Differential Transcriptome Responses to Aflatoxin B\(_1\) in the Cecal Tonsil of Susceptible and Resistant Turkeys

Kent M. Reed \(^1,\,*\), Kristelle M. Mendoza \(^1\) and Roger A. Coulombe Jr. \(^2\)

\(^1\) Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN 55108, USA; mendo008@umn.edu
\(^2\) Department of Animal, Dairy and Veterinary Sciences, College of Agriculture and Applied Sciences, Utah State University, Logan, UT 84322, USA; roger@usu.edu

* Correspondence: reedx054@umn.edu; Tel.: +1-612-624-1287

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Abstract: The nearly-ubiquitous food and feed-borne mycotoxin aflatoxin B\(_1\) (AFB\(_1\)) is carcinogenic and mutagenic, posing a food safety threat to humans and animals. One of the most susceptible animal species known and thus a good model for characterizing toxicological pathways, is the domesticated turkey (DT), a condition likely due, at least in part, to deficient hepatic AFB\(_1\)-detoxifying alpha-class glutathione S-transferases (GSTAs). Conversely, wild turkeys (Eastern wild, EW) are relatively resistant to the hepatotoxic, hepatocarcinogenic and immunosuppressive effects of AFB\(_1\) owing to functional gene expression and presence of functional hepatic GSTAs. This study was designed to compare the responses in gene expression in the gastrointestinal tract between DT (susceptible phenotype) and EW (resistant phenotype) following dietary AFB\(_1\) challenge (320 ppb for 14 days); specifically in cecal tonsil which functions in both nutrient absorption and gut immunity. RNAseq and gene expression analysis revealed significant differential gene expression in AFB\(_1\)-treated animals compared to control-fed domestic and wild birds and in within-treatment comparisons between bird types. Significantly upregulated expression of the primary hepatic AFB\(_1\)-activating P450 (CYP1A5) as well as transcriptional changes in tight junction proteins were observed in AFB\(_1\)-treated birds. Numerous pro-inflammatory cytokines, TGF-\(\beta\) and EGF were significantly down regulated by AFB\(_1\) treatment in DT birds and pathway analysis suggested suppression of enteroendocrine cells. Conversely, AFB\(_1\) treatment modified significantly fewer unique genes in EW birds; among these were genes involved in lipid synthesis and metabolism and immune response. This is the first investigation of the effects of AFB\(_1\) on the turkey gastro-intestinal tract. Results suggest that in addition to the hepatic transcriptome, animal resistance to this mycotoxin occurs in organ systems outside the liver, specifically as a refractory gastrointestinal tract.

Keywords: Poultry; Turkey; Transcriptome; Aflatoxin B\(_1\); Cecal Tonsil; Cecum; RNAseq

Key Contribution: This study is the first to examine the transcriptome of the turkey cecal tonsil region of gastro-intestinal tract. Importantly it combines RNAseq and gene expression analysis and identifies key gene transcripts modulated in response to dietary AFB\(_1\) treatment.

1. Introduction

Aflatoxin B\(_1\) (AFB\(_1\)) is a hepatotoxic, hepatocarcinogenic and immunosuppressive mycotoxin commonly found in food and feed, especially corn [1]. Poultry are particularly sensitive to the toxic effects of AFB\(_1\) and commercial domesticated turkeys are perhaps the most susceptible animal thus far studied [2,3]. Exposure to AFB\(_1\) through contaminated feed is practically unavoidable and can
result in reduced feed intake, weight gain and feed efficiency and increased mortality, hepatotoxicity and GI hemorrhaging (reviewed in Monson et al. [4]). As a potent immunotoxin, AFB$_1$ suppresses cell-mediated, humoral and phagocytic immunological functions, thereby increasing susceptibility to bacterial and viral diseases [5–7].

In contrast to their modern domesticated counterparts, wild turkeys are relatively resistant to aflatoxicosis [8]. Metabolism of AFB$_1$ requires bioactivation by hepatic cytochrome P450s (CYPs) to the electrophilic exo-AFB$_1$-8,9-epoxide (AFBO), which is catalyzed primarily, at pharmacological concentrations by the high-efficiency CYP1A5 and to a minor extent by the lower-affinity CYP3A37 which predominates only at high, environmentally-irrelevant substrate concentrations [9]. In most animals, AFBO is detoxified primarily by hepatic glutathione S-transferases (GSTs) [3]. The most likely mechanism for the extreme susceptibility in domesticated turkeys is dysfunctional hepatic GSTs rendering them unable to detoxify AFB$_1$ [10–14]. In this regard, domesticated turkeys closely resemble humans in that they also lack hepatic alpha-class GSTs (GSTA) with high activity toward AFB$_1$ (seen in mice and rats) suggesting that turkeys may represent a better model to study aflatoxin toxicology than either of these rodent species [9]. Expression of GSTA in the intestine and the potential for extra-hepatic bioactivation and metabolism of AFB$_1$ in turkeys is unknown.

To better understand the response of the domestic turkey to AFB$_1$ exposure, we initiated transcriptomic analysis of AFB$_1$-challenged domestic birds [15], where genes and gene pathways in the liver were significantly dysregulated by dietary AFB$_1$ challenge, such as pathways associated with cancer, apoptosis, cell cycle and lipid regulation. These changes reflect the molecular mechanisms underlying DNA alkylation and mutation, inflammation, proliferation and liver damage in aflatoxicosis. Analysis of spleen tissues from the same birds examined in the Monson et al. [15] study found that short AFB$_1$ exposure suppressed innate immune transcripts, especially from antimicrobial genes associated with either increased cytotoxic potential or activation-induced cell death during aflatoxicosis [16].

The differential response of domestic and wild turkey to AFB$_1$ was examined in a controlled feeding trial [17]. Analysis by RNAseq of the hepatic transcriptome found genes dysregulated as a response to toxic insult with significant differences observed between these genetically distinct birds in the expression of Phase I and Phase II drug metabolism genes. Genes important in cellular regulation, modulation of apoptosis and inflammatory responses were also affected. Unique responses in wild birds were seen for genes that negatively regulate cellular processes, serve as components of the extracellular matrix or modulate coagulation factors. Wild turkey embryos also showed differential AFB$_1$ effects compared to their commercial counterparts presumably due to lower levels of AFBO [18]. When treated with AFB$_1$, embryos showed up-regulation in cell cycle regulators, Nrf2-mediated response genes and coagulation factors [18]. Results of these studies supported the hypothesis that the reduced susceptibility of wild turkeys is related to higher constitutive expression of GSTA3, coupled with an inherited (genetic) difference in functional gene expression in domesticated birds.

The molecular basis for the differences in AFB$_1$ detoxification observed between domesticated commercial and wild birds has been extensively studied in our laboratories. However, extra-hepatic effects, such as those occurring at the site of initial toxicant exposure, the intestine, are needed to fully understand the systemic effects of AFB$_1$ in this susceptible species. Unlike many mycotoxins, AFB$_1$ is efficiently absorbed (>80%) in the avian upper gastrointestinal tract (GIT) [19]. Recent studies of broiler chickens have found conflicting evidence for the potential impact of AFB$_1$ on gut permeability, from no effect [20] to increased permeability [21]. The avian small intestine is a primary site of nutrient absorption [22] but is often overlooked from an immunological perspective. The cecal tonsils are the largest aggregates of avian gut-associated lymphoid tissue, yet basic information on gene expression in the cecal tonsil is lacking in the turkey. This study focused on the effects of dietary AFB$_1$ on gene expression in the turkey GIT and specifically the region at the junction of the distal ileum and cecum (the cecal tonsil region) that functions in AFB$_1$ absorption and gut immunity. The purpose of this study
was to examine the transcriptomic response of the cecal tonsil region of the turkey intestine to dietary AFB₁ treatment and contrast these in susceptible (domesticated) and resistant (wild) birds.

2. Results

The effects of AFB₁ on body weight and liver mass are summarized in a companion study of hepatic gene expression [17]. Sequencing produced from 9.8M to 14.2M reads per library (average 12.7 million) (Table S1). Data are deposited in the NCBI’s Gene Expression Omnibus (GEO) repository as SRA BioProject 346253. Median Q scores of the trimmed and filtered reads ranged from 36.5 to 37.7 among the forward and reverse reads. The number of reads per treatment group ranged from 10.9 to 12.8M with the mean number for EW birds being slightly higher than for the DT birds (12.6M verses 11.2M). Over 90% of the quality-trimmed reads mapped to the annotated turkey gene set (NCBI Annotation 101) and the vast majority of reads (average 85.2%) mapped concordantly (Table S1). Based on mapping, the estimated mean insert size of the libraries was 195.4 ± 15.8 bp. Variation in mapped reads among the treatment groups was visualized by PCA (Figure 1). Samples (AFB₁ treatment/CNTL) generally clustered distinctly by treatment group within the space defined by the first two principal components. The exceptions were two EW AFB₁ samples (EW1C and EW3C) that clustered with the control birds. The relationships among groups was reiterated in the hierarchical clustering of groups by Euclidean distance and heat map of co-expressed genes (Figure S1). This indicates the main effect underlying this study is AFB₁ treatment.

![Figure 1. Principal component analysis (PCA) of by-total normalized RNAseq read counts. For each treatment group, sample to sample distances (within- and between-treatments) are illustrated on the first two principle components.](image)

Evidence of expression (mapped reads ≥ 1.0 in at least one individual) was detected for 19,754 genes (tRNAs excluded) with an average of 17,261 genes observed per individual (Tables S1 and S2). When qualified (by-total normalized read count ≥ 3.0), the number of expressed genes averaged...
16,132 per individual (76.79% of the turkey gene set) with an average of 17,877 expressed genes per treatment group. The numbers of observed and expressed genes were higher for control groups than for AFB1-treatment groups of both EW and DT. A total of 16,097 genes (84.4%) was co-expressed among all groups and the number of co-expressed genes within the EW and DT lines was 17,833 and 16,277, respectively (Figure 2). Each treatment group had a distinct set of uniquely expressed genes, with the numbers being greater for the control groups (200 and 185) compared to the AFB1 groups (80 and 113) (Figure 2).

Figure 2. Distribution of expressed genes in turkey cecal tonsil by treatment group.

2.1. Differential Gene Expression

2.1.1. AFB1 Treatment Effects

The full list of genes showing significant differential expression (DE) in pairwise treatment comparisons is provided in Table S3. In comparison of DT birds exposed to AFB1 (DTAFB) with control-fed birds (CNTL) DE was observed for 11,237 genes in the cecal tonsil (FDR p-value < 0.05). Of these, 7568 had |log2FC| > 1.0 and 4515 had |log2FC| > 2.0 (Table 1). The number of DE genes was considerably fewer for the AFB1-treated EW turkeys (703 with FDR p-value < 0.05 and 687 genes with |log2FC| > 2.0). In DT birds, the majority (65.4%) of DEGs were down regulated (Figure 3) although 48 of the 50 genes with the greatest fold change were up regulated (Table S4). In contrast, 98% of the DEGs in AFB1-treated EW birds were up regulated. Combined, 655 DEGs were shared in comparisons for both bird types, with 3860 being unique to DT birds and 32 unique to EW birds (Figure 3). Functional interpretation of many avian genes is based on sequence and syntenic similarity with human and other model organisms and therefore many functions are necessarily posited.

Shared Transcriptome Response

Among the 655 shared genes were the two phase I enzymes important in AFB1 metabolism (Table S3). The first, CYP1A5 (cytochrome P450, family 1, subfamily A, polypeptide 5) was highly up regulated in both EW and DT birds treated with AFB1 (log2FC = 7.66 and 9.67, respectively). Secondly, CYP3A37 (cytochrome P450 3A37) was significantly up regulated in only the DT birds (log2FC = 2.73). Studies from our laboratory have identified these as the principal turkey hepatic cytochromes responsible for efficient epoxidation of AFB1; CYP1A5 has highest affinity toward AFBO (low Km, high Vmax/Kcat) and bioactivates > 99% of AFB1 in turkey liver. In turkey, CYP3A37 (high Km, low Vm, Kcat) is only active at high environmentally-irrelevant substrate (i.e., AFB1).
concentrations [9]. Although potential biochemical activity of GSTAs in the intestine (cecal tonsil) of turkeys is unknown, expression of GSTA4 was significantly upregulated in both the EW and DT birds with AFB1 exposure (log2FC = 4.53 and 5.89, respectively).

Table 1. Summary of genes with significant differential expression (DE) in pair-wise comparisons of treatment groups.

| Comparison     | Groups                  | Expressed Genes | Shared Genes | Unique Genes (Each Group) | FDR p-Value < 0.05 | |log2FC| >1.0 | |log2FC| >2.0 | Up/Down Regulated |
|----------------|-------------------------|-----------------|--------------|-------------------------|--------------------|----------------|---------|--------|------------|----------------|
| AFB1 Line      | EW vs CNTL              | 18744           | 17833        | 402/509                 | 703                | 703            | 687      | 674/13 |            |                |
|                | DT vs CNTL              | 18654           | 16277        | 304/2073                | 11237              | 7568           | 4515     | 1563/2952 |            |                |
|                | CNTL vs EW              | 18736           | 17956        | 386/394                 | 679                | 348            | 67       | 37/30  |            |                |
|                | AFB vs DT               | 18447           | 16369        | 1866/212                | 1666               | 1666           | 1410     | 1308/102 |            |                |

For each comparison, the treatment groups, total number of expressed, shared and unique genes, genes with significant FDR p-value and the numbers of significant DE genes that also had |log 2 FC| > 2.0 are given. For the DE genes with |log 2 FC| >2.0 the number of genes up and down regulated are given. Genes were considered expressed in a treatment group if by-total normalized read count ≥ 3.0 in any individual within the group.

DE was also observed for several members of the claudin protein family. Claudins are integral components forming the backbone of the tight junctions of epithelial and endothelial cells [23]. In EW birds, CLDN1 (claudin 1) was upregulated by AFB1 (log2FC = 4.55), whereas CLDN18 was down regulated (log2FC = −6.57) (Table S3). In DT birds, CLDN1, CLDN2 and CLDN11 were up regulated (log2FC = 0.04, 4.01 and 2.17, respectively) and CLDN3, CLDN10, CLDN19 and CLDN23 were down regulated (log2FC = −2.52, −7.17, −4.11, −8.05, respectively). Expression of other key tight-junction proteins, tricellulin (MARVEL domain-containing protein 2, LOC104915344) and occludin (LOC104915505), were also significantly altered in DT but with smaller fold changes (Table S3). Uregulation of membrane tight-junction proteins such as claudins, is indicative of an epithelial response in the gut lumen to AFB1 and may suggest that AFB1 could alter gut permeability and perhaps stimulate a protective response in the gut to diminish mucosal inflammation/immune defense and repair processes.
Expression differences in CLDN1 observed in RNAseq read counts were further tested by qRT-PCR where expression of CLDN1 transcripts was significantly higher in EW birds compared to controls regardless of AFB1-treatment (Figure 4). Relative CLDN1 expression was also similarly variable in other wild-type birds (Rio Grande Wild, RGW) where expression was comparable to that of EW birds and significantly elevated with AFB1 treatment. Expression in other domestic birds (broad breasted white, BB) was more similar to that of the wild birds than the Nicholas DT suggesting that the lower CLDN1 expression observed in the Nicholas DT birds may have a genetic component.

Only two of the 655 shared DEGs (ATP12A and RSAD2) in the RNAseq data showed differences in the directionality of expression. ATP12A (ATPase, H+\textasciitildeK+ transporting, non-gastric, alpha polypeptide) was down regulated (log2FC = −2.83) in DT and up regulated (log2FC = 4.69) in EW birds. Similarly, RSAD2 (radical S-adenosyl methionine domain containing 2) was down regulated (log2FC = −3.47) in DT and up regulated (log2FC = 3.23) in EW. Two additional loci (SCD, stearoyl-CoA desaturase [delta-9-desaturase]) and a ncRNA (LOC104914677) had a similar directional expression pattern, with significant up regulation in EW with AFB1 treatment and down regulation in DT, however the log2FC in the DT birds was below 2.0. ATP12A is a member of the P-type cation transport ATPase family and in humans is involved in tissue-specific potassium absorption [24]. RSAD2 is an interferon inducible antiviral protein and has been shown in human cell lines to inhibit secretion of soluble proteins [25]. In mammals, SCD has a regulatory role in the expression of genes involved in lipogenesis and is important in mitochondrial fatty acid oxidation and energy homeostasis [26].

Nine of the 655 DEGs were significantly down regulated in both DT and EW with AFB1 treatment. These included GGT1 (gamma-glutamyltransferase 1), OTOR (otoraplin), PLIN1 (perilipin 1), RSPH14 (radial spoke head 14 homolog), SLC34A2 (solute carrier family 34, member 2), LOC100550279 (fatty acid-binding protein, adipocyte-like [FABP4-like]), LOC104909385 (erythroblastic NAD(P)(+)-arginine
ADP-ribosyltransferase pseudogene), LOC104913555 (gamma-glutamyltranspeptidase 1-like) and TNFRSF13C (tumor necrosis factor receptor superfamily, member 13C). Genes of particular interest in the GI tract include Perilipin 1 and fatty acid-binding protein (LOC100550279) that are involved in lipid transport and metabolism in human adipocytes [27]. SLC34A2 is a sodium-dependent phosphate transporter with an inverse pH dependence [28]. It is expressed in several mammalian tissues of epithelial origin including lung and small intestine and may be the main phosphate transporter in the brush border membrane. The B-cell activating factor TNFRSF13C is known to promote survival of mammalian B-cells in vitro and is a regulator of the peripheral B-cell population [29].

Functional gene classification of the 655 shared DEGs with DAVID identified 10 enriched gene clusters (Table S5). The cluster with the highest enrichment score included members of the serpin family of protease inhibitors (SERPINA10, SERPINC1, SERPIND1, SERPINF2 and SERPING1) that control many inflammation and coagulation processes. Other enriched clusters included complement components, mannan-binding lectin serine peptidase 1 and 2 (MASP1, MASP2), the (C4/C2 activating components) and coagulation factors F2, F7, F9 and F10. PANTHER overrepresentation tests found greatest fold enrichment for biological processes indicative of the dual absorption/immunity roles of the small intestine. Complement activation (GO:0001867) and regulation of intestinal absorption (GO:1904729, 1904478, 0030300) were significantly enriched as was cholesterol homeostasis (GO:0042632) as exemplified by up regulation of several genes (ABCG5, ABCG8, ANGPTL3, APOA1, APOA4, APOA5, CETP, EPHX2, G6PC, LIPC and LPL).

Unique Transcriptome Responses

Domesticated birds showed the greatest AFB1 gene response with 3860 unique DEGs (Figure 3). Genes showing the highest differential response (Table S4) were enriched for those encoding proteins with signal peptides and Serpins. DEGs with the greatest up regulation included INHBC (inhibin, beta C, log2FC = 13.63), claudin-19-like (LOC100544298, log2FC = 12.56), TTC36 (tetratricopeptide repeat domain 36, log2FC = 12.28) and three ncRNAs (LOC104913410, LOC104915491, LOC10491649, log2FC =12.74 to 13.15), SMIM24 (small integral membrane protein 24, log2FC = −12.48) and SLC10A2 (solute carrier family 10 [sodium/bile acid cotransporter], member 2, log2FC = −12.07). Expression of GSTA3 was significantly lower in DT birds treated with AFB1 compared to controls (log2FC = −2.33). Other αGSTs (GSTA1 and GSTA2) were significantly up regulated but with lower fold change (log2FC < 2.0, Table S3).

Over 650 of the 3860 DEGs were functionally clustered (DAVID enrichment score 24.96) as having membrane or transmembrane UniProt keywords. The majority of these (518, 77.9%) were down regulated as an effect of AFB1 treatment. Several alpha-1-antitrypsin-like loci were significantly up regulated consistent with a response to acute inflammation. Analysis of the 3860 unique genes in IPA found the most significant canonical pathways to be Axonal Guidance Signaling (−log(p-value) = 8.65), Hepatic Fibrosis / Hepatic Stellate Cell Activation (8.24), GPCR-Mediated Integration of Enterodendocrine Signaling Exemplified by an L Cell (7.33) and Calcium Signaling (7.28). DEGs in these pathways were almost exclusively down regulated in AFB1-treated birds. This effect is dramatically illustrated for the in the IPA canonical pathway “GPCR-Mediated Integration of Enterodendocrine Signaling Exemplified by an L Cell” (Figure 5) suggesting suppression in domesticated birds of enterodendocrine cells that produce and release gastrointestinal hormones such as glucagon-like peptides, peptide YY and oxyntomodulin that participate in nutrient sensing and appetite regulation and peptides to activate nervous responses [30].
Toxins were further tested in eight genes by qRT-PCR. These included ADCYAP1 and (glucagon-like peptide 2 receptor), GRPR. Unique genes were also DE in the liver transcriptomes obtained from the same individuals [17] but among these are genes involved in lipid synthesis and metabolism (exemplified by comparison (Figure 3). The majority (28, 87.5%) were up regulated in the AFB1-treated birds. Included in the pathway were genes such as NMB, CCKAR (cholecystokinin A receptor), GALR1 (galanin receptor 1), GLP2R (glucagon-like peptide 2 receptor), GRPR (gastrin-releasing peptide receptor), NMB (neuromedin B), NPT2R (neuropeptide Y receptor Y2) and VIPR1 (LOC100303683, vasoactive intestinal polypeptide receptor). With the exception of VIPR1, each of these genes showed lower expression in AFB1-treated DT birds as compared to treated EW birds. The VIPR1 receptor was selected as it is downstream of ADCYAP1 receptor. With the exception of NMB and VIPR1, expression of the selected genes in EW birds was greater than in DT (domestic Nicholas turkey) consistent with RNAseq results (Figure 4). Disparate results between qRT experiments and RNAseq may be attributed to the higher efficiency of qRT-PCR in sampling genes with low average expression such as NMB. In the case of ADCYAP1, CCKAR and GRPR expression was also greater in the untreated EW birds relative to untreated DT birds. As expected, little variation was observed in VIPR1. Relative expression of these genes was also tested in the other commercial-type (breast white, BB) and wild-type birds (Rio Grande subspecies, RGW). Comparable expression results were seen for ADCYAP1 and GRPR. Expression of 3 genes in the BB birds (CCKAR, GALR1 and NPY2R) was elevated as compared to the DT group with levels more similar to the EW and RGW groups (Figure 4).

Only 32 DEGs were found unique to the wild turkey in the AFB1 versus CNTL RNAseq comparison (Figure 3). The majority (28, 87.5%) were up regulated in the AFB1-treated birds. Included among these are genes involved in lipid synthesis and metabolism (exemplified by ACSBG2, ANGPTL4 and SCD) and immune response (IRG1 [immunoresponsive 1 homolog], PI3 [peptidase inhibitor 3]). A single annotation cluster (GO:0016021 integral component of membrane) was identified in DAVID that included 5 genes (CLDN18, FAXDC2, PTPRQ, SCD and SLC23A1). Interestingly, 29 of the 32 unique genes were also DE in the liver transcriptomes obtained from the same individuals [17] but showed opposite directional change in response to AFB1.

**Figure 5.** Differential expression of genes in the IPA canonical pathway “GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell.” Genes with significantly lower expression in domesticated turkeys relative to Eastern wild birds after AFB1 treatment are denoted in green. Genes tested by qRT-PCR are outlined in orange (Figure 4).
2.2. Wild versus Domesticated Turkey

2.2.1. Control Birds

Comparison of the transcriptomes of EW and DT birds in the control groups found 679 DEGs (FDR p-value < 0.05, \( \log_{2} FC = -7.882 \) to 6.715, Table 1 and Table S3), with 67 having \(|\log_{2} FC| > 2.0\) (Figure 6, Table S6). Of the 67 genes, 13 were shared in common in the EW versus DT AFB1 comparisons (Figure 6). The shared loci included 7 genes up regulated in EW birds; (CAMK4 [calcium/calmodulin-dependent protein kinase IV], LOC100548321 [Pendrin], NEFM [neurofilament, medium polypeptide], LOC104914065 [pendrin-like] LING02 [leucine rich repeat and Ig domain containing 2], LOC100538933 [probable ATP-dependent RNA helicase DDX60] and the uncharacterized LOC100549340 [ncRNA]). This differential expression may have implications for both epithelial function and inflammatory response. For example, as an anion exchange protein, Pendrin may function to regulate active chloride transport across epithelial membranes as a chloride-formate exchanger [31]. CAMK4 is implicated in transcriptional regulation in immune and inflammatory responses [32] and DDX60 is thought to positively regulate DDX58/RIG-I- and IFIH1/MDA5-dependent type I interferon and interferon inducible gene expression [33].

Down regulated genes among the 13 shared DE loci in the EW/DT comparison included LOC100540418 (BPI fold-containing family C protein-like [BPIFC]), LOC104915630 (3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase-like [HSD3B1]), LOC104917314 (14-3-3 protein gamma-B) and 3 uncharacterized ncRNA loci. Two of these genes have direct implication in gut homeostasis. BPIFC is a lipid transfer/lipopolysaccharide binding protein that may help provide defense against microorganisms [34]. In humans, HSD3B1 is an important gene in the biosynthesis of hormonal steroids as it catalyzes oxidative conversion of delta-5-3-beta-hydroxysteroid precursors. Altered expression of hormones in the gut may directly influence gene expression in the gut microbiota [35].

![Wild vs Domesticated](image)

**Figure 6.** Distribution of differentially expressed genes between turkey types (wild and domesticated). For each comparison, the number of significant genes (FDR p-value < 0.05 and \(|\log_{2} FC| > 2.0\) shared or unique to each treatment group are indicated. Circle size is proportional to the number of genes and direction of expression change (↑ or ↓) is given for each group.

Of the 54 DEGs unique to the control group birds slightly more (55%) were up regulated in the EW birds compared to the DT birds (Table S6). These 54 unique DEGs included integral membrane proteins (e.g., AQP10), cytoplasmic enzymes (NME8), nuclear transcriptional regulators (HOXB5) and secretory proteins (GKN2) that are typical of intestinal epithelium but without significant enrichment for any particular biological process. Greatest differential expression was seen for claudin 18 (CLDN18), a membrane protein that is a component of tight junction strands with higher expression in EW.
(log2FC = 6.72) than DT. Also represented were genes with immune system roles such as DNTT (DNA nucleotidylexotransferase), which functions in generating antigen receptor diversity and NOS1 (nitric oxide synthase 1), a host defense effector with antimicrobial activity.

2.2.2. AFB1 Treatment

The greatest number gene expression differences observed between the EW and DT birds occurred in the AFB1-treatment groups. A total of 1666 DEGs (FDR p-value < 0.05) were observed with 1410 having |log2FC| > 2.0 (Table 1). As discussed above, 13 DEGs were shared with the control comparison and 1397 were unique (Figure 6, Table S7). Interestingly, 93% of the DEGs showed higher expression in the EW birds compared to DT. Non-coding RNAs comprised 29.4% of the down regulated genes (n = 30) and 5% of the up regulated DEGs (n = 66). Greatest differential expression (up regulation) in EW compared to DT was seen for LOC104912821 (ovostatin homolog, log2FC = 11.84), LOC104915655 (alpha-2-macroglobulin, A2M, log2FC = 11.4) and genes such as SLC10A2 (solute carrier family 10, log2FC = 11.06) and FABP6 (fatty acid binding protein 6, log2FC = 10.26). Ovostatin and A2M both have endopeptidase inhibitor activity, whereas SLC10A2 and FABP6 function in bile acid metabolism. Greatest down regulation in EW compared to DT was seen for GYG2 (Glycogenin 2, log2FC = −7.19) and LOC104916581 (7-dehydrocholesterol reductase-like, log2FC = −5.56). In humans, GYG2 is expressed mainly in the liver and heart and is involved in initiating reactions of glycogen biosynthesis; 7-dehydrocholesterol reductase is ubiquitously expressed and helps catalyze the production of cholesterol [36,37].

Functional analysis of the 1397 unique DEGs in DAVID found highest enrichment score (14.11) for the annotation cluster “Membrane” (p = 4.1 × 10^-16), which included 284 genes (Table S7). The second annotation cluster (enrichment = 5.39) contained 50 genes with immunoglobulin-like domains or Ig-like fold (homologous superfamily IPR013783, p = 5.7 × 10^-7). Included were several complement proteins, interleukins and Ig superfamily members (Table S7). Additional clusters identified in DAVID included “extracellular exosome” (136 DEGs, p = 6.5 × 10^-3) and “signal” (118 DEGs, p = 2.3 × 10^-8). Calcium signaling was the most expressively represented Kegg pathway containing 29 DEGs (p = 1.8 × 10^-6, Figure S2), followed by “Focal adhesion” (28 DEGs, p = 6.1 × 10^-4) and “Neuroactive ligand-receptor interaction” (28 DEGs, p = 7.4 × 10^-2).

Among the 1397 unique DEGs were two olfactory receptor genes, LOC100546335 (OR51E2-like) and LOC1005546179 (OR51G2-like). Both of these loci were up regulated in the EW birds compared to DT with AFB1-treatment (log2FC = 8.15 and 8.46, respectively). Expression of functional taste and olfactory receptors has been observed in human enteroeendocrine cells [38,39] and a survey of RNAseq data from multiple human tissues identified expressed olfactory receptors with broad and tissue-exclusive expression [40]. An interesting aspect of LOC100546335 and LOC1005546179 is that based on read count, expression of both loci was roughly similar. These loci are adjacent in the turkey genome and are annotated as sharing two non-coding 5’ exons (Figure 7). A total of seven transcript variants for the two genes were predicted by NCBI’s automated computational analysis gene prediction method (Gnomon). Examination of RNAseq reads from 3 individuals in the present study (EW1, EW9 and NC11) found split RNAseq reads (intron spanning) that support each of the predicted variants with the exception of the variant 51E2- -X4. However, RNAseq reads did map to the non-coding upstream (5’) exon of variant 4 (Figure 7). Interestingly, split reads were also identified in each individual that indicated splicing events between the two small 5’ exons, not predicted in the NCBI models.
AFBO epoxide has been implicated in the rat intestine [42] and even nasal mucosal cells [43]. Studies of cultured human intestinal epithelial cells (Caco-2) found AFB1 decreases trans-epithelial electrical resistance (TEER) [44]. Similarly, Romero et al. [45] reported that AFB1 treatment caused a reduction in TEER and mitochondrial viability and increased cell permeability. By contrast, the detoxified AFB1 metabolite AFM1 did not permanently compromise the integrity of Caco-2 cells grown on microporous filter supports [46]. In poultry, AFB1 is efficiently absorbed in the upper GI tract and thus exposure of the intestinal mucosa is greater than in other organs. While we have not quantified AFB1 bioactivation in the turkey gut, expression of the primary hepatic AFB1-activating CYP1A5 was highly upregulated by AFB1 in the turkey cecum. Increased CYP1A5 expression in AFB1-treated turkeys was also observed in the liver [17] and is a common observation in animals, as this and other CYPs are known to be induced by AFB1 and other foodborne and environmental toxicants [47]. Similarly, expression of GSTAs (particularly GSTA4), were up regulated by AFB1. In contrast, a prior study found expression of GSTAs in the liver were oppositely affected; GSTA1, GSTA2 and GSTA4 were down regulated after 2 weeks exposure to AFB1 and expression of GSTA3 was significantly lower in EW birds compared to DT after AFB1 treatment [17].

The gastrointestinal epithelium provides an important physical barrier to foreign antigens and pathogens and disruptions thereof are increasingly associated with diseases [48]. Although few studies have specifically investigated the ability of aflatoxin to compromise intestinal permeability [19,49], the potential for mycotoxins to cause dysfunction of the intestinal barrier has come under increased study. Mycotoxins modulate the composition of gut microbiota, often eliminating beneficial bacteria, which leads to increased colonization by gut pathobionts and pathogens [50,51]. Exposure to AFB1 has been shown to induce changes in gut microbiota in rodents [52,53] and to modify barrier function in intestinal epithelial cells [49]. Probiotic gram-positive strains of Lactobacillus, Propionibacterium and Bifidobacterium have been proposed as feed additives to attenuate AFB1-induced toxicity in poultry due to their ability to bind AFB1, thereby reducing its bioavailability [54–57]. Gene expression in AFB1-treated birds is modulated by probiotics but the negative effects of AFB1 are not fully mitigated [15,16]. It is possible that in addition to binding AFB1, these probiotics exert positive effects by acting to decrease gut permeability and other protective functions [58].

**Figure 7.** Alignment of NCBI predicted sequence variants to the predicted genes for two olfactory receptor loci.

### 3. Discussion

Naturally-occurring dietary toxins such as AFB1 pose significant public health risk throughout the world but especially in locales characterized by high contamination levels of dietary staples such as corn. One of most significant is AFB1 which primarily targets the liver, the organ with the highest concentration of bioactivating CYPs. Extra-hepatic metabolism and bioactivation of this mycotoxin is a much-studied topic [41] but comparatively few studies have focused on the gastrointestinal tract, even though dietary exposure is the principal route for people and animals. Conversion of AFB1 to the AFBO epoxide has been implicated in the rat intestine [42] and even nasal mucosal cells [43]. Studies of cultured human intestinal epithelial cells (Caco-2) found AFB1 decreases trans-epithelial electrical resistance (TEER) [44]. Similarly, Romero et al. [45] reported that AFB1 treatment caused a reduction in TEER and mitochondrial viability and increased cell permeability. By contrast, the detoxified AFB1 metabolite AFM1 did not permanently compromise the integrity of Caco-2 cells grown on microporous filter supports [46]. In poultry, AFB1 is efficiently absorbed in the upper GI tract and thus exposure of the intestinal mucosa is greater than in other organs. While we have not quantified AFB1 bioactivation in the turkey gut, expression of the primary hepatic AFB1-activating CYP1A5 was highly upregulated by AFB1 in the turkey cecum. Increased CYP1A5 expression in AFB1-treated turkeys was also observed in the liver [17] and is a common observation in animals, as this and other CYPs are known to be induced by AFB1 and other foodborne and environmental toxicants [47]. Similarly, expression of GSTAs (particularly GSTA4), were up regulated by AFB1. In contrast, a prior study found expression of GSTAs in the liver were oppositely affected; GSTA1, GSTA2 and GSTA4 were down regulated after 2 weeks exposure to AFB1 and expression of GSTA3 was significantly lower in EW birds compared to DT after AFB1 treatment [17].

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Of interest in the present study is the potential of AFB1 to disrupt tight junction proteins allowing for increased translocation of substances from the lumen to the blood and lymphatic circulation [49]. Transmembrane tight junctions consist of claudins, occludin, tricellulin and a group of junction adhesion molecules that form the horizontal barrier at the apical lateral membrane [59]. Claudins are a family of transmembrane proteins that are essential components in the apical junctional complex of epithelia and endothelia cells [60], the expression of which in humans, is modulated by aflatoxins [45, 61]. Romero et al. [45] found dose-dependent down regulation in CLDN3 and occludin in human Caco-2 cells treated with AFB1 consistent with an observed decrease in gut barrier properties. Gao et al. [61] found decreased expression of TJ proteins (CLDN3, CLDN4, occludin and zonula occludens-1) and disrupted structures following exposure to aflatoxin M1 (4-hydroxylated metabolite of AFB1).

Dietary AFB1 treatment in the present study elicited transcriptional changes in several claudin transcripts including up regulation of CLDN1 in both EW and DT, down regulation of CLDN3 in DT, down regulation of CLDN18 in EW and up regulation of CLDN10 and CLDN23 in EW birds. Transcriptional modifications of claudins may indicate a response to restore impaired TJ proteins and potentially compromised gut permeability. In vivo studies in poultry have produced inconsistent results. In broilers, AFB1 increased gut permeability as measured by the serum lactose/rhamnose ratio (dual sugar test), as well as increases in expression of CLDN1, multiple jejunal amino acid transporters and the translation initiation factor 4E [21]. A second study [20] found no evidence for increased gut permeability in broilers as measured by GI leakage of FITC-d following exposure to varying concentrations of AFB1. Annotation of avian claudin genes is based on similarities to mammalian orthologs and in many cases function has not been experimentally demonstrated. Results of the present study indicate that additional studies of the effect of AFB1 on gut permeability in turkey are needed.

Exposure to AFB1 has widespread adverse physiologic effects. In poultry, AFB1 adversely affects production characteristics causing poor performance, decreased growth rate, body weight, weight gain, egg production, reproductive performance and feed efficiency [62]. Humoral and cell-mediated immune functions in poultry are also impaired by AFB1 in keeping with its well-known immunotoxicity [3,5,6,16,41,63–65]. Altered humoral response to fowl cholera and Newcastle Disease (ND) virus has been described in chickens where correlation was observed between outbreaks of ND and AFB1-contaminated feeds (reviewed in Reference [65]). Effects on cell-mediated immunity are evident as decreased phagocytic activity in leukocytes [66–69]. Exposure to AFB1 in turkeys causes suppression of humoral and cellular immunity resulting in compromised immune response in hatchlings making them more susceptible to disease [6]. In this respect, AFB1 is a “force-multiplier” synergizing the adverse effects of other agents and pathogens detrimental to poultry health.

Compromised epithelial barrier is associated with increased paracellular permeability that may lead to overstimulation of the gut immune system and a non-specific systemic inflammatory response [48,70]. The cecal tonsil is the major lymphoid tissue in the avian cecum that provides important and unique immune functions. Detailed studies in poultry have demonstrated impairment of the normal function of the cecal tonsil caused by AFB1 through depletion of lymphocytes and lesions in the absorptive cells [71]. AFB1 significantly decreases intestinal IgA(+) cells and the expression of immunoglobulins in the intestinal mucosa [72]. Dietary AFB1 exposure decreases cell-mediated immunity while inducing the inflammatory response. Immune activation and inflammation result in mucosal recruitment of activated cells, modulated by cytokines. Cytokine-mediated dysfunction of tight junctions is important in gastrointestinal disease [48] as cytokines and other growth factors may act to alternatively decrease (e.g., IL-10) or increase (e.g., IL-6) gut permeability [58]. In the commercial DT birds, numerous pro-inflammatory cytokines, TGF-β and EGF were significantly down regulated by AFB1 treatment. In contrast, the interleukin 6 (IL6R) and interleukin 13, alpha 2 (IL13RA2) receptors and the interleukin 1 receptor accessory protein (IL1RAP) were significantly up regulated in both EW and DT birds. In humans, IL13RA2 functions to internalize the immunoregulatory cytokine IL-13. Dysregulation of IL6 impacts CLDN2 expression (significantly up regulated by AFB1 in DT in this study) and can undermine the integrity of the intestinal barrier [73].
In response to the luminal environment, chemical receptors of intestinal epithelial and neuroendocrine cells modulate the function of these cells and ultimately systematic metabolism and homeostasis [38,74]. For example, ingestion of food results in signaling to the brain to regulate food intake and detection of bacterial metabolites may induce host defense responses. Part of this gut-brain axis is performed by enteroendocrine L-cells with specific nutrient-sensing receptors [30]. These include intestinal olfactory receptors that recognize ingested odor compounds and alter glucose homeostasis through induced secretion of gut-peptides [75]. In pigs, the olfactory receptor OR51E1 has been localized to enteroendocrine cells along the GI tract. Expression of the gene encoding this receptor was significantly altered following modulation of the intestinal microbiota, presumably in response to microbial metabolites [76]. Differential expression of OR genes in the turkey GIT may be caused by a direct action of AFB₁ on the intestinal epithelial cells or secondarily through changes in the intestinal microbiota induced by AFB₁.

Intensive breeding and genetic selection to produce the modern domesticated turkey has dramatically affected performance metrics. For example, growth rate to market age has essentially doubled in the past 40 years and feed efficiency of contemporary tom turkeys is approximately 50% better when compared to non-growth selected birds fed modern diets [77]. Under normal conditions, commercial birds typically reach 19 lbs. by 20 weeks of age, with a feed conversion ratio of approximately 2.5 [78]. Our results suggest that selection for production traits, such as increased nutrient conversion, may have contributed to the extreme sensitivity of DT to AFB₁. In the same way, the relative resistance of WT, in addition to expression of AFB₁-detoxifying GSTAs, may also involve extra-hepatic mechanisms such as a more refractory gastrointestinal tract, in addition to the presence of functional hepatic GST-mediated AFB₁ detoxifying capability [12,13]. Possibly related to this, studies of production performance in chickens suggest that sensitivity to AFB₁ has increased since the 1980s, concomitant with industry selection for increased nutrient conversion and demands for greater metabolism (reviewed in Yunus et al. [65]). Elucidation of extra-hepatic routes of pathogenesis provides a clearer picture of the complexity of species resistance and susceptibility to this potent mycotoxin that may also suggest analogous mechanisms in humans.

4. Materials and Methods

This study used turkeys previously found to vary in AFB₁-detoxifying GST activity. Animal husbandry and the AFB₁ protocol were as described in Reed et al. [17]. Birds included AFB₁-treated and control animals from the Eastern Wild (EW, Meleagris gallopavo silvestris) subspecies and domesticated Nicholas turkeys (DT). Male turkey poults were subjected to a short-term AFB₁-treatment protocol in which the diet of challenge birds was supplemented beginning on day 15 of age with 320 ppb AFB₁ and continued for 14 days. Previous studies with higher AFB₁ dosing (1 ppm) caused an unacceptable mortality rate. Birds serving as experimental controls received a standard AFB₁-free diet. At the end of the trial, birds were euthanized and a section of the cecum corresponding to the cecal tonsil was removed and placed in RNAlater (ThermoFisher Scientific, Waltham, MA, USA) for RNA isolation and RNAseq analysis. All procedures were approved by Utah State University’s Institutional Animal Use and Care Committee (Approval #2670, date of approve: 26 September 2016).

4.1. RNA Isolation and Sequencing

Total RNA was isolated from cecal tonsils by TRIzol extraction (ThermoFisher), treated with DNase (Turbo DNA-freeTM Kit, ThermoFisher) and stored at −80°C. Library preparation and sequencing was performed at the University of Minnesota Genomics Center. Briefly, concentration and quality of RNA was assessed on a 2100 Bioanalyzer (Agilent Technologies) and RNA Integrity Numbers (RIN) averaged 6.7. Replicate samples (n = 4) from each treatment group were examined. Indexed libraries (n = 16) were constructed, multiplexed, pooled and sequenced (101-bp paired-end reads) on the HiSeq 2000 using v3 chemistry (Illumina, Inc., San Diego, CA, USA). Sequence reads
were groomed, assessed for quality and mapped to turkey genome (UMD 5.0, NCBI Annotation 101) as described in Reed et al. [17].

4.2. Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed on both domesticated and wild turkeys. Samples included the Eastern Wild (EW; M. g. silvestris) and domesticated Nicholas turkey (DT) birds, plus domesticated Broad Breasted White (BB) and birds of the Rio Grande subspecies of wild turkey (RGW; M. g. intermedia) from a parallel AFB1-challenge experiment. Of the 6 samples from the DT and EW groups used for qRT-PCR, four were in common with the RNAseq study. Synthesis of cDNA was performed on DNase-treated mRNA using Invitrogen Super Script IV First-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). The iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA, SA) was used for quantitative analysis of gene-specific amplicons with the CFX96 touch real time detection system (BioRad, Hercules, CA, USA). Primers were designed using the turkey genome sequence (UMD5.0) and Primer3 software [79]. Primer sets were designed so the amplicon spanned an exon/exon junction and at least one intron. Several normalizing genes were tested for uniformity and the most stable reference gene (hypoxanthine guanine phosphoribosyl transferase, HPRT) was determined with Reffinder [80]. Target gene reactions were conducted in triplicate and HPRT normalization reactions, no template and gDNA controls were run in duplicate. Disassociation curves were used to confirm single product amplification and to preclude the possibility of dimer amplification.

4.3. Statistical Analysis

For expression analysis of RNAseq data, read counts were by-total normalized and expressed as reads per 11.9M (CLC Genomics Workbench v. 8.0.2, CLC Bio, Aarhus, Denmark). Principal component analysis (PCA) and hierarchical clustering of samples based on Euclidean distance was performed (with single linkage) in CLCGWB using by-total normalization. Empirical analysis of differential gene expression (EdgeR) and ANOVA were performed in CLCGWB on mapped read counts with TMM (Trimmed Mean of M-values) normalization (Bonferroni and FDR corrected). Pair-wise comparisons between treatment groups were made following the standard workflow Wald test. Significant differentially expressed (DE) genes were used to investigate affected gene pathways with Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA). Gene Ontology (GO) and functional classification was performed in DAVID (v6.8, [81]) and overrepresentation tests for gene enrichment were performed with PANTHER (GO Consortium release 20150430) [82]. For analysis of qRT-PCR data, expression was normalized first to HPRT, then interpreted using the Double Delta Ct Analysis (ΔΔCt, [83]) and a comparative Ct approach. Expression analysis was performed using the standard ΔΔCt workflow within the CFX Maestro software package (Biorad, Hercules, CA, USA).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/11/1/55/s1.
Figure S1: Hierarchical clustering of samples based on Euclidean distance reiterated relationships shown by PCA. Figure S2: Kegg calcium-signaling pathway. Table S1: Summary of RNAseq data for turkey cecal tonsil transcriptomes. Table S2: Mean quality-trimmed RNAseq read counts for turkey cecal tonsil from two turkey types (Wild and Domesticated). Table S3: Summary of pairwise differential gene expression analysis of cecal tonsil transcriptomes. Table S4: Fifty genes showing the greatest differential expression in each pairwise comparison of treatment groups. Table S5: Functional annotation gene clusters identified in DAVID among the 655 DEGs shared between EW and DT birds in AFB1 versus CNTL comparisons. Table S6: Significant differentially expressed genes (FDR p-values < 0.05 and |log2FC | > 2.0) identified in comparison of Eastern Wild versus domesticated turkeys in the CNTL groups. Table S7: Genes showing differential expression that were unique in the comparison of AFB1-treated Eastern wild turkeys versus domesticated turkeys.

Author Contributions: K.M.R. and R.A.C. wrote and edited the manuscript; R.A.C. designed and performed the AFB1-treatment experiments; K.M.R. and K.M.M. collected and analyzed data; K.M.R. and K.M.M. performed data analysis and interpretation.

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Abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| AFB          | aflatoxin B<sub>1</sub>                          |
| AFBO         | exo-AFB1-8,9-epoxide                             |
| BB           | Broad Breasted White                             |
| Ct           | threshold cycle                                  |
| CYP          | cytochrome P450                                   |
| DE           | differentially expressed                         |
| DEG          | differentially expressed gene                    |
| DT           | domesticated turkey                              |
| EW           | Eastern wild turkey (Meleagris gallopavo silvestris) |
| FC           | fold change                                      |
| FDR          | false discovery rate                             |
| GO           | gene ontology                                    |
| GST          | glutathione S-transferase                        |
| IPA          | Ingenuity Pathway Analysis                       |
| ncRNA        | non-coding RNA                                   |
| PCA          | principal component analysis                     |
| qRT-PCR      | quantitative real-time polymerase chain reaction  |
| RGW          | Rio Grande wild turkey (Meleagris gallopavo intermedia) |

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