions of DNA damage and oxidative stress, *CDKN1A* is induced (Wang et al., 1997). Loss of *CDKN1A* may contribute to tumor suppression through sensitized apoptotic response (Wang et al., 1997). Additionally, phosphorylation of *STAT1* leads to enhanced gene expression of *CDKN1A* (Hikasa, et al., 2003). The up-regulation seen in d 7 liver tissues in LOW and HIGH barrows compared to CON suggests *CDKN1A* may be an early indicator of apoptosis activity due to aflatoxin administration.

Zinc finger matrin type 3 (ZMAT3). Zinc finger matrin type 3 is regulated by *p53*, and positively regulates *p53* mRNA by binding and stabilizing the mRNA to prevent its deadenylation. This act forms a positive feedback loop and increases *p53* protein levels, allowing enhanced *p53* response to DNA damage (Vilborg et al., 2009; Vilborg et al., 2010). Similar to *CDKN1A*, changes in *ZMAT* expression may be an early indication of apoptotic response to aflatoxins.

Kininogen 1 (KNG1). Kininogen 1 (high-molecular-weight kininogen (HK)) releases nonapeptide bradykinin, released from the interaction with plasma kallikrein. This release causes the generation of 2-chain high-molecular-weight kininogen (HKa), which can induce apoptosis of proliferating endothelial cells and inhibit angiogenesis (Merkulov et al., 2008).

Pim-1 oncogene (PIM1). Pim-1 oncogene is a serine/threonine kinase that is involved in cell survival, proliferation, differentiation, apoptosis, and tumorigenesis (Hu et al., 2009). Multiple cytokines activate *PIM1* through the JAK/STAT signaling pathway, and *PIM1* can also negatively regulate the JAK/STAT pathway. Pim-1 oncogene helps regulate cell apoptosis and anti-apoptotic activity by phosphorylating *Bcl-xL*/*BCL2*-associated death promoter (*BAD*), which is a pro-apoptotic member of the *BCL2* family (Hu et al., 2009). Zhang et al. (2007) reported that *PIM1* phosphorylates and stabilizes *CDKN1A*, promoting cell proliferation and contributing to tumorigenesis. In this way, *PIM1* is important to anti-apoptosis signaling, which may lead to increased tumor growth. In agreement with previously mentioned research, *PIM1* expression is decreased, and *CDKN1A* is increased, indicating increased apoptosis and decreased cell survival in barrows administered AFB1.

Signal transducer and activator of transcription 1 (STAT1). Signal transducer and activator of transcription 1 induces apoptosis and is considered a tumor suppressor (Regis et al., 2008). Apoptosis can be regulated by *STAT1* through negative regulation of Mdm2, an anti-apoptotic gene, and a *p53* interaction. Along with apoptosis, *STAT1* negatively regulates the cell cycle through *CDKN1A* up-regulation, and negatively regulates cell survival genes *Bcl-xl* and *BCL*2, both anti-apoptotic genes (Regis et al., 2008).

Protein phosphatase 2 (catalytic subunit, beta isoform) (PP2CB). Protein phosphatase 2 (catalytic subunit, beta isoform) is activated by unsaturated fatty acids, and also requires *BAD* to influence apoptosis. Phosphorylation of *BAD* is anti-apoptotic, but dephosphorylation is pro-apoptotic. The dephosphorylation of *BAD* by *PP2CB* leads to apoptosis (Klumpp et al., 2006). Reduced expression of *PP2CB* was also reported in prostate cancer (Hornstein et al., 2008).

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide interacts with TP53 to enhance the transcriptional activity of *p53* and inhibit apoptosis. Binding of *YWHAZ* (and/or its family of genes) to *BAD*, *Bax*, and other apoptotic genes prevents further binding, inhibiting apoptosis (Xing et al., 2000; Tsuruta et al., 2004). Our results indicate a different response in *YWHAZ* expression on d 7 compared to d 70. Other genes that are up-regulated in the d 7 groups (*CDKN1A*, *ZMAT3*, and *AEN*) may decrease the d 7 expression of *YWHAZ*, although the d 70 expression is up-regulated.

B-cell Lymphoma 2 (BCL2)/adenovirus E1B 19kDa interacting protein 2 (BNIP2). B-cell lymphoma 2/adenovirus E1B 19kDa interacting protein 2 is pro-apoptotic. An interaction of *BNIP2* with the pro-survival proteins *BCL2* and *E1B 19kDa* decreases signaling and increases apoptosis (Zhang et al., 2003).

Fem-1 homolog b (FEM1B). Fem-1 homolog b is pro-apoptotic and acts as a death receptor-associated protein to regulate apoptosis (Oyhenart et al., 2005). In cancer cells, apoptosis is induced with increased *FEM1B* expression (Subauste et al., 2009).

Cullin 2 (CUL2). Cullin 2 has been predicted to function as a tumor suppressor, but little research investigating the specific role of *CUL2* has been conducted. Cullin 2 binds to form a complex that is a subunit on the RNA polymerase II transcriptional machinery, so *CUL2* could potentially influence RNA expression (Maeda et al., 2008).

Insulin-like growth factor 2 (IGF2). Insulin-like growth factor 2 is typically considered a growth promoter important for fetal development, but it has also been demonstrated that *IGF2* is important for tumor formation (Sun et al., 2006). Sakatani et al. (2005) reported that a loss of imprinting, or activation of the normally silent allele of *IGF2*, may predispose mammals to tumor development.

NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3). NADH dehydrogenase (ubiquinone) Fe-S protein 3 is a subunit of the mitochondrial respiratory chain complex 1, which is necessary for the transfer of electrons from NADH to ubiquinone during the ATP production process (Huang et al., 2007).

Collagen (type II, alpha 1) (COL2A1). Collagen (type II, alpha 1), the gene responsible for coding type II collagen, has been used as a measure of apoptosis through caspase function (Nuttall et al., 2001).

Apoptosis enhancing nuclease (AEN). Apoptosis enhancing nuclease encodes a DNase that enhances apoptosis by breaking DNA strands (Lee et al., 2005). The *p53* gene phosphorylation status regulates *AEN* expression, and *AEN* also regulates *p53* expression (Kawase et al., 2008). The up-regulation seen in our results indicates increased apoptosis in the treated barrows beginning at or before d 7 of treatment.

Neural precursor cell expressed developmentally down-regulated 8 (NEDD8) activating enzyme E1 subunit 1 (NAE1). Neural precursor cell expressed developmentally down-regulated 8 (NEDD8) activating enzyme E1 subunit 1 is a regulatory subunit for the *NEDD8* pathway, which regulates cullin activity and has a role in degradation of proteins important for cell-cycle progression, DNA damage, and stress response (Swords et al., 2010). Over-expression of *NAE1* leads to apoptosis due to *NEDD8* conjugation deregulation (Soucy et al., 2009). Up-regulation of *NAE1* may increase *CUL2* expression, as seen in the RNA-Seq results, but not in the d 7 real-time RT-PCR analysis.

Our results demonstrate that performance and blood parameters in young growing barrows are affected by consumption of an aflatoxin-contaminated diet, especially when the concentration of aflatoxin is high (≥ 500 ppb); however, even lower concentrations (250 ppb) are detrimental to performance when administered for a more chronic period. Limited histological changes were observed. Genomic analysis indicates considerable variation in the number of transcripts, with most probed sequences having low levels of transcription. Changes in the expression of genes involved in a variety of functions related to cellular stress and toxicity responses, such as apoptosis, regulation of cell growth and proliferation, and mRNA processing, are differentially regulated in response to AFB1. Of specific interest, 15 genes with apoptotic roles were differentially expressed after long-term aflatoxin exposure (70 d). Of those 15 genes, 4 (*CDKN1A*, *ZMAT3*, *YWHAZ*, and *AEN*) were also differentially expressed after only a short-term exposure (7 d), indicating those genes may be early indicators of an apoptotic response to aflatoxins. Further study of these differentially expressed genes may lead to prevention strategies and treatments for aflatoxicosis. Additionally, early response genes may prove to be useful for early diagnosis of aflatoxicosis, or even indicate differences in tolerance to dietary aflatoxins.

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***Objective 1*- Tables**

Table 1. Percentage composition of the basal diet (as-fed basis)[1]

Item	d 0 to 20 (Starter)	d 21 to 70 (Grower)
Ingredient, %		
Ground corn	56.70	77.80
Soybean meal (46% CP)	27.40	19.25
Dried whey	10.00	-
Blood meal	2.50	-
Mono-Calcium phosphate	1.10	1.30
Calcium carbonate	0.85	0.98
Salt	0.30	0.25
Vitamin premix	0.25[2]	0.20[3]
Trace mineral premix	0.15[4]	0.13[5]
Zinc oxide	0.25	0.00
L-Lysine HCl	0.25	0.15
DL-Methionine	0.18	0.00
L-Threonine (98%)	0.13	0.00
Chemical composition		
DE, kcal/kg	3,400	3,400
CP, %	21.42	16.00
Lysine, %	1.49	0.90
Methionine, %	0.50	0.26
Threonine, %	1.05	0.65
Ca, %	0.76	0.70
P, %	0.65	0.60
P Available, %	0.51	0.43

[1]Basal diet formulated according to Custom Ag Products, Inc. (Beloit, KS 67420).
[2]Vitamin premix supplied (per kg of diet): vitamin A, 55,000 IU; vitamin D_3, 8,250 IU; vitamin E, 275 IU; menadione, 22 mg; vitamin B_{12}, 0.22 mg; biotin, 0.85 mg; folic acid, 5.5 mg; niacin, 275 mg; d-pantothenic acid, 165 mg; vitamin B_6, 16.5 mg; riboflavin, 49.5 mg; and thiamine, 16.5mg.
[3]Vitamin premix supplied (per kg of diet): vitamin A, 44,000 IU; vitamin D_3, 6,600 IU; vitamin E, 220 IU; menadione, 17.6 mg; vitamin B_{12}, 0.18 mg; biotin, 0.68 mg; folic acid, 4.4 mg; niacin, 220 mg; d-pantothenic acid, 132 mg; vitamin B_6, 13.2 mg; riboflavin, 39.6 mg; and thiamine 13.2 mg.
[4]Trace mineral premix provided (per kg of diet): Cu, 16.5 mg, from copper sulfate; I, 0.36 mg, from ethylenediamine dihydriodide; Fe, 165 mg from ferrous sulfate; Mn, 43.5 mg from manganese sulfate; Se, 0.30 mg, from sodium selenite; and Zn, 165 mg from zinc sulfate.
[5]Trace mineral premix provided (per kg of diet): Cu, 13.75 mg, from copper sulfate; I, 0.30 mg, from ethylenediamine dihydriodide; Fe, 137.5 mg from ferrous sulfate; Mn, 36.25 mg from manganese sulfate; Se, 0.25 mg, from sodium selenite; and Zn, 137.5 mg from zinc sulfate.

Table 2. Weekly ADFI (kg) of barrows fed corn-soybean diets containing different concentrations of aflatoxin[1,2]

Week[3]	CON	LOW	HIGH	SEM
1	2.06	2.03	1.97	0.01
2	2.74	2.48	2.49	0.10
3	3.10	2.76	2.77	0.10
4	3.32	2.92	2.93	0.10
5	3.92[a]	3.15[b]	3.13[b]	0.14
6	4.10[a]	3.46[ab]	3.21[b]	0.14
7	4.36[a]	3.86[ab]	3.45[b]	0.14
8	5.04[a]	3.72[b]	3.42[b]	0.14
9	5.24[a]	4.10[b]	3.52[b]	0.14
10	5.63[a]	4.31[b]	3.47[c]	0.15

[a-c]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]CON = 0 ppb aflatoxin B1 (AFB1) diet; LOW = 250 ppb AFB1 diet; HIGH = 500 ppb AFB1 diet.
[2]Main effect of treatment $P < 0.001$; main effect of week $P < 0.001$; interaction of week × treatment $P < 0.001$.
[3]wk 1, n = 3 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 2 to 4, n = 2 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 5 to 10, n = 1 pen with 10 pigs each for CON, LOW, and HIGH treatments.

Table 3. Weekly ADG (kg) of barrows fed corn-soybean diets containing different concentrations of aflatoxin[1,2]

Week[3]	CON	LOW	HIGH	SEM
1	0.48	0.54	0.46	0.04
2	0.69	0.62	0.70	0.04
3	0.68	0.62	0.58	0.04
4	0.71	0.63	0.51	0.04
5	0.64	0.59	0.49	0.06
6	0.65	0.47	0.50	0.06
7	0.68	0.72	0.53	0.06
8	0.77[a]	0.61[ab]	0.40[b]	0.06
9	0.92	0.82	0.61	0.06
10	0.84[a]	0.59[ab]	0.43[b]	0.06

[a-c]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]CON = 0 ppb aflatoxin B1 (AFB1) diet; LOW = 250 ppb AFB1 diet; HIGH = 500 ppb AFB1 diet.
[2]Main effect of treatment $P = 0.001$; main effect of week $P < 0.001$; interaction of week × treatment $P = 0.002$.
[3]wk 1, n = 3 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 2 to 4, n = 2 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 5 to 10, n = 1 pen with 10 pigs each for CON, LOW, and HIGH treatments.

Table 4. Weekly average serum concentrations of aspartate amino transferase, alanine amino transferase, bilirubin, and urea nitrogen in barrows fed corn-soybean diets containing different concentrations of aflatoxin[1]

Week[2]	CON	LOW	HIGH	SEM
Aspartate amino transferase, U/L[3]				
1	48.13	47.23	43.80	3.60
2	64.45	50.50	50.68	4.64
3	43.75	50.00	53.60	4.52
4	42.25	48.74	48.42	4.60
5	36.00[a]	75.56[b]	54.80[ab]	6.51
6	41.20	51.90	56.90	6.39
7	40.10	49.30	49.60	6.39
8	67.60	53.78	71.90	6.51
9	53.56	57.33	57.20	6.62
10	44.80	61.89	56.00	6.62
Alanine amino transferase, U/L[4]				
1	39.23	43.43	41.93	2.02
2	48.53	54.89	53.42	2.40
3	49.28	55.09	56.02	2.40
4	55.28	58.79	60.77	2.40
5	54.76	62.73	62.12	3.22
6	49.66	51.03	54.92	3.22
7	47.46	47.73	50.62	3.22
8	55.66	50.13	52.42	3.22
9	52.96	49.83	49.52	3.22
10	51.96	49.96	49.02	3.27
Bilirubin, mg/dL[5]				
1	0.20	0.16	0.16	0.04
2	0.22	0.20	0.21	0.05
3	0.19	0.18	0.22	0.05
4	0.21	0.19	0.29	0.05
5	0.20[a]	0.79[b]	0.27[a]	0.06
6	0.23	0.29	0.22	0.06
7	0.23	0.20	0.22	0.06
8	0.20	0.20	0.31	0.06
9	0.22	0.22	0.37	0.06
10	0.21	0.27	0.51	0.07
Urea N, mg/dL[6]				
1	12.97	10.00	10.30	0.70
2	12.46	9.06	10.13	0.82
3	14.76[a]	9.56[b]	10.88[ab]	0.82
4	16.21	9.16	9.68	0.82
5	9.86	8.40	9.23	1.08
6	11.86	8.10	9.33	1.08
7	12.36	8.20	10.53	1.08
8	11.86	7.50	10.33	1.08
9	11.96	10.00	10.53	1.08
10	11.86	8.24	10.23	1.10

[a-c]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1]CON = 0 ppb aflatoxin B1 (AFB1) diet; LOW = 250 ppb AFB1 diet; HIGH = 500 ppb AFB1 diet.

[2]wk 1, n = 3 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 2 to 4, n = 2 pens of 10 pigs each for CON, LOW, and HIGH treatments; wk 5 to 10, n = 1 pen with 10 pigs each for CON, LOW, and HIGH treatments.
[3]Main effect of treatment $P = 0.050$; main effect of week $P < 0.001$; interaction of week × treatment $P = 0.020$.
[4]Main effect of treatment $P = 0.506$; main effect of week $P < 0.001$; interaction of week × treatment $P = 0.571$.
[5]Main effect of treatment $P = 0.080$; main effect of week $P < 0.001$; interaction of week × treatment $P < 0.001$.
[6]Main effect of treatment $P = 0.032$; main effect of week $P = 0.003$; interaction of week × treatment $P = 0.002$.

Table 5. Average organ weights (kg) as a percent of body weight of barrows fed corn-soybean diets containing different concentrations of aflatoxin[1]

Organ	CON	LOW	HIGH	Mean SEM
d 7[2]				
Liver	3.04	3.17	3.19	0.11
Kidney	0.56	0.58	0.51	0.02
Pancreas	0.24	0.22	0.41	0.07
d 28[2]				
Liver	2.63	2.80	3.05	0.11
Kidney	0.50	0.47	0.45	0.02
Pancreas	0.13	0.13	0.13	0.07
d 70[2]				
Liver	2.54[a]	3.00[ab]	3.20[b]	0.11
Kidney	0.42	0.43	0.43	0.02
Pancreas	0.08	0.10	0.08	0.07

[ab]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]CON = 0 ppb aflatoxin B1 (AFB1) diet; LOW = 250 ppb AFB1 diet; HIGH = 500 ppb AFB1 diet.
[2]d 7, n = 10 pigs each for CON, LOW, and HIGH treatments; d 28, n = 10 pigs each for CON, LOW, and HIGH treatments; d 70, n = 10 pigs each for CON, LOW, and HIGH treatments.

Table 6. Number of barrows within treatment groups fed corn-soybean diets containing different concentrations of aflatoxin[1] that graded 0, 1, or 2 for histological analysis

	Grade[2]		
Treatment	0	1	2
CON[2]	6	2	0
LOW[3]	4	5	0
HIGH[4]	7	2	1

[1]CON = 0 ppb aflatoxin B1 (AFB1) diet; LOW = 250 ppb AFB1 diet; HIGH = 500 ppb AFB1 diet.
[2]Sections were assigned grades of 0 to 5. No grades of 3, 4, or 5 were assigned. A grade of 0 indicates no detection of changes in the liver tissue, and a grade of 1 indicates that portal tracts were infiltrated by very small numbers of lymphocytes, macrophages, and eosinophils. A grade of 2 indicates liver damage as evidenced by the presence of bile duct proliferation in the portal areas.

***Objective 2*- Tables**

Table 7. Number of genes in functional categories identified using DAVID

Functional Group	Number of Genes[1]
Ion Binding	42
Nucleotide Binding	21
ATP Binding	17
Catabolic Processes	15
Apoptosis	15
Positive Apoptosis Regulation	8
Negative Apoptosis Regulation	7
Vesicle Membrane	7
Protein Kinase Activity	6
Zinc Finger	5
Protein Kinase Activity	5
Actin Binding	5
GTP Binding	5
Reproduction	5
Regulation of Metabolism	5
Peroxisome	4
mRNA Metabolism/ Splicing	4
Metabolic Processes	4
Repeat: WD40	4
Negative Gene Regulation	4
Positive Gene Regulation	4
Chromosome Replication	3
Metabolism	3
Binding	3
Metabolism Regulation	3
Oxygenase	3
Ubiquitin Regulation	3
Muscle Function	3
Blood Regulation	3
PDZ	3
Cell Membrane	3
Immune Cell Regulation	3
Lysosome	3
Metabolism Regulation	3
Kinase Regulation	3
PH	3
Mitosis	3
Endoplasmic Reticulum Membrane	3
Reproduction	3
Transcription Regulation	3
Cell Replication	3
Cell Homeostasis	3
Positive Transcription Regulation	3

[1]Genes detected as differentially expressed by RNA-Seq, and with high correlations ($r > |0.80|$; $P < 0.01$) between transcript copy number and treatment level.

Table 8. Apoptosis regulating genes[1] with treatment differences ($P < 0.05$) using PROC GLM in transcript copy number detected by RNA-Seq

Gene	CON	LOW	HIGH
CDKN1A	187.62 ± 77.81[a]	1,117.23 ± 95.30[b]	1,481.85 ± 82.53[c]
BNIP2	12.22 ± 1.48[a]	22.15 ± 1.82[b]	31.24 ± 1.57[c]
ZMAT3	6.04 ± 9.69[a]	76.72 ± 11.86[b]	129.43 ± 10.27[c]
AEN	109.53 ± 29.94[a]	408.22 ± 36.67[b]	507.84 ± 31.75[b]
IGF2	105.57 ± 6.76[a]	42.73 ± 8.28[b]	28.70 ± 7.17[b]
STAT1	75.65 ± 17.83[a]	192.26 ± 21.84[b]	251.85 ± 18.91[b]
PP2CB	192.69 ± 19.50[a]	309.36 ± 23.88[b]	380.87 ± 20.68[b]
CUL2	76.04 ± 8.71[a]	135.22 ± 10.67[b]	159.77 ± 9.24[b]
NAE1	26.39 ± 3.33[a]	51.76 ± 4.08[b]	59.38 ± 3.53[b]
COL2A1	67.37 ± 4.55[a]	94.42 ± 5.57[b]	108.93 ± 4.82[b]
NDUFS3	153.80 ± 10.95[a]	212.02 ± 13.41[b]	252.01 ± 11.61[b]
PIM1	67.95 ± 4.27[a]	36.50 ± 5.23[b]	27.26 ± 4.53[b]
YWHAZ	258.67 ± 47.76[a]	540.10 ± 58.50[b]	687.45 ± 50.66[b]
KNG1	589.33 ± 27.04[a]	303.43 ± 33.11[b]	292.79 ± 28.68[b]
FEM1B	47.02 ± 7.94[a]	81.88 ± 9.72[b]	115.99 ± 8.42[c]

[a-c]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]Least squares means ± standard error; CON = 0 ppb AFB1; LOW = 250 ppb AFB1; HIGH = 500 ppb AFB1.

Table 9. Forward and reverse primers for real-time RT-PCR

Gene	Forward Primer	Reverse Primer
[1]*GAPDH*	5'gggcatgaaccatgagaagt3'	5'aagcagggatgatgttctgg3'
[1]*CDKN1A*	5'ctcttttctagccgggctct3'	5'gctgcctgaggtagaactgg3'
[1]*KNG1*	5'tggctgccccagagatatac3'	5'tggccgtgaacacaatagaa3'
[1]*ZMAT3*	5'agcttgtcctgcctgttgtt3'	5'tggggtaggtaggtcacagg3'
[1]*PIM1*	5'ttggtagggtgatgggactc3'	5'caggctacatgctgctcaaa3'
[2]*STAT1*	5'ccttgagaccacctctctgc3'	5'tgaaagctgagaccatcgtg3'
[2]*PP2CB*	5'gtggcctctctccatccata3'	5'cagttggtgagcacgagaaa3'
[2]*YWHAZ*	5'agcaggctgagcgatatgat3'	5'tctcagcaccttccgtcttt3'
[2]*BNIP2*	5'gtccccatggaatatgttgg3'	5'gcactgcttcatcagcacat3'
[2]*FEM1B*	5'gattcacctcgatcccagaa3'	5'ggcactgttcccttcattgt3'
[2]*CUL2*	5'ctgtgcctccgtaatttggt3'	5'caaacgtgaaggggagaaaa3'
[2]*IGF-2*	5'cgtgctgctatgctgcttac3'	5'aagcagcactcttccacgat3'
[2]*NDUFS3*	5'gctctctttgcgcttcaact3'	5'cgcaactcaacatagccaga3'
[2]*COL2A1*	5'tcttcatgcggacttcagtg3'	5'ccccctggggtaggtataga3'
[2]*AEN*	5'ggaatagcagagccaacagg3'	5'tcccagaactcacactgctg3'
[2]*NAE1*	5'ggggtgagttccagagatga3'	5'tgcaggaagaccagtgtgag3'

[1]Used for validation with d 70 samples and on d 7 samples.
[2]Used on d 7 samples only.

Table 10. Comparison of fold changes (FC) estimated from RNA-Seq and real-time RT-PCR of four apoptosis genes chosen for validation

	RNA-Seq[1]			Real-time RT-PCR[2]		
Gene	FC[3] (L/C)	FC[3] (H/C)	FC[3] (H/L)	FC[3] (L/C)	FC[3] (H/C)	FC[3] (H/L)
CDKN1A	5.95	7.90	1.33	4.14	4.47	1.08
ZMAT3	12.70	21.43	1.69	4.35	6.05	1.39
KNG1	-1.94	-2.02	-1.04	-2.13	-2.47	-1.16
PIM1	-1.86	-2.49	-1.34	-2.65	-3.71	-1.40

[1]Nine samples tested in CON (0 ppb AFB1) and HIGH (500 ppb AFB1) groups, 6 samples tested in LOW (250 ppb AFB1) group.
[2]Eight samples tested in all three treatment groups.
[3]FC values expressed as a ratio between LOW and CON groups (L/C), HIGH and CON groups (H/C), and HIGH and LOW groups (H/L). FC > 1.00 indicates up-regulation of gene; FC < -1.00 indicates down-regulation of gene.

Table 11. Relative expression of d 70 apoptosis genes[1] selected for validation using real-time RT-PCR

Gene	CON[2]	LOW[3]	HIGH[2]
CDKN1A	1.68 ± 0.42^{a}	6.96 ± 0.51^{b}	7.50 ± 0.42^{b}
ZMAT3	1.75 ± 1.12^{a}	7.59 ± 1.37^{b}	10.57 ± 1.12^{b}
KNG1	6.55 ± 0.51^{a}	3.08 ± 0.63^{b}	2.65 ± 0.51^{b}
PIM1	9.80 ± 1.31^{a}	3.70 ± 1.60^{b}	2.64 ± 1.31^{b}

[ab]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]Least squares means ± standard error; CON = 0 ppb AFB1; LOW = 250 ppb AFB1; HIGH = 500 ppb AFB1.
[2]Nine samples tested.
[3]Six samples tested.

Table 12. Relative expression of d 7 apoptosis genes[1] determined by real-time RT-PCR

Gene	CON	LOW	HIGH	FC[2] L/C	FC[2] H/C	FC[2] H/L
CDKN1A	2.11 ± 1.36^{a}	14.36 ± 1.36^{b}	19.09 ± 1.36^{b}	6.81	9.05	1.33
ZMAT3	1.51 ± 0.50^{a}	4.53 ± 0.50^{b}	6.99 ± 0.50^{c}	3.00	4.63	1.54
KNG1	2.24 ± 0.27	2.05 ± 0.27	1.57 ± 0.27	-1.09	-1.43	-1.31
PIM1	2.48 ± 0.64	4.04 ± 0.64	2.79 ± 0.64	1.63	1.13	-1.45
STAT1	2.55 ± 0.33	2.47 ± 0.33	1.96 ± 0.33	-1.03	-1.30	-1.26
YWHAZ	1.56 ± 0.12^{a}	1.38 ± 0.12^{ab}	1.11 ± 0.12^{b}	-1.13	-1.41	-1.24
PP2CB	1.73 ± 0.15	1.57 ± 0.15	1.62 ± 0.15	-1.10	-1.07	1.03
BNIP2	1.92 ± 0.16	1.96 ± 0.16	1.52 ± 0.16	1.02	-1.26	-1.29
FEM1B	2.54 ± 0.29	2.88 ± 0.29	2.32 ± 0.29	1.13	-1.09	-1.24
CUL2	1.88 ± 0.14	2.07 ± 0.14	1.67 ± 0.14	1.10	-1.13	-1.24
IGF2	3.02 ± 0.27	2.79 ± 0.27	2.16 ± 0.27	-1.08	-1.40	-1.29
NDUFS3	1.55 ± 0.13	1.90 ± 0.13	1.77 ± 0.13	1.23	1.14	-1.07
COL2A1	2.15 ± 0.26	1.78 ± 0.26	1.57 ± 0.26	-1.21	-1.37	-1.13
AEN	1.69 ± 0.46^{a}	5.93 ± 0.46^{b}	7.76 ± 0.46^{c}	3.51	4.59	1.31
NAE1	1.98 ± 0.21	2.20 ± 0.21	1.66 ± 0.21	1.11	-1.19	-1.33

[ab]Within a row, means without a common superscript letter differ ($P < 0.05$).
[1]Least squares means ± standard error; CON = 0 ppb AFB1; LOW = 250 ppb AFB1; HIGH = 500 ppb AFB1.
[2]FC values expressed as a ratio between LOW and CON groups (L/C), HIGH and CON groups (H/C), and HIGH and LOW groups (H/L). FC > 1.00 indicates up-regulation of gene, FC < -1.00 indicates down-regulation of gene.