

**Whole transcriptome response of chicken spleen and peripheral blood leukocytes to  
avian pathogenic *Escherichia coli***

by

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## ABSTRACT

A greater understanding of the immune response after infection can form the necessary foundation of knowledge needed to enhance immunity through genetic selection. Whole genome microarrays allow for comprehensive analysis of the transcriptome. The transcriptomic responses of spleen and peripheral blood leukocytes (PBL) each displayed differential expression in broiler chickens infected with avian pathogenic *Escherichia coli* (APEC), the causative agent for colibacillosis. This differential expression was associated with treatment factors of infection status, pathology level and day post-infection. Within the spleen, the largest number of significantly differentially expressed genes was between chickens with a severe pathology and those uninfected: 1,101 genes at 1 day post-infection and 1,723 genes at 5 days post-infection. Significant differences in splenic expression between mild and severe pathology was only noted at 5 days post-infection, for 799 genes. Within PBL, the largest number of significantly differentially expressed genes was between mild and severe pathology on day 5, for 1,914 genes. Significant differences in expression were also noted between severe pathology and uninfected chickens, for 1,097 genes at 1 day post-infection and for 506 genes at 5 days post-infection. In both tissues, a severe pathological state resulted in more induction of gene expression response than repression. Several immune-related gene families, including the Toll-like receptors, cytokines and beta-defensins, were differentially expressed in both tissues. Combining results from the two tissues revealed potential pathway regulation between tissues over time. MAPK pathway signaling in PBL at 1 day post-infection, could be causative for the downstream cytokine and p53 pathway signaling observed in the spleen at 5 days post-infection. Vaccination against an APEC virulence factor generated no discernible difference in gene expression in either

tissue, with or without other factors of day, challenge, or pathology, although it was efficacious in reducing pathology, indicating further research is necessary to identify the impact of APEC vaccination on the transcriptome. Combining this knowledge with genotypic markers could help to reveal the genomic locations responsible for conveying APEC resistance, allowing breeders to use this information to reduce the incidence of APEC infection in poultry.

## CHAPTER 1. GENERAL INTRODUCTION

### Introduction

For poultry producers, avian pathogenic *Escherichia coli* (APEC) infection results in losses due to reduced productivity, discarded product, animal death and increased costs to prevent and treat disease, and to exterminate pathogens within their flocks (Barnes et al., 2008). Producers utilize a variety of mechanisms to combat disease within their flocks, such as antimicrobials, vaccination and the purchase of more robust birds from breeding companies, developed through genetic selection. Global concern over antibiotic use in food animals has led to strict limitations being placed on their use and a greater need for research into alternative methods of disease control. Vaccination against APEC can be problematic since, because of differences between disease causing serogroups, many vaccines do not protect well against a heterologous challenge. Therefore, another method for disease control may be required: immune enhancement through genetic selection.

Genetic improvement requires greater knowledge about how a host is able to effectively combat infection and the genetic architecture behind it, as testing disease resistance often removes animals from the breeding population. The chicken is a pioneer in animal agriculture as a historically important model in genetics, developmental biology, and immunology, and the first agricultural animal to have its genome fully sequenced in 2004 (Wallis et al., 2004). The genome sequence has since been improved, with further builds and continuing efforts to map previously unknown regions. This sequence information is invaluable in locating single nucleotide polymorphisms (SNP) for use in marker assisted

selection, quantitative trait loci (QTL) mapping and general annotation of genes and their function (as reviewed by Dodgson et al., 2011).

Gene expression of host tissues to infection is commonly utilized to assess and enhance understanding of response to infection. Microarray technology allows for simultaneous evaluation of thousands of genes. Information from multiple tissues and thousands of genes facilitates the deduction of critical pathways, both within and between tissues, which are important in immune response. The goal of the research summarized in this dissertation was to gain greater understanding of chicken host response to APEC. Transcriptomic response of spleen and peripheral blood leukocytes was assessed by using microarrays to identify host response mechanisms and elucidate the means through which a chicken is able to mount a successful defense against APEC.

## **Organization of Dissertation**

This dissertation is arranged in the alternative journal paper format that consists of five chapters containing three manuscripts for publication. Chapter 1 is a general introduction and a review of relevant literature on the dissertation topic. Chapter 2 is a manuscript published in *BMC Genomics*. Chapter 3 is a manuscript prepared for *Developmental and Comparative Immunology*. Chapter 4 is a manuscript prepared for *Avian Diseases*. Chapter 5 is the discussion of the combined results of the dissertation, general conclusions and future directions of this research. Figures and tables related to each manuscript appear at the end of each chapter. Reference sections are at the end of each chapter. References for manuscripts have been re-formatted from specific journal guidelines into a single format for consistency.

## Literature Review

### Avian pathogenic *Escherichia coli* causes colibacillosis in chickens

*Escherichia coli* is a Gram-negative bacterium that is ubiquitously found in the environment and the gastrointestinal tract of animals. The majority of *E. coli* encountered are harmless and are commonly part of healthy gut microflora, but others can result in disease. In chickens, colibacillosis is characterized as an infection caused by an extra-intestinal infection with avian pathogenic *E. coli* or APEC (Barnes et al., 2008). It is a concern at all ages of poultry production, spreads through vertical and horizontal transmission, and results in a variety of manifestations of disease, with septicemia the most prevalent (as reviewed by Lutful Kabir, 2010). Chickens are typically exposed to APEC orally into the gastrointestinal tract or through the respiratory tract (Matthijs et al., 2003), where it can quickly gain access to the bloodstream and colonize in other tissues (as reviewed by Dziva and Stevens, 2008). This allows APEC access to the entire body, where it can colonize in a specific tissue, resulting in local infection, or remain in the bloodstream and colonize multiple regions for a systemic infection. Respiratory exposure results in airsacculitis, pericarditis and perihepatitis, with macro- and microscopic lesions identifiable within the respiratory system (Dwars et al., 2009, Peighambari et al., 2000). Yolk sac infection of incubating and young broilers is a major cause of early mortality associated with APEC infection (Cortés et al., 2004). In laying hens, APEC-induced septicemia, pericarditis and perihepatitis are still common, with ruptured follicles causing egg yolk peritonitis (Dhillon and Jack, 1996). Poultry production is a multi-billion dollar industry annually in the U.S. Colibacillosis results in large monetary losses through decreased production, death and carcass condemnation (Barnes et al., 2008), along with associated costs of disease treatment

and prevention. Body weight and feed efficiency both decrease in broilers infected with APEC (Huff et al., 2006). Several APEC isolates show resistance to antimicrobials, forcing the discovery of new control mechanisms (Gyles, 2008). Research suggests transfer of antibiotic resistance or virulence plasmids between APEC and *Salmonella* is possible (Fricke et al., 2009), showing the need to control both so as to not enhance the virulence of the other. Bacterial infections are especially problematic for organic farms, where antimicrobials are strictly regulated. *E. coli* was found to be the most common cause of mortality in an investigation of 15 organic Danish poultry flocks (Stokholm et al., 2010). Although not as widely studied as other bacterial diseases, such as *Salmonella*, for the aforementioned reasons, APEC has become recognized as a major concern within the poultry industry.

A zoonotic bacterium is any bacterium which is able to be transmitted between animals and humans, resulting in disease. Infection in food-production animals allows for potential foodborne transmission of zoonotic bacteria. Fully cooked egg products and meat cooked to an internal temperature of 165°F, as recommended by the USDA, helps to protect consumers against bacterial exposure. Poor kitchen practices can allow for recontamination of cooked products or contamination of uncooked ingredients (de Jong et al., 2008). Standard sanitation methods at processing plants are not enough to completely eliminate the risk of recontamination after cooking (Keeratipibul et al., 2009). During slaughter, carcasses can be contaminated through contact with gut content or viscera and eggs through vertical transmission or fecal contamination of the shell (as reviewed by Lutful Kabir, 2010). Among chicken carcasses at slaughter, over 99% contain some form of *E. coli* before evisceration and 38.7% still test positive after chilling (USDA FSIS, 2008). Extra-intestinal pathogenic *E. coli* (ExPEC) can be isolated from retail meats, most commonly from poultry meat, that

show phylogenetic similarity to human ExPEC and resistance to multiple antimicrobials (Xia et al., 2011). In addition to direct contact with poultry products, contaminated manure can spread bacteria into soil and water sources, providing routes of contamination for produce as well (as reviewed by Santamaría and Toranzos, 2003). Therefore, prevention of infection and colonization by APEC in the chicken is key to reducing a potential food borne risk for humans. APEC is a concern for human health as it shows many similarities to human ExPEC and improper handling of poultry products provides a route of exposure. APEC genomic sequence displays strong sequence similarities to currently available human ExPEC sequences (Johnson et al., 2007). Transcriptional similarities exist between APEC and ExPEC, with little evidence of host specificity discovered (Bauchart et al., 2010). Many virulence genes are common to both APEC and uropathogenic *E. coli* isolates in type and rate of occurrence (Zhao et al., 2009). Evidence of zoonotic potential for APEC has been established through its ability to cause disease in a rat model for human meningitis (Tivendale et al., 2010). APEC has been shown to invade and survive in human cells (Chanteloup et al., 2011). Transcriptional and virulence factor similarities between APEC and human ExPECs suggest the potential of APEC to act as a zoonotic source of human ExPEC or as a supply for ExPEC virulence genes.

The vast majority of chicken bacterial infection research to date has focused on *Salmonella*. APEC, however, is gaining ground as it is now acknowledged as an important poultry and human health issue. APEC is considered both a primary and secondary pathogen, with greater ability to cause disease in chickens already infected with other pathogens (as reviewed by Lutful Kabir, 2010). Although APEC on its own is able to cause disease, prior infection with infectious bronchitis virus (IBV) can further impact host

response, reducing ability to combat new infection with APEC (Matthijs et al., 2009; Peighambari et al., 2000). Bacteria, like APEC, are difficult to defend against because of the variety of strains and acquired resistances. Such virulent bacteria can acquire the same resistance to host defenses as commensal bacteria, because plasmid transfer from bacteria located in the gut to those located in the respiratory tract has been documented (Dheilly et al., 2011). APEC O1:K1:H7 is sequenced, showing virulence genes associated with a common disease causing serotype (Johnson et al., 2007). Knowledge of these virulence genes is important to understand how APEC is able to avoid conventional host immune responses, and what can be done to better prepare the host to resist infection. Sequence data have allowed for successful identification of genes necessary for APEC O1 survival and growth in chicken serum (Li, G. et al., 2011), providing potential mechanisms to target to inhibit invasion.

APEC typically enters the host through ingestion or inhalation, after which it is able to translocate across mucosal layers and invade the bloodstream (as reviewed by Dziva and Stevens, 2008). The internal environment of the chicken is typically a hazardous environment for foreign microorganisms. APEC utilizes a variety of genes that allow it to adapt to stressful environments (Li, G. et al., 2011). APEC virulence factors can also be located on plasmids which can transfer to other bacteria including non-pathogenic strains (Mellata et al., 2010). APEC O1 is host to a variety of virulence factors that aid in survival, adhesion and invasion into host cells (Johnson et al., 2007). APEC is able to resist complement (Nolan et al., 2002) and phagocytosis by macrophages (Bastiani et al., 2005). APEC requires the correct balance of iron to function. Genes involved in iron acquisition and regulation are important to APEC survival and virulence (Li, G. et al., 2011). Adhesion



genes, such as *fimC*, are more common among APEC than non-pathogenic *E. coli* (McPeake et al., 2005). Although Shiga toxin genes have been discovered among APEC, they are not expected to be primary contributors to virulence (Parreira and Gyles, 2002). Instead, other types of toxins, such as colicin (Dias da Silveira et al., 2002), and induction of apoptosis (Bastiani et al., 2005) contribute to virulence.

Chickens have been utilized to better understand the human immune system. The B-cell is named after its discovery in the chicken's bursa of Fabricius. The similarities between APEC and human ExPEC, along with zoonotic potential, make the chicken an excellent candidate as a model organism for the study of human ExPEC. The chicken has been a successful model organism for the study of *Staphylococcus aureus* induced arthritis (Zhou et al., 2007), vitiligo (Smyth et al., 1981), and ovarian cancer (Seo et al., 2011). Uropathogenic *E. coli* (UPEC) has also shown marked similarities to APEC and is able to cause disease in the chicken (Zhao et al., 2009). Because of similarities in the infectious agent, colibacillosis in chickens may serve as a model for human infection with ExPEC.

### **Chicken host immune response to APEC infection**

Chickens utilize a variety of mechanisms to protect against and combat pathogenic infection. The chicken lacks traditional lymph nodes and must therefore rely on other methods of antigen presentation, such as gut- and bronchus-associated lymphoid tissues and an organ unique to Aves, the bursa of Fabricius. The bursa of Fabricius is the primary site of B-cell development and proliferation of successfully rearranged IgM positive B-cells after day 15 of embryogenesis (Narabara et al., 2009). Removal of the bursa from young results in failure to produce antibodies, but it plays a reduced role later in life because it begins to involute as the bird matures (Taylor and McCorkle, 2009). Bursal weight, often measured as

an indicator of immune system health, and relative size can increase with immunomodulator supplementation (Bakayaraj et al., 2011). Because APEC is typically inhaled or ingested, it bypasses the skin as a protective element. Instead, the first lines of defense are the mucosal layers of the gastrointestinal and respiratory tracts. Both the gastrointestinal tract and respiratory epithelium utilize IgA, a mucosal antibody. Along with invading pathogenic bacteria, commensal bacteria reside in the gastrointestinal tract. Commensal bacteria are beneficial to the host by aiding in digestion and have immune roles. They are able to competitively exclude the colonization of harmful bacteria and suppress gut inflammatory responses, to protect themselves and the host from excessive inflammation (as reviewed by Brisbin et al., 2008). Gut-associated lymphoid tissues (GALT) within the gastrointestinal tract help to store lymphocytes and produce immunoglobulins (Befus et al., 1980). Commensal and probiotic bacteria can interact with GALT cells to increase natural antibodies (Haghighi et al., 2006). The respiratory system is a primary site of APEC infection as the main route of entry. The chicken respiratory system does not contain resident immune cells and therefore relies more heavily on recruited heterophils and macrophages (as reviewed by Dziva and Stevens, 2008). Within the bronchus form nodules called, bronchus-associated lymphoid tissue (BALT), which are a site of lymphocyte aggregation and antigen presentation (Fagerland and Arp, 1993). BALT also contains phagocytic cells, which can be a source of recruitment during respiratory infection (Fagerland and Arp, 1993).

In chickens, the spleen is the largest discrete lymphoid organ and plays an important role in innate and adaptive response (Oláh and Vervelde, 2008). The spleen has many functions, including storage and a place for lymphocytes to aggregate with antigen presenting

cells such as dendritic cells and macrophages (Glick, 2000). Isotype switching from the primary IgM to secondary IgG occurs in the germinal centers of the spleen (Jeurissen, 1993). Gene expression in the spleen is commonly studied and functions as an indicator of immune response (Abasht et al., 2009, Li, X. et al., 2011). APEC can localize and colonize the spleen of chickens, as such the spleen has successfully been used for re-isolation of APEC after infection (Caza et al., 2008; Dheilly et al., 2011; Mellata et al., 2010). Stimulation of chickens with a cell surface component of APEC, lipopolysaccharide (LPS), causes an increase in relative size of the spleen (Shini et al., 2008). The spleen's importance as an immunological organ can also be attributed to its interaction with leukocytes.

White blood cells, or leukocytes, are immune cells that protect the host against disease. There are a variety of leukocytes; lymphocytes such as B- and T- cells, granulocytes such as heterophils, basophils and eosinophils, along with macrophages, dendritic cells and natural killer cells. Heterophils are the first cell type to rapidly localize to sites of infection, where they play an important role in bacterial killing (Kogut et al., 1995). Heterophils kill bacteria through a variety of mechanisms, such as extracellular traps (Chuammitri et al., 2009), degranulation and release of enzymes and peptides (Kogut et al., 2001), and oxidative burst (Farnell et al., 2003), although the lack of myeloperoxidase results in a weakened oxidative burst (Conlon et al., 1991). Improvements in heterophil function increase overall resistance to bacterial infections (Swaggerty et al., 2003). Heterophil abundance and function has been shown to be variable and greatly dependent on genetic background of the population (Kogut et al., 2006; Redmond et al., 2011; Swaggerty et al., 2006). Macrophages are involved in both the innate and acquired immune response. Few tissues host resident macrophages, so recruitment and activation is required to respond to most sites of infection

(Qureshi, 2003). Recognition of pathogen by cell surface receptors, such as toll-like receptors (TLR), results in rapid gene expression changes involved in macrophage phagocytosis and bacterial killing (Bliss et al., 2005). Macrophages can also function as an antigen presenting cell for lymphocytes (Bliss et al., 2005). Intra-tracheal inoculation of APEC resulted in macrophages localizing to site of infection, with subsequent clearance of bacteria (Matthijs et al., 2009). Lymphocytes are primarily divided into two groups; B-cells which function in humoral immunity and T-cells with roles in cell mediated immunity. B-cells originate from the yolk sac and bone marrow before migrating to the bursa of Fabricius for further development (Lebacq and Ritter, 1979; Oláh and Vervelde, 2008). They are producers of immunoglobulins and antibodies, products responsible for broad and targeted protection. T-cells are found in the thymus and spleen, and are producers of cytokines (Lowenthal et al., 1994). The number of mature T-cells, as indicated by cell surface markers CD3, 4, and 8, become more abundant in the first 2 weeks after hatch, but are not yet fully functionally active, contributing to the weaker immune system early in life (Lowenthal et al., 1994). Maturation involves generation of a functional T-cell receptor by recombination of gene segments, which is able to recognize antigen without recognizing self (Viertlboeck and Göbel, 2008). T-cell receptor type and cluster of differentiation molecules help to distinguish different groups of T-cells, with proposed functions similar to mammalian T-cell classes (Viertlboeck and Göbel, 2008). The ratio of heterophils to lymphocytes is commonly used as an indicator of stress in poultry, with increased heterophils and decreased lymphocytes indicative of a stressed state (Gross and Siegel, 1983). Natural killer cells are classified as a lymphocyte and have cytotoxic capabilities which they use to kill foreign or infected cells (Juul-Madsen et al., 2008). Dendritic cells are antigen presenting cells,

transitioning from immature to mature once an antigen is ready to present. Maturation of dendritic cells is associated with a decrease in CC chemokine receptor (CCR) 6 and an increase in CCR7 expression (Wu et al., 2011).

Different peripheral blood cell types use the spleen as an area of interaction and a source of cell development and education (Oláh and Vervelde, 2008). By the time of hatch, B- and T-lymphocytes, and macrophages are already dispersed in the red pulp of the spleen (Mast and Goddeeris, 1999.) T-cells rapidly multiply within the spleen during the first week of life (Lowenthal et al., 1994.). IgM positive B-cells begin to appear in the spleen after 1 week of age (Narabara et al., 2009). CCR7 expression is much higher in the spleen than in other immune organs (Annamalai and Selvaraj, 2011), where migrating T-cells can interact with presented antigens from dendritic cells. Populations of leukocytes in the spleen differ between lines selected for high or low antibody response, with more CD4<sup>+</sup> and B-cells in the high antibody line and more CD8<sup>+</sup> cells in the low antibody line (Parmentier et al., 1995).

Both the spleen and peripheral blood leukocytes respond to and help protect the host from APEC infection through their independent functions and interactions in whole organism signaling. Macrophages exposed to APEC in culture increase their expression of cytokines and oxidative burst genes (Lavrič et al., 2008). Cultured macrophages not only illustrate the importance of cytokines and chemokines, but show the importance of TLR pathways in APEC response (Keeler et al., 2007). Although virulence factors can protect APEC, heterophils and macrophages are able to reduce APEC by phagocytosis (Mellata et al., 2003). Insufficient recruitment of heterophils and macrophages in response to APEC infection is associated with chickens that are susceptible to cellulitis (Olkowski et al., 2005). LPS stimulation results in a rapid reduction of CCR7 expression in the spleen (Annamalai and

Selvaraj, 2011), suggestive of a migration of stored leukocytes from the spleen to sites of infection.

Along with select tissue types, several select genes and gene families hold important roles in immune response. The major histocompatibility complex (MHC) was amongst the first major genes discovered for its role in the immune system and recognition of self. It is involved in the training of leukocytes to prevent self-identification and autoimmunity. In chickens, the MHC was discovered due to its association with the B blood group (Schierman and Nordskog, 1961). The B blood group was first described by Briles et al. (1950) and because of its association with the MHC, certain B group haplotypes are associated with resistance to disease (as reviewed by Davison, 2003; Hunt et al., 2010; Kim et al., 2008). Compared to mammals, the chicken MHC is quite small (Kaufman et al., 1999). Due to its small size and complexing of disease resistance traits, it is not surprising that other genes outside of MHC have been identified for their importance in immune response.

Another early recognized gene family of importance for immune function was that responsible for immunoglobulins. Immunoglobulins, also known as antibodies when specific to an antigen, are produced by B-cells. Immunoglobulins (Ig) are separated into three different isotypes: IgM, the first antibody type produced; IgG, which is also referred to as IgY and is the second type produced, and IgA (as reviewed by Ratcliffe, 2006). There is no clear consensus as to the existence of mammalian IgE and IgD homologues in the chicken. Early reports of an IgD homologue have not been replicated or expanded upon (Chen et al., 1982). IgY is the predicted evolutionary precursor to mammalian IgG and IgE (Aveskog and Hellman, 1998), and shows structural similarities to IgE, although conducting primarily IgG type function (Taylor et al., 2008). Chickens do not have a large repertoire of light and

heavy chains for antibody construction compared to other species, so genetic variability for Ig molecules primarily comes from gene conversion and somatic hypermutation, rather than chain rearrangement (Ratcliffe, 2008).

Toll-like receptors (TLR) are a type of pattern recognition receptor that detect molecular components common to many pathogens. Each TLR recognizes a specific agonist that is unique to a particular pathogen. In chickens, TLR4 and 5 recognize lipopolysaccharide (Dil and Qureshi, 2002; Leveque et al., 2003) and flagellin (Iqbal et al., 2005; Kestra et al., 2008), respectively, which are both common to Gram-negative bacteria like APEC. Other TLRs, such as TLR7, 15, and 21, are not typically associated with bacterial infection, but more often with antigens, imiquimod (Brownlie et al., 2009) and ssRNA (Philbin et al., 2005) for TLR7, and with CpG oligodeoxynucleotides for TLR15 (Ciraci and Lamont, 2011) and TLR21 (Brownlie et al., 2009). Despite this, there is evidence that these TLRs will also respond to Gram-negative bacteria like APEC (Ciraci and Lamont, 2011; de Zoete et al., 2010; Nerren et al., 2010). Consistent with their role in immune response, TLR expression is greater among immune-related tissues such as spleen and peripheral blood leukocytes than in other tissues (Philbin et al., 2005). TLRs help mediate oxidative burst response in heterophils (Farnell et al., 2003). Several TLRs are differentially expressed in response to bacterial infection (Abasht et al., 2009). Different breeding lines have shown differential TLR expression following *Salmonella* challenge (Abasht et al., 2009). TLR signaling initiates down-stream responses, including changing expression of cytokine genes.

Cytokines are cell signaling molecules of the immune system involved in regulating pro- and anti-inflammatory responses and recruitment of leukocytes to sites of infection,

chemokines. Pro-inflammatory cytokines, such as interleukin-1 $\beta$  and interleukin-6, aide in the destruction of pathogens, while anti-inflammatory cytokines, such as interleukin-10, help to protect the host against excessive inflammation. With the completion of the genome sequence, greater knowledge has been gained about the location and structure of cytokines and chemokines (Kaiser et al., 2005). Elevated levels of pro-inflammatory cytokines and chemokines were noted in heterophils from a *Salmonella* resistant chicken line compared to a susceptible line, while anti-inflammatory TGF- $\beta$ 4 was reduced (Swaggerty et al., 2006). Increases in pro-inflammatory cytokines have also been associated with LPS injection (De Boever et al., 2009). Administration of recombinant interferon gamma has been shown to increase immune response to *E. coli* through increased phagocyte activity, increased interleukin 6 production and increased recruitment of MHC II cells (Janardhana et al., 2007).

Avian beta-defensins (AvBD), previously referred to as gallinacins, are a family of cationic antimicrobial peptides with broad-spectrum activity found in epithelial cells and leukocytes (Milona et al., 2007). Fourteen different genes have been discovered in this family within a small region on chromosome 3 (Lynn et al. 2007; Xiao et al., 2004). Avian beta-defensins act by binding to components of the bacterial membrane surface, resulting in depolarization, membrane disruption and death (Brogden, 2005; Sugiarto and Yu, 2007). Heterophil derived AvBD exhibit the ability to kill *Escherichia coli* (Evans et al., 1994). AvBD4,7, and 9 have proven ability to kill *Salmonella* (Milona et al., 2007). Chicken populations resistant to bacterial infection from *Salmonella* exhibit higher basal levels of AvBD (Derache et al., 2009). Allelic differences have been identified among AvBD genes, which are associated with differences in immune response and functionality. SNPs within AvBD genes are associated with antibody response to *Salmonella* vaccination and with



bacterial load in the cecum and spleen of advanced intercross lines derived from commercial breeds (Hasenstein et al., 2006; Hasenstein and Lamont, 2007). In song birds, specifically the great tit, alternate alleles of AvBD7 were associated with different levels of killing ability between Gram-positive and Gram-negative bacteria (Hellgren et al., 2010). Targeted amino acid substitution within AvBD8 can increase its effectiveness against *Escherichia coli* (Higgs et al., 2007). Like many immune genes, unchecked activity can have a deleterious effect. AvBD activity past levels necessary for bacterial killing can result in haemolysis up to 20% (Milona et al., 2007).

### **Current practices to combat infection: What can we do against APEC?**

The shift in production from small scale flocks to large, enclosed production systems influences how disease can be introduced and spread. Good biosecurity practices are the first step to avoid exposure to pathogens. Comparisons of biosecurity recommendations illustrate many common practices, such as wearing disposable boots or utilizing footbaths, clean clothing in facilities, disinfecting equipment, restricted animal access and utilizing all-in/all-out systems (Moore et al., 2008). Many inconsistencies in delivery of information exist however, which may reduce implementation and effectiveness of these practices (Moore et al., 2008). Along with larger flocks, poultry production has become geographically more condensed (Graham et al., 2008). Areas of high density production can increase risk through more frequent exchanges of personnel and equipment associated with individual facilities (Vieira et al., 2009). Although following recommended biosecurity practices reduces the risk of disease spread, the potential for disease introduction through insects, rodents, contaminated feed or other sources merits use of other control mechanisms.

Antimicrobial treatment is a common method to combat bacterial infection. APEC is difficult to combat because many isolates display resistance against many of the commonly used antimicrobials (Zhao et al., 2005). Commensal *E. coli* shows resistance to several antimicrobials as well (Krueger et al., 2011), making it a potential source of resistance genes for APEC. Use of multiple antimicrobials, oxytetracycline, trimethoprim-sulfadimethoxin, and enrofloxacin, has been shown to control extent of pathology after challenge with several resistant APEC strains (Dheilly et al., 2011). Oxytetracycline administered in drinking water alone has not been able to control the spread of APEC in layer houses, although chlorine was able to control water borne spread (Dhillon and Jack, 1996). Therapeutic and sub-therapeutic levels of antibiotics have been fed to chickens for the purpose of maintaining good health and increasing production performance. Concern about generating antimicrobial-resistant bacteria through the use of antibiotics as growth promoters, use in continuous sub-therapeutic doses, and increases in organic farming are leading to the ban of antimicrobial use at sub-therapeutic levels for enhancement of production. Organic poultry farming operations have continued to rise in number since 1997 (USDA ERS, 2008). Organic farming creates an increased demand for disease control options other than the administration of antibiotics. The European Union has already banned the use of antibiotics as growth promoters (Casewell et al., 2003). Several antimicrobials have been banned by the FDA for poultry usage, such as flouroquinolones like enrofloxacin. Studies evaluating current and past levels of antimicrobial resistance during drug use and following subsequent drug bans have shown mixed results. Although some studies illustrate increases in the percentage of antimicrobial resistant strains following feed supplementation of antibiotics, it is difficult to differentiate a change in bacterial population structure from an increase in newly acquired antibiotic

resistances by bacteria (da Costa et al., 2008; Diarra et al., 2007; Thibodeau et al., 2008).

Resistance rates for *Campylobacter* following the enrofloxacin ban appear to have decreased (Han et al., 2009).

Administration of bacteriophage, viruses that kill bacteria, has been effective at reducing mortality in chickens (Huff et al., 2010; Lau et al., 2010). Bacteriophage administration is limited due to antibody production against the phage after first exposure, decreasing effectiveness (Huff et al., 2010). So while bacteriophage administration may make an effective one time post-infection treatment, it may have limitations as a preventative measure. Dietary immunomodulators such as pre- and probiotics have also come into use to improve chicken health. Prebiotics ascorbic acid and  $\beta$ -glucans fed in the diet of broilers induce changed expression of cytokines in the spleen (Redmond et al., 2010). Probiotic supplementation of chicks can reduce incidence and colonization of pathogenic bacteria (Menconi et al., 2011) and change cytokine expression in the spleen (Brisbin et al., 2010).

Vaccination is a preventative technique by which an introduced antigen induces an adaptive immune response. Vaccines developed and tested against a single serogroup of APEC have proven effective at increasing anti-lipopolysaccharide IgG and IgA levels, along with a decrease in circulating bacteria levels (Yaguchi et al., 2009). The difficulty is finding a vaccine that is effective against a heterologous challenge. Previous vaccines developed against APEC adhesion domain of FimH were not able to induce a protective response against APEC challenge (Vandemaele et al., 2005). Other virulence proteins, such as *iss*, increased serum survival, are much more common among APEC isolated from diseased birds than *E. coli* from healthy birds (McPeake et al., 2005), and are suspected to be an outer

membrane protein (Barondess and Beckwith, 1995). A vaccine developed against *iss* has shown protective capabilities against heterogonous challenge (Lynne et al., 2006).

Breeding populations to increase resistance to disease is an attractive alternative to antibiotics. Commercial have focused on increasing production of commercial traits instead of immune response, so it may be necessary to utilize non-commercial populations to improve response. Genetic selection impacts management decisions, because genetic background impacts the effectiveness of vaccines (Chang et al., 2010) and other immunomodulators (Redmond et al., 2010; Redmond et al., 2011). Populations bred to utilize vaccination and dietary supplementation more effectively will be better protected from disease and reduce costs for producers. Genetic selection requires intensive phenotyping and research, which are typically associated with high costs. New technologies to measure phenotypes and genotypes will hopefully reduce costs, which, when combined with effective breeding strategies, will allow for breeding programs that can effectively utilize phenotypic training data over multiple generations.

### **Principles and techniques behind genetic improvements**

The basic principle behind evolution and genetic selection is change over time. Historically, breeders relied on phenotypic information that could be measured on the breeding stock. This presents a difficulty in the instance of sex-limited traits or traits that would result in the sacrifice of breeding stock to measure, requiring extrapolation based on pedigree and family records. The use of selection indices and BLUP estimation of breeding values allows for even better selection of breeding animals with improved genetic response, than selection based purely on phenotypic data (Morris and Pollott, 1997). New technology allows breeders to measure and utilize pedigree and genetic information, such as genotypes

for genetic markers for quantitative trait loci associated with traits of interest and gene expression data. Marker assisted selection allows for more rapid and economical selection by eliminating the need to wait for progeny data in sex limited traits or sacrifice of breeding stock for disease testing (as reviewed by Dekkers, 2004). Genome-wide association studies use genetic markers, such as SNPs, across the entire genome to look for associations with a particular trait of interest. Genome-wide association studies predict that large numbers of loci are responsible for complex traits, such as immune response, and can be captured through whole-genome scans with large SNP panels (Hayes and Goddard, 2010). Provided enough phenotypic and genotypic data is available, predicted phenotypes based on genome-wide marker data can be accurate for traits unable to be observed on breeding animals, like disease resistance (Lee et al., 2008). Genomic selection, which fits the estimated effects for all markers, has proven to be a more beneficial selection method than standard BLUP for traits with low heritability (Piyasatian et al., 2007). Genomic estimated breeding values, breeding values estimated from genomic associations to QTL, are expected to be even more effective than reliance on strictly on pedigree data (Wolc et al., 2011). For any of these selection techniques to be applied, extensive phenotype data is required on genotyped individuals.

It is difficult to identify an animal with a superior immune response due to genetics. *In vivo* experimentation can be utilized to measure pathology and survival rate of the flock, but at the cost of potential breeding animals. Mock-infection with a non-pathogenic antigen can yield information about immune response, but can be antigen specific and give limited information related to survival (De Boever et al., 2009). *In vitro* stimulation of cell cultures, with bacteria or other agonists, allows for investigation of a particular cell type and utilize

techniques such as RNAi to investigate gene roles (Lavrič et al., 2008; Ciraci and Lamont, 2011). For traits to be practical in breeding, they need to be heritable and either associated with markers in order to use pedigree information or measurable on the breeding animal. Traditional immune traits measured through *in vivo* study have found associated genetic markers. Resource populations exhibiting high and low antibody response to *E. coli* vaccination have been successfully utilized to discover microsatellite markers associated with antibody production (Yunis et al., 2002) and survival rate (Yonash et al., 2001). Another trait that can be measured in *in vitro* or *in vivo* studies is gene expression. Gene expression is influenced by infection and can be measured on multiple tissues to give insight into the regulation of genes and pathways during infection.

Multiple technologies have been developed to assay gene expression response. Microarray technology allows for the investigation of several thousands of genes at the same time. Microarray platforms exist for multiple species, including chickens, some targeted to specific cell types (Bliss et al., 2005) or gene sets (Keeler et al., 2007; Smith et al., 2006), while others are more global. The chicken currently has a global microarray available, with 42,034 features, which is able to detect expression levels across a variety of tissues (Li et al., 2008). Transcriptomic response to bacterial infection has been assessed utilizing this microarray to better understand chicken, host, response to *Campylobacter* (Li et al., 2010; Li, X. et al., 2011), *Salmonella* (Chiang et al., 2008), and laryngotacheitis virus (Lee et al., 2010). Global gene expression platforms allow for the discovery of novel genes involved in immune response, as a non-specific assay, giving greater insight into pathways and signal cascades that change during infection.

Quantitative PCR (QPCR) is another technology utilized to measure gene expression in a more targeted manner. Quantitative PCR targeted expression is useful when specific genes or gene families are known to be involved in response and has been used successfully in the chicken (Abasht et al., 2009; Ciraci and Lamont, 2011; Redmond et al., 2009). QPCR is regarded as technically more precise and repeatable than microarrays, and for this reason it is the standard to use in technical validation of microarray results (Larkin et al., 2005).

High-throughput sequencing machines have allowed for the recent development of RNA-sequencing (RNA-seq) technology. Although a new technology, RNA-seq appears to be a very reliable method of measuring RNA expression which, unlike microarrays, also allows for detection of genes with low levels of expression, splice variants and novel transcripts (Marioni et al., 2008). Most RNA-seq studies were not published on until 2008, but its recognition as a ground-breaking new tool for transcriptomic research has led to over 300 published manuscripts in the Pub-Med database using this technology in the past 3 years. RNA-seq technology has been successfully utilized in humans to investigate transcriptomic response to disease (Twine et al., 2011). As of August 2011, only one paper has utilized RNA-seq in chickens (Wolf and Bryk, 2011), illustrating its ability to be utilized within a chicken transcriptome and the need to utilize the technology more.

### **Creating an enhanced immune response through genetic selection**

First efforts at breeding for enhanced immune response were through the MHC by selecting for B blood groups resistant to disease. As MHC haplotype is a discrete trait, it is easily selected for and can be introgressed into commercial populations. Various MHC haplotypes have been associated with resistance to *Eimeria maxima* (Kim et al., 2008), avian influenza (Hunt et al., 2010) and Marek's disease (as reviewed by Davison, 2003). The

disadvantage to single gene selection is that a given allele isn't always beneficial for all traits. The B<sup>21/B21</sup> haplotype, resistant to Marek's disease virus, is associated with a higher incidence of *E. coli* derived cellulitis in broilers than the B<sup>13/B13</sup> haplotype, but surprisingly no change in severity of pathology (Macklin et al., 2009). This is contradictory to a study showing that the B<sup>21/B21</sup> haplotype is associated with greater survival after APEC challenge in layers (Cavero et al., 2009). This contradiction may be explained by differences in selection history and genetics of the two populations assessed, layers compared to broilers. While the MHC plays a large role, disease resistance is expected to be polygenic in nature, requiring investigation into what genes contribute to this resistance.

Phenotype based selection has been utilized to improve polygenic immune traits. Mice populations have been successfully divergently selected for antibody response to sheep and pigeon red blood cells (Biozzi et al., 1970). These "high" and "low" responder lines were found to divergently respond to multiple types of antigen. Selection for a specific antigen resulted in an increase in antibody production against other antigens, allowing for broad spectrum performance increases. Selection for high antibody production appeared to decrease emphasis on cellular immunity, as the low line saw higher macrophage catabolic activity than the high responder line (Mouton et al., 1984). Unfortunately for production species, breeding for enhanced commercial production traits has resulted in reduced pressure on immunity and commercial breeds have been shown to be weaker responders to infection than non-production breeds (Redmond et al., 2009). However, even within commercial broiler chicken populations, resistant and susceptible genetic lines have been discovered against an APEC challenge for mortality and lesions (Ask et al., 2006). Studies on APEC resistance have found no significant association between mortality and production traits



(Cavero et al., 2009). Heritability estimates for resistance against a particular pathogen are typically low, 0.12 for mortality to *Salmonella* (Janss and Bolder, 2000) and 0.17 for resistance to APEC (Cavero et al., 2009). Taking advantage of the new breeding techniques can help to improve heritability estimates and accuracy. Resistance to disease is a very complex trait, resulting in hard to define characteristics and low heritability. Focusing breeding efforts on a particular component of disease resistance can increase heritability. Heritability estimates for heterophil to lymphocyte ratio are high at 0.59, along with heritability estimates for heterophil and leukocyte numbers individually (Campo and Davila, 2002). Increase in antibody response to *E. coli* vaccination has a high heritability of 0.44 (Yonash et al., 1996). Gene expression has been shown to be moderately heritable in human studies (Monks et al., 2004; Price et al., 2011), making selection on gene expression an option to explore for breeders.

Gene expression profiles help to identify and quantify pathways regulated in response to infection. Investigation into differentially expressed genes can allow investigators to decipher the optimal expression profile for pathogen elimination without causing self-inflicted pathology. Expression of immune related genes has been shown to be heritable in the chicken. Parental chickens with high and low expression levels of immune related genes produced chicks with increased or decreased expression levels (Swaggerty et al., 2008), although heritability was not estimated. Populations bred for resistance or susceptibility to *Salmonella* show different expression patterns both before and after infection, illustrating a role for expression profiles in disease resistance (Derache et al., 2009; Sadeyen et al., 2004). Cytokines from distinct genetic lines, meat type, egg type, and an Egyptian breed that hasn't undergone commercial selection, differ in their response to *Salmonella in vitro* (Redmond et

al., 2009). Background genetics impacts gene expression, so extensive research of current production lines is necessary to determine the best method to increase immune response. SNP studies have identified QTLs and genes thought to be responsible for expression differences, allowing producers to use SNP genotype information of parental stock in their breeding programs (Le Bihan-Duval et al., 2011, Smith et al., 2011).

The tools necessary for developing a successful breeding program against APEC exist. Genetic background has proven to be a factor in disease resistance. Gene expression is a valuable assay to measure the ideal basal and response expression levels to infection. Very little is known about *in vivo* chicken gene expression response to APEC infection. Many disease studies choose to focus on cell culture responses; while valuable, they may leave gaps in our knowledge of whole organism responses. Multiple tissue analysis taken from whole organisms for estimation of disease response should provide insight into systems biology. This foundational research is necessary for discovery of new targets of interest for future studies and MAS programs. Proper application of genetics research should increase resistance to disease while making an effort to maintain production levels.

### **Experimental design and sample selection for this study**

A large-scale animal experiment was carried out to generate the necessary samples for this study. The overall goal of the study was to increase the scientific understanding of host genomics of response to APEC. In total, approximately 720 chickens were used with 120 birds in each of 6 replicates conducted at different times. Within each replicate, half of the birds were vaccinated or non-vaccinated at 2 weeks of age, that is, 60 birds into each partition. Treatment groups that were challenged with APEC at 4 weeks of age had a larger proportion of chickens, 48 vaccinated birds were challenged and 48 non-vaccinated birds

were challenged. Twelve birds were included in the non-challenged, vaccinated group, and 12 birds in the non-challenged, non-vaccinated group. Half of the birds from each treatment group were harvested for sample collection at each of two time points, 1 and 5 days post challenge. The large number of birds in APEC-challenged groups allowed for identification of a large range of pathology phenotypes, which was used to further divide the non-vaccinated, challenged treatment groups into two phenogroups each: mild and severe pathology. Therefore, the two vaccine treatments, two APEC-challenge statuses, two tissue-harvest times, and the further division of the two non-vaccinated, challenged groups into two phenogroups each, resulted in a total of ten treatment groups for analysis.

Individuals, rather than pools, were used for the microarray analysis performed within this dissertation study, to allow for a clear connection to pathology phenotype and the ability to analyze multiple tissues from the same individual birds. Four of the 6 replicates were utilized; two replicates were not used because they were subject to non-treatment factors. One individual was selected as a treatment group representative for each of the four replicates, resulting in a total of 40 samples assayed on the microarrays. Selection of the samples from the non-vaccinated, challenged groups was based on pathology scores, identifying the most extreme mild and severe scores, scores closest to the minimum of 0 and the maximum of 7. For the other treatment groups, birds were selected to be the best average representative of that treatment group. A limiting factor for inclusion in the study was the availability and quantity of spleen and leukocyte tissues for RNA isolation. Sufficient isolated quantity of tissue was necessary for good RNA yield and quality required for microarray analysis.

## References

- Abasht, B., M. G. Kaiser, J. van der Poel, and S. J. Lamont. 2009. Genetic lines differ in Toll-like receptor gene expression in spleens of chicks inoculated with *Salmonella enterica* serovar Enteritidis. *Poult. Sci.* 88:744-749.
- Annamalai, T., and R. K. Selvaraj. 2011. Chemokine receptor CCR7 and CXCR5 mRNA in chickens following inflammation or vaccination. *Poult. Sci.* 90:1695-1700.
- Ask, B., E. H. van der Waaij, A. Stegeman and J. A. M. van Arendonk. 2006. Genetic variation among broiler genotypes in susceptibility to colibacillosis. *Poult. Sci.* 85:415-421.
- Aveskogh, M., and L. Hellman. 1998. Evidence for an early appearance of modern post-switch isotypes in mammalian evolution; cloning of IgE, IgG and IgA from the marsupial *Monodelphis domestica*. *Eur. J. Immunol.* 28:2738-2750.
- Bakyaraj, S., S. K. Bhanja, S. Majumdar, and B. Dash. 2011. Modulation of post-hatch growth and immunity through in ovo supplemented nutrients in broiler chickens. *J. Sci. Food Agric.* Doi: 10.1002/jsfa.4577. [Epub ahead of print]
- Barnes, H. J., L. K. Nolan, and J. P. Vaillancourt. 2008. Colibacillosis, in Y. M. Saif (ed.), *Diseases of Poultry*. 12th ed. p.691-732. Blackwell Publ.
- Barondess, J. J., and J. Beckwith. 1995. *bor* gene of phage  $\lambda$ , involved in serum resistance, encodes a widely conserved outer membrane lipoprotein. *J. Bacteriol.* 177:1247-1253.
- Bastiani, M., M. C. Vidotto, and F. Horn. 2005. An avian pathogenic *Escherichia coli* isolate induces caspase 3/7 activation in J774 macrophages. *FEMS Microbiol. Lett.* 253:133-140.

- Bauchart, P., P. Germon, A. Brée, E. Oswald, J. Hacker, and U. Dobrindt. 2010. Pathogenic comparison of human extraintestinal and avian pathogenic *Escherichia coli* – Search for factors involved in host specificity or zoonotic potential. *Microb. Pathog.* 49:105-115.
- Befus, A D., N. Johnston, G. A. Leslie and J. Bienenstock. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J. Immunol.* 125:2626-2632.
- Biozzi, G., R. Asofsky, R. Lieberman, C. Stiffel, D. Mouton, and B. Benacerraf. 1970. Serum concentrations and allotypes of immunoglobulins in two lines of mice genetically selected for "high" or "low" antibody synthesis. *J. Exp. Med.* 132:752-764.
- Bliss, T. W., J. E. Dohms, M. G. Emara, and C. L. Keeler Jr. 2005. Gene expression profiling of avian macrophage activation. *Vet. Immunol. Immunopathol.* 105:289-299.
- Briles, W. E., W. H. McGibbon, and M. R. Irwin. 1950. On multiple alleles effecting cellular antigens in the chicken. *Genetics.* 35:633-652.
- Brisbin, T. J., J. Gong, P. Parvizi, and S. Sharif. 2010. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. *Clin. Vaccine Immunol.* 17:1337-1343.
- Brisbin, T. J., J. Gong, and S. Sharif. 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Anim. Health Res. Rev.* 9:101-110.
- Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3:238-250.

- Brownlie, R., J. Zhu, B. Allan, G. K. Mutwiri, L. A. Babiuk, A. Potter, and P. Griebel. 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Mol. Immunol.* 46:3163-3170.
- Campo, J. L., and S. G. Davila. 2002. Estimation of heritability for heterophil:lymphocyte ratio in chickens by restricted maximum likelihood. Effects of age, sex and crossing. *Poult. Sci.* 81:1448-1453.
- Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* 52:159-161.
- Cavero, D., M. Schmutz, H. C. Philipp, and R. Preisinger. 2009. Breeding to reduce susceptibility to *Escherichia coli* in layers. *Poult. Sci.* 88:2063-2068.
- Caza, M., F. Lépine, S. Milot, and C. M. Dozois. 2008. Specific roles of the *iroBCDEN* genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. *Infect. Immun.* 76:3539-3549.
- Chang, S., J. R. Dunn, M. Heidari, L. F. Lee, J. Song, C. W. Ernst, Z. Ding, L. D. Bacon, and H. Zhang. 2010. Genetics and vaccine efficacy: host genetic variation affecting Marek's disease vaccine efficacy in White Leghorn chickens. *Poult. Sci.* 89:2083-2091.
- Chanteloup, N. K., G. Porcheron, B. Delaleu, P. Germon, C. Schouler, M. Moulin-Schouler and P. Gilot. 2011. The extra-intestinal avian pathogenic *Escherichia coli* strain BEN 2908 invades avian and human epithelial cells and survives intracellularly. *Vet. Microbiol.* 147:435-539.

- Chen, C. L., J. E. Lehmeyer, and M. D. Cooper. 1982. Evidence for an IgD homologue on chicken lymphocytes. *J. Immunol.* 129:2580-2585.
- Chiang, H. I., C. L. Swaggerty, M. H. Kogut, S. E. Dowd, X. Li, I. Y. Pevzner and H. Zhou. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44K Agilent microarray. *BMC Genomics.* 9:526.
- Chuammitri, P., J. Ostojić, C. B. Andreasen, S. B. Redmond, S. J. Lamont, and D. Palić. 2009. Chicken heterophil extracellular traps (HETs): Novel defense mechanism of chicken heterophils. *Vet. Immunol. Immunopathol.* 129:126-131.
- Ciraci, C., and S. J. Lamont. 2011. Avian-specific TLRs and downstream effector responses to CpG-induction in chicken macrophages. *Dev. Comp. Immunol.* 35:392-398.
- Conlon, P., D. Smith and T. Gowlett. 1991. Oxygen radical production by avian leukocytes. *Can. J. Vet. Res.* 55:193-195.
- Cortés, C. R., G. Téllez Isaías, C. López Cuello, J. M. Villaseca-Flores, R. C. Anderson, and C. Eslava Campos. 2004. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. *Rev. Latinoam. Microbiol.* 46:12-16.
- da Costa, P. M., A. Bica, P. Vaz-Pires, and F. Bernardo. 2008. Effects of antimicrobial treatment on selection of resistant *Escherichia coli* in broiler fecal flora. *Microb. Drug Resist.* 14:299-306.
- Davison, T. F. 2003. The immunologists' debt to the chicken. *Br. Poult. Sci.* 44:6-21.
- De Boever, S., S. Croubels, E. Meyer, S. Sys, R. Beyaert, R. Ducatelle, and P. De Backer. 2009. Characterization of an intravenous lipopolysaccharide inflammation model in broiler chickens. *Avian Pathol.* 38:403-411.

- de Jong, A. E., L. Verhoeff, M. J. Nauta, and R. de Jonge. 2008. Cross-contamination in the kitchen: effect of hygiene measures. *J. Appl. Microbiol.* 105:615-624.
- Dekkers, J. C. 2004. Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. *J. Anim. Sci.* 82 E-Suppl:E313-328.
- Derache, C., E. Esnault, C. Bonsergent, Y. Le Vern, P. Quéré, and A. C. Lalmanach. 2009. Differential modulation of  $\beta$ -defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chicken inbred lines. *Dev. Comp. Immunol.* 33:959-966.
- de Zoete, M. R., A. M. Keesstra, P. Roszczenko, and J. P. van Putten. 2010. Activation of human and chicken Toll-like receptors by *Campylobacter* spp. *Infect. Immun.* 78:1229-1238.
- Dheilly, A., A. Boudier, L. Le Devendec, G. Hellard, and I. Kempf. 2011. Clinical and microbial efficacy of antimicrobial treatments of experimental avian colibacillosis. *Vet. Microbiol.* 149:422-429.
- Dhillon, A. S., and O. K. Jack. 1996. Two outbreaks of colibacillosis in commercial caged layers. *Avian Dis.* 40:742-746.
- Diarra, M. S., F. G. Silversides, F. Diarrassouba, J. Pritchard, L. Masson, R. Brousseau, C. Bonnet, P. Delaquis, S. Bach, B. J. Skura, and E. Topp. 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, *Clostridium perfringens* and *enterococcus* counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. *Appl. Environ. Microbiol.* 73:6566-6576.



- Dias da Silveira, W., A. Ferreira, M. Brocchi, L. Maria de Hollanda, A. F. Pestana de Castro, A. Tatsumi Yamada, and M. Lancellotti. 2002. Biological characteristics and pathogenicity of avian *Escherichia coli* strains. *Vet. Microbiol.* 85:47-53.
- Dil, N., and M. A. Qureshi. 2002. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. *Vet. Immunol. Immunopathol.* 84:191-207.
- Dodgson, J. B., M. E. Delany, and H. H. Cheng. 2011. Poultry genome sequences: progress and outstanding challenges. *Cytogenet. Genome Res.* 134:19-26.
- Dwars, R. M., M. G. R. Matthijs, A. J. J. M. Daemen, J. H. H. van Eck, L. Vervelde, and W. J. M. Landman. 2009. Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus. *Vet. Immunol. Immunopathol.* 127:65-76.
- Dziva, F., and M. P. Stevens. 2008. Colibacillosis in poultry: unraveling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. *Avian Pathol.* 37:355-366.
- Evans, E. W., G. G. Beach, J. Wunderlich, and B. G. Harmon. 1994. Isolation of antimicrobial peptides from avian heterophils. *J. Leukoc. Biol.* 56:661-665.
- Fagerland, J. A., and L. H. Arp. 1993. Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. *Avian Dis.* 37:10-18.
- Farnell, M. B., T. L. Crippen, H. He, C. L. Swaggerty, and M. H. Kogut. 2003. Oxidative burst mediated by toll like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial toll agonists. *Dev. Comp. Immunol.* 27:423-429.

- Fricke, W. F., P. F. McDermott, M. K. Mammel, S. Zhao, T. J. Johnson, D. A. Rasko, P. J. Fedorka-Cray, A. Pedroso, J. M. Whichard, J. E. Leclerc, D. G. White, T. A. Cebula, and J. Ravel. 2009. Antimicrobial resistance-conferring plasmids with similarity to virulence plasmids from avian pathogenic *Escherichia coli* strains in *Salmonella enterica* serovar Kentucky isolates from poultry. *Appl. Environ. Microbiol.* 75:5963-5971.
- Glick, B. 2000. Immunophysiology, in G. Causey Whittow (ed.), *Sturkie's Avian Physiology*. 5th ed. p.657-670. Academic Press.
- Graham, J. P., J. H. Leibler, L. B. Price, J. M. Otte, D. U. Pfeiffer, T. Tiensin, and E. K. Silbergeld. 2008. The animal-human interface and infectious disease in industrial food animal production: rethinking biosecurity and biocontainment. *Public Health Rep.* 123:282-299.
- Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis.* 27:972-979.
- Gyles, C. L. 2008. Antimicrobial resistance in selected bacteria from poultry. *Anim. Health Res. Rev.* 9:149-158.
- Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, H. Zhou, B. Sanei, J. R. Chambers, and S. Sharif. 2006. Probiotics stimulate production of natural antibodies in chickens. *Clin. Vaccine Immunol.* 13:975-980.
- Han, F., S. I. Lestari, S. Pu, and B. Ge. 2009. Prevalence and antimicrobial resistance among *Campylobacter* spp. in Louisiana retail chickens after the enrofloxacin ban. *Foodborne Pathog. Dise.* 6:163-171.

- Hasenstein, J. R., and S. J. Lamont. 2007. Chicken gallinacin gene cluster associated with *Salmonella* response in advanced intercross line. *Avian Dis.* 51:561-567.
- Hasenstein, J. R., G. Zhang, and S. J. Lamont. 2006. Analyses of five gallinacin genes and the *Salmonella enterica* serovar Enteritidis response in poultry. *Infect. Immun.* 74:3375-3380.
- Hayes, B., and M. Goddard. 2010. Genome-wide association and genomic selection in animal breeding. *Genome.* 53:876-883.
- Hellgren, O., Sheldon, B. C., and A. Buckling. 2010. *In vitro* tests of natural allelic variation of innate immune genes (avian  $\beta$ -defensins) reveal functional differences in microbial inhibition. *J. Evol. Biol.* 23:2726-2730.
- Higgs, R., D. J. Lynn, S. Cahalane, I. Alaña, C. M. Hewage, T. James, A. T. Lloyd, and C. O'Farrelly. 2007. Modification of chicken avian  $\beta$ -defensin-8 at positively selected amino acid sites enhances specific antimicrobial activity. *Immunogenetics.* 59:573-580.
- Huff, W. E., G. R. Huff, N. C. Rath, and A. M. Donoghue. 2010. Immune interference of bacteriophage efficacy when treating colibacillosis in poultry. *Poult. Sci.* 89:895-900.
- Hunt, H. D., S. Jadhao, and D. E. Swayne. 2010. Major histocompatibility complex and background genes in chickens influence susceptibility to high pathogenicity avian influenza virus. *Avian Dis.* 54:572-575.
- Iqbal, M., V. J. Philbin, G. S. Withanage, P. Wigley, R. K. Beal, M. J. Goodchild, P. Barrow, I. McConnell, D. J. Maskell, J. Young, N. Bumstead, Y. Boyd, and A. L. Smith. 2005. Identification and functional characterization of chicken toll-like receptor 5

- reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar typhimurium. Infect. Immun. 73:2344-2350.
- Janardhana, V., M. E. Ford, M. P. Bruce, M. M. Broadway, T. E. O'Neil, A. J. Karpala, M. Asif, G. F. Browning, K. A. Tivendale, A. H. Noormohammadi, J. W. Lowenthal, and A. G. Bean. 2007. IFN- $\gamma$  enhances immune responses to *E. coli* infection in the chicken. J. Interferon Cytokine Res. 27:937-946.
- Janss, L. L., and N. M. Bolder. 2000. Heritabilities of and genetic relationships between salmonella resistance traits in broilers. J. Anim. Sci. 78:2287-2291.
- Jeurissen, S. H. M. 1993. The role of various compartments in the chicken spleen during an antigen-specific humoral response. Immunology. 80:29-33.
- Johnson, T. J., S. Kariyawasam, Y. Wannamuehler, P. Mangiamale, S. J. Johnson, C. Doetkott, J. A. Skyberg, A. M. Lynne, J. R. Johnson, and L. K. Nolan. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189:3228-3236.
- Juul-Madsen, H. R., B. Viertlboeck, A. L. Smith and T. W. F. Göbel. 2008. Avian innate immune responses, in F. Davison, B. Kaspers and K. A. Schat (ed.), Avian Immunology. 1st ed. p.129-158. Elsevier Ltd.
- Kaiser, P., T. Y. Poh, L. Rothwell, S. Avery, S. Balu, U. S. Pathania, S. Hughes, M. Goodchild, S. Morrell, M. Watson, N. Bumstead, J. Kaufman, and J. R. Young. 2005. A genomic analysis of chicken cytokines and chemokines. J. Interferon. Cytokine Res. 25:467-484.

- Kaufman, J., S. Milne, T. W. Göbel, B. A. Walker, J. P. Jacob, C. Auffray, R. Zoorob, and S. Beck. 1999. The chicken B locus is a minimal essential major histocompatibility complex. *Nature*. 401:923-925.
- Keeratipibul, S., T. Oupaichit, and P. Techaruwichit. 2009. Contamination profiles of *Escherichia coli* and enterococci in steamed chicken meat products. *J. Food Prot.* 72:1821-1829.
- Keeler, C. L. Jr, T. W. Bliss, M. Lavrič, and M. N. Maughan. 2007. A functional genomics approach to the study of avian innate immunity. *Cytogenet. Genome Res.* 117:139-145.
- Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten. 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. *Mol. Immunol.* 45:1298-1307.
- Kim, D. K., H. S. Lillehoj, Y. H. Hong, D. W. Park, S. J. Lamont, J. Y. Han, and E. P. Lillehoj. 2008. Immune-related gene expression in two B-complex disparate genetically inbred Fayoumi chicken lines following *Eimeria maxima* infection. *Poult. Sci.* 87:433-443.
- Kogut, M. H., K. J. Genovese, and V. K. Lowry. 2001. Differential activation of signal transduction pathways mediating phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. *Inflammation*. 25:7-15.
- Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. In vivo activation of heterophil function in chickens following injection with *Salmonella enteritidis*-immune lymphokines. *J. Leukoc. Biol.* 57:56-62.

- Kogut, M. H., C. Swaggerty, H. He, I. Pevzner, and P. Kaiser. 2006. Toll-like receptor agonists stimulate differential functional activation and cytokine and chemokine gene expression in heterophils isolated from chickens with differential innate responses. *Microbes Infect.* 8:1866-1874.
- Krueger, A. L., J. Foster, F. Medalla, K. Joyce, M. B. Perri, L. Johnson, M. Zervos, J. M. Whichard, and E. J. Barzilay. 2011. Commensal *Escherichia coli* isolate resistant to eight classes of antimicrobial agents in the United States. *Foodborne Pathog. Dis.* 8:329-332.
- Larkin, J. E., B. C. Frank, H. Gavras, R. Sultana, and J. Quackenbush. 2005. Independence and reproducibility across microarray platforms. *Nat. Methods.* 2:337-344.
- Lau, G. L., C. C. Sieo, W. S. Tan, M. Hair-Bejo, A. Jalila, and Y. W. Ho. 2010. Efficacy of a bacteriophage isolated from chickens as a therapeutic agent for colibacillosis in broiler chickens. *Poult. Sci.* 89:2589-2596.
- Lavrič, M., M. N. Maughan, T. W. Bliss, J. E. Dohms, D. Bencina, C. L. Keeler, and M. Narat. 2008. Gene expression modulation in chicken macrophages exposed to *Mycoplasma synoviae* or *Escherichia coli*. *Vet. Microbiol.* 126:111-121.
- Lebacqz, A. M., and M. A. Ritter. 1979. B-cell precursors in early chicken embryos. *Immunology.* 37:123-134.
- Le Bihan-Duval, E., J. Nadaf, C. Berri, F. Pitel, B. Graulet, E. Godet, S. Y. Leroux, O. Demeure, S. Lagarrigue, C. Duby, L. A. Cogburn, C. M. Beaumont, and M. J. Duclos. 2011. Detection of a cis eQTL controlling BMCO1 gene expression leads to the identification of a QTG for chicken breast meat color. *PLoS One.* 6:e14825.

- Lee, J. Y., J. J. Song, A. Wooming, X. Li, H. Zhou, W. G. Bottje, and B. W. Kong. 2010. Transcriptional profiling of host gene expression in chicken embryo lung cells infected with laryngotracheitis virus. *BMC Genomics*. 11:445.
- Lee, S. H., J. H. van der Werf, B. J. Hayes, M. E. Goddard, and P. M. Visscher. 2008. Predicting unobserved phenotypes for complex traits from whole-genome SNP data. *PLoS Genet*. 4:e1000231.
- Leveque, G., V. Forgetta, S. Morroll, A. L. Smith, N. Bumstead, P. Barrow, J. C. Loredon-Osti, K. Morgan, and D. Malo. 2003. Allelic variation in *TLR4* is linked to susceptibility to *Salmonella enterica* serovar Typhimurium infection in chickens. *Infect. Immun*. 71:1116-1124.
- Li, G., K. A. Tivendale, P. Liu, Y. Feng, Y. Wannemuehler, W. Cai, P. Mangiamale, T. J. Johnson, C. Constantinidou, C. W. Penn, L. K. Nolan, and S. M. Payne. 2011. Transcriptome analysis of avian pathogenic *Escherichia coli* O1 in chicken serum reveals adaptive responses to systemic infection. *Infect. Immun*. 79:1951-1960.
- Li, X., H. I. Chiang, J. Zhu, S. E. Dowd, and H. Zhou. 2008. Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics*. 9:60.
- Li, X., C. L. Swaggerty, M. H. Kogut, H. I. Chiang, Y. Wang, K. J. Genovese, H. He, F. M. McCarthy, S. C. Burgess, I. Y. Pevzner, and H. Zhou. 2011. Systemic response to *Campylobacter jejuni* infection by profiling gene transcription in the spleen of two genetic lines of chickens. *Immunogenetics*. Doi: 10.1007/s00251-011-057-1. [Epub ahead of print]
- Li, X., C. L. Swaggerty, M. H. Kogut, H. I. Chiang, Y. Wang, K. J. Genovese, H. He, and H. Zhou. 2010. Gene expression profiling of the local cecal response of genetic chicken

- lines that differ in their susceptibility to *Campylobacter jejuni* colonization. PLoS One. 5:e11827.
- Lowenthal, J. W., T. E. Connick, P. G. McWaters, and J. J. York. 1994. Development of T cell immune responsiveness in the chicken. Immunol. Cell Biol. 72:115-122.
- Lutful Kabir, S. M. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. Int. J. Environ. Res. Public Health. 7:89-114.
- Lynn, D. J., R. Higgs, A. T. Lloyd, C. O'Farrelly, V. Harvé-Grépinet, Y. Nys, F. S. Brinkman, P. L. Yu, A. Soulier, P. Kaiser, G. Zhang and R. I. Lehrer. 2007. Avian beta-defensin nomenclature: a community proposed update. Immunol. Lett. 110:86-89.
- Lynne, A. M., S. L. Foley and L. K. Nolan. 2006. Immune response to recombinant *Escherichia coli* Iss protein in poultry. Avian Dis. 50:273-276.
- Macklin, K. S., R. A. Norton, and S. J. Ewald. 2009. Effect of the major histocompatibility complex on the inhibition of induced cellulitis development in a broiler chicken model. Avian Dis. 53:297-300.
- Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens and Y. Gilad. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 18:1509-1517.
- Mast, J., and B. M. Goddeeris. 1999. Development of immunocompetence of broiler chickens. Vet. Immunol. Immunopathol. 70:245-256.
- Matthijs, M. G. R., M. P. Ariaans, R. M. Dwars, J. H. H. van Eck, A. Bouma, A. Stegeman, and L. Vervelde. 2009. Course of infection and immune responses in the respiratory



- tract of IBV infected broilers after superinfection with *E. coli*. Vet. Immunol. Immunopathol. 127:77-84.
- Matthijs, M. G. R., J. H. H. van Eck, W. J. M. Landman, and J. A. Stegeman. 2003. Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: A comparison between vaccine and virulent field virus. Avian Pathol. 32:473-481.
- McPeake, S. J. W., J. A. Smyth, and H. J. Ball. 2005. Characterisation of avian pathogenic *Escherichia coli* (APEC) associated with colisepticaemia compared to faecal isolates from healthy birds. Vet. Microbiol. 110:245-253.
- Mellata, M., K. Ameiss, H. Mo, R. Curtiss 3<sup>rd</sup>. 2010. Characterization of the contribution to virulence of three large plasmids of avian pathogenic *Escherichia coli*  $\chi$ 7122 (O78:K80:H9). Infect. Immun. 78:1528-1541.
- Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss 3<sup>rd</sup>, B. Lehoux, and J. M. Fairbrother. 2003. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect. Immun. 71:494-503.
- Menconi, A., A. D. Wolfenden, S. Shivaramaiah, J. C. Terraes, T. Urbano, J. Kuttel, C. Kremmer, B. M. Hargis, and G. Tellez. 2011. Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella enterica* serovar Heidelberg in neonatal broiler chickens and turkey poults. Poult. Sci. 90:561-565.
- Milona, P., C. L. Townes, R. M. Bevan, and J. Hall. 2007. The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars. Biochem. Biophys. Res. Commun. 356:169-174.

- Monks, S. A., A. Leonardson, H. Zhu, P. Cundiff, P. Pietrusiak, S. Edwards, J. W. Phillips, A. Sachs, and E. E. Schadt. 2004. Genetic inheritance of gene expression in human cell lines. *Am. J. Hum. Genet.* 75:1094-1105.
- Moore, D. A., M. L. Merryman, M. L. Hartman, and D. J. Klingborg. 2008. Comparison of published recommendations regarding biosecurity practices for various production animal species and classes. *J. Am. Vet. Med. Assoc.* 233:249-256.
- Mouton, D., Y. Bouthillier, J. C. Mevel, and G. Biozzi. 1984. Genetic selection for antibody responsiveness in mice: further evidence for inverse modification of macrophage catabolic activity without alteration of the expression of T-cell-mediated immunity. *Ann. Immunol. (Paris)*. 135D:173-186.
- Narabara, K., A. Abe, Gerilechaogetu, H. Hanieh, and Y. Kondo. 2009. B cell differentiation in the bursa of Fabricius and spleen of embryos and chicks immediately after hatching. *Anim. Sci. J.* 80:669-677.
- Nerren, J. R., H. He, K. Genovese, and M. H. Kogut. 2010. Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by Gram-negative and Gram-positive bacteria, but not TLR agonists. *Vet. Immunol. Immunopathol.* 136:151-156.
- Nolan, L. K., C. W. Giddings, S. M. Horne, C. Doetkott, P. S. Gibbs, R. E. Wooley, and S. L. Foley. 2002. Complement resistance, as determined by viable count and flow cytometric methods, and its association with the presence of iss and the virulence of avian *Escherichia coli*. *Avian Dis.* 46:386-392.
- Oláh, I., and L. Vervelde. 2008. Structure of the avian lymphoid system, in F. Davison, B. Kaspers and K. A. Schat (ed.), *Avian Immunology*. 1st ed. p.13-50. Elsevier Ltd.

- Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, B. M. Wurtz and L. Kumor. 2005. The role of first line of defence mechanisms in the pathogenesis of cellulitis in broiler chickens: skin structural, physiological and cellular response factors. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* 52:517-524.
- Parmentier, H. K., M. B. Kreukniet, B. Goree, T. F. Davison, S. H. Jeurissen, E. G. Harmsen and M. G. Nieuwland. 1995. Differences in distribution of lymphocyte antigens in chicken lines divergently selected for antibody responses to sheep red blood cells. *Vet. Immunol. Immunopathol.* 48:155-168.
- Parreira, V. R., and C. L. Gyles. 2002. Shiga toxin genes in avian *Escherichia coli*. *Vet. Microbiol.* 87:341-352.
- Peighambari, S. M., R. J. Julian and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. *Avian Dis.* 44:759-769.
- Philbin, V. J., M. Iqbal, Y. Boyd, M. J. Goodchild, R. K. Beal, N. Bumstead, J. Young, and A. L. Smith. 2005. Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology.* 114:507-521.
- Piyasatian, N., R. L. Fernando, and J. C. Dekkers. 2007. Genomic selection for marker-assisted improvement in line crosses. *Theor. Appl. Genet.* 115:665-674.
- Price, A. L., A. Helgason, G. Thorleifsson, S. A. McCarroll, A. Kong, and K. Stefansson. 2011. Single-tissue and cross-tissue heritability of gene expression via identity-by-descent in related or unrelated individuals. *PLoS Genet.* 7:e1001317.
- Qureshi, M. A. 2003. Avian macrophage and immune response: an overview. *Poult. Sci.* 82:691-698.

- Ratcliffe, M. J. 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. *Dev. Comp. Immunol.* 30:101-118.
- Ratcliffe, M. J. H. 2008. B cells, the bursa of Fabricius and the generation of antibody repertoires, in F. Davison, B. Kaspers and K. A. Schat (ed.), *Avian Immunology*. 1st ed. p.129-158. Elsevier Ltd.
- Redmond, S. B., P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2009. Chicken heterophils from commercially selected and non-selected genetic lines express cytokines differently after *in vitro* exposure to *Salmonella enteritidis*. *Vet. Immunol. Immunopathol.* 132:129-134.
- Redmond, S. B., P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2011. Proportion of circulating chicken heterophils and CXCLi2 expression in response to *Salmonella enteritidis* are affected by genetic line and immune modulating diet. *Vet. Immunol. Immunopathol.* 140:323-328.
- Redmond, S. B., R. M. Tell, D. Coble, C. Mueller, D. Palić, C. B. Andreasen, and S. J. Lamont. 2010. Differential splenic cytokine responses to dietary immune modulation by diverse chicken lines. *Poult. Sci.* 89:1635-1641.
- Sadeyen, J. R., J. Trotureau, P. Velge, J. Marly, C. Beasumont, P. A. Barrow, N. Bumstead, and A. C. Lalmanach. 2004. *Salmonella* carrier state in chicken: comparison of expression of immune response genes between susceptible and resistant animals. *Microbes Infect.* 6:1278-1286.
- Santamaría, J., and G. A. Toranzos. 2003. Enteric pathogens and soil: a short review. *Int. Microbiol.* 6:5-9.

- Schierman, L. W., and A. W. Nordskog. 1961. Relationship of blood type to histocompatibility in chickens. *Science*. 134:1008-1009.
- Seo, H. W., D. Rengaraj, J. W. Choi, K. J. Park, G. Song, and J. Y. Han. 2011. The expression profile of apoptosis-related genes in the chicken as a human epithelial ovarian cancer model. *Oncol. Rep.* 25:49-56.
- Shini, S., P. Kaiser, A. Shini, and W. L. Bryden. 2008. Biological response of chickens (*Gallus gallus domesticus*) induced by corticosterone and a bacterial endotoxin. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 149:324-333.
- Smith, J., J. R. Sadeyen, I. R. Paton, P. M. Hocking, N. Salmon, M. Fife, V. Nair, D. W. Burt and P. Kaiser. 2011. Systems analysis of immune responses in Mark's Disease Virus infection chickens identifies a gene involved in susceptibility and highlights a possible novel pathogenicity mechanism. *J. Virol.* 85:11146-11158. [Epub ahead of print]
- Smith, J., D. Speed, P. M. Hocking, R. T. Talbot, W. G. Degen, V. E. Schijns, E. J. Glass, and D. W. Burt. 2006. Development of a chicken 5 K microarray targeted towards immune function. *BMC Genomics*. 7:49.
- Smyth, J. R. Jr., R. E. Boissy, and K. V. Fite. 1981. The DAM chicken: a model for spontaneous postnatal cutaneous and ocular amelanosis. *J. Hered.* 72:150-156.
- Stokholm, N. M., A. Permin, M. Bisgaard, and J. P. Christensen. 2010. Causes of mortality in commercial organic layers in Denmark. *Avian Dis.* 54:1241-1250.
- Sugiarto, H. and P. L. Yu. 2007. Mechanisms of action of ostrich beta-defensins against *Escherichia coli*. *FEMS Microbiol. Lett.* 270:195-200.

- Swaggerty, C. L., I. Y. Pevzner, P. Kaiser, and M. H. Kogut. 2008. Profiling pro-inflammatory cytokine and chemokine mRNA expression levels as a novel method for selection of increased innate immune responsiveness. *Vet. Immunol. Immunopathol.* 126:35-42.
- Swaggerty, C. L., I. Y. Pevzner, V. K. Lowry, M. B. Farnell and M. H. Kogut. 2003. Functional comparison of heterophils isolated from commercial broiler chickens. *Avian Pathol.* 32:95-102.
- Taylor, A.I., H. J. Gould, B. J. Sutton, and R. A. Calvert. 2008. Avian IgY binds to monocyte receptor with IgG-like kinetics despite an IgE-like structure. *J. Biol. Chem.* 283:16384-16390.
- Taylor, R. L. Jr., and F. M. McCorkle Jr. 2009. A landmark contribution to poultry science—Immunological function of the bursa of Fabricius. *Poult. Sci.* 88:816-823.
- Thibodeau, A., S. Quessy, E. Guévremont, A. Houde, E. Topp, M. S. Diarra, and A. Letellier. 2008. Antibiotic resistance in *Escherichia coli* and *Enterococcus* spp. isolates from commercial broiler chickens receiving growth-promoting doses of bacitracin or virginiamycin. *Can. J. Vet. Res.* 72:129-136.
- Tivendale, K. A., C. M. Logue, S. Kariyawasam, D. Jordan, A. Hussein, G. Li, Y. Wannemuehler, and L. K. Nolan. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. *Infect. Immun.* 78:3412-3419.
- Twine, N. A., K. Janitz, M. R. Wilkins, and M. Janitz. 2011. Whole transcriptome sequencing reveals gene expression and splicing differences in brain regions affected by Alzheimer's disease. *PLoS One.* 6:e16266.

United States Department of Agriculture Economic Research Service (USDA ERS). 2008.

U.S. certified organic farmland acreage, livestock numbers, and farm operations.

<<http://www.ers.usda.gov/Data/organic/Data/Farmlandlivestockandfarm.xls>>

United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS).

2008. The nationwide microbiological baseline data collection program: young chicken survey. Accessed August 2011.

<[http://www.fsis.usda.gov/PDF/Baseline\\_Data\\_Young\\_Chicken\\_2007-2008.pdf](http://www.fsis.usda.gov/PDF/Baseline_Data_Young_Chicken_2007-2008.pdf)>

Vandemaele, F., C. Ververken, N. Bleyen, J. Geys, C. D'Hulst, T. Addwebi, P. van Empel, and B. M. Goddeeris. 2005. Immunization with the binding domain of FimH, the adhesion of type 1 fimbriae, does not protect chickens against avian pathogenic *Escherichia coli*. *Avian Pathol.* 34:264-272.

Vieira, A. R., C. L. Hofacre, J. A. Smith, and D. Cole. 2009. Human contacts and potential pathways of disease introduction on Georgia poultry farms. *Avian Dis.* 53:55-62.

Viertlboeck, B., and T. W. F. Göbel. 2008. Avian T cells: Antigen recognition and lineages, in F. Davison, B. Kaspers and K. A. Schat (ed.), *Avian Immunology*. 1st ed. p.91-106. Elsevier Ltd.

Wallis, J.W., J. Aerts, M.A. Groenen, R.P. Crooijmans, D. Layman, T.A. Graves, D.E.

Scheer, C. Kremitzki, M.J. Fedele, N.K. Mudd, M. Cardenas, J. Higginbotham, J.

Carter, R. McGrane, T. Gaige, K. Mead, J. Walker, D. Albracht, J. Davito, S. P.

Yang, S. Leong, A. Chinwalla, M. Sekhon, K. Wylie, J. Dodgson, M. N. Romanov,

H. Cheng, P. J. de Jong, K. Osoegawa, M. Nefedov, H. Zhang, J. D. McPherson, M.

Krzywinski, J. Schein, L. Hillier, E. R. Mardis, R. K. Wilson, and W. C. Warren.

2004. A physical map of the chicken genome. *Nature* 432: 761-764.

- Wolc, A., J. Arango, P. Settar, J. E. Fulton, N. P. O'Sullivan, R. Preisinger, D. Habier, R. Fernando, D. J. Garrick, and J. C. Dekkers. 2011. Persistence of accuracy of genomic estimated breeding values over generations in layer chickens. *Genet. Sel. Evol.* 43:23.
- Wolf, J. B., and J. Bryk. 2011. General lack of global dosage compensation in ZZ/ZW systems? Broadening the perspective with RNA-seq. *BMC Genomics.* 12:91.
- Wu, Z., T. Hu, and P. Kaiser. 2011. Chicken CCR6 and CCR7 are markers for immature and mature dendritic cells respectively. *Dev. Comp. Immunol.* 35:563-567.
- Xia, X., J. Meng, S. Zhao, S. Bodeis-Jones, S. A. Gaines, S. L. Ayers, and P. F. McDermott. 2011. Identification and antimicrobial resistance of extraintestinal pathogenic *Escherichia coli* from retail meats. *J. Food Prot.* 74:38-44.
- Xiao, Y., A. L. Hughes, J. Ando, Y. Matsuda, J. F. Cheng, D. Skinner-Noble, and G. Zhang. 2004. A genome-wide screen identifies a single  $\beta$ -defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. *BMC Genomics.* 5:56.
- Yaguchi, K., T. Ohgitani, T. Noro, T. Kaneshige, and Y. Shimizu. 2009. Vaccination of chickens with liposomal inactivated avian pathogenic *Escherichia coli* (APEC) vaccine by eye drop or coarse spray administration. *Avian Dis.* 53:245-249.
- Yonash, N., H. H. Cheng, J. Hillel, D. E. Heller, and A. Cahaner. 2001. DNA microsatellites linked to quantitative trait loci affecting antibody response and survival rate in meat-type chickens. *Poult. Sci.* 80:22-28.
- Yonash, N., G. Leitner, R. Waiman, E. D. Heller, and A. Cahaner. 1996. Genetic differences and heritability of antibody response to *Escherichia coli* vaccination in young broiler chicks. *Poult. Sci.* 75:683-690.



- Yunis, R., E. D. Heller, J. Hillel, and A. Cahaner. 2002. Microsatellite markers associated with quantitative trait loci controlling antibody response to *Escherichia coli* and *Salmonella enteritidis* in young broilers. *Anim. Genet.* 33:407-414.
- Zhao, S., J. J. Maurer, S. Hubert, J. F. De Villena, P. F. McDermott, J. Meng, S. Ayers, L. English, and D. G. White. 2005. Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. *Vet. Microbiol.* 107:215-224.
- Zhao, L., S. Gao, H. Huan, X. Xu, X. Zhu, W. Yang, Q. Gao, and X. Liu. 2009. Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model. *Microbiology.* 155:1634-1644.
- Zhou, Q., Q. Gu, X. Y. Hu, D. H. Wang, X. M. Li, S. Q. Zhou, and G. F. Cheng. 2007. Role of interleukin-6 in the pathogenesis of an avian model of *Staphylococcus aureus* arthritis. *Poult. Sci.* 86:1245-1250.

## CHAPTER 2. SPLEEN TRANSCRIPTOME RESPONSE TO INFECTION WITH AVIAN PATHOGENIC *ESCHERICHIA COLI* IN BROILER CHICKENS

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### Abstract

#### Background

Avian pathogenic *Escherichia coli* (APEC) is detrimental to poultry health and its zoonotic potential is a food safety concern. Regulation of antimicrobials in food-production animals has put greater focus on enhancing host resistance to bacterial infections through genetics. To better define effective mechanism of host resistance, global gene expression in the spleen of chickens, harvested at two times post-infection (PI) with APEC, was measured using microarray technology, in a design that will enable investigation of effects of vaccination, challenge, and pathology level.

#### Results

There were 1,101 genes significantly differentially expressed between severely infected and non-infected groups on day 1 PI and 1,723 on day 5 PI. Very little difference

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was seen between mildly infected and non-infected groups on either time point. Between birds exhibiting mild and severe pathology, there were 2 significantly differentially expressed genes on day 1 PI and 799 on day 5 PI. Groups with greater pathology had more genes with increased expression than decreased expression levels. Several predominate immune pathways, Toll-like receptor, Jak-STAT, and cytokine signaling, were represented between challenged and non-challenged groups. Vaccination had, surprisingly, no detectable effect on gene expression, although it significantly protected the birds from observable gross lesions. Functional characterization of significantly expressed genes revealed unique gene ontology classifications during each time point, with many unique to a particular treatment or class contrast.

## **Conclusions**

More severe pathology caused by APEC infection was associated with a high level of gene expression differences and increase in gene expression levels. Many of the significantly differentially expressed genes were unique to a particular treatment, pathology level or time point. The present study not only investigates the transcriptomic regulations of APEC infection, but also the degree of pathology associated with that infection. This study will allow for greater discovery into host mechanisms for disease resistance, providing targets for marker assisted selection and advanced drug development.

## **Background**

Maintaining proper food-animal health is important from an animal welfare, animal production and food safety standpoint. Avian pathogenic *Escherichia coli* (APEC) are a group of extraintestinal *E. coli* that commonly infect poultry. Infection can lead to colibacillosis, a disease that can be localized or systemic, with the more acute and serious

forms ending in septicemia and death (Barnes et al., 2008; as reviewed by Lutful Kabir, 2010). Colibacillosis is one of the most frequent diseases in poultry resulting in mortality losses at all stages of life and decreased production efficiency in older birds (Barnes et al., 2008; Russell, 2003). Mortality, decreased production and condemnation of contaminated product may result in significant economic losses to the poultry industry worldwide (Barnes et al., 2008; Bisaiillon et al., 1988; as reviewed by Lutful Kabir, 2010; Sams, 2001).

APEC can enter the food supply through contaminated eggs and meat (Johnson et al., 2009; as reviewed by Lutful Kabir, 2010; Sams, 2001), generating a path for human exposure. APEC has zoonotic potential, demonstrated by common virulence factors (Moulin-Schouleur et al., 2006; Rodriguez-Siek et al., 2005), genome similarities to human extraintestinal *E. coli* (Johnson et al., 2007), and capacity to cause disease in animal models (Tivendale et al., 2010). As such, APEC has been implicated as a possible source of *E. coli* responsible for urinary tract infections and meningitis in humans (Ewers et al., 2007; Rodriguez-Siek et al., 2005).

There is increasing pressure to reduce antimicrobial usage in livestock production. Other methods to maintain healthy, disease-free populations, such as enhanced host genetic resistance, have become a topic of great interest. Previous research has demonstrated the potential for genetic resistance to disease in poultry (Kramer et al., 2003; Schou et al., 2003). Greater understanding of host response to infection and resulting pathology will allow researchers to identify the genes that best convey protection.

The spleen is involved in both the humoral and cellular immune responses through its role in the generation, maturation and storage of lymphocytes (Jeurissen, 1993; Smith and Hunt, 2004). Gene expression in the chicken spleen is commonly used as an indicator of

immune response (Lu et al., 2009; Redmond et al., 2010). Here, we characterize the differences in splenic gene expression profiles between birds with mild and severe pathology, and of differing vaccination status over two time points in order to identify host traits that are associated with colibacillosis resistance.

## **Methods**

### **Animals**

In six replicates, 120 male non-vaccinated commercial broiler chicks were used at 1 day of age from a local hatchery (total n = 720). Birds were reared on wire-floored cages with *ad libitum* access to food and water, and a 22:2 hour light:dark cycle for the first 15 days and a 16:8 hour cycle thereafter. Half of the chicks were vaccinated with the increased serum survival protein (Iss) vaccine (Lynne et al., 2006), given intramuscularly, at a dose of 0.5ml/bird, containing 2 $\mu$ g of vaccine and 50 $\mu$ g of Quil A adjuvant in phosphate buffered saline (PBS) at 2 weeks of age. Non-vaccinated birds received 50 $\mu$ g of Quil A adjuvant in 0.5ml PBS at 2 weeks of age via the same route. The Iss vaccine is generated from a recombinant Iss protein fused to glutathione S-transferase (GST). *iss* is a gene encoding the increased serum survival outer membrane protein that is common in many APEC serotypes (Johnson et al., 2006). At 4 weeks of age, chicks were challenged with 0.1ml containing 10<sup>8</sup> colony forming units of APEC O1, or PBS, by the intra-air sac route into the left thoracic air sac. Birds were euthanized and necropsied at 2 time points, 1 day and 5 days post-challenge, using half of the birds on each day. All animal research protocols were approved by the Iowa State University Institutional Animal Care and Use Committee.

### **Lesion scoring**

At necropsy, lesions were characterized and scores assigned for 3 internal tissues, air sacs, pericardium and liver, as described by Peighambari et al. (2000). Air sacs had a score range of 0 to 3, pericardium and liver had a score range of 0 to 2. A total lesion score was generated from the summation of lesion scores from each of the 3 tissues, with scores ranging from 0 to 7. Within each experimental replicate, the birds with the highest and lowest total lesion scores were designated as having mild or severe lesions. Birds with mild pathology had scores ranging from 0 to 2 with an average of 0.375 while those with severe pathology had scores ranging from 4 to 7 with an average of 6.125. Birds from the vaccinated, challenged group were not further subdivided by pathology and a bird with an average total lesion score for that day and experimental replicate were selected for analysis. Treatment groups are abbreviated by vaccination status (V for vaccinated or NV for non-vaccinated), challenge status (C for challenged or NC for non-challenged), day of necropsy (D1 for 1 day post challenge or D5 for 5 days post challenge), and, where utilized, pathology (M for mild or S for severe).

### **Bacteria**

APEC O1 strain (O1:K1:H7) was previously isolated from the lung of a turkey that died due to colisepticemia (NCBI Reference Sequence: NC\_008563.1). It has been fully characterized and its genomic sequence is the only total APEC sequence presently in the public domain (Johnson et al., 2007). It was stored in Brain Heart Infusion (BHI) broth with 10% glycerol at -80°C. Two days prior to bacterial challenge, APEC O1 culture was streaked on Luria Bertani (LB) agar and incubated overnight at 37°C. After incubation, 10ml of LB broth was inoculated with an isolated colony of APEC O1 from LB agar and incubated overnight at 37°C with shaking. On the day of challenge, the bacteria were pelleted by

centrifugation at 5000g for 15 minutes and the bacterial pellet washed 3 times with PBS.

Bacteria were enumerated based on spectrometric reading at 600 nm, then the inoculum was adjusted to the desired bacterial concentration in PBS. Counts were confirmed through serial dilution plating of the inocula onto MacConkey agar overnight.

### **Splenic RNA isolation**

One sample from each of the 10 treatment groups, over 4 replicates, was selected for RNA isolation and microarray analysis. Spleen tissue was removed, diced and placed into 3.5mL of RNeasy lysis buffer (Qiagen, 69903). Tissues were kept at 4°C for 7 days, then excess RNeasy lysis buffer was poured off, tissue transferred to 1.5mL tube and stored in -80°C freezer. Isolation of RNA from spleen samples was performed using the Ambion MagMAX-96 for Microarrays Kit (AM1839) (Applied Biosystems, Foster City, CA). For each sample, 25 to 30mg of tissue was placed into 600µL of TRI Reagent Solution (Ambion). Tissues were then homogenized in the TRI Reagent. Following homogenization, 300µL of homogenate was processed according to manufacturer's instructions, using the Spin Procedure. Total RNA was eluted using 50µL of Elution Buffer and stored at -80°C. Quality and quantity of total RNA was assessed by Nanodrop (Thermo Scientific) and agarose gel electrophoresis. Across all 40 samples, the average 260/280 ratio was 2.06 with a standard deviation of 0.055. For 12 random samples, RNA Integrity Numbers were measured using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). An average RIN of 9.21 was achieved across all 12 samples.

### **Microarray experiments**

Four hundred nanograms of RNA was reverse transcribed into cDNA. During reverse transcription, a T7 promoter primer region was included into the cDNA. The cDNA

was then transcribed back into cRNA, using T7 RNA polymerase, labeled with either Cy3 or Cy5 dye. The labeled cRNA was then purified using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Labeled samples were assessed by Nanodrop for sufficient quantity and a minimum specificity of 8, where specificity is the concentration of the dye (pmol/ $\mu$ g) times 1000 divided by the concentration of the cRNA (ng/ $\mu$ L).

Each labeled dye (825ng) was hybridized to Agilent 4x44 Chicken Microarray (Li et al., 2008) for 17 hours at 65°C. Samples were arranged in a reference design, using the NV-NC-D1 sample from each experimental replicate as the reference to which all other samples within the replicate were hybridized. After hybridization, slides were washed using commercial Agilent Wash Buffer and Stabilization and Drying Buffer (Agilent Technologies, Santa Clara, CA) and scanned using GenePix 4100A scanner (Molecular Devices Inc., Sunnyvale, CA)

### **Microarray analysis**

Median signal intensities for each spot were background-corrected and log-transformed. The Locally Weighted Scatterplot Smoothing (LOWESS) procedure was used to correct the intensity-dependent dye bias for each 2-color array (Dudoit et al., 2002). All technical control spots and any genes exhibiting an average signal to noise ratio (SNR) less than 3 over all 36 arrays were removed from analysis, where SNR is calculated as (median foreground – median background)/background SD for each dye. Likelihood ratio tests were conducted in R to determine the necessity of including random effects of array position, slide and experimental replicate in the model. The results of these tests showed no evidence of the presence of array position or slide effects and therefore only experimental replicate was included as a random effect. Treatment means were parameterized (Table 1) and estimated



by fitting a linear mixed model to the difference of normalized signal intensities between the Cy3 and Cy5 channels for each array. The fixed effects of the linear mixed model include the effects of challenge, vaccination, severity (mild or severe), time point and interactions among them using the parameterization shown in Table 1. *P* values were obtained for all contrasts of interest and converted to q-values for false discovery rate control using the R package q-value that implements the method proposed by Storey and Tibshirani (2003). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (Barrett et al., 2009; Edgar et al., 2002) and are accessible through GEO Platform accession number GPL6413 and Series accession number GSE25511 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25511>).

Functional analysis of biological processes category was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b). Lists of significant genes were analyzed against the background of all 24,851 genes included for further study.

### **Quantitative PCR**

Ten genes, *interleukin 1 beta*, *interleukin 6*, *avian beta-defensin 2*, *avian beta-defensin 6*, *avian beta-defensin 7*, *interferon gamma*, *toll-like receptor 2-type I*, *toll-like receptor 4*, *myeloid differentiation protein 2*, and *interleukin receptor 1-type II* were utilized to validate microarray results. Genes were selected based on their functions in immune response and significance within microarray results. Primer sequences are listed in Additional File 1. New primer sequences were designed using sequences from NCBI and PRIMER3 (Rozen and Skaletsky, 2000). Individual spleen samples were run in triplicate, with each triplicate randomly distributed on the 96-well plate. RNA was quantified using

QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA) as previously described by Redmond et al. (2010). Cycle threshold (Ct) values were recorded for each sample. Ct values were adjusted for starting concentration and reaction efficiency using the formula:  $40 - (\text{Sample Mean Ct Target} + (\text{Median 28S} - \text{Sample Mean 28S}) * (\text{Slope Target} / \text{Slope 28S}))$ . Values were analyzed using the Fit Model procedure in JMP software (SAS Institute Inc., Cary, NC). Contrasts with significant differential expression in microarray analysis had fold change compared to qPCR results.

## Results

### Microarray

Ten groups were generated by dividing birds into vaccinated (V) and non-vaccinated (NV), challenged (C) and non-challenged (NC), sampled on 1 (D1) or 5 (D5) days post challenge, and the non-vaccinated challenged birds on both days were subdivided into mild (M) and severe (S) pathology (Fig. 1). Birds were assigned to vaccination, challenge and time post-harvest groups *a priori*, and to pathology severity groups *a posteriori*. Samples from 40 individual birds (four biological replicates from each of 10 treatment groups) were used for gene expression study. After the removal of genes with low signal to noise ratio, 24,851 genes were included for further analysis. There was no detectable vaccination effect on spleen gene expression levels. Vaccination status had, however, a significant impact on total lesion score on day 1 (Fig. 2a) and day 5 (Fig. 2b) ( $P$  value  $< 0.001$ ), reducing the total lesions observed in vaccinated birds. The number of significantly differentially expressed (DE) genes ( $q$ -value  $< 0.05$ ) between treatment groups and NV-NC control groups were analyzed (Fig. 3a). There were a large number of DE genes between NV-C severe and NV-NC control groups on day 1 ( $n=1,101$ ) and day 5 ( $n=1,723$ ). There were few DE genes

between NV-C mild and NV-NC control groups on day 1 (n=29) and (n=0) on day 5. The change in gene expression over the two post-infection days was analyzed within each treatment group (Fig. 3b). The NV-C severe group showed the most difference between the two days with 248 DE genes. The differences between the two pathologies, NV-C mild and NV-C severe, were analyzed (Fig. 3c). More DE genes were seen on day 5 (n=799) between pathologies than on day 1, (n=2).

Direction and degree of difference between treatment groups was analyzed (Fig. 4). Given a threshold of a minimum fold change of 1.5, there was a greater number of up-regulated genes due to increased pathology than the number of down-regulated genes. Visual comparison between multiple contrasts was generated. When comparing the change over time for all treatment groups, the NV-C mild pathology group clustered with both the NV-NC and V-NC groups; the NV-C severe pathology group and the V-C group clustered together (Fig. 5). When comparing each treatment group to the NV-NC control group on each day, the NV-C mild group on day 5 clustered with the V-NC groups, while the NV-C mild group on day 1 clustered with the challenged groups (Fig. 6).

### **Gene ontology analysis**

All contrasts in which there were sufficient DE genes (n>29) were further analyzed for common biological processes (BP). For GO analysis, seven contrasts had significantly enriched BP terms. There were 156 unique BP terms, of which 95 were uniquely represented once across all 7 contrasts (See Additional Files 2, 3, 4, 5, 6, 7, 8). Many of the repeated biological process terms focused on white blood cell regulation along with defense/immune response to bacteria, and metabolic processes. The groups with the highest number of DE genes had the most biological terms common to these genes (Table 2). In contrasting NV-C

severe and NV-NC control groups, on day 1 PI many biological processes were centered around immune and defense response, while day 5 PI heavily focused on regulation of white blood cells. The two most influenced KEGG pathways were the Jak-STAT signaling pathway and the cytokine-cytokine receptor interaction pathway, which occurred in the contrast between NC-C severe and NV-NC control on day 5 and in the contrast between NV-C severe and NV-C mild on day 5.

### **Quantitative PCR validation**

Quantitative real time PCR (qPCR) was carried out on ten significantly DE genes, using a subset of the same RNA samples used for microarray analysis, to confirm the results seen on the microarray. Samples from vaccinated groups were not included in qPCR analysis due to no DE genes from vaccination effect, allowing analysis of 24 samples (4 replicates from 6 treatment groups). A normalizer gene, ribosomal 28S, was used to correct for starting template amount. Results from qPCR validation show general agreement with microarray results in direction of expression and significance (Table 3).

## **Discussion**

### **Experimental design**

This was a novel experimental design, allowing a contrast not only between challenged and non-challenged individuals, but also varying degrees of pathology. The large number of birds allotted into the challenged groups allowed the identification of a sufficient spread of lesion scores to separate pathology groups (Fig. 1). These commercial birds were raised in a homogenous environment and exhibited a large spread in pathological response, suggesting that a mechanism other than environment, genetic variation, is responsible for the resistant or susceptible phenotypes and is available to select upon.

The common reference design was selected for this microarray experiment. Although there are loop designs that could gain some efficiency for some contrasts, the efficiency gain of could be eliminated in the instance of a loss of a microarray slide. This is because a rather big loop would be needed for our study of 10 treatment groups and the design of the microarray slide with 4 arrays per 1 slide would result in a large loss of data if a slide were to fail. However, reference design is very robust in this sense. Statistical calculation also shows that the reference design using the most naïve group, the NC-NV-D1 group, as the reference provides comparable variance estimates for our contrasts of interest with the loop design. The common reference design was suggested by Dobbin and Simon (2002) because it produces better results than does a loop design for multiple comparisons and clustering analysis, both of which were applied in data analysis.

### **Differential gene expression**

Surprisingly, there was no significant vaccination effect on gene expression in the spleen. The vaccine, however, was clearly effective in protecting the chicks, as evidenced by the lesion scores. There are several variables that could contribute to no detection of DE genes in response to vaccination: statistical power, the tissue examined or the timeframe selected, in regard to age vaccinated, age challenged, and time points tissues were collected. Fold change calculations of these non-significant genes reveal few changes of 2 fold or greater, suggesting that inter-animal variation was not responsible for lack of detection of differential expression. The spleen is an important immunological organ in the chicken and has been successfully utilized to detect differences in cytokine gene expression after immunization Marek's disease vaccines and DNA vaccines (Abdul-Careem et al., 2007; Abdul-Careem et al., 2008; Gimeno and Cortes, 2011; Kano et al., 2009; Song et al., 2010).

The selection of vaccination at 2 weeks of age has also been successfully utilized to detect splenic expression differences (Abdul-Careem et al., 2008), with several other vaccination times used successfully: day 18 of incubation, 1 day of age, and 2 weeks of age with a 4 week booster (Abdul-Careem et al., 2007; Gimeno and Cortes, 2011; Kano et al., 2009; Song et al., 2010). The time period selected between immunization and sampling can greatly impact the ability to detect expression differences, with later sampling times, 10 days post vaccination, showing reduced expression differences compared to earlier sampling times (Abdul-Careem et al., 2008; Gimeno and Cortes, 2011), though detection 26 days post vaccination is still possible (Kano et al., 2009). It is likely that the selection of 1 and 5 days post challenge, 15 and 20 days after initial vaccination, was not optimal for expression discovery in the spleen utilizing an Iss vaccine.

There were more DE genes in the NV-C severe group than the V-C group on both days when contrasted against NV-NC control. Analysis of host-pathogen interaction genes (Kano et al., 2009) and cytokines (Abdul-Careem et al., 2007) in response to Marek's Disease virus challenge revealed more DE genes in the spleens of non-vaccinated birds than vaccinated. This trend was again seen in birds challenged with *Salmonella* Enteritidis in the cecum of vaccinated and non-vaccinated birds (Carvajal et al., 2008). Non-vaccinated birds that receive a pathogen challenge may rely more on significant changes in gene regulation to fight off bacteria than vaccinated birds. The greater amount of DE genes detected may also be attributed to the selection of average lesioned birds for representatives of the V-C group, though the number of DE genes is still a small fraction of that generated by the severe group.

Effect of APEC challenge on splenic gene expression varied with level of pathology. The contrasts in which there were the largest numbers of DE genes involved the NV-C

severe group. Severe pathology may produce the largest changes in gene expression levels making them easier to detect, as observed with serum cytokine levels (Sevimli et al., 2008). The NV-C mild group varied little from the NV-NC control, suggesting that the bacteria may have been cleared and the bird returned to homeostasis by day 5, while still exhibiting a small response on day 1. In contrast, the number of DE genes increased from day 1 to day 5 in the NV-C severe group compared to the NV-NC control. Past studies utilizing *Salmonella* and two distinct genetic populations illustrated differences in gene expression patterns between mild and severe pathologies unique to a genetic background (Zhou and Lamont, 2007), demonstrating the importance of assessing responses in lines of interest, such as a commercial line used in this study, for potential application. Commercial type birds, broiler chickens and broiler x Leghorn chickens, noted the same increase in gene expression over time under severe pathology, while chickens with a more robust chicken background, broiler x Fayoumi, had fewer DE genes that decreased over time under severe pathology (Zhou and Lamont, 2007). Due to the clear difference in number of DE genes seen between either NV-C mild or NV-C severe and the NV-NC control group, it is unusual that there is not a higher number of DE genes detected between the NV-C mild and NV-C severe groups on day 1. Levels of serum amyloid A and cytokines showed a linear trend between control, mild, moderate and severe conditions (Sevimli et al., 2008). This suggests that this is the trend for splenic gene expression level between NV-NC control, NV-C mild and NV-C severe groups. This intermediate expression could make it more difficult to detect expression differences, while the large expression level changes in the NV-C severe group make it easy to detect them against NV-NC control.

At both tissue harvest times, more genes increased expression in challenged groups compared to NV-NC control groups (55-78%) and more increased in severe compared to mild pathology group on day 5 (66%). Bacterial challenge has been demonstrated to cause more gene induction than repression (Zhou and Lamont, 2007), particularly within cytokines (Brisbin et al., 2010). Direction stayed consistent for genes that were significant on both days. Of the genes that were differentially expressed in NV-C severe compared to NV-NC control on both days, 385 of 387 were in the same direction of regulation and all 6 DE genes on both days in the V-C groups were in the same direction of regulation.

In generation of the heatmaps, genes included were largely driven by the NV-C severe group, requiring a minimum q-value of 0.05 across all contrasts included. Comparing the change over time for each treatment group (Fig. 5), groups clustered as expected based on earlier contrast comparisons. The NV-C mild pathology group showed little difference compared to NV-NC control and also showed more similar expression pattern changes to the V-NC group than to the NV-C severe and V-C group. As expected, both the NV-NC and the V-NC groups exhibit minimal changes of 2 fold or greater, further illustrating the lack of expression changes over time without a pathogen stimulus. When comparing each treatment with the NV-NC control (Fig. 6), the NV-C mild group on day 1 was most similar to the other challenged groups, while on day 5 it clustered with non-challenged groups, supporting the hypothesis of returning to homeostasis, or a non-challenged state, by day 5. Contrasts that represented the same day post challenged clustered together, demonstrating strong similarities in expression changes over time among groups.

### **Gene ontology investigation**



The advantage of utilizing a global microarray is the potential to investigate and discover a wide range of gene ontology (GO) terms hidden within the dataset. Although it is difficult to differentiate cause and effect in gene expression in a pathogen challenge, functional terms enriched in the biological processes may grant some insights. Terms related to immune response, regulation of immune related cells, and metabolic processes commonly appeared in contrasts involving the NV-C severe group on both days or V-C group on day 1, consistent with bacterial infection as detected in multiple tissues (Lan et al., 2010; Li et al., 2010; Tsai et al., 2010; Zhou et al., 2009).

The NV-C severe group on day 1 had many terms related to response to bacteria, inflammation and circulatory processes, along with a few receptor signaling terms. Particular genes among these groups included the avian beta-defensins, known peptides with antimicrobial activity that have demonstrated induction patterns in various tissues in response to *E.coli* derived lipopolysaccharide and *Salmonella* (Meade et al., 2009; Milona et al., 2007; Subedi et al., 2007). Toll-like receptors (TLR) recognize conserved molecular patterns common to many pathogens. Changes in TLR expression, in response to bacterial infection, have also been demonstrated (Lu et al., 2009; Abasht et al., 2009). Induction of pro-inflammatory response after pathogen challenge is common (Jarosinski et al., 2005). The changes seen in inflammation and circulatory processes may be responsible for observed lesion phenotype.

At day 5, many of the terms found in the NV-C severe group had changed, focusing more on regulation of white blood cells, localization and transport, with lesser emphasis on metabolic and biosynthetic processes than day 1. The severe status of these birds may be attributed to a slower response by these defense mechanisms. Cytokines and chemokines

help signal white blood cells and attract them to sites of infection. Many cytokines have reported expression differences in less than 5 days post infection (Kim et al., 2008; Zhou et al., 2009). The main detectable differences between NV-C mild and NV-C severe groups on day 5 involved response mechanism and regulation of WBC. The Jak-STAT and cytokine-cytokine receptor interaction pathways have several overlapping elements, 6 and 9 overlapping elements in contrasts of NV-C severe vs. NV-C mild on day 5 and NV-C severe vs. NV-NC control on day 5 respectively. Bacterial infection has previously noted changes in the Jak-STAT pathway in granulosa cells (Tsai et al., 2010) and cecal tissue (Li et al., 2010). Changes in genes in these pathways have also been discovered in spleen after *Clostridium* infection: signal transducer and activator of transcription, growth factor receptor-bound protein, cytokines and cytokine receptor genes (Zhou et al., 2009).

The low number of DE genes found between several contrasts of interest limited GO analysis. Only one biological process term was significantly enriched in two contrasts examined: intracellular signaling cascade, between NV-C mild and NV-NC control groups on day 1 and transcription, between V-C and NV-NC control on day 5. As with many current microarrays, annotation has limited the extent of GO analysis, illustrating the urgent need to increase our knowledge in gene functions of genome sequences which have been discovered (van den Berg et al., 2010).

## Conclusions

There is a large difference in splenic transcriptome profiles between birds with mild and severe lesions in response to APEC infection, revealing gene networks potentially associated with disease resistance. The response of birds with severe lesions is much larger, both in magnitude and number of differentially expressed genes, than birds with mild lesions.

Time post-challenge with APEC also resulted in significant differences in gene expression. Few differences were detected between the NV-C mild group and NV-NC control at day 1 and zero at day 5, suggesting that immune response to APEC was very rapid, occurring before day 1 sampling, or involved few detectable gene expression changes. Vaccination generated an efficacious protective effect, but no expression differences were detected at 2 weeks post vaccination. The gene ontology terms found within uniquely differentially expressed genes of birds with severe lesions helped provide insight into what genes are different as well as the overall processes defined by those genes. Changes in the Jak-STAT pathway and cytokine-cytokine receptor signaling highlight the importance of proper signaling cascades to fight infection. The results from this study add greater depth to the knowledge base about chicken host response to APEC.

### **Authors' contributions**

ES participated in necropsy, processed tissues, performed microarray experiments, analyzed data, conducted qPCR validation and drafted the manuscript. MO participated in the development of the microarray experimental design and assisted in data analysis. EB participated in qPCR validation. NB participated in qPCR validation. XL mentored and supervised microarray experiments. HZ provided equipment and annotation files for microarray experiments. TJ participated in the design of the study and necropsy. SK participated in the design of the study, purified Iss-GST fusion protein, and oversaw animal experiments (prepared Institutional Animal Care and Use Committee protocols and, performed vaccination, bacterial challenge and necropsy). PL participated in design of the study, necropsy and data analysis. LN participated in the design of the study, necropsy and oversaw animal experiments and bacterial challenge. SL participated in the design of the

study, necropsy and interpretation of the results. All authors read, edited and approved the final manuscript.

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## References

- Abasht, B., M. Kaiser, J. van der Poel, and S. J. Lamont. 2009. Genetic lines differ in Toll-like receptor gene expression in spleens of chicks inoculated with *Salmonella enterica* serovar Enteritidis. *Poult. Sci.* 88:744-749.
- Abdul-Careem, M. F., B. D. Hunter, M. D. Lambourne, L. R. Read, P. Parvizi, and S. Sharif. 2008. Expression of cytokine genes following pre- and post-hatch immunization of chickens with herpesvirus of turkeys. *Vaccine.* 26:2369-2377.
- Abdul-Careem, M. F., B. D. Hunter, P. Parvizi, H. R. Haghighi, N. Thanthrige-Don, and S. Sharif. 2007. Cytokine gene expression patterns associated with immunization against Marek’s disease in chickens. *Vaccine.* 25:424-432.

- Barnes, H. J., L. K. Nolan, and J. P. Vaillancourt. 2008. Colibacillosis, in Y. M. Saif (ed.), Diseases of Poultry. 12th ed. p.691-732. Blackwell Publ.
- Barrett, T., D. B. Troup, S. E. Wilhite, R. Ledoux, D. Rudnev, C. Evangelista, I. F. Kim, A. Soboleva, M. Tomashevsky, K. A. Marshall, K. H. Phillippy, P. M. Sherman, R. N. Muertter, and R. Edgar. 2009. NCBI GEO: archive for high-throughput functional genomic data. Nucleic Acids Res. 37:D5-D15.
- Bisaillon, J. R., A. H. Meek and T. E. Feltmate. 1988. An assessment of condemnations of broiler chicken carcasses. Can. J. Vet. Res. 52:269-276.
- Brisbin, J. T., J. Gong, P. Parvizi, and S. Sharif. 2010. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin. Vaccine Immunol. 17:1337-1343.
- Carvajal, B. G., U. Methner, J. Pieper, and A. Berndt. 2008. Effects of *Salmonella enterica* serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens. Vaccine. 26:5423-5433.
- Chiang, H. I., C. L. Swaggerty, M. H. Kogut, S. E. Dowd, X. Li, I. Y. Pevzner and H. Zhou. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44K Agilent microarray. BMC Genomics. 9:526.
- Dobbin, K., and R. Smith. 2002. Comparison of microarray designs for class comparison and discovery. Bioinformatics. 18:1438-1445.
- Dudoit, S., Y. H. Yang, M. J. Callow, and T. P. Speed. 2002. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Statistica Sinica. 12:111-139.

- Edgar, R., M. Domrachev, and A. E. Lash. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30:207-210.
- Ewers, C., G. Li, H. Wilking, S. Kießling, K. Alt, E. M. Antão, C. Laternus, I. Diehl, S. Glodde, T. Homeier, U. Böhnke, H. Steinrück, H. C. Philipp, and L. H. Wieler. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? *Int. J. Med. Microbiol.* 297:163-176.
- Gimeno, I. M., and A. L. Cortes. 2011. Chronological study of cytokine transcription in the spleen and lung of chickens after vaccination with serotype 1 Marek's disease vaccines. *Vaccine.* 29:1583-1594.
- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009a. Bioinformatics enrichment tools: paths towards the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37:1-13.
- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009b. Systemic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nat. Protoc.* 4:44-57.
- Jarosinski, K. W., B. L. Njaa, P. H. O'Connell, and K. A. Schat. 2005. Pro-inflammatory responses in chicken spleen and brain tissues after infection with very virulent plus Marek's disease virus. *Viral Immunol.* 18:148-161.
- Jeurissen, S. H. M. 1993. The role of various compartments in the chicken spleen during an antigen-specific humoral response. *Immunology.* 80:29-33.
- Johnson, T. J., S. Kariyawasam, Y. Wannamuehler, P. Mangiamale, S. J. Johnson, C. Doetkott, J. A. Skyberg, A. M. Lynne, J. R. Johnson, and L. K. Nolan. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong

- similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189:3228-3236.
- Johnson, T. J., C. M. Logue, Y. Wannemuehler, S. Kariyawasam, C. Doetkott, C. DebRoy, D. G. White, and L. K. Nolan. 2009. Examination of the source and extended virulence genotypes of *Escherichia coli* contaminating retail poultry meat. Foodborne Pathog. Dis. 66:657-667.
- Johnson, T. J., K. E. Siek, S. J. Johnson, and L. K. Nolan. 2006. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. J. Bacteriol. 188:745-758.
- Kano, R., S. Konnai, M. Onuma, and K. Ohashi. 2009. Microarray analysis of host immune responses to Marek's disease virus infection in vaccinated chickens. J. Vet. Med. Sci. 71:603-610.
- Kim, D. K., H. S. Lillehoj, Y. H. Hong, D. W. Park, S. J. Lamont, J. Y. Han, and E. P. Lillehoj. 2008. Immune-related gene expression in two B-complex disparate genetically inbred Fayoumi chicken lines following *Eimeria maxima* infection. Poult. Sci. 87:433-443.
- Kramer, J., A. H. Visscher, J. A. Wagenaar, J. B. Cornelissen, and S. H. Jeurissen. 2003. Comparison of natural resistance in seven genetic groups of meat-type chicken. Br. Poult. Sci. 44:577-585.
- Lan, D., C. Tang, M. Li, and H. Yue. 2010. Screening and identification of differentially expressed genes from chickens infected with Newcastle disease virus by suppression subtractive hybridization. Avian Pathol. 39:151-159.

- Li, X., H. I. Chiang, J. Zhu, S. E. Dowd, and H. Zhou. 2008. Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics*. 9:60.
- Li, X., C. L. Swaggerty, M. H. Kogut, H. I. Chiang, Y. Wang, K. J. Genovese, H. He, and H. Zhou. 2010. Gene expression profiling of the local cecal response of genetic chicken lines that differ in their susceptibility to *Campylobacter jejuni* colonization. *PLoS One*. 5:e11827.
- Lu, Y., A. J. Sarson, J. Gong, H. Zhou, W. Zhu, Z. Kang, H. Yu, S. Sharif, and Y. Han. 2009. Expression profiles of genes in Toll-like receptor-mediated signaling of broiler infected with *Clostridium perfringens*. *Clin. Vaccine Immunol*. 16:1639-1647.
- Lutful Kabir, S. M. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int. J. Environ. Res. Public Health*. 7:89-114.
- Lynne, A. M., S. L. Foley and L. K. Nolan. 2006. Immune response to recombinant *Escherichia coli* Iss protein in poultry. *Avian Dis*. 50:273-276.
- Meade, K. G., F. Narciandi, S. Cahalane, C. Reiman, B. Allan, and C. O'Farrelly. 2009. Comparative *in vivo* infection models yield insights on early host immune response to *Campylobacter* in chickens. *Immunogenetics*. 61:101-110.
- Milona, P., C. L. Townes, R. M. Bevan, and J. Hall. 2007. The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars. *Biochem. Biophys. Res. Commun*. 356:169-174.
- Moulin-Schouleur, M., C. Schouler, P. Tailliez, M. R. Kao, A. Bree, P. Germon, E. Oswald, J. Mainil, M. Blanco, and J. Blanco. 2006. Common virulence factors and genetic



- relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. J. Clin. Microbiol. 44:3484-3492.
- Peighambari, S. M., R. J. Julian, and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. Avian Dis. 44:759-769.
- Redmond, S. B., R. M. Tell, D. Coble, C. Mueller, D. Palić, C. B. Andreasen, and S. J. Lamont. 2010. Differential splenic cytokine responses to dietary immune modulation by diverse chicken lines. Poult. Sci. 89:1635-1641.
- Rodrigues-Siek, K. E., C. W. Gidding, C. Doetkott, T. J. Johnson, M. K. Fakhr, and L. K. Nolan. 2005. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology. 151:2097-2110.
- Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biology programmers. Methods Mol. Bio. 132:365-386.
- Russell, S. M. 2003. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors, and populations of *Campylobacter* spp. and *Escherichia coli*. Poult. Sci. 82:1326-1331.
- Sams, A. R. 2001. First processing: Slaughter through chilling, in A. R. Sams (ed.), Poultry Meat Processing. p.19-34. CRC Press.
- Schou, T., A. Permin, A. Roepstorff, P. Sørensen, and J. Kjaer. 2003. Comparative genetic resistance to *Ascaridia galli* infections of 4 different commercial layer-lines. Br. Poult. Sci. 44:182-185.
- Sevimli, A., D. Misirloğlu, A. Yağci, A. Bülbül, A. Yilmaztepe, and K. Altunbas. 2008. The role of chicken IL-1 beta, IL-6 and TNF-alpha in the occurrence of amyloid arthropathy. Vet. Res. Commun. 32:499-508.

- Smith, K. G., and J. L. Hunt. 2004. On the use of spleen mass as a measure of avian immune system strength. *Oecologia*. 138:28-31.
- Song, H., X. Song, L. Xu, R. Yan, M. A. Shah, and X. Li. 2010. Changes of cytokines and IgG antibody in chickens vaccinated with DNA vaccines encoding *Eimeria acervulina* lactate dehydrogenase. *Vet. Parasitol.* 173:219-227.
- Storey, J. D., and R. Tibshirani. 2003. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA*. 100:9440-9445.
- Subedi, K., N. Isobe, M. Nishibori, and Y. Yoshimura. 2007. Changes in the expression of gallinacins, antimicrobial peptides, in ovarian follicles during follicular growth and in response to lipopolysaccharide in laying hens (*Gallus domesticus*). *Reproduction*. 133:127-133.
- Tivendale, K. A., C. M. Logue, S. Kariyawasam, D. Jordan, A. Hussein, G. Li, Y. Wannemuehler, and L. K. Nolan. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. *Infect. Immun.* 78:3412-3419.
- Tsai, H. J., C. H. Chiu, C. L. Wang, and C. H. Chou. 2010. A time-course study of gene responses of chicken granulosa cells to *Salmonella* Enteritidis infection. *Vet. Microbiol.* 144:325-333.
- van den Berg, B. H., F. M McCarthy, S. J. Lamont, and S. C. Burgess. 2010. Re-annotation is an essential step in systems biology modeling of functional genomics data. *PLoS One*. 5:e10642.

Zhou, H., J. Gong, J. Brisbin, H. Yu, A. J. Sarson, W. Si, S. Sharif, and Y. Han. 2009.

Transcriptional profiling analysis of host response to *Clostridium perfringens* infection in broilers. Poult. Sci. 88:1023-1032.

Zhou, H., and S. J. Lamont. 2007. Global gene expression profile after *Salmonella enterica*

Serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. Cytogenet.

Genome Res. 117:131-138.

**Table 1** - Parameterization of treatment groups

Group	Parameterization
NV-NC-D1	$\mu$
NV-NC-D5	$\mu + \tau_1$
NV-C-D1-M	$\mu + \beta$
NV-C-D5-M	$\mu + \beta + \lambda$
NV-C-D1-S	$\mu + \beta + \gamma$
NV-C-D5-S	$\mu + \beta + \lambda + \gamma + \theta$
V-NC-D1	$\mu + \alpha$
V-NC-D5	$\mu + \alpha + \tau_2$
V-C-D1	$\mu + \alpha + \beta + \phi$
V-C-D5	$\mu + \alpha + \beta + \phi + \tau_3$

**Table 2** - Number of GO biological terms found in each contrast

Number of differentially expressed (DE) genes found between contrasts of interest and the number of biological terms found using those genes.

Contrast	DE Genes	Biological Terms
NV-C Mild Day 1 vs. NV-NC Control Day 1	29	2
NV-C Severe Day 1 vs. NV-NC Control Day 1	1101	27
NV-C Severe Day 5 vs. NV-NC Control Day 5	1723	73
V-C Day 1 vs. NV-NC Control Day 1	182	27
V-C Day 5 vs. NV-NC Control Day 5	137	1
NV-C Severe Day 1 vs. NV-C Severe Day 5	248	4
NV-C Severe Day 5 vs. NV-C Mild Day 5	799	63

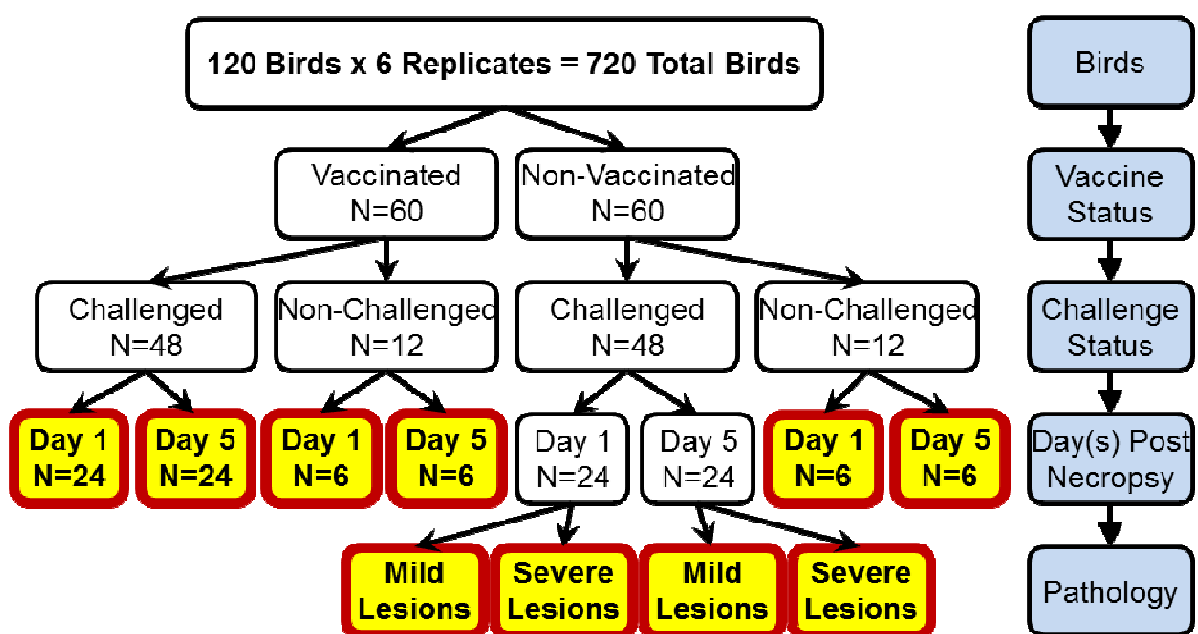
**Table 3** - Quantitative PCR validation

Log2 fold change between contrasts presented. + values indicate higher expression in the first group, - values indicate higher expression in the second group. \*\* *P* value < 0.05 in qPCR, q-value < 0.05 in microarray, \* *P* value < 0.10 in qPCR

Gene	Contrast	qPCR	Microarray
AvBD 2	NV-C Severe Day 5 vs. NV-C Mild Day 5	-3.035*	-2.038**
	NV-C Severe Day 1 vs. NV-NC Control Day 1	2.954**	1.552**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	-3.206**	-1.863**
	NV-C Severe Day 1 vs. NV-C Severe Day 5	5.525**	2.931**
AvBD 6	NV-C Severe Day 1 vs. NV-NC Control Day 1	2.592**	1.719**
	NV-C Severe Day 1 vs. NV-C Severe Day 5	4.726**	2.194**
AvBD 7	NV-C Severe Day 5 vs. NV-C Mild Day 5	-4.259	-3.440**
	NV-C Severe Day 1 vs. NV-NC Control Day 1	1.214	1.731**
	NV-C Severe Day 1 vs. NV-C Severe Day 5	4.446*	3.346**
IL1B	NV-C Mild Day 1 vs. NV-NC Control Day 1	0.567	3.826**
	NV-C Severe Day 1 vs. NV-NC Control Day 1	1.290	1.836**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	2.296	1.960**
IL6	NV-C Severe Day 5 vs. NV-C Mild Day 5	2.263	1.117**
	NV-C Severe Day 1 vs. NV-NC Control Day 1	3.027*	1.227**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	2.611	1.138**
IFNG	NV-C Severe Day 5 vs. NV-C Mild Day 5	2.399**	1.154**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	2.427**	2.000**
TLR 2	NV-C Severe Day 1 vs. NV-NC Control Day 1	-0.070	1.140**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	0.699	0.984**
TLR 4	NV-C Severe Day 5 vs. NV-NC Control Day 5	1.396*	1.336**
MD-2	NV-C Severe Day 5 vs. NV-C Mild Day 5	0.028	5.311**
	NV-C Severe Day 5 vs. NV-NC Control Day 5	0.034	1.230**

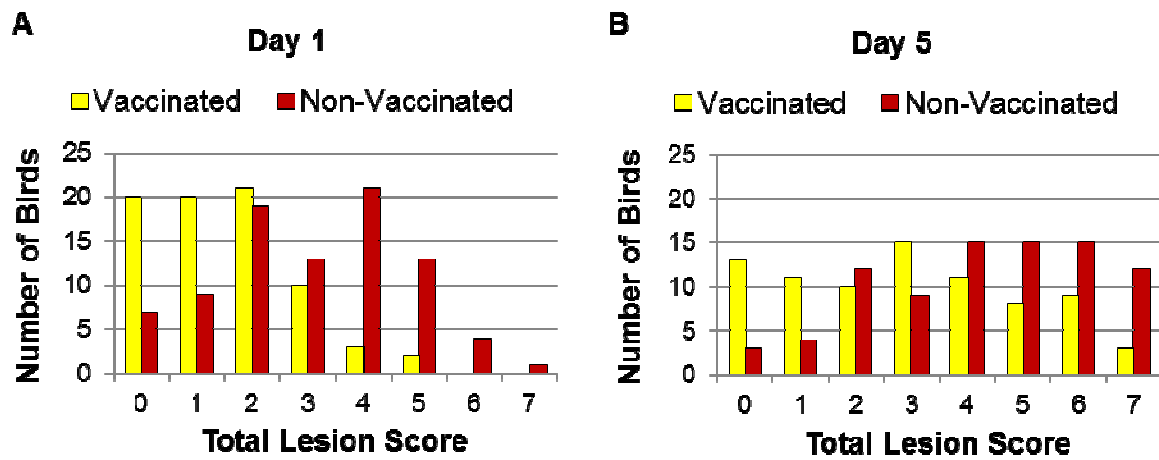
**Table 3** (continued)

<b>Gene</b>	<b>Contrast</b>	<b>qPCR</b>	<b>Microarray</b>
IL1-R, type II	NV-C Severe Day 1 vs. NV-C Mild Day 1	1.574 **	1.263**
	NV-C Severe Day 1 vs. NV-NC Control Day 1	1.241	2.676**



**Figure 1** – Experimental design

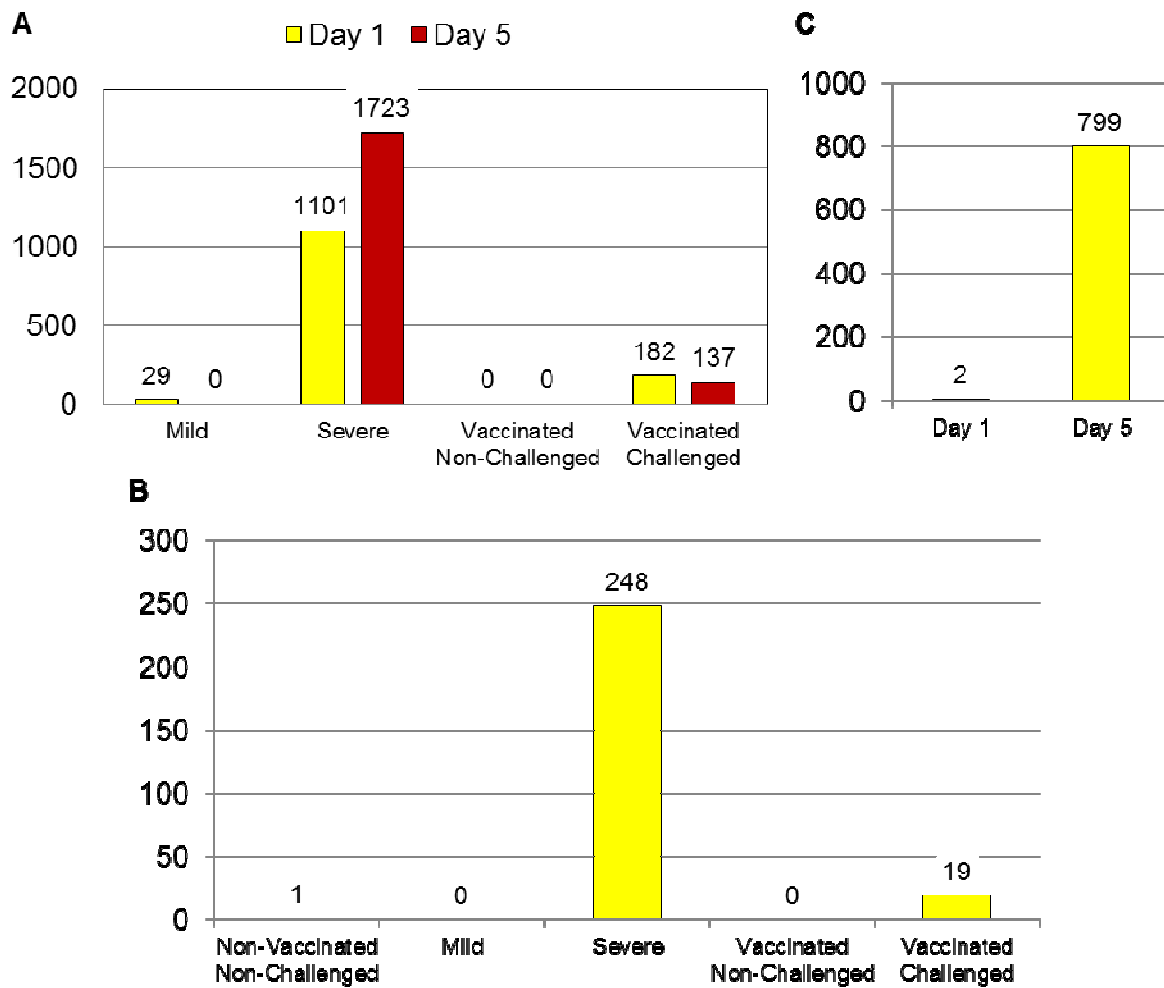
Flow chart of experimental design and 10 treatment groups showing how bird numbers within each replicate were placed into each of the 10 treatment groups



**Figure 2** - Total lesion score distribution

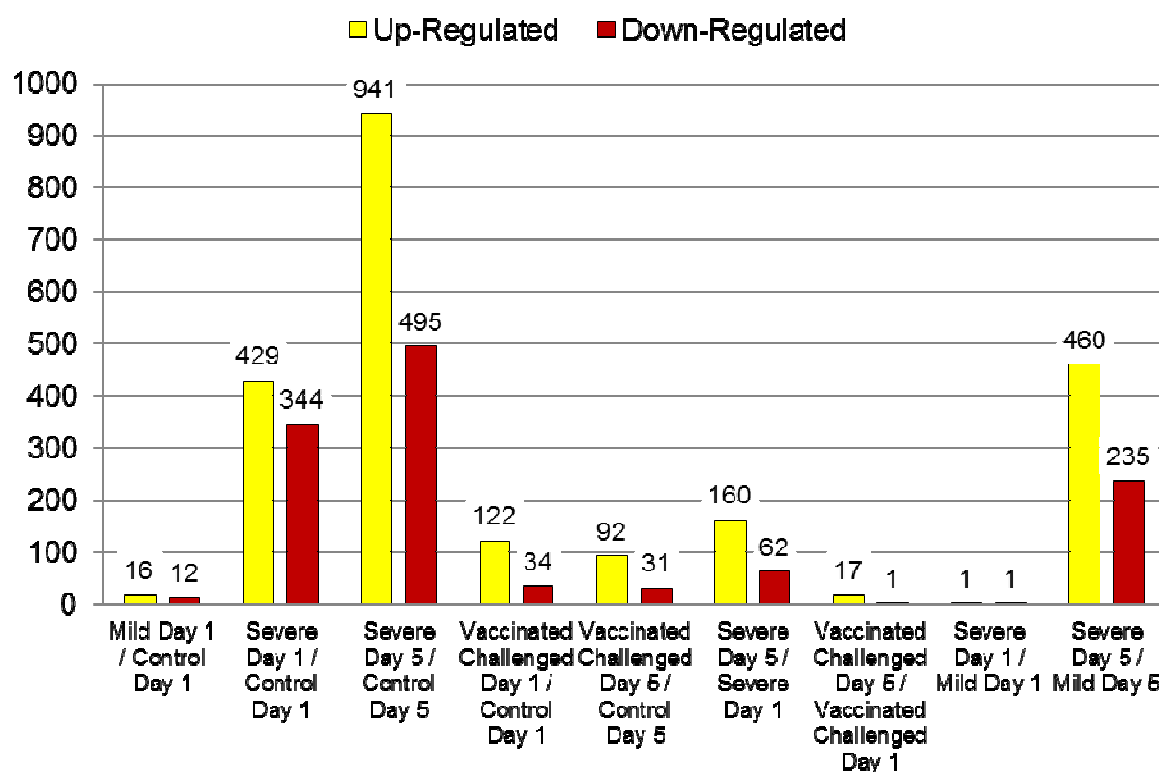
Comparison of the distribution of lesion scores of challenged birds taken on day 1 between birds that received the vaccine and birds that did not receive the vaccine for all 4 replicates on (a) day 1 and (b) day 5 ( $P < 0.001$ )





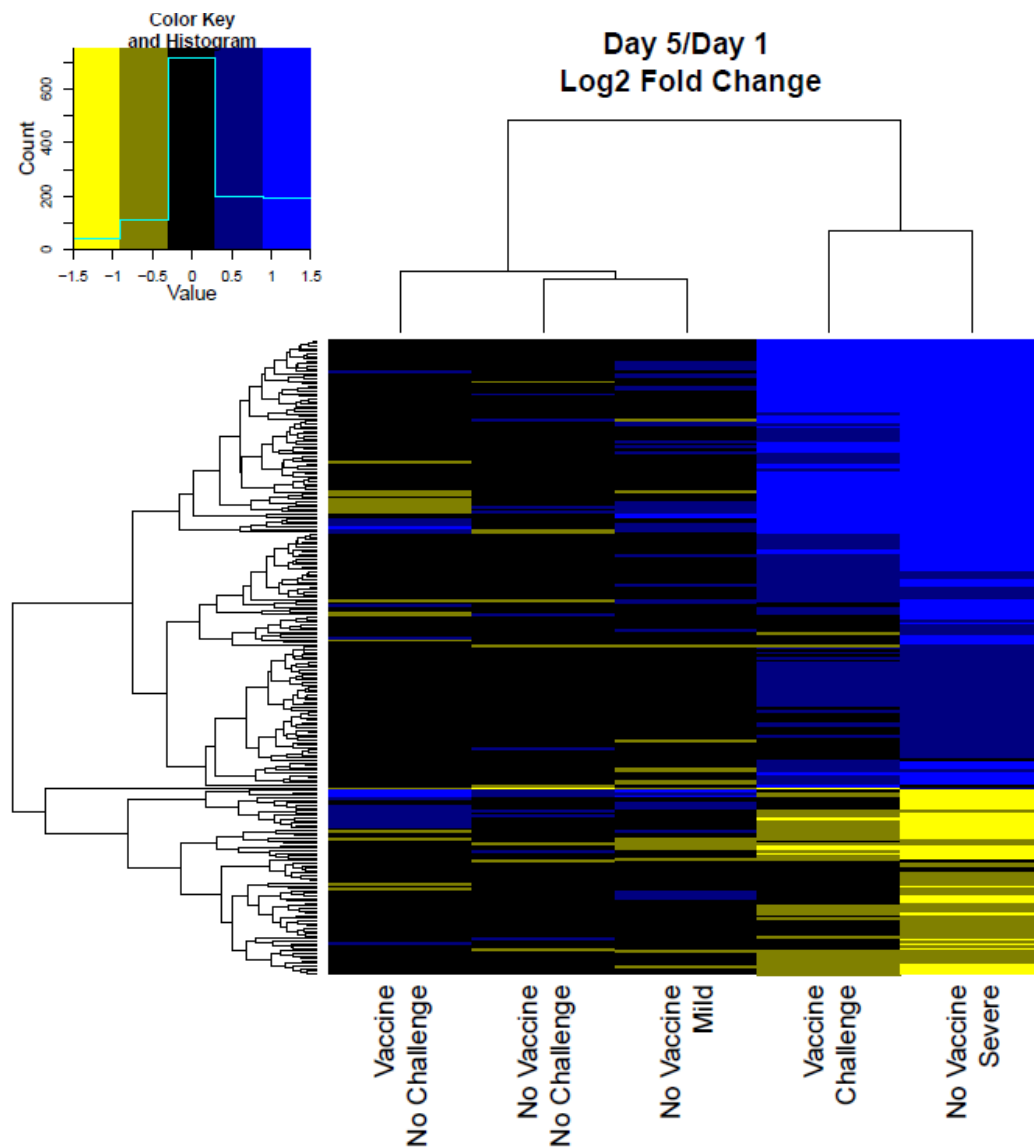
**Figure 3** – Significantly differentially expressed genes for contrasts of interest

Number of significantly differentially expressed genes between (a) treatment and control groups, (b) day 1 and day 5 within treatment, and (c) mild and severe groups (q-value < 0.05)



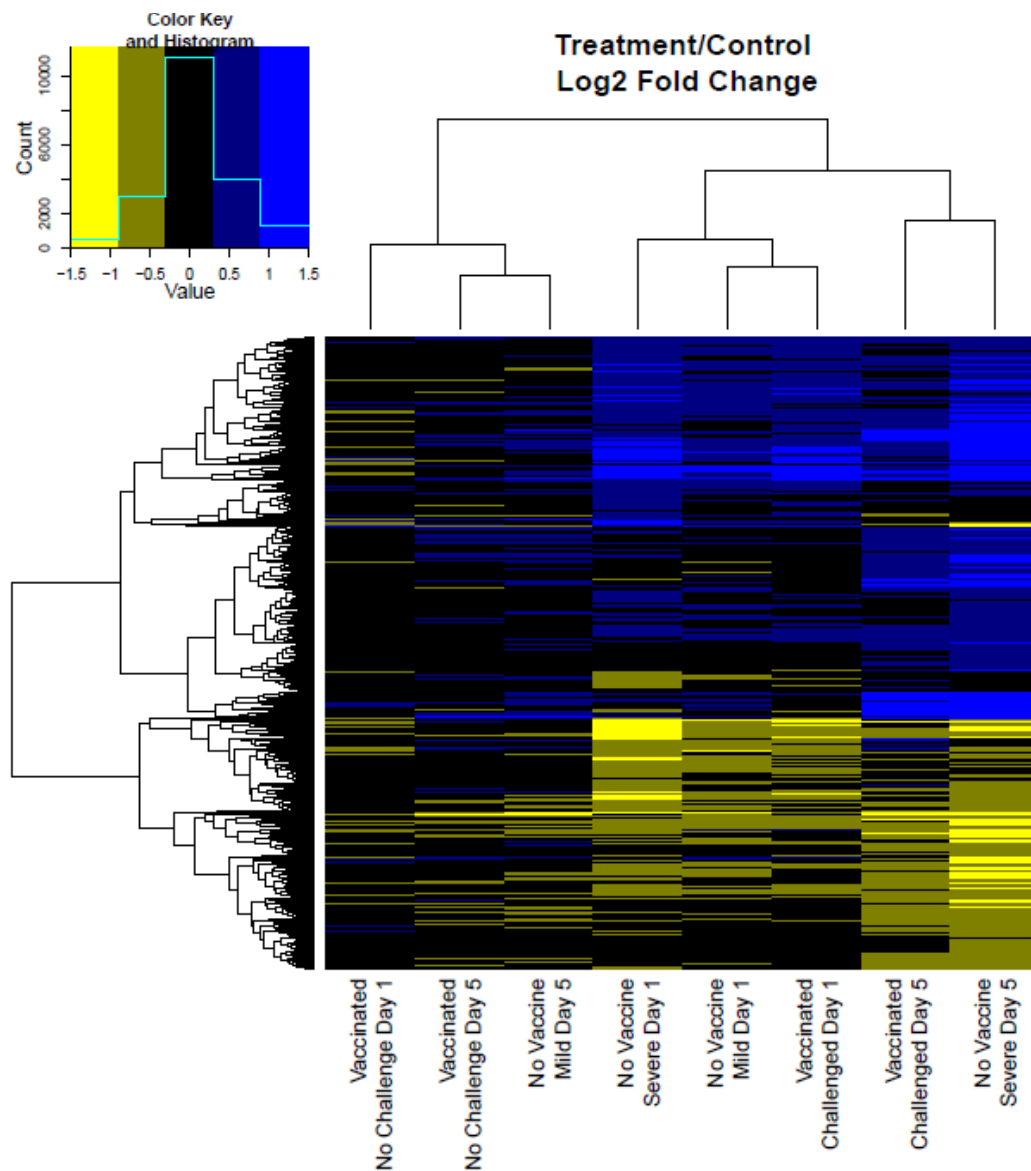
**Figure 4** – Direction of response

Directionality of significantly differentially expressed genes found in various contrasts with a minimum fold change of 1.5. For each contrast, up-regulated means there is greater expression in the first group, down-regulated means there is greater expression in the second group listed (q-value < 0.05)



**Figure 5** - Heatmap comparison of day 1 and day 5

Visual representation of log2 fold change differences for all treatment groups between day 1 and day 5. Genes included were significant in at least one contrast presented (q-value < 0.05). Positive fold change indicates greater expression on day 5.



**Figure 6** - Heatmap comparison of treatments and controls

Visual representation of log<sub>2</sub> fold change differences between all treatment groups and day appropriate NV-NC control. Genes included were significant in at least one contrast presented ( $q\text{-value} < 0.05$ ). Positive fold change indicates greater expression in treatment group.

## Additional files

### Additional file 1 – Primers utilized for qPCR analysis

Forward and reverse primer sequences used for quantitative PCR analysis.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Reference
AvBD2	TTTCTCCAGGGTTGTC TTCG	AGCAGCTTCCGACTTT GATT	Designed for Current Study
AvBD6	TGCAGGTCAGCCCTAC TTTT	GTCCACTGCCACATGA TCC	Designed for Current Study
AvBD7	CTGCTTTCCAGGGATC TGTC	GCCAGAGAAGCCATTT GGTA	Designed for Current Study
IL1 $\beta$	GCTCTACATGTCGTGT GTGATGAG	TGTCGATGTCCCGCAT GA	Withanage et al., 2004 Infection and Immunity, 72:2152-2159.
IL6	GCTCGCCGGCTTCGA	GGTAGGTCTGAAAGGC GAACAG	Kaiser et al., 2000 Microbiology, 146:3217-3226.
IFN $\gamma$	GTGAAGAAGGTGAAA GATATCATGGA	GCTTTGCGCTGGATTC TCA	Kaiser et al., 2000 Microbiology, 146:3217-3226.
TLR2	CCGGTGCTTCATTCAC AGAT	CATATCCCATGCTCCT TTCC	Designed for Current Study
TLR4	GGATCTTTCAAGGTGC CACA	CAAGTGTCGATGGGT AGGT	Abasht et al., 2008 Veterinary Immunology and Immunopathology, 123:314-323
MD2	ATGAAGCATCCCACCC AATA	AATGAGCTGAAGAATC ACAAAAAGT	Designed for Current Study
IL1R	TCCAGTGGTACAAGGA TGCTC	GCCGGAGTCTGTCTGGT AATA	Designed for Current Study
28S	GGCGAAGCCAGAGGA AACT	GACGACCGATTTGCAC GTC	Kaiser et al., 2000 Microbiology, 146:3217-3226.

### Additional file 2 – Excel file – Mild vs. Control, Day 1

Results of DAVID analysis of significant genes using a custom background of genes

included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 3** – Excel file – Severe vs. Control, Day 1

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 4** – Excel file – Severe vs. Control, Day 5

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 5** – Excel file – Vaccinated Challenged vs. Control, Day 1

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 6** – Excel file – Vaccinated Challenged vs. Control, Day 5

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 7** – Excel file – Severe vs. Mild, Day 5

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

**Additional file 8** – Excel file – Severe Day 1 vs. Severe Day 5

Results of DAVID analysis of significant genes using a custom background of genes included in the microarray analysis. Results presented are of the Biological Processes ALL category

# CHAPTER 3. TRANSCRIPTOME RESPONSE OF LEUKOCYTES FROM CHICKENS INFECTED WITH AVIAN PATHOGENIC *ESCHERICHIA COLI* IDENTIFIES PATHWAYS ASSOCIATED WITH RESISTANCE

A paper prepared for *Developmental and Comparative Immunology*<sup>10</sup>

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## Abstract

Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis, which is responsible for morbidity, mortality and condemnation of processed birds, and is a human health risk. Genetic selection to increase host immune response is a promising method to reduce reliance on antimicrobial therapy. This approach requires in depth knowledge of what constitutes and controls a successful response. Broiler chicks were experimentally vaccinated at 2 weeks of age and challenged at 4 weeks of age with APEC. At 1 and 5 days post-infection, whole blood was collected and peripheral blood leukocytes isolated. Gross lesions in the air sacs, pericardium and liver at necropsy were used to assign pathology category, either mild or severe, to non-vaccinated, challenged chicks. Ten treatment groups were classified with *a priori* factors of vaccination status, challenge status, day post-infection, and *a posteriori* factor of pathology. The transcriptome of blood leukocytes was evaluated utilizing a chicken

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44K Agilent microarray. Large numbers of expression differences occurred in 4 pair-wise contrasts, all of which included the severe pathology groups. More genes were up-regulated than down-regulated in response to infection. There was no detectable vaccination effect, despite a significant reduction in total gross lesions in vaccinated chicks. Gene ontology analysis of significant genes revealed enrichment of immune response and metabolic processes. This study not only analyzed the effect of vaccination and challenge on transcriptomic response, but also investigated the differences in host response associated with mild and severe outcomes of colibacillosis, providing the framework for future studies on genetic resistance to APEC.

## **Introduction**

Avian pathogenic *Escherichia coli* (APEC) are a subpathotype of extraintestinal pathogenic *E. coli* (ExPEC) that cause extraintestinal diseases in poultry that are collectively known as colibacillosis. APEC may also be a food safety concern, with current research showing strong genetic similarities between APEC and human ExPEC (Johnson et al., 2007; Moulin-Schouler et al., 2006; Rodrigues-Siek et al., 2005a). These similarities suggest zoonotic potential for APEC and APEC as a source for human ExPEC virulence genes (Ewers et al., 2007; Jakobsen et al., 2010; Rodrigues-Siek et al., 2005a). Recently, APEC has been shown to cause disease in a rat model for human meningitis (Tivendale et al., 2010). Through horizontal and vertical transmission, APEC can cause disease and death in infected birds, resulting in large monetary losses through condemnation of carcasses and reduced production (Barnes et al., 2008; Bisaiillon et al., 1988; Russell, 2003). Though there are many serogroups of APEC that cause disease in birds (Barnes et al., 2005; Rodrigues-Siek et al., 2005b), O1 has been a focal point for study of pathogen and host response to infection, as

it is one of the major serogroups responsible for colibacillosis worldwide (as reviewed by Dziva and Stevens, 2008). In addition, APEC O1 is one of the most well characterized APEC strains in the literature (Johnson et al., 2007; Li, G. et al., 2011).

Antimicrobials are commonly used to control colibacillosis in the poultry industry. With rising concern and demonstration of drug resistant bacteria (as reviewed by Lutful Kabir, 2010), other control methods, such as enhanced host genetics, are a growing area of research. Past experimentation has illustrated the potential for breeding for colibacillosis resistance (Ask et al., 2006; Caverio et al., 2009), indicating that greater research surrounding the mechanisms of resistance is needed as a foundation for more effective breeding programs. Gene expression analysis of immune tissues is commonly used to characterize immune response (Chiang et al., 2008; Redmond et al., 2009) and provides potential candidate genes for disease resistance. Gene expression levels of immune genes have been shown to be heritable (Swaggerty et al., 2008), evidence that this tactic could be successful in a breeding program.

The chicken immune system is equipped with several mechanisms to combat pathogens. Peripheral blood leukocytes (PBL) are comprised of a diverse group of cell types that serve in both the innate and adaptive immune responses (Erf, 2004; Sharma, 2008). Heterophils, the avian equivalent of neutrophils, are an innate responder and typically the first cell type to fight infection. Heterophils destroy susceptible bacteria through phagocytosis, oxidative burst and extracellular traps (Chuammitri et al., 2009; Farnell et al., 2006). Proper signaling by cytokines and T-helper cells can increase the effectiveness of immune response. The defensive mechanisms of APEC can, however, reduce the effectiveness of the innate immune response and may include resistance to the detrimental

effects of phagocytosis and complement, or decreasing the antimicrobial activity of heterophils (Harmon, 1998; Kottom et al., 1997; Mellata et al., 2003; Nolan et al., 2003; Qureshi, 2003). Enhanced genetics of the host immune response may allow chickens to overcome APEC's defenses. This study reports genes expression differences in PBL due to challenge status and pathology differences, identifying factors that contribute to a successful host response to APEC challenge.

## **Materials and Methods**

### **Ethics Statement**

All animal research protocols were performed under animal biosafety level 2 protocols and were approved by the Iowa State University Institutional Animal Care and Use Committee (approval # 11-07-6460-G).

### **Bacteria Preparation**

APEC O1 strain O1:K1:H7 (NCBI Reference Sequence: NC\_008563.1) was kept in Brain Heart Infusion (BHI) broth with 10% glycerol at -80°C. Its genomic sequence is available and has been completely characterized (Johnson et al., 2007). Two days prior to bacterial challenge, bacteria were removed from the freezer and streaked onto Luria Bertani (LB) agar then incubated overnight at 37°C. An isolated colony was then placed into 10 ml of LB broth and incubated overnight at 37°C with shaking. On day of APEC challenge, bacteria were pelleted by centrifugation at 5000xg for 15 minutes. The pellet was then washed in phosphate buffered saline (PBS) 3 times before being enumerated by spectrometric reading at 600 nm. The inoculum was adjusted to the desired bacterial concentration, and counts were confirmed through serial dilution plating onto MacConkey agar overnight.

## Animal Experiments

Non-vaccinated, commercial male broiler chicks were purchased at 1 day of age from a local hatchery. In six replicates, 720 birds (120 per replicate) were raised on wire-floor cages with *ad libitum* access to food and water. Challenged birds were housed separately from non-challenged birds. At 2 weeks of age, 50% of the chicks were intramuscularly vaccinated with 0.5ml/bird of Iss vaccine (Lynne et al., 2006), containing 2µg of vaccine and 50µg of Quil A adjuvant in PBS. Non-vaccinated chicks received 50µg of Quil A adjuvant in PBS via the same route. Increased serum survival, *iss*, encodes an outer membrane lipoprotein and is a virulence factor common to most APEC serotypes (Johnson et al., 2006; Pfaff-McDonough et al., 2000; Rodrigues-Siek et al., 2005b). At 4 weeks of age, 80% of the chicks, half vaccinated and half non-vaccinated, were challenged with 0.1ml containing  $10^8$  colony forming units of APEC O1 injected into the left thoracic air sac. Non-challenged chicks received 0.1ml of PBS via the same route. Birds were sampled and euthanized at 2 time points, 1 and 5 days post-infection (PI), equally splitting birds within each group between the two times.

Blood samples were collected from the jugular vein into 5mL vacuum tubes containing EDTA and placed on ice until PBL isolation. Birds were then euthanized and internal lesion scores assigned for 3 tissues, air sacs, pericardium and liver, as described by Peighambari et al. (2000). Scores from 0 to 2 were assigned for pericardium and liver; scores from 0 to 3 were assigned for air sacs. A summation of all 3 lesions scores for each individual bird was used to generate a total lesion score. Non-vaccinated, challenged birds were split into two pathology categories based upon total lesion score: mild and severe. Birds with low lesion scores were used to represent mild pathology, with an average lesion

score of 0.375, and those with high lesion scores were used to represent severe pathology, with an average lesion score of 6.125. Ten treatment groups were generated from vaccinated (V) or non-vaccinated (NV), challenged (C) or non-challenged (NC), day 1 or day 5 necropsy treatments and 2 pathological categories of mild and severe within the non-vaccinated, challenged groups (Figure 1).

### **PBL Isolation**

PBL were separated from whole blood samples. Phosphate buffered saline was combined with approximately 1-3 ml of blood sample to a total volume of 10 ml. A Histopaque 1077/1119 (Sigma Aldrich, St. Louis, MO) discontinuous gradient was created by placing 10 ml of Histopaque 1119 into a 50 ml tube, overlaying 10 ml of Histopaque 1077, then overlaying 4 ml of blood/PBS mixture. The gradient mixture was centrifuged at 700xg for 30 min at room temperature. Cell layers were removed from the plasma/Histopaque 1077 interface (mononuclear cells) and from the 1077/1119 interface (heterophils) with a Pasteur pipette, combined and transferred to a 15 ml tube. Cells were washed by adding 6 ml of PBS and centrifuged at 600xg for 10 min at room temperature. Supernatant was discarded and the cell pellet washed a second time with PBS. Supernatant was discarded and PBL resuspended in 1.5 ml of RNAlater (AM7021) (Applied Biosystems, Foster City, CA). Cells were refrigerated in RNAlater for 7 days then excess RNAlater was decanted and cells were stored at -80°C until RNA isolation.

### **RNA Isolation**

RNA samples were isolated using the Ambion MagMax-96 kit for Microarrays (AM1839) (Applied Biosystems, Foster City, CA). Briefly, PBL were added to 0.6 ml of TRI Reagent Solution (Ambion, Austin, TX). Samples were homogenized and split into two

300 µl aliquots, with 1 aliquot further processed and the other held in reserve. Samples were then processed using the Spin Procedure according to manufacturer's instructions. Total RNA was eluted with 30 µl of Elution Buffer and stored at -80°C. Quality and quantity of RNA were assessed by Nanodrop (Thermo Scientific, West Palm Beach, FL).

### **Microarray Experiments**

Gene expression was assessed utilizing the global 2-color chicken 44K Agilent Microarray (Li et al., 2008). A total of 40 samples, one from each of 4 experimental replicates of 10 treatment groups, were hybridized to the microarray in a reference design, using the NV-NC-day 1 sample as the reference for each experimental replicate, on 36 arrays. Within each replicate, the NV-NC-day 1 sample was hybridized to the other 9 treatment groups. Dye assignments were swapped between replicates. Briefly, 400 ng of total RNA was reverse transcribed into cDNA with a T7 promoter region incorporated, then transcribed back into cRNA labeled with either Cy3 or Cy5 dye. Before hybridization, 825 ng of each labeled sample, Cy3 and Cy5, a blocking agent and fragmentation buffer were mixed together and incubated for 30 minutes at 60°C. Following incubation, gex hybridization buffer was added and samples were hybridized to the microarray slide for 17 hours at 65°C. After hybridization, slides were washed in Agilent Wash Buffer, Acetonitrile, and Stabilization and Drying Buffer (Agilent Technologies, Santa Clara, CA) then scanned using GenePix 4100A scanner and GenePix Pro software (Molecular Devices Inc., Sunnyvale, CA).

The median backgrounds were subtracted from the median Cy3 and Cy5 foreground intensity reads for each spot and were log<sub>2</sub>-transformed. Technical control spots and spots exhibiting an average signal to noise ratio of less than 3 over all 36 arrays were excluded

from further analysis. Signal to noise ratios were calculated as (median foreground – median background)/background standard deviation for each dye. Locally Weighted Scatterplot Smoothing (LOWESS) procedure was utilized to correct for intensity dependent dye bias (Dudoit et al., 2002). A linear mixed model approach was used to estimate treatment means by fitting the difference of Cy3 and Cy5 normalized signal intensities with each treatment groups' parameterization for each gene as described by Sandford et al. (2011). Only one random effect, experimental replicate, was included in the model, as likelihood ratio testing determined no effect of slide or array position. *P* values were obtained for contrasts of interest. False discovery rate was controlled by converting *P* values to *q* values using the R package *qvalue* (Story and Tibshirani, 2003). Gene ontology analysis of biological processes for significant genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a, Huang et al., 2009b).

### **Quantitative PCR Validation**

Quantitative real time PCR (qRT-PCR) was performed as described by Redmond et al. (Redmond et al., 2009) to confirm microarray results. The following fifteen genes were selected because of significance in the microarray study: clusters of differentiation (CD) 3ε, CD4, CD5, CD28, toll-like receptors (TLR) 7, TLR15, TLR21, heat shock protein 70 (HSP70), P20K, Rab11a, avian beta-defensins (AvBD) 2, AvBD4, AvBD5, AvBD6, and AvBD7. 28S was utilized as a housekeeping gene to normalize for starting concentration of RNA. Primer sequences for CD4, CD5, TLR7, Rab11a, AvBD2, AvBD4, AvBD5, AvBD6, and AvBD7 were designed using sequences from NCBI and PRIMER3 (Rozen and Skaletsky, 2000). Primer sequences for 28S, TLR15, and TLR21 have been previously reported (28S (Kaiser et al., 2000); TLR15 (Higgs et al., 2006); TLR21 Brownlie et al.,

2009)). CD3ε, CD28, and P20K were previously utilized (Zhou and Lamont, 2007) but primer sequences were not published. All unpublished primer sequences can be found in Table 1. Each sample was run in three wells. Cycle threshold (Ct) values were recorded for each well and each sample triplicate was averaged. Slopes representing reaction efficiency for each gene were generated through amplification of a serial dilution. CT values were adjusted for RNA concentration and reaction efficiency using the formula:  $40 - [\text{Sample Meant CT Target Gene} + (\text{Median 28S for All Samples} - \text{Sample Mean 28S}) * (\text{Slope Target Gene} / \text{Slope 28S})]$ . Adjusted Ct values were analyzed using the Fit Model procedure in JMP software (SAS Institute Inc., Cary, NC). Validation was carried out utilizing RNA extracted from different birds than those included in the microarray analysis representing the same treatment groups and replicates, allowing for both technical and biological replication.

#### **Microarray Data Accession Number**

The microarray data for this experiment have been deposited in NCBI's Gene Expression Omnibus (GEO) (Barrett et al., 2009; Edgar et al., 2002) database and are accessible through the series accession GSE31387 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31387>).

## **Results**

### **Transcriptome Analysis of PBL**

Forty individual samples were analyzed by microarray; 1 bird from each of 10 treatment groups, with 4 replications. After removal of spots with a signal to noise ratio less than 3, a total of 24,387 genes were included in the statistical analysis. Effects of treatment were analyzed through contrasts of treatment groups. Contrasts measured tested effect of time for each treatment group, comparisons of each group to control, effect of vaccination,



and effect of pathology. Large numbers (>100) of significantly differentially expressed genes ( $q$  value < 0.05) were detected in 4 contrasts of interest, all involving the NV-C severe groups. The largest number of differentially expressed genes (1,914 genes) was found in the contrast of NV-C severe day 5 vs. NV-C mild day 5. The second largest number of differentially expressed genes (1,097 genes) was found in the contrast of NV-C severe group vs. NV-NC control group on day 1. This number was reduced to 506 differentially expressed genes for the same contrast on day 5. The number of differentially expressed genes in the contrast of NV-C severe day 1 vs. NV-C severe 5 was 107 genes.

Numbers of shared and unique differentially expressed genes between 3 contrasts analyzing differences in treatment group on the same day are displayed in Figure 2. A total of 417 differentially expressed genes found in the contrast of NV-C severe day 5 vs. NV-NC control day 5 were also differentially expressed in the NV-C severe day 5 vs. NV-C mild day 5 contrast; 280 genes had increased expressed in the severe group in both contrasts, 137 genes had decreased expressed in the severe group in both contrasts. In all of the shared genes between the contrasts represented in Figure 2, 99% of genes were expressed in the same direction relative to the severe group; either all more highly expressed in the severe group across all contrasts or more lowly expressed among all contrasts.

The differences between internal lesion scores of vaccinated and non-vaccinated, challenged birds were tested through two-sample  $t$ -tests for each day. There was a significant ( $P$  value < 0.001) reduction in internal lesions among vaccinated, challenged birds compared to those non-vaccinated, challenged birds on both days, however, no differentially expressed genes due to vaccination effect were detected. On day 1, the mean $\pm$ standard deviation of lesion scores for vaccinated birds was  $1.50\pm1.27$  (N=76) and unvaccinated birds

3.06±1.68 (N=87). On day 5, the mean±standard deviation of lesion scores for vaccinated birds was 2.94±2.08 (N=80) and unvaccinated birds 4.28±1.94 (N=85). Vaccination effect on gene expression was tested both through the V-NC group vs. NV-NC contrast on both days and through a contrast utilizing a combination of all treatment groups, non-challenged and challenged, on both days.

Samples from individuals with severe pathology or collected 5 days post-infection exhibited more gene induction than repression. Of differentially expressed genes with a minimum fold change of 1.5, 60-80% of genes exhibited induction in the 4 contrasts that include: NV-C severe day 5 vs. NV-C mild day 5, NV-C severe day 1 vs. NV-NC day 1, NV-C severe day 5 vs. NV-NC day 5, NV-C severe day 1 vs. NV-C severe day 5. Many differentially expressed genes showed large fold changes. In the 4 contrasts described, 25-31% of differentially expressed genes had a fold change of 3 or greater.

Heatmaps were generated to characterize patterns of gene expression between similar contrasts by including genes with a minimum q value of 0.05 in any contrast (Figures 3 and 4). The NV-C mild group on day 5 showed more similarities to the V-NC groups than to the challenged groups (Figure 3). The remaining challenged groups, both vaccinated and non-vaccinated, exhibited similar expression patterns. The only group with notable expression changes over time was the NV-C severe group (Figure 4).

### **Gene Ontology Analysis of Significant Genes**

Gene ontology analysis focused on biological process terms among significant genes. Larger numbers of significantly enriched GO terms were found in contrasts with a higher number of genes with differential expression. Three GO terms related to response (response to stimulus, response to stress and defense response) were discovered among significantly

differentially expressed genes in the contrast of NV-C severe day 1 vs. NV-C severe 5. Among the other 3 contrasts described (NV-C severe day 5 vs. NV-C mild day 5, NV-C severe day 1 vs. NV-NC control day 1, NV-C severe day 5 vs. NV-NC control day 5), a variety of metabolic and biosynthetic processes were common. Prominent within NV-C severe day 5 vs. NV-C mild day 5, were GO terms for signal transduction, immune system processes, ion homeostasis and, surprisingly, several GO terms centered on reproduction. Within NV-C severe vs. NV-NC control, response terms were prominent on day 1, and ion homeostasis and DNA structural terms were prominent on day 5.

GO analysis of unique and shared differentially expressed genes for 3 contrasts (Figure 2) was carried out; NV-C severe day 5 vs. NV-C mild day 5, NV-C severe day 1 vs. NV-NC control day 1, NV-C severe day 5 vs. NV-NC control day 5. Many of the genes shared among contrasts were related to immune response. These included (a) CD4, tumor necrosis factor receptor, and Rab11a shared by all 3 contrasts; (b) ATPase, CD5, interferon gamma receptor, and toll-like receptor 15 shared by NV-C severe day 1 vs. NV-NC control day 1 and NV-C severe day 5 vs. NV-NC control day 5; (c) ATPase, CD3ε, CD200R1, toll-like receptor 7 shared by NV-C severe day 5 vs. NV-NC control day 5 and NV-C severe day 5 vs. NV-NC control day 5. Avian beta-defensins, CD74 and interleukin-8 were unique to NV-C severe vs. NV-NC control on day 1, (e). Unique to NV-C severe vs. NV-C mild on day 5 (f) were genes related to ion transport and energy (ATPases and ATP synthases), immune response (CD28, CD79b, interleukin 4 receptor, interleukin 10 receptor beta, toll-like receptor 21), and reproduction.

Only two contrasts, NV-C severe day 5 vs. NV-C mild day 5 and NV-C severe day 1 vs. NV-NC control day 1, had significantly enriched KEGG pathways detected by DAVID

( $P$  value  $< 0.10$ , Tables 2 and 3). Many of the pathways exhibited relatedness to defense mechanisms, including lysosome pathway, signaling pathways, apoptosis and NK cell mediated toxicity.

The majority of pathways had higher expression in the severe group compared to either mild or control groups, though some, like the cell adhesion molecules pathway and the regulation of actin cytoskeleton, had less expression among severe groups.

### **Microarray Validation by Quantitative Real Time PCR**

Fifteen significant genes from the microarray study were validated through qRT-PCR analysis: CD 3 $\epsilon$ , CD4, CD5, CD28, TLR 7, TLR15, TLR21, HSP70, P20K, Rab11a, AvBD2, AvBD4, AvBD5, AvBD6, and AvBD7. These genes had significant differential expression in microarray analysis ( $q$  value  $< 0.05$ ). Validation was performed on two contrasts of interest, NV-C severe day 5 vs. NV-C mild day 5 for CD 3 $\epsilon$ , CD4, CD5, CD28, TLR 7, TLR15, TLR21, HSP70, P20K, and Rab11a, and NV-C severe day 1 vs. NV-NC day 1 for AvBD2, AvBD4, AvBD5, AvBD6, and AvBD7. Results show similar trends in direction of fold change for 12 of the 15 genes analyzed; TLR7, TLR15, and Rab11a were expressed in the opposite direction from the microarray, although the results were non-significant.

### **Discussion**

Gene expression differences associated with challenge, pathology, or vaccination status, can provide insight into the molecular genetic architecture of host response. The largest amount of gene expression differences occurred between pathology categories classified as mild and severe in the NV-C day 5 group. Several prominent receptor types and clusters of differentiation important to immune response and signaling were differentially expressed between the mild and severe groups. The toll-like receptors (TLR) 7, 15 and 21 all

experienced high expression in the severe group compared to the mild group. Surprisingly, two of these TLRs showed fold change in the opposite direction in the qRT-PCR results. There are several differences in the technical and statistical approaches between microarray and qRT-PCR. These mixed results, however, do still indicate an importance of the TLR family with regards to response to infection and illustrate the need for further interrogation. TLRs have shown expression changes due to pathogen challenge in multiple tissues (Abasht et al., 2008; Lu et al., 2009; Redmond et al., 2009). *In vitro* *Salmonella* stimulation of heterophils from a *Salmonella*-resistant population of birds revealed higher expression of TLR15 than heterophils from a *Salmonella* susceptible population (Nerren et al., 2009), consistent with the results of the current qRT-PCR validation. Cultured macrophages have also shown up-regulation of TLR15 when stimulated with *E. coli*- or *Salmonella*- derived LPS (Ciraci and Lamont, 2011). It is surprising that in the current study, TLR4 and TLR5 were not among those genes differentially expressed, because they are considered to be the main TLRs for recognizing features of Gram-negative bacteria such as APEC (Brownlie and Allan, 2011). TLR4 expression differences have been reported in heterophils due to bacterial challenge and genetic background (Redmond et al., 2009). TLR4 and TLR5 had differential expression in the cecum, TLR4 in the spleen and TLR5 within males in the spleen of *Salmonella* infected chicks (Abasht et al., 2008).

Interleukin receptors 4 and 10, and interferon gamma (IFN $\gamma$ ) receptor 2 all experienced high expression in the severe group compared to the mild group. Changes in interleukin (IL) receptor genes have been noted in response to pathogen challenge (Crowley et al., 2011), although expression changes in IL4R and IL10R have not been extensively studied. IFN $\gamma$ R2 in macrophages exposed to *Salmonella* endotoxin *in vitro* was up-regulated

4 hours post stimulation (Ciraci et al., 2010). Tregs, which control the expression of IL-4 and IFN $\gamma$  to prevent autoimmunity, have been shown to fail under high antigen dose *in vitro* (George et al., 2003). Higher expression of receptors for these pro-inflammatory cytokines may allow for greater downstream signaling triggered by IL-4 and IFN $\gamma$ , promoting the severe pathology through autoimmunity. Cytokines are commonly produced by PBL after pathogen challenge and have shown differential expression due to bacterial challenge and genetic background in heterophils *in vitro* (Redmond et al., 2009). Although several receptors were up-regulated, no cytokine genes were significantly differentially expressed.

Clusters of differentiation (CD) are cell surface molecules common to leukocytes that have roles in the immune response. Differential expression of CD molecules in response to *Salmonella* and *Campylobacter* infections has been observed in multiple chicken tissues: heterophils (Chiang et al., 2008) and jejunum (Schokker et al., 2010) with *Salmonella* and ceca with *Campylobacter* (Li et al., 2010). In the current study, many CD molecules had higher expression among the mild versus severe pathology group, including CD3 $\epsilon$ , CD4, CD5, CD28, CD79b and CD200R1. CD81 was also differentially expressed, but showed higher expression in the severe pathology (susceptible) group. Previous expression studies have reported higher levels of CD4 among heterophils from *Salmonella* resistant chickens compared to susceptible lines (Chiang et al., 2008). This is particularly noteworthy as this difference was noted in non-challenged birds, presenting CD4 expression level as a potential pre-challenge assessment of susceptibility. The current study utilized bacterial challenge to assess pathology and found higher levels of CD4 among birds showing mild pathology (resistant) than in birds demonstrating severe pathology (susceptible). Many CD molecules are associated with or have higher prevalence of specific PBL types (CD3 $\epsilon$  among T-cells

(Göbel and Fluri, 1997), CD4 among T-cells (Koskinen et al., 2002), ggCD200R-B1 among macrophages (Viertlboeck et al., 2008)), suggestive of differences in PBL population composition between mildly and severely affected birds that influence downstream pathology. Higher CD4 expression may be indicative of a higher T-helper population within birds with mild pathology. In humans, CD200R1 acts as a regulator of myeloid cell activation and pro-inflammatory response (Mukhopadhyay et al., 2010). Avian CD200R, specifically ggCD200R-B1, has high homology to mammalian CD200R (Viertlboeck et al., 2008). Lower expression among the severe pathology group would allow an unchecked pro-inflammatory response, leading to greater host damage.

The Ras superfamily can be divided into five major family groups: Ras, Rab, Rho, Ran and Arf. Within the realm of immune response, Ras genes can cause regulatory changes in cell proliferation, differentiation and survival, Rho are Ras homologous proteins with roles in the cell cycle, Rab proteins have roles in vesicle formation and transport and Arf also has roles in vesicle transport (Wennerberg et al., 2005). Ten members of the Ras superfamily, Ras, Rab, Rho and Arf groups, were differentially expressed between pathology groups on day 5, introducing a new family of genes to be explored in greater depth in the role of immune response. Seven had higher expression among the severe group and 3 among the mild group. Ras p21 protein activator 3, Rasa3, which is involved in a signaling pathway for B-cells to avoid pro-apoptotic signals (Maréchal et al., 2007), was higher amongst the mild group. Rab11a has been shown to have roles in TLR4 trafficking to phagosome and control interferon regulatory factor-3 in human monocytes (Husebye et al., 2010). The conflicting result of the qRT-PCR validation in Rab11a, along with the lack of literature on the Ras

family in chickens, illustrates the need for more attention to this gene group in the investigation of immune response.

Differences between the NV-C severe group and the NV-NC group were observed on both days. The number of differentially expressed genes in this contrast decreased over time from 1,097 genes on day 1 to 506 on day 5. This may be due to the rapid response of PBL to infection. Unique to the day 1 comparison of the NV-C severe and NV-NC group for PBL, genes encoding avian beta-defensins (AvBD) and interleukin 8 were up-regulated. The genes for AvBD2, 4, 5, 6, and 7 were all rapidly up-regulated by APEC infection. The antimicrobial properties of beta-defensins have been well described (van Dijk et al., 2008). AvBD2, 5, 6, and 7 have been found to be expressed in leukocytes (Chiang et al., 2008; van Dijk et al., 2008). TLR agonists, such as LPS, increase AvBD2 in heterophils (Kannan et al., 2009). Additionally, structural variants in AvBD genes have been associated with response to *Salmonella* in chickens (Hasenstein et al., 2006; Hasenstein and Lamont, 2007), indicating the feasibility of their use in marker-assisted selection to enhance the anti-bacterial response on a population level. The *in vitro* response of macrophages to *Salmonella* endotoxin is typified by a significant induction of IL8 at 1, 2, 4, and 8 hours post stimulation (Ciraci et al., 2010). The chemotactic ability of IL8 for other peripheral blood mononuclear cells and heterophils (Barker et al., 1993) is consistent with a role in early response to infection, as seen here, with IL8 only significantly induced in the day 1 response.

Differential expression was seen among clusters of differentiation genes and in the Rab and Rho family groups on day 1 between the NV-C severe and NV-NC group, similar to the differential expression between mild and severe pathologies at day 5. All differentially expressed CD genes were down-regulated following APEC challenge: CD4, CD5, CD74,



CD82, CD83 and CD247. A strain of APEC (APEC17) was previously shown to activate caspase 3/7 in macrophages, inducing apoptosis (Bastiani et al., 2005). APEC O1 in the current study may result in APEC-induced PBL death, shifting the PBL population structure compared to basal (non-challenged) levels. CD247, also known as the T-cell receptor (TCR)  $\zeta$ -chain, is well conserved between chickens and mammals (Göbel and Bolliger, 1998), and is responsible for aiding in assembly of the TCR complex and receptor signaling. *In vitro* studies of the human  $\zeta$ -chain have shown degradation by activated caspases (Gastman et al., 1999), indicating a possible mechanism by which APEC could reduce the abilities of T-cells and of the cell-mediated response, resulting in more severe pathology. Among the Rab and Rho genes that were differently expressed, only RhoB was down-regulated in the severe pathology group. Under stress, RhoB inhibits apoptosis and activates NF- $\kappa$ B in rats (Li, Y. et al., 2011), such that decrease of expression in severe pathology would allow greater apoptosis and limit NF- $\kappa$ B activation. Rab11a was again higher in the severe pathology group in this contrast, along with Rab18, 32 and 35.

Fewer significantly differentially expressed genes limited GO analysis and interpretation of the NV-C severe day 5 and NV-NC day 5 group comparison. Similar to other contrasts, three CD groups were significantly differentially expressed. CD3 $\epsilon$ , CD4, and CD200R1 showed less expression in the NV-C severe group, suggestive of continued reduction in CD4<sup>+</sup> leukocytes, such as T-cells, and in regulators of pro-inflammatory response. Expression patterns within prominent GO groupings for ion homeostasis and cellular developmental processes were inconsistent, with no clear trend of greater expression in one treatment group compared to the other.

Many genes were significantly expressed in more than one contrast (Figure 2), which is reinforced by the common patterns seen within the treatment/control heatmap (Figure 3). Similarities between NV-C severe and NV-C mild on day 5 and NV-C severe vs. NV-NC group on day 1 suggest similarities between mild pathology on day 5 and the control groups. This could be the result of a return to homeostasis after a successful defense against APEC. The changes between the severe pathology group and the control non-challenged group over time appear to be driven by the NV-C severe group, as this was the only group to exhibit large changes between day 1 and day 5 (Figure 4).

Only two contrasts, NV-C severe day 5 vs. NV-C mild day 5 and NV-C severe day 1 vs. NV-NC control day 1, had significantly enriched KEGG pathways, as detected by DAVID ( $P$  value  $< 0.10$ , Tables 2 and 3). Between pathology states on day 5, genes that enriched metabolic pathways were more highly expressed in the severe group, potentially mobilizing more energy to fight infection. The effect of severe status compared to the non-challenged control on day 1 illustrates the importance of signaling pathways during early response to infection.

The lack of a detectible vaccination effect, given the large impact on total lesion scores and the tissue analyzed, is surprising. Vaccination against Newcastle disease virus increases serum antibody titers and can impact T cell populations in the 9 weeks following vaccination (Dalgaard et al., 2010). Significant changes in IFN $\alpha$  and IFN $\gamma$  mRNA expression have been reported in peripheral blood at 1 and 7 days post-vaccination to Marek's disease vaccine in 6-8 week old chickens (Qu  r   et al., 2005). These chickens were then sampled only 4 hours after challenge to observe differential expression patterns due to vaccination and challenge (Qu  r   et al., 2005). Several cytokines showed significant

expression changes 1 day post-vaccination with complete Freund's adjuvant (Hangalapura et al., 2006). Time of vaccination, time of challenge and time of tissue sampling all impact the observed mRNA expression patterns. Our sampling at 15 and 19 days post-vaccination may have been too late to observe expression changes in PBL due to vaccination alone, and sampling at 1 and 5 days post-infection may have been too late to observe rapid, vaccine-induced effects in response to infection.

Great insights about how particular cell types react to a foreign agent can be gained through targeted *in vitro* experimentation. The limitation of *in vitro* experimentation, however, is in knowing how well the information will translate to response at the whole organism level. The *in vivo* aspect of this experiment is invaluable in its comparison to *in vitro* studies. By utilizing samples taken from whole blood, the current study detected expression differences that are caused by changes in circulating populations, instead of focusing on effects in an isolated cell type(s) in culture. Understanding the systemic nature of APEC infections requires study of a whole organism response, which is better measured through an *in vivo* experiment. It is, however, through targeted *in vitro* experiments we are able to discern between cause and effect of expression, through technologies such as RNAi and gene knockdown.

Peripheral blood leukocytes are comprised of cells with roles in innate/adaptive response and cell/humoral-mediated responses. Transcriptome interrogation of this system reveals which gene expression patterns play an important role in immune response. Additionally, these cells can be collected from live birds without the need to harvest the breeding animal to assay the cellular response. Through this greater understanding of host response, candidate genes to improve genetic resistance to APEC infection can be identified.

Applying selection for beneficial genotypes in breeding populations will generate enhanced host responses against APEC infection.

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## **Author Contributions**

ES participated in necropsy, processed tissues, performed microarray experiments, analyzed data, conducted qPCR validation and drafted the manuscript. MO participated in the development of the microarray experimental design and assisted in data analysis. MS participated in qPCR validation. XL mentored and supervised microarray experiments. HZ provided equipment and annotation files for microarray experiments. TJ participated in the design of the study and necropsy. SK participated in the design of the study, purified Iss-GST fusion protein, and oversaw animal experiments (prepared Institutional Animal Care and Use Committee protocols and, performed vaccination, bacterial challenge and necropsy). PL participated in design of the study, necropsy and data analysis. LN participated in the design of the study, necropsy and oversaw animal experiments and bacterial challenge. SL participated in the design of the study, necropsy and interpretation of the results. All authors read, edited and approved the final manuscript.

## References

- Abasht, B., M. G. Kaiser, and S. J. Lamont. 2008. Toll-like receptor gene expression in cecum and spleen of advanced intercross line chicks infected with *Salmonella enterica* serovar Enteritidis. *Vet. Immunol. Immunopathol.* 123:314-323.
- Ask, B., E. H. van der Waaij, J. A. Stegeman, and J. A. van Arendonk. 2006. Genetic variation among broiler genotypes in susceptibility to colibacillosis. *Poult. Sci.* 85:415-21.
- Barker, K. A., A. Hampe, M. Y. Stoeckle, and H. Hanafusa. 1993. Transformation-associated cytokine 9E3/CEF4 is chemotactic for chicken peripheral blood mononuclear cells. *J. Virol.* 67:3528-3533.
- Barnes, H. J., L. K. Nolan, and J. P. Vaillancourt. 2008. Colibacillosis, In Y. M. Saif (ed.), *Diseases of Poultry*. 12<sup>th</sup> ed. p.691-732. Blackwell Publ.
- Barrett, T., D. B. Troup, S. E. Wilhite, R. Ledoux, D. Rudnev, C. Evangelista, I. F. Kim, A. Soboleva, M. Tomashevsky, K. A. Marshall, K. H. Phillippy, P. M. Sherman, R. N. Muerter, and R. Edgar. 2009. NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res.* 37:D5-D15.
- Bastiani, M., M. C. Vidotto, and F. Horn. 2005. An avian pathogenic *Escherichia coli* isolate induces caspase 3/7 activation in J774 macrophages. *FEMS Microbiol. Lett.* 253:133-140.
- Bisaillon, J. R., A. H. Meek and T. E. Feltmate. 1988. An assessment of condemnations of broiler chicken carcasses. *Can. J. Vet. Res.* 52:269-276.
- Brownlie, R., and B. Allan. 2011. Avian toll-like receptors. *Cell Tissue Res.* 343:121-130.

- Brownlie, R., J. Zhu, B. Allan, G. K. Mutwiri, L. A. Babiuk, A. Potter, and P. Griebel. 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Mol. Immunol.* 46:3163-3170.
- Cavero, D., M. Schmutz, H. C. Philipp, and R. Preisinger. 2009. Breeding to reduce susceptibility to *Escherichia coli* in layers. *Poult. Sci.* 88:2063-2068.
- Ciraci, C., and S. J. Lamont. 2011. Avian-specific TLRs and downstream effector responses to CpG-induction in chicken macrophages. *Dev. Comp. Immunol.* 35:392-398.
- Ciraci, C., C. K. Tuggle, M. J. Wannemuehler, D. Nettleton, and S. J. Lamont. 2010. Unique genome-wide transcriptome profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin. 11:545.
- Chiang, H. I., C. L. Swaggerty, M. H. Kogut, S. E. Dowd, X. Li, I. Y. Pevzner, and H. Zhou. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44K Agilent microarray. *BMC Genomics.* 9:526.
- Chuammitri, P., J. Ostojić, C. B. Andreasen, S. B. Redmond, S. J. Lamont, and D. Palić. 2009. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Vet. Immunol. Immunopathol.* 129:126-131.
- Crowley, T. M., V. R. Haring, and R. Moore. 2011. Chicken anemia virus: an understanding of the in-vitro host response over time. *Viral Immunol.* 24:3-9.
- Dalgaard, T. S., L. R. Norup, A. R. Pedersen, K. J. Handberg, P. H. Jørgensen, and H. R. Juul-Madsen. 2010. Flow cytometric assessment of chicken T cell-mediated immune responses after Newcastle disease virus vaccination and challenge. *Vaccine.* 28:4506-4514.

- Dudoit, S., Y. H. Yang, M. J. Callow, and T. P. Speed. 2002. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. *Statistica Sinica*. 12:111-139.
- Dziva, F., and M. P. Stevens. 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. *Avian Pathol.* 37:355-366.
- Edgar, R., M. Domrachev, and A. E. Lash. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30:207-210.
- Erf, G. F. 2004. Cell-mediated immunity in poultry. *Poult. Sci.* 83:580-590.
- Ewers, C., G. Li, H. Wilking, S. Kießling, K. Alt, E. M. Antão, C. Laternus, I. Diehl, S. Glodde, T. Homeier, U. Böhnke, H. Steinrück, H. C. Philipp, and L. H. Wieler. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? *Int. J. Med. Microbiol.* 297:163-176.
- Farnell, M. B., A. M. Donoghue, F. S. de Los Santos, P. J. Blore, B. M. Hargis, G. Tellez, and D. J. Donoghue. 2006. Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poult. Sci.* 85:1900-1906.
- Gastman, B. R., D. E. Johnson, T. L. Whiteside, and H. Rabinowich. 1999. Caspase-mediated degradation of T-cell receptor  $\zeta$ -chain. *Cancer Res.* 59:1422-1427.
- George, T. C., J. Bilsborough, J. L. Viney, and A. M. 2003. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression *in vitro*. *Eur. J. Immunol.* 33:502-511.
- Göbel, T. W., and L. Bolliger. 1998. The chicken TCR  $\zeta$ -chain restores the function of a mouse T cell hybridoma. *J. Immunol.* 160:1552-1554.

- Göbel, T. W., and M. Fluri. 1997. Identification and analysis of the chicken CD3 $\epsilon$  gene. *Eur. J. Immunol.* 27:194-198.
- Hangalapura, B. N., M. G. Kaiser, J. J. Poel, H. K. Parmentier, and S. J. Lamont. 2006. Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. *Dev. Comp. Immunol.* 30:503-511.
- Harmon, B. G. 1998. Avian heterophils in inflammation and disease resistance. *Poult. Sci.* 77:972-977.
- Hasenstein, J. R., and S. J. Lamont. 2007. Chicken gallinacin gene cluster associated with *Salmonella* response in advanced intercross line. *Avian Dis.* 51:561-567.
- Hasenstein, J. R., G. Zhang, and S. J. Lamont. 2006. Analysis of five gallinacin genes and the *Salmonella enterica* serovar Enteritidis response in poultry. *Infect. Immun.* 74:3375-3380.
- Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk and C. O'Farrelly. 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* 74:1692-1698.
- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009a. Bioinformatics enrichment tools: paths towards the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37:1-13.
- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009b. Systemic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nat. Protoc.* 4:44-57.
- Husebye, H., M. H. Aune, J. Stenvik, E. Samstad, F. Skeldal, O. Halaas, N. J. Nilsen, H. Stenmark, E. Latz, T. E. Mollnes, O. Bakke, and T. Espevik. 2010. The Rab11a



- GTPase controls Toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes. *Immunity*. 33:583-596.
- Jakobsen, L., D. J. Spangholm, K. Pedersen, L. B. Jensen, H. D. Emborg, Y. Agersø, F. M. Aarestrup, A. M. Hammerum, and N. Frimodt-Møller. 2010. Broiler chickens, broiler chicken meat, pigs and pork as sources of ExPEC related virulence genes and resistance in *Escherichia coli* isolates from community-dwelling humans and UTI patients. *Int. J. Food Microbiol.* 142:264-272.
- Johnson, T. J., K. E. Siek, S. J. Johnson, and L. K. Nolan. 2006. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. *J. Bacteriol.* 188:745-758.
- Johnson, T. J., S. Kariyawasam, Y. Wannamuehler, P. Mangiamele, S. J. Johnson, C. Doetkott, J. A. Skyberg, A. M. Lynne, J. R. Johnson, and L. K. Nolan. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. *J. Bacteriol.* 189:3228-3236.
- Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella gallinarum*. *Microbiology*. 12:3217-3226.
- Kannan, L., R. Liyanage, J. O. Lay Jr., and N. C. Rath. 2009. Evaluation of beta defensin 2 production by chicken heterophils using direct MALDI mass spectrometry. *Mol. Immunol.* 46:3151-3156.

- Koskinen, R., J. Salomonsen, C. A. Tregaskes, J. R. Young, M. Goodchild, N. Bumstead, and O. Vainio. 2002. The chicken CD4 gene has remained conserved in evolution. *Immunogenetics*. 54:520-525.
- Kottom, T. J., L. K. Nolan, M. Robinson, J. Brown, T. Gustad, S. M. Horne, and C. W. Giddings. 1997. Further characterization of a complement-sensitive mutant of a virulent avian *Escherichia coli* isolate. *Avian Dis.* 41:817-823.
- Li, G., K. A. Tivendale, P. Liu, Y. Feng, Y. Wannemuehler, W. Cai, P. Mangiamele, T. J. Johnson, C. Constantinidou, C. W. Penn, and L. K. Nolan. 2011. Transcriptome analysis of avian pathogenic *Escherichia coli* O1 in chicken serum reveals adaptive responses to systemic infection. *Infect. Immun.* 79:1951-1960.
- Li, X., H. I. Chiang, J. Zhu, S. E. Dowd, and H. Zhou. 2008. Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics*. 9:60.
- Li, X., C. L. Swaggerty, M. H. Kogut, H. I. Chiang, Y. Wang, K. J. Genovese, H. He, and H. Zhou. 2010. Gene expression profiling of the local cecal response of genetic chicken lines that differ in their susceptibility to *Campylobacter jejuni* colonization. *PLoS One*. 5:e11827.
- Li, Y. D., Y. P. Liu, D. M. Cao, Y. M. Yan, Y. N. Hou, J. Y. Zhao, R. Yang, Z. F. Xia, and J. Lu. 2011. Induction of small G protein RhoB by non-genotoxic stress inhibits apoptosis and activates NF- $\kappa$ B. *J. Cell. Physiol.* 226:729-738.
- Lu, Y., A. J. Sarson, J. Gong, H. Zhou, W. Zhu, Z. Kang, H. Yu, S. Sharif, and Y. Han. 2009. Expression profiles of genes in Toll-like receptor-mediated signaling of broiler infected with *Clostridium perfringens*. *Clin. Vaccine Immunol.* 16:1639-1647.

- Lutful Kabir, S. M. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int. J. Environ. Res. Public Health*. 7:89-114.
- Lynne, A. M., S. L. Foley and L. K. Nolan. 2006. Immune response to recombinant *Escherichia coli* Iss protein in poultry. *Avian Dis.* 50:273-276
- Maréchal, Y., X. Pesesse, Y. Jia, V. Pouillon, D. Pérez-Morga, J. Daniel, S. Izui, P. J. Cullen, O. Leo, H. R. Luo, C. Erneux, and S. Schurmans. 2007. Inositol 1,3,4,5-tetrakisphosphate controls proapoptotic Bim gene expression and survival in B cells. *Proc. Natl. Acad. Sci. U. S. A.* 104:13978-13983.
- Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss III, B. Lehoux, and J. M. Fairbrother. 2003. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. *Infect. Immun.* 71:494-503.
- Moulin-Schouleur, M., C. Schouler, P. Tailliez, M. R. Kao, A. Bree, P. Germon, E. Oswald, J. Mainil, M. Blanco, and J. Blanco. 2006. Common virulence factors and genetic relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. *J. Clin. Microbiol.* 44:3484-3492.
- Mukhopadhyay, S., A. Plüddemann, J. C. Hoe, K. J. Williams, A. Varin, K. Makepeace, M. L. Akin, D. M. Bowdish, S. T. Smale, A. N. Barclay, and S. Gordan. 2010. Immune inhibitory ligand CD200 induction by TLRs and NLRs limits macrophage activation to protect the host from meningococcal septicemia. *Cell Host Microbe.* 8:236-247.
- Nerren, J. R., C. L. Swaggerty, K. M. MacKinnon, K. J. Genovese, H. He, I. Pevzner, and M. H. Kogut. 2009. Differential mRNA expression of the avian-specific toll-like receptor

- 15 between heterophils from *Salmonella*-susceptible and -resistant chickens. Immunogenetics. 61:71-77.
- Nolan, L. K., S. M. Horne, C. W. Giddings, S. L. Foley, T. J. Johnson, A. M. Lynne and J. Skyberg. 2003. Resistance to serum complement, *iss*, and virulence of avian *Escherichia coli*. Vet. Res. Commun. 27:101-110.
- Peighambari, S. M., R. J. Julian, and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. Avian Dis. 44:759-769.
- Pfaff-McDonough, S. J., S. M. Horne, C. W. Giddings, J. O. Doetkott, M. H. Smith and L. K. Nolan. 2000. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. Avian Dis. 44:23-33.
- Quéré, P., C. Rivas, K. Ester, R. Novak, and W. L. Ragland. 2005. Abundance of IFN- $\alpha$  and IFN- $\gamma$  mRNA in blood of resistant and susceptible chickens infected with Marek's disease virus (MDV) or vaccinated with turkey herpesvirus; and MDV inhibition of subsequent induction of IFN gene transcription. Arch. Virol. 150:507-519.
- Qureshi, M. A. 2003. Avian macrophage and immune response: an overview. Poult. Sci. 82:691-698.
- Redmond, S. B., P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2009. Chicken heterophils from commercially selected and non-selected genetic lines express cytokines differently after *in vitro* exposure to *Salmonella enteritidis*. Vet. Immunol. Immunopathol. 1132:129-134.
- Rodrigues-Siek, K. E., C. W. Gidding, C. Doetkott, T. J. Johnson, M. K. Fakhr, and L. K. Nolan. 2005a. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology. 151:2097-2110.

- Rodrigues-Siek, K. E., C. W. Gidding, C. Doetkott, T. J. Johnson, and L. K. Nolan. 2005b. Characterizing the APEC pathotype. *Vet. Res.* 36:241-256.
- Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biology programmers. *Methods Mol. Bio.* 132:365-386.
- Russell, S. M. 2003. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors, and populations of *Campylobacter* spp. and *Escherichia coli*. *Poult. Sci.* 82:1326-1331.
- Sandford, E. E., M. Orr, E. Balfanz, N. Bowerman, X. Li, H. Zhou, T. J. Johnson, S. Kariyawasam, P. Liu, L. K. Nolan, and S. J. Lamont. 2011. Spleen transcriptome response to infection with avian pathogenic *Escherichia coli* in broiler chickens. *BMC Genomics.* 12:469. [Epub ahead of print]
- Schokker, D., M. A. Smits, A. J. Hoekman, H. K. Parmentier, and J. M. Rebel. 2010. Effects of *Salmonella* on spatial-temporal processes of jejunal development in chickens. *Dev. Comp. Immunol.* 34:1090-1100.
- Sharma, J. M. 2008. Host factors for disease resistance, In Y. M. Saif (ed.), *Diseases of Poultry*. 12<sup>th</sup> ed. p.47-58. Blackwell Publishing.
- Storey, J. D., and R. Tibshirani. 2003. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA.* 100:9440-9445.
- Swaggerty, C. L., I. Y. Pevzner, P. Kaiser, and M. H. Kogut. 2008. Profiling pro-inflammatory cytokine and chemokine mRNA expression levels as a novel method for selection of increased innate immune responsiveness. *Vet. Immunol. Immunopathol.* 126:35-42.

Tivendale, K. A., C. M. Logue, S. Kariyawasam, D. Jordan, A. Hussein, G. Li, Y.

Wannemuehler, and L. K. Nolan. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. *Infect. Immun.* 78:3412-3419.

van Dijk, A., E. J. Veldhuizen, and H. P. Haagsman. 2008. Avian defensins. *Vet. Immunol. Immunopathol.* 124:1-18.

Viertlboeck, B. C., M. A. Hanczaruk, F. C. Schmitt, R. Schmitt, and T. W. Göbel. 2008. Characterization of the chicken CD200 receptor family. *Mol. Immunol.* 45:2097-2105.

Wennerberg, K., K. L. Rossman, and C. J. Der. 2005. The Ras superfamily at a glance. *J. Cell Sci.* 118:843-846.

Zhou, H., and S. J. Lamont. 2007. Global gene expression profile after *Salmonella enterica* Serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. *Cytogenet. Genome Res.* 117:131-138.

**Table 1** - Primers utilized for qRT-PCR validation experiments

<b>Gene</b>	<b>Accession</b>	<b>Forward Sequence 5' - 3'</b>	<b>Reverse Sequence 5' - 3'</b>
CD3ε	Y08917	CTGCTGTGTGTGGTTGGTG	CGGATCTGGCTTCCATTTTA
CD4	AY528652	ATACCGTGGAGGAAGCTCAT	TGCCACCTCATACCAGTGAT
CD5	Y12011	ACAGGAGGCTGATGAAGAGG	CTCTGCTGCTCCTCCACTCT
CD28	X67915	CAGTCTTTATAATCTACCGGCAAAA	TTGTTCTTCTGGTGAGGTGGA
TLR7	AJ720504	CGGAAAATGGTACATCATGC	AAAGTTTTGGGAAACCAACG
Rab11a	AJ720402	GCAAAAGCACCATTGGAGTA	GCACCTCGATAGTACGCTGA
P20K	M25784	CTAGGGAGCGGAACACTACACG	GTTTGGGAAGCAGCATTTCAT
HSP70	NM_001006685	ATAGGGTGGGAGCCAAGAAC	GGTTTCGGTCAAGCCAAC
AvBD2	AF033336	TTTCTCCAGGGTTGTCTTCG	AGCAGCTTCCGACTTTGATT
AvBD4	AY621306	TTTCATCGTGCTCCTCTTTG	CATAGCCCCAGGTAAGCATT
AvBD5	AY621307	CCACAAGTCATGTCCTCCAG	CATGGAGATGAACGTGAAGG
AvBD6	AY621308	TGCAGGTCAGCCCTACTTTT	GTCCACTGCCACATGATCC
AvBD7	AY621309	CTGCTTTCCAGGGATCTGTC	GCCAGAGAAGCCATTTGTA

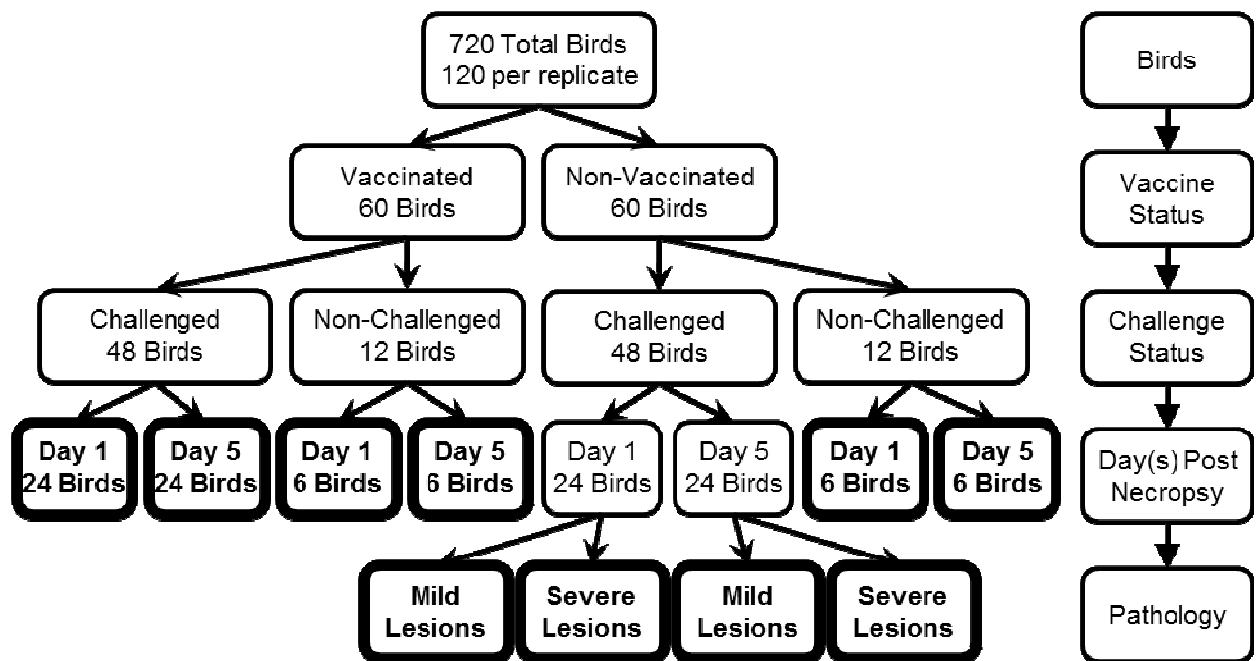
**Table 2** - Effect of severity of pathology at day 5 in non-vaccinated, APEC challenged birds on KEGG pathway enrichment ( $P$  value < 0.10)

KEGG Pathway	Gene Count	Accessions
Lysosome gga04142	15	AJ721124, X95502, AJ851595, AJ719840, AJ719682, AJ289021, X07775, AJ719710, BX931548, AJ720066, AJ720880, AJ721067, AJ719318, AJ720351, AJ720226
Oxidative phosphorylation gga00190	12	BX933021, BX932247, AJ721124, AJ720085, AF461018, AJ720322, CR382422, CR385488, BX950606, AJ719669, AJ289021, BX934066
Galactose metabolism gga00052	5	AJ719970, AJ721042, AJ719347, AF538281, AF534111, AJ719710
Amino sugar and nucleotide sugar metabolism gga00520	7	AJ719970, AJ719291, AJ721042, AJ719347, AB071038, AF538281, AJ720584, AF534111
Cell adhesion molecules (CAMs) gga04514	8	CR390246, M14049, X56559, X67915, AY159125, Y08823, BX950651, AY528652, X71786
Calcium signaling pathway gga04020	10	BX930007, Y12601, AF020315, AY393849, AF205066, AJ720104, AJ012579, AJ719540, M66385, NM_001031553
Pentose phosphate pathway gga00030	5	AJ719291, AJ721042, AF538281, AJ720644, AJ720046
Regulation of actin cytoskeleton gga04810	15	AJ719895, X56559, NM_001031553, BX933901, X06387, AJ720675, AY046915, BX933838, M24637, M14049, AY159125, AJ720866, AY460177, X71786, CR523532, AJ851398
Starch and sucrose metabolism gga00500	5	AJ719970, AJ719291, AJ720880, AJ721042, AF538281, AF534111
Nicotinate and nicotinamide metabolism gga00760	4	BX930007, AJ851590, CR386613, DQ150102
Jak-STAT signaling pathway gga04630	11	CR407301, BX933215, X68073, AJ719433, AJ720866, AY957508, AF082666, AF034576, AJ851398, U40844, AF177875



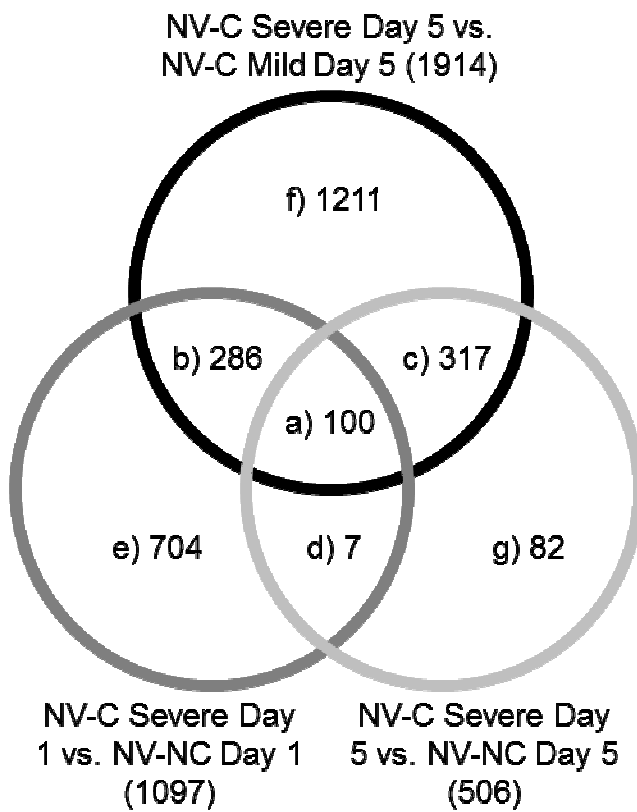
**Table 3** - Effect of severe pathology at day 1 compared to non-vaccinated, non-challenged birds on KEGG pathway enrichment ( $P$  value < 0.10)

KEGG Pathway	Gene Count	Accessions
MAPK signaling pathway gga04010	12	AJ851529, AY033635, CR387146, AJ720776, AJ851546, X68073, BX933236, AJ719540, AF296875, AF167296, U10329, AJ851639
VEGF signaling pathway gga04370	6	AJ851529, AY033635, AJ720776, AJ719540, M64990, U10329
Natural killer cell mediated cytotoxicity gga04650	7	AJ851529, AY033635, AJ720191, AJ719540, AF296875, AY957508, AJ002317
NOD-like receptor signaling pathway gga04621	5	AY033635, AJ720776, BX935364, AY057939, M16199
Apoptosis gga04210	6	AJ720191, BX931997, U26645, AJ719540, AF296875, AY057939
Arachidonic acid metabolism gga00590	3	AJ006405, M64990, U10329
DNA replication gga03030	4	AJ720172, AJ719992, AJ719763, AJ719578



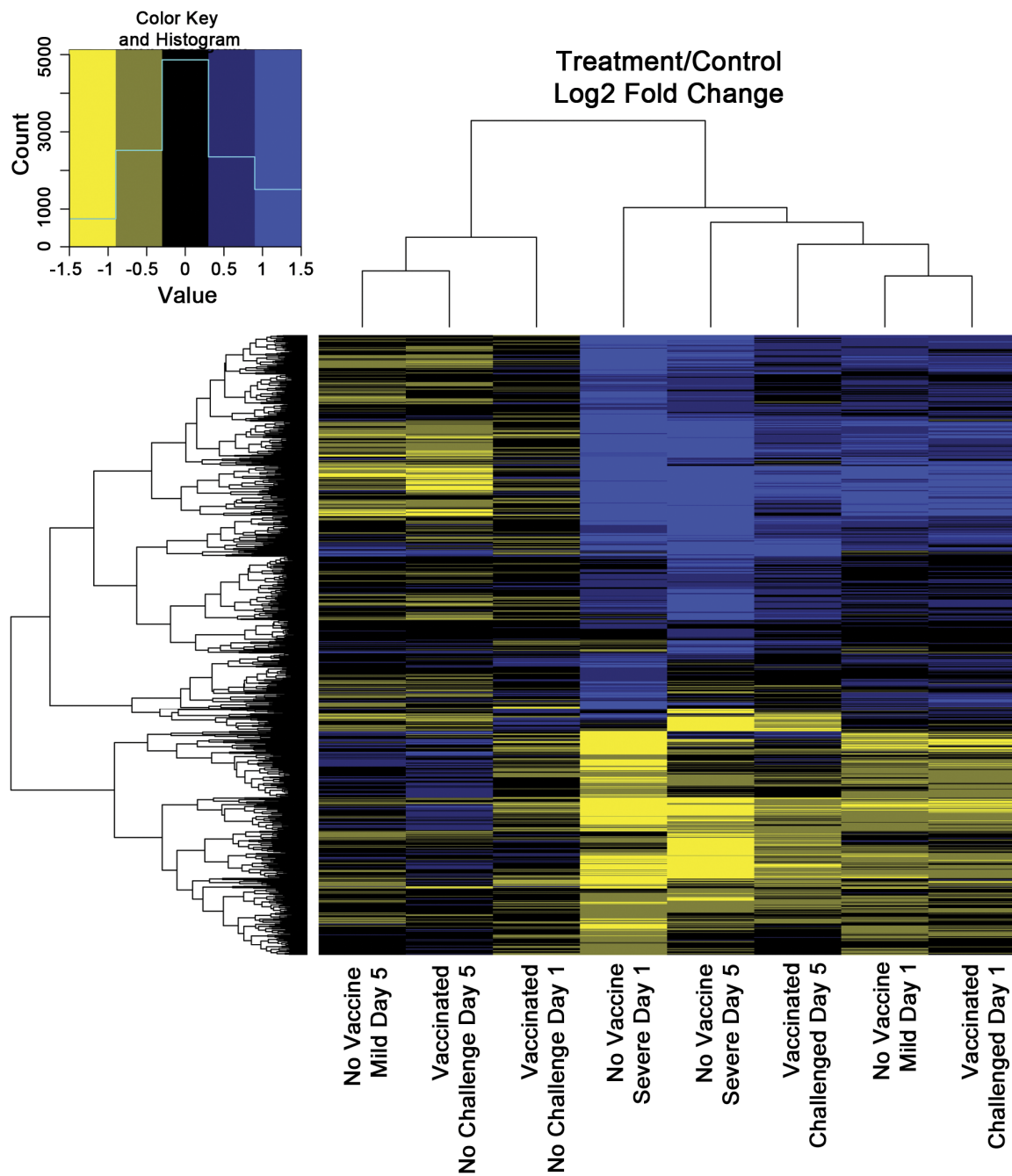
**Figure 1 - Treatment Groups**

Chicks were divided into treatment groups at 2 weeks of age by vaccination status, at 4 weeks of age by challenge status, by day of necropsy post challenge, and by lesion scores assigned at necropsy. Treatment groups are bolded.



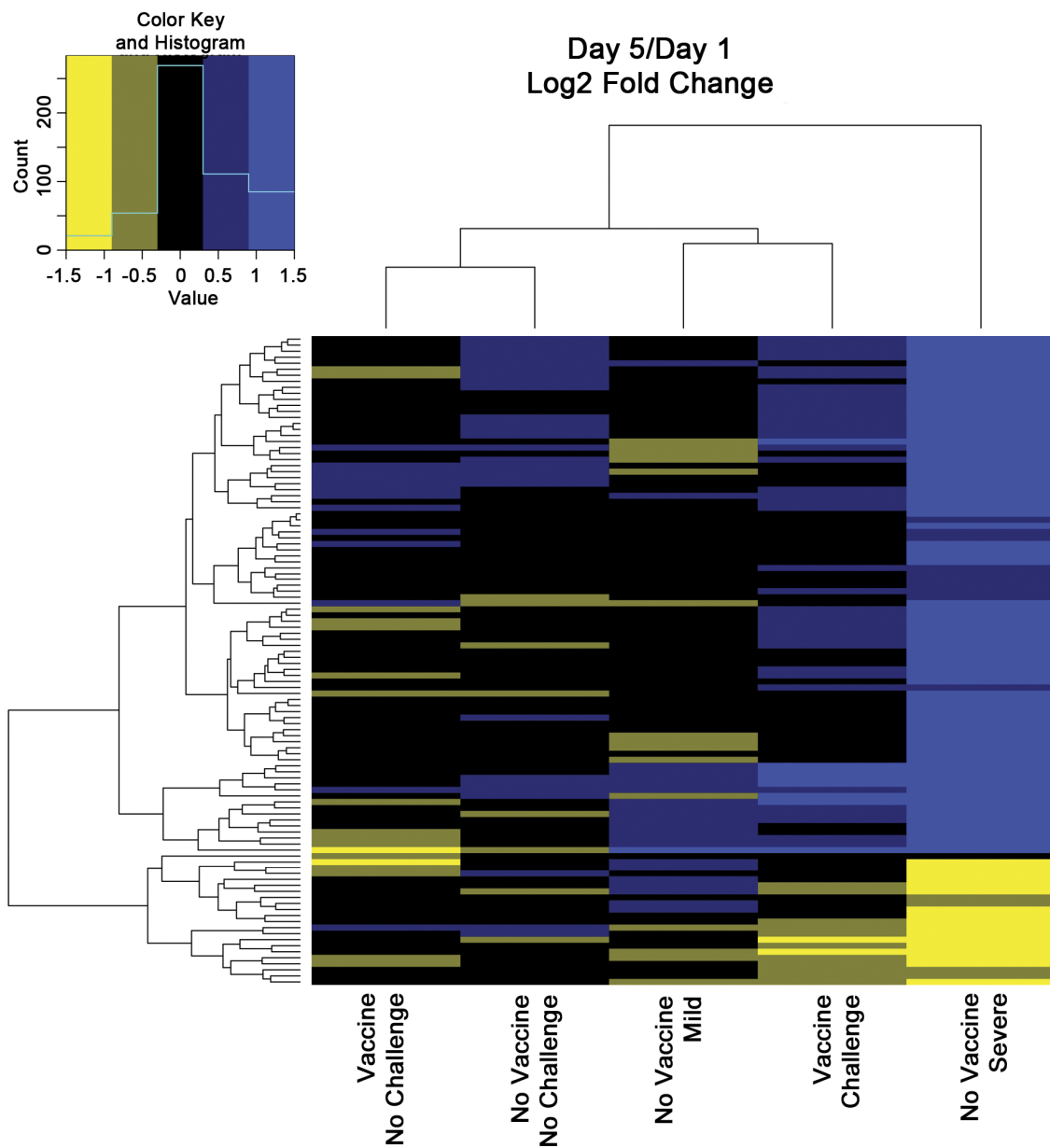
**Figure 2** - Shared Significant Genes

Comparison of shared significant genes within the 3 contrasts of NV-C Mild day 5 vs. NV-C Severe day 5, NV-C Severe day 1 vs. NV-NC group day 1, and NV-C Severe day 5 vs. NV-NC group day 5. a) represents shared genes over all 3 contrasts, b-d) represent shared genes between 2 contrasts, and e-g) represent genes unique to a contrast. The numbers in parenthesis represent the total number of significant genes within each contrast.



**Figure 3 - Treatments Compared to Controls**

Heatmap comparison of fold change for each treatment group compared to the day appropriate NV-NC control. Fold change was calculated as  $\log_2(\text{treatment}) - \log_2(\text{control})$ .



**Figure 4 - Day 5 Compared to Day 1**

Heatmap comparison of fold change for each day 5 treatment group compared the day 1 treatment group. Fold change was calculated as  $\log_2(\text{day 5}) - \log_2(\text{day 1})$ .

# CHAPTER 4. STRONG CONCORDANCE BETWEEN TRANSCRIPTOMIC PATTERNS OF SPLEEN AND PERIPHERAL BLOOD LEUKOCYTES IN RESPONSE TO AVIAN PATHOGENIC *ESCHERICHIA COLI* INFECTION

A paper prepared for *Avian Diseases*<sup>19</sup>

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## Summary

Avian pathogenic *Escherichia coli* (APEC) is a concern for poultry producers and exhibits zoonotic potential. Understanding host transcriptional responses to infection aids understanding of protective mechanisms and serves to inform future colibacillosis-control strategies. Transcriptomic results of the splenic and peripheral blood leukocytes (PBL) responses to APEC infection in the same individual birds were compared in order to identify common patterns and connecting pathways. Over 100 genes in three contrasts examining pathology and infection status were significantly differentially expressed in both tissues and similarly regulated. Differences in catalytic activity appear between mild and severe pathology responses. Early expression differences in the MAPK pathway in PBL precede spleen responses in the p53 and cytokine-cytokine receptor pathways. This type of bi-analysis is useful in identifying genes and pathways important to the response to APEC, whose significance might otherwise go unappreciated.

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Key Words: transcriptome, spleen, leukocyte, meta-analysis, APEC

Abbreviations: APEC = avian pathogenic *Escherichia coli*; Ct = cycle threshold; DPI = day post-infection; IRG1 = immunoresponsive homolog 1; MAPK = mitogen activated protein kinase; PBL = peripheral blood leukocyte; PCR = polymerase chain reaction

## Introduction

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of colibacillosis and has zoonotic potential, making it a concern for producers and consumers alike. APEC infection results in extra-intestinal infections, causing decreased body weight, condemnations of carcasses and potential animal death (Barnes et al., 2008). Recent studies show marked structural similarities between APEC and human extra-intestinal pathogenic *E. coli* (ExPEC) strains, suggesting that APEC has zoonotic potential and may be a source of virulence genes for human ExPEC (Johnson et al., 2007; Johnson et al., 2008; Johnson et al., 2009). These genomic similarities appear to translate to functional similarities, as certain APEC strains are able to cause disease in a rat model of human neonatal meningitis (Tivendale et al., 2010).

Microarray technology has enabled great advances in our understanding of gene function in both microbial pathogens and their hosts. Small tissue- or pathway-specific platforms allow for more targeted research, while use of large global arrays, such as the chicken 44K Agilent microarray (Li et al., 2008), can yield more comprehensive data. Indeed, the larger arrays are more likely to reveal novel genes or pathways since their probes are not restricted to a few elements, often of known function and selected for a particular purpose. Most microarray studies analyze the expression patterns of a particular tissue after

disease exposure, but few combine information from multiple tissues to understand on how tissues interact to fight infection. Meta-analyses combine the information gathered from multiple studies addressing similar questions, but often the data from such studies originate from different individuals or use mRNA that was collected under different experimental conditions (Biswas et al., 2011; Daves et al., 2011; Genini et al., 2011). In the current study, transcriptome patterns in spleen and peripheral blood leukocytes (PBL) from the same individual chickens were compared in a bi-analysis to better understand the host response to APEC. Better understanding of concurrent function and interaction of tissues can provide insight into host response mechanisms and elucidate the best methods to combat disease. Analysis of tissues from the same individuals is expected to yield a more refined assessment of gene action.

## **Materials and Methods**

Previously published microarray experiments examined two separate tissues; spleen (Sandford et al., 2011a) and PBL (Sandford et al., 2011b). Microarray data are available in GEO; Series 25511 and Series 31387 respectively. Tissues were collected from the same experimental study, using the same individual chickens. Commercial male broiler chickens were vaccinated or non-vaccinated at 2 weeks of age, then challenged or non-challenged at 4 weeks of age with APEC O1 delivered via intra-air sac injection. Tissues were then harvested at 1 and 5 days post-infection (DPI). Within the non-vaccinated, challenged chickens, pathology status was assigned by internal lesion scores. Three contrasts from these two experiments were selected for meta-analysis based on large numbers of significantly differentially expressed genes in both tissues: severe pathology vs. mild pathology at 5 DPI, severe pathology vs. control at 1 DPI and severe pathology vs. control at 5 DPI (Table 1).



Significance for the number of significantly expressed genes in both tissues was established through a Chi square test based on 2x2 contingency table. Pathway analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b).

## **Results**

### Transcriptome meta-analysis

In each of the three contrasts examined, a large number of genes were significantly differentially expressed in both tissues: 168 genes in severe pathology vs. mild pathology at 5 DPI, 163 genes severe pathology vs. control at 1 DPI and 114 genes severe pathology vs. control at 5 DPI (Fig. 1). In each instance, the number of genes observed was significantly higher than the number expected ( $P$  value  $< 0.0001$ ). There was a high level of concordance, agreement in direction, of expression patterns ( $>90\%$ ) for genes that were significantly expressed in both tissues (Fig. 2). Amongst these gene sets, severe pathology was associated with more induction than repression of gene expression ( $>70\%$ ) (Fig. 3).

A large number of significantly differentially expressed genes were discovered in both tissues at 1 versus 5 days post-infection (Fig. 4). More genes, 174, were found in common between severe pathology and control birds at 1 DPI in PBL (34%) and between severe pathology and control at 5 DPI in the spleen (16%), than between severe pathology and control at 5 DPI in PBL (6%) and between severe pathology and control at 1 DPI in the spleen (4%), 66 genes.

### DAVID

Molecular function and biological processes of genes significant to both tissues were examined using DAVID (Tables 2-4). Analysis of significantly differentially expressed

genes in severe pathology vs. mild pathology contrast revealed high amounts of genes with function in catalytic, lyase and transporter activity (Table 2). These activities worked primarily within metabolic and biosynthetic processes (Table 3). Greater than 90% of genes with catalytic activity had increased expression in the severe pathology compared to the mild pathology. The majority of biological processes associated with severe pathology vs. control 1 DPI were for response to infection (Table 4), but no molecular functions were found for this contrast. Only one biological process term and one molecular function term were significantly enriched among significantly differentially expressed genes between severe pathology and control 5 DPI; ‘Generation of precursor metabolites and energy’ was enriched with 3 genes and ‘SH3 domain binding’ was enriched with 2 genes.

KEGG pathways within each tissue were examined for the contrasts of severe pathology compared to control at both 1 and 5 DPI. Results showed pathway networks connecting these two tissues over time. The MAPK pathway in PBL at 1 DPI experienced a 12 element enrichment transitioning to downstream pathways in the spleen at 5 DPI, the p53 and cytokine-cytokine receptor pathways experienced a 6 and 14 element enrichment respectively.

## **Discussion**

Examination revealed similarity in gene expression patterns between spleen and PBL. Although genes significantly expressed in both tissues were in high concordance, only 6-22% of significantly differentially expressed genes from the individual tissue analysis were also significantly differentially expressed in the other tissue. This is surprisingly low, because spleen and leukocyte function often work together, with the spleen serving as site of leukocyte storage and education (Oláh and Vervelde, 2008). Results comparing basal

expression patterns of 8 different chicken tissues found that the majority of genes assessed were expressed in all 8 tissues, 49%, while few were tissue-specific, 14% (Nie et al., 2010). The low percentage of significant genes in both tissues in the current study is likely due to how each tissue responds to an infection stimulus and not a reflection of if the gene is expressed in both tissues. Expression in response to stimulus illustrates the importance of each tissue as an individual site within the whole organism.

Comparison of tissues across time points revealed the PBL at 1 DPI shared more significant genes with the spleen at 5 DPI than PBL at 5 DPI shared with the spleen at 1 DPI. This could be an indication of tissues that respond early or late to infection, or differences in the flow of cascading signals. Time course experiments of other bacterial infections have shown increasing rates of colonization over the first 48 hours after infection (Meade et al., 09). Many reports of spleen colonization of APEC are measured 48 hours or more after infection (Caza et al., 2008; Dheilily et al., 2011; Mellata et al., 2010); whereas, APEC has been shown to gain access to the bloodstream hours after intra-air sac inoculation (Stordeur et al., 2004; Pourbakhsh et al., 1997). There has been at least one study showing early splenic colonization, but data comparing bacterial counts to those found in blood were not reported (Pourbakhsh et al., 1997). The spleen may not exhibit as strong of an early response as the PBL due to differences in time to colonization.

KEGG pathway networks were discovered across time points and tissues. Enrichment of the MAPK pathway within the PBL at 1 DPI could be responsible for the downstream changes in cytokine-cytokine and p53 signaling seen in the spleen at 5 DPI. MAPK and p53 pathways are traditionally associated with apoptosis, tumor suppression and

viral infection (Lin et al., 2009; Xing et al., 2010). The increase in genes related to apoptotic activity in the severe pathology may contribute to the observed pathology.

In both tissues, pathology differences are associated with differences in transport and reaction rate. mRNA expressed from catalytic activity genes were elevated in severe pathology group compared to mild pathology group in both tissues. Increases in catalytic activity have previously been associated with greater pathology. Mouse mutants with an inactivated catalytic gene show reduced incidence and severity of lipopolysaccharide induced arthritis (Zack et al., 2009). Few of these catalytic genes have been well studied, especially in response to infection, but one gene, IRG1, immunoresponsive 1 homolog, was highly expressed the severe pathology groups for both tissues in the current study and has recently been associated with infection response. Expression differences between chickens that are resistant and susceptible to Marek's disease were observed in IRG1 after infection with Marek's virus, with greater expression in the susceptible line (Smith et al., 2011). Genotypes of SNPs found within the promoter region of IRG1 were associated with susceptibility and viral counts (Smith et al., 2011). In the current study, transporter gene levels were elevated in the severe pathology, including a copper transporter, solute carrier family 31, member 2. Analysis of APEC O1 survival in chicken serum revealed that copper-uptake by APEC is necessary for survival and that copper may be of low quantity in serum (Li et al., 2011). Thus individuals showing severe pathology may be in competition with APEC for copper reserves.

Both tissues had increases in expression of antimicrobial genes in the severe pathology group compared to the control at 1 DPI. Several avian beta-defensins were significantly increased in both tissues after APEC infection. The beta-defensins are a well-

documented antimicrobial gene family with protective responses against bacterial infection and their significance was reported on in the original separate tissue specific studies (Sandford et al., 2011a, Sandford et al., 2011b). Two other antimicrobial genes, cathelicidin-2 and cathelicidin-3, were significantly increased in both tissues after APEC infection. Cathelicidin-2 has bacterial killing ability (van Dijk et al., 2009a) and is released by heterophils after exposure to LPS (van Dijk et al., 2009b). Consistent with the current study's reports of spleen and PBL, cathelicidin expression increases after infection with *Salmonella* in cecal tonsils (Akbari et al., 2008).

Few DAVID terms were discovered for genes significantly differentially expressed between severe pathology and control at 5 DPI in both tissues. This may indicate that later responses to infection are more tissue specific.

A disadvantage of large scale microarray studies is interpreting the meaning of potentially thousands of significant gene expression signals. Combining information from multiple studies or tissues allows refining of the data set and reveal gene sets that may have been previously unidentified. This bi-analysis utilized samples from not only the same experiment, but from the same individual birds, giving a strong connection between tissues to allow for whole organism examination of transcriptional patterns. This study provides better understanding of the relationship of spleen and PBL in host response to APEC infection.

## References

- Akbari, M. R., H. R. Haghighi, J. R. Chambers, J. Brisbin, L. R. Read, and S. Sharif. 2008. Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with *Salmonella enterica* serovar typhimurium. Clin. Vaccine Immunol. 15:1689-1693.

- Barnes, H. J., L. K. Nolan, and J. P. Vaillancourt. 2008. Colibacillosis, in Y. M. Saif (ed.), Diseases of Poultry. 12th ed. p.691-732. Blackwell Publ.
- Biswas, S., J. Manikandan, and P. N. Pushparaj. 2011. Decoding the differential biomarkers of Rheumatoid arthritis and Osteoarthritis: A functional genomics paradigm to design disease specific therapeutics. *Bioinformation*. 6:153-157.
- Caza, M., F. Lépine, S. Milot, and C. M. Dozois. 2008. Specific roles of the iroBCDEN genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. *Infect. Immun*. 76:3539-3549.
- Daves, M. H., S. G. Hilsenbeck, C. C. Lau, and T. K. Man. 2011. Meta-analysis of multiple microarray datasets reveals a common gene signature of metastasis in solid tumors. *BMC Med. Genomics*. 4:56.
- Dheilly, A., A. Boudier, L. Le Devendec, G. Hellard, and I. Kempf. 2011. Clinical and microbial efficacy of antimicrobial treatments of experimental avian colibacillosis. *Vet. Microbiol*. 149:422-429.
- Genini, S., B. Badaoui, G. Sclep, S. C. Bishop, D. Waddington, M. H. Pinard van der Laan, C. Klopp, C. Cabau, H. M. Seyfert, W. Petzl, K. Jensen, E. J. Glass, A. de Greeff, H. E. Smith, M. A. Smits, I. Olsaker, G. M. Boman, G. Pisoni, B. Castiglioni, P. Cremonesi, M. Del Corvo, E. Foulon, G. Foucras, R. Rupp, and E. Giuffra. 2011. Strengthening insights into host responses to mastitis infection in ruminants by combining heterogeneous microarray data sources. *BMC Genomics*. 12:225.
- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc*. 4:44-57.

- Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37:1-13.
- Johnson, T. J., S. Kariyawasam, Y. Wannamuehler, P. Mangiamele, S. J. Johnson, C. Doetkott, J. A. Skyberg, A. M. Lynne, J. R. Johnson, and L. K. Nolan. 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. *J. Bacteriol.* 189:3228-3236.
- Johnson, T. J., C. M. Logue, Y. Wannamuehler, S. Kariyawasam, C. Doetkott, C. DebRoy, D. G. White, and L. K. Nolan. Examination of the source and extended virulence genotypes of *Escherichia coli* contaminating retail poultry meat. *Foodborne Pathog. Dis.* 6:657-667. 2009.
- Johnson, T. J., Y. Wannamuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *J. Clin. Microbiol.* 46:3987-3996. 2008.
- Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella gallinarum*. *Microbiology.* 12:3217-3226.
- Li, G., K. A. Tivendale, P. Liu, Y. Feng, Y. Wannemuehler, W. Cai, P. Mangiamele, T. J. Johnson, C. Constantinidou, C. W. Penn and L. K. Nolan. 2011. Transcriptome analysis of avian pathogenic *Escherichia coli* O1 in chicken serum reveals adaptive responses to systemic infection. *Infect. Immun.* 79:1951-1960.

- Li, X., H. I. Chiang, J. Zhu, S. E. Dowd, and H. Zhou. 2008. Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics*. 9:60.
- Lin, P. Y., J. W. Lee, M. H. Liao, H. Y. Hsu, S. J. Chiu, H. J. Liu, and W. L. Shih. 2009. Modulation of p53 by mitogen-activated protein kinase pathways and protein kinase C delta during avian reovirus S1133-induced apoptosis. *Virology*. 385:323-334.
- Mellata, M., K. Ameiss, H. Mo, R. Curtiss 3<sup>rd</sup>. 2010. Characterization of the contribution to virulence of three large plasmids of avian pathogenic *Escherichia coli*  $\chi$ 7122 (O78:K80:H9). *Infect. Immun.* 78:1528-1541.
- Nie, H., R. P. Crooijmans, A. Lammers, E. M. van Schothorst, J. Keijer, P. B. Neerincx, L. A. Leunissen, H. J. Megens, and M. A. Groenen. 2010. Gene expression in chicken reveals correlation with structural genomic features and conserved patterns of transcription in terrestrial vertebrates. *PLoS One*. 5:e11990.
- Oláh, I., and L. Vervelde. 2008. Structure of the avian lymphoid system, in F. Davison, B. Kaspers and K. A. Schat (ed.), *Avian Immunology*. 1st ed. p.13-50. Elsevier Ltd.
- Pourbakhsh, S. A., M. Boulianne, B. Martineau-Doizé, and J. M. Fairbrother. 1997. Virulence mechanisms of avian fimbriated *Escherichia coli* in experimentally inoculated chickens. *Vet. Microbiol.* 58:195-213.
- Sandford, E. E., M. Orr, E. Balfanz, N. Bowerman, X. Li, H. Zhou, T. J. Johnson, S. Kariyawasam, P. Liu, L. K. Nolan, and S. J. Lamont. 2011. Spleen transcriptome response to infection with avian pathogenic *Escherichia coli* in broiler chickens. *BMC Genomics*. 12:469. [Epub ahead of print]
- Sandford, E. E., M. Orr, M. Shelby, X. Li, H. Zhou, T. J. Johnson, S. Kariyawasam, P. Liu, L. K. Nolan, and S. J. Lamont. 2011. Transcriptome response of leukocytes from



- chickens infected with avian pathogenic *Escherichia coli* identifies pathways associated with resistance. Dev. Comp. Immunol. [To Be Submitted]
- Smith, J., J. R. Sadeyen, I. R. Paton, P. M. Hocking, N. Salmon, M. Fife, V. Nair, D. W. Burt and P. Kaiser. 2011. Systems analysis of immune responses in Mark's Disease Virus infected chickens identifies a gene involved in susceptibility and highlights a possible novel pathogenicity mechanism. J. Virol. 85:11146-11158. [Epub ahead of print]
- Stordeur, P., A. Brée, J. Mainil, and M. Moulin-Schouleur. 2004. Pathogenicity of pap-negative avian *Escherichia coli* isolated from septicemic lesions. Microbes Infect. 6:637-645.
- Tivendale, K. A., C. M. Logue, S. Kariyawasam, D. Jordan, A. Hussein, G. Li, Y. Wannemuehler, and L. K. Nolan. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. Infect. Immun. 78:3412-3419.
- van Dijk, A., E. M. Molhoek, E. J. A. Veldhuizen, J. L. M. Tjeerdsma-van Bokhoven, E. Wagendorp, F. Bikker, and H. P. Haagsman. 2009a. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. Mol. Immunol. 46:2465-2473.
- van Dijk, A., M. H. G. Tersteeg-Zijderveld, J. L. M. Tjeerdsma-van Bokhoven, A. J. M. Jansman, E. J. A. Veldhuizen and H. P. Haagsman. 2009b. Chicken heterophils are recruited to the site of *Salmonella* infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. Mol. Immunol. 46:1517-1526.

- Xing, Z., C. J. Cardona, J. Anunciacion, S. Adams, and N. Dao. 2010. Roles of the ERK MAPK in the regulation of proinflammatory and apoptotic responses in chicken macrophages infected with H9N2 avian influenza virus. *J. Gen. Virol.* 91:343-351.
- Zack, M. D., M. A. Melton, J. L. Stock, C. E. Storer, R. A. Barve, J. C. Minnerly, D. J. Weiss, J. A. Stejskal, M. D. Tortorella, J. R. Turk, K. M. Shevlin, and A. M. Malfait. 2009. Reduced incidence and severity of experimental autoimmune arthritis in mice expression catalytically inactive A disintegrin and metalloproteinase 8 (ADAM8). *Clin. Exp. Immunol.* 158:246-256.

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**Table 1** - Number of significantly differentially expressed genes in each contrast of interest for each tissue

Contrast of Interest	Spleen	PBL
Severe Pathology vs. Mild Pathology at 5 DPI	799	1914
Severe Pathology vs. Control at 1 DPI	1101	1097
Severe Pathology vs. Control at 5 DPI	1723	506

**Table 2** - Enriched molecular function terms between severe pathology and mild pathology at 5 DPI in both tissues ( $P$  value < 0.05)

Term	Count	Genes
hydro-lyase activity GO:0016836	3	IRG1, ENO2, ACO1
inorganic cation transmembrane transporter activity GO:0022890	4	ATP6V0D2, ATP6V1E1, SLC31A2, TCIRG1
hydrogen ion transmembrane transporter activity GO:0015078	3	ATP6V0D2, ATP6V1E1, TCIRG1
hydrolase activity, acting on glycosyl bonds GO:0016798	3	BST1, GUSB, CHIA
iron ion binding GO:0005506	4	CYP8B, HBG1, ACO1, HEBP1
catalytic activity GO:0003824	21	BST1, PNAT3, ABHD12, CAMK2D, DALRD3, GALM, ENO2, DIO2, CYP8B, CHIA, TCIRG1, ACO1, USP18, RNASET2, IRG1, ATP6V1E1, GUSB, NUS1, AKR7A2, GSTA3, GCH1

**Table 3** - Enriched biological process terms between severe pathology and mild pathology at 5 DPI in both tissues ( $P$  value < 0.05)

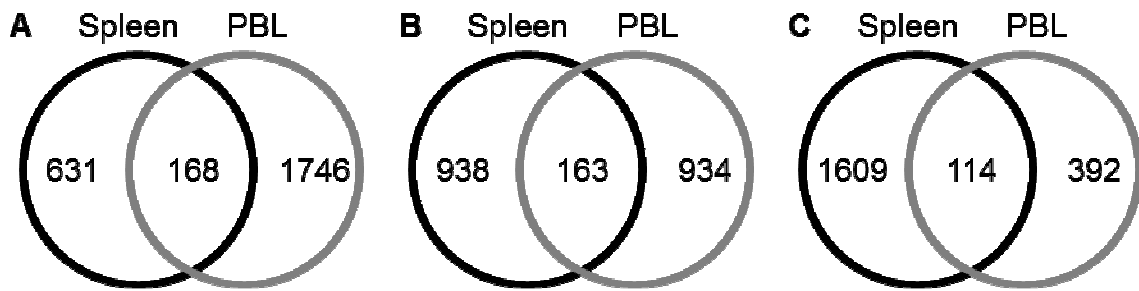
<b>Term</b>	<b>Count</b>	<b>Genes</b>
generation of precursor metabolites and energy GO:0006091	5	ATP6V0D2, ATP6V1E1, ENO2, TCIRG1, ACO1
purine ribonucleoside triphosphate metabolic process GO:0009205	4	ATP6V0D2, ATP6V1E1, GCH1, TCIRG1
ATP synthesis coupled proton transport GO:0015986	3	ATP6V0D2, ATP6V1E1, TCIRG1
purine nucleotide metabolic process GO:0006163	4	ATP6V0D2, ATP6V1E1, GCH1, TCIRG1
catabolic process GO:0009056	6	IRG1, ENO2, GCH1, CHIA, ACO1, USP18
oxidative phosphorylation GO:0006119	3	ATP6V0D2, ATP6V1E1, TCIRG1
alcohol metabolic process GO:0006066	4	VLDLR, ENO2, GALM, GCH1
metabolic process GO:0008152	21	BST1, PNAT3, ATP6V0D2, CAMK2D, DALRD3, GALM, ENO2, DIO2, CHIA, FOXP1, TCIRG1, ACO1, USP18, IRG1, VLDLR, ATP6V1E1, GUSB, AKR7A2, NFIL3, GSTA3, GCH1
amine metabolic process GO:0009308	4	DALRD3, DIO2, GCH1, CHIA
ATP biosynthetic process GO:0006754	3	ATP6V0D2, ATP6V1E1, TCIRG1
nucleoside phosphate metabolic process GO:0006753	4	ATP6V0D2, ATP6V1E1, GCH1, TCIRG1
ATP metabolic process GO:0046034	3	ATP6V0D2, ATP6V1E1, TCIRG1
nucleobase, nucleoside and nucleotide metabolic process GO:0055086	4	ATP6V0D2, ATP6V1E1, GCH1, TCIRG1
purine ribonucleoside triphosphate biosynthetic process GO:0009206	3	ATP6V0D2, ATP6V1E1, TCIRG1
heterocycle metabolic process GO:0046483	4	ATP6V0D2, ATP6V1E1, GCH1, TCIRG1

**Table 3** (continued)

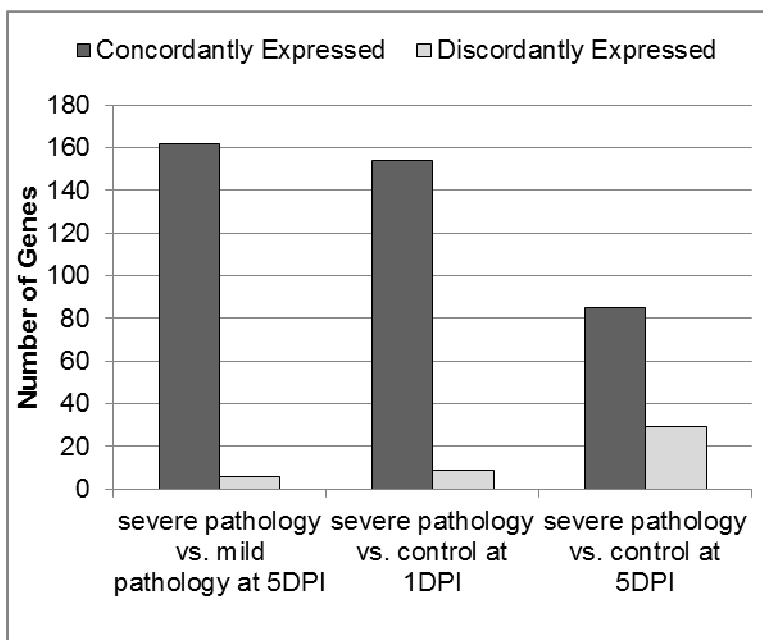
<b>Term</b>	<b>Count</b>	<b>Genes</b>
nucleoside triphosphate biosynthetic process GO:0009142	3	ATP6V0D2, ATP6V1E1, TCIRG1

**Table 4** - Enriched biological process terms between severe pathology and control at 1 DPI  
in both tissues ( $P$  value < 0.05)

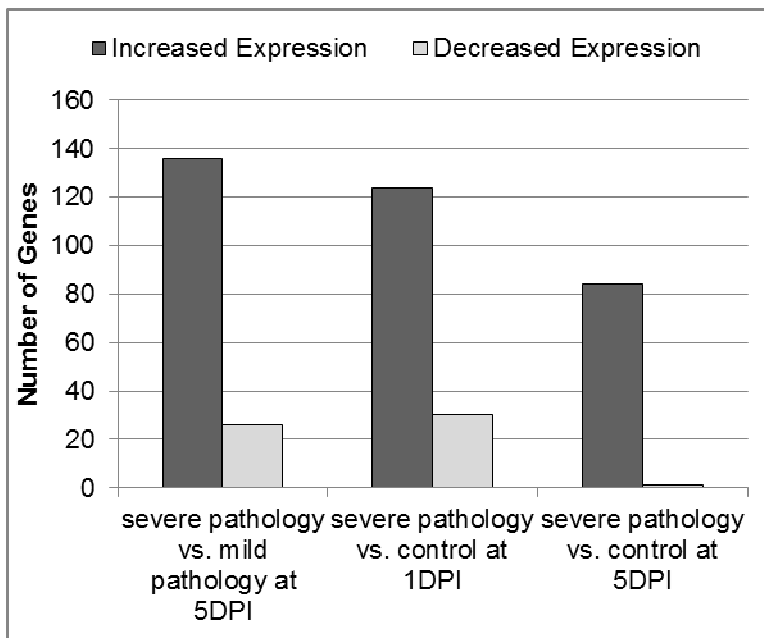
<b>Term</b>	<b>Count</b>	<b>Genes</b>
defense response to bacterium GO:0042742	8	GAL7, GAL6, GAL5, CATHL3, GAL4, LYG2, CATHL2, GAL2
defense response GO:0006952	9	GAL7, GAL6, GAL5, CATHL3, GAL4, LYG2, CATHL2, TLR15, GAL2
response to other organism GO:0051707	8	GAL7, GAL6, GAL5, CATHL3, GAL4, LYG2, CATHL2, GAL2
response to stress GO:0006950	12	F13A1, LYG2, CATHL2, PTGS2, MMP2, TLR15, GAL7, GAL6, GAL5, GAL4, CATHL3, GAL2
response to stimulus GO:0050896	14	F13A1, LYG2, CATHL2, PTGS2, MMP2, TLR15, GAL7, GAL6, GAL5, GAL4, CATHL3, BCL2L1, GAL2, AGRP
innate immune response GO:0045087	3	CATHL3, CATHL2, TLR15
prostaglandin biosynthetic process GO:0001516	2	HPGDS, PTGS2
growth GO:0040007	3	BCL2L1, PTGS2, LEFTY2
icosanoid biosynthetic process GO:0046456	2	HPGDS, PTGS2



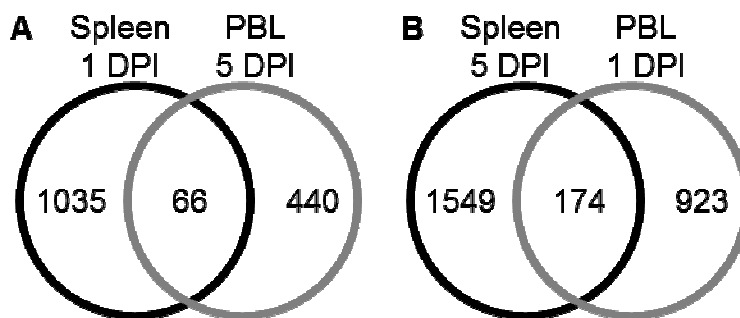
**Figure 1** - The number of genes significantly expressed in both spleen and PBL tissue in the contrasts of (A) severe pathology vs. mild pathology at 5 DPI, (B) severe pathology vs. control at 1 DPI, and (C) severe pathology vs. control at 5 DPI.



**Figure 2** - The number of concordantly and discordantly significantly differentially expressed genes in each contrast across both tissues. Many more significant genes were concordantly expressed in both tissues.



**Figure 3** - Regulation of expressed in concordantly expressed genes. Gene expression was primarily increased among concordantly expressed genes.



**Figure 4** - The number of genes significantly expressed between severe pathology and control at different days post-infection in spleen and PBL. (A) spleen at 1 DPI compared with PBL at 5 DPI, (B) spleen at 5 DPI compared with PBL at 1 DPI

## **CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS**

### **Expanding knowledge of host response with large scale, robust animal experiments**

One difficulty in studying response to disease in animals is the ability to use an effective experimental design. Often, few contrasts or few replicates are examined due to the economic and logistic constraints of the experiment. Here we conducted one of the largest disease studies available in the chicken, utilizing approximately 720 individuals, collecting thousands of tissues, including spleen, blood, thymus, bursa of Fabricius, bone marrow, liver, brain, and air sacs. With this large size, 10 different experimental groups could be represented. The number of challenged animals included in this study gave us the ability to see a large spread in pathological response, thereby allowing maximum separation between individuals to characterize mild and severe phenotypes. The number of disease measurements and tissues collected will be valuable resources for future scientists to analyze to enhance knowledge about both host and pathogen.

### **Infection with avian pathogenic *Escherichia coli* has a major impact on transcriptomic responses**

The spleen and PBL of chickens infected with APEC exhibited significant changes in transcriptomic responses (Chapters 2 and 3). These changes were reflected in the effects of challenge status, day and pathology. Within the spleen, challenge at any level, regardless of pathology or vaccination, produced differential expression from the naïve control birds. The severe pathology group had the largest number of genes differentially expressed compared to the control group in both tissues. This is perhaps reflective of the most dynamic contrast in pathological phenotype: non-infected, average basal state, versus susceptible plus infected. Overall, greater pathology resulted in more induction of gene expression than repression of



gene expression. This pattern of more abundant induction response has also been recorded in the spleens of chickens infected with *Salmonella enterica* (Zhou and Lamont, 2007), but is contrary to results in the spleens of chickens infected with *Campylobacter jejuni* (Li et al., 2011). This may be reflective of the greater amount of phylogenetic similarity between *E. coli* and *Salmonella*, and potential similar actions within the host, indicating that *Salmonella* is a better comparative infection model for APEC, than *Campylobacter*.

Choice of time post-challenge is important in understanding APEC infection; status of lesions can be milder if measured too early or late in the course of infection (Dwars et al., 2009). Time post-challenge played an important role in the identification of differentially expressed genes in the current study. For both tissues, pathology differences were not evident until 5 days post-challenge (Chapter 2 and 3). Time also generated differences in tissue roles and infection response. PBL from circulating blood exhibited high expression response due to infection early, which then waned to fewer differentially expressed genes later in infection (Chapter 3). The opposite trend was seen in the spleen, with later responses exhibiting higher numbers of differentially expressed genes (Chapter 2). Early and late sampling after infection is necessary to learn more about how these responses are being regulated. Sampling at early, day 1, and late, day 5, response times allowed for the potential identification of cascading pathways, early signaling in PBL that influenced later signaling in the spleen (Chapter 4). These studies and others have illustrated the importance of choice of time point and the advantage of sampling at multiple time points in a gene expression study (Ciraci et al., 2010).

Several contrasts of interest saw surprisingly little or no change in expression. Detectable differences between the mild pathology and the control only existed in the first

day post-challenge in the spleen. This might be a result of low power to detect, if the differences between groups were subtle. Subtle expression changes may be better detected with multiple platforms rather than increased sampling numbers, due to efficiency of binding of the probes, or by eliminating pre-filtering of the dataset to control noise. Our choice of a two-color array, however, is considered more sensitive than one-color microarray platforms to small changes (Pedotti et al., 2008). Given that the initial bacterial dosage was the same for all infected individuals and yet these individuals supported no lesions, it is possible that response to bacteria was rapid enough that a homeostatic state resumed within 24 hours. However, gene expression differences between severe and mild pathology were different than those found between severe pathology and control, indicating some level of undetectable expression differences between mild pathology and control. Neither tissue experienced detectable expression differences due to a vaccine effect despite its observed efficacy in reducing lesion severity. Time course studies have shown that time post-vaccination impacts detectable expression profiles (Gimeno and Cortes, 2011), indicating that 15 and 19 days post-vaccination may be a difficult time to observe expression differences.

### **Spleen and peripheral blood leukocytes are important immunological tissues in the fight against APEC infection**

The spleen displayed differential expression in 9 unique contrasts examined (Chapter 2). PBL experienced fewer contrasts with differential expression than the spleen, only 4 (Chapter 3). The ability of various leukocytes to phagocytize or use other killing methods is important to the clearance of APEC infection. Isolated heterophils and macrophages are often assessed *in vitro* to determine expression patterns in response to bacteria (Bliss et al.,

2005; Redmond et al., 2009). PBL analysis allows for assessment of the entire dynamic population of circulating leukocytes. These tissues have not only illustrated important independent function, but also joint expression patterns and pathways. A significantly greater than expected number of genes were significantly expressed in both tissues, with high levels of concordance (Chapter 4). Early signaling pathways in PBL appear to trigger downstream pathways in the spleen, illustrating the importance of coordination between tissues for proper immune signaling.

The response of classical immune family genes, TLRs, AvBDs, and cytokines between the two tissues was thought-provoking. Well-characterized Gram-negative receptors, TLR2 and TLR4, were increased in the spleen but not in the PBL, whereas more recently identified receptors, TLR7, TLR15, and TLR21, were stimulated in PBL (Chapters 2 and 3). TLR7 and TLR15 have been differentially regulated in the spleen in response to *Clostridium perfringens* infection (Lu et al., 2009), but TLR21 has not been extensively studied in the spleen, so the extent of TLR21 expression in the spleen is unknown. AvBDs were differentially expressed between pathologies in the spleen but only between the severe and control group day 1 post-infection in PBL (Chapters 2 and 3). Spleen expression supports previous work showing differential AvBD expression patterns in intestinal epithelial cells of *Salmonella* resistant and susceptible populations (Derache et al., 2009). Expression changes in cytokine genes seemed limited to the spleen (Chapter 2), although several cytokine receptor genes were differentially expressed in PBL (Chapter 3). The spleen may be functioning in a regulatory capacity over PBL, releasing signaling molecules while the PBL prepare themselves to more readily bind them and transduce signals. Macrophages and heterophils have shown *in vitro* stimulation of cytokines hours after challenge, indicating that

the later time of sampling may have missed induction in PBL, although the overall immune process in the whole organism might be postulated to be slower than *in vitro* exposure of isolated cells to bacteria (Bliss et al., 2005; Redmond et al., 2009).

**Pathology differences exist within commercial broiler populations and are associated with differing gene expression**

In both tissues examined, large gene expression differences existed between pathology states, similar to the large expression differences seen in populations characterized as resistant or susceptible to *Salmonella* or *Campylobacter* (Chiang et al., 2008; Li et al., 2011). A higher number of significant genes were found in the PBL (1,914) than the spleen (799) (Chapters 2 and 3). The differences between mild and severe pathology are important for deducing what constitutes a resistant or susceptible phenotype. A difficulty with gene expression profiles is deciphering whether the observed expression is in response to the bacterial infection and pathology or the cause of the pathology. Further investigation into the functions of these genes seems to imply that it is both. Increases in catalytic activity in the severe pathology appear to be responsible for host inflicted pathology (Chapter 4). Antimicrobial peptide genes were more abundant in the mild group in the spleen, enforcing that AvBD are important genes in the control of APEC and expression is increased in response to infection.

Within this population of male commercial broiler chickens, a large spread of pathology was recorded (Chapter 2). Although these pathology differences can have many causes, including environment and bacterial load, there is reason to postulate that genetics is also a factor, despite concerns over limited genetic variability within commercial stock (Muir et al., 2008). Genotype has been associated with susceptibility to colibacillosis in broiler

lines (Ask et al., 2006), although not associated with any particular genes. The gene expression differences found here can serve as a base for targeted genotype association studies in broilers in order to find a possible genetic cause for these expression differences.

### **Future directions for researching resistance to APEC**

Gene expression in this research was assessed using the most cost effective and comprehensive available technology at the time, a global microarray. In the past few years, RNA-seq technology has been revolutionary in the quantification of RNA transcripts. Microarrays rely on specific probe sequences to capture transcript expression levels, but RNA-seq sequences the whole transcript, not limiting itself to preselected genes and allowing for identification and quantification of splice variants or allele specific expression (Malone and Oliver, 2011). Utilization of this technology will allow for more precise measurements, the ability to examine the role of microRNAs, and better measurement of lowly expressed sequences. Although sequenced, much of the chicken genome still remains unannotated with unknown function. To fully utilize the information provided by these large transcriptomic studies, further annotation is required (van den Berg et al., 2010).

Analysis of SNP genotype information with phenotypic data can be used to identify regions of the genome associated with expression levels. The chicken has available a 60K SNP chip (Groenen et al., 2011), with 650K chips in development (Gheyas et al., 2011), allowing for good full genome coverage. Utilization of these SNP data in combination with microarray data will help locate markers associated with expression QTL and aid in the attempt to identify causal mutations. These data can also be utilized to search for SNPs associated with lesion scores, to locate QTL or genes associated with phenotype.

Discovering the reason behind the observed pathology will allow researchers to learn if susceptibility is primarily the result of an overactive host response or an inadequate response to infection. If the genetic basis for pathology differences can be discerned, it will be the first step in identifying resistant animals pre-infection rather than post-infection. Further work on expression of other immune tissues can help to complete the story of cascading responses. Meta-analysis of multiple tissues has been shown to help identify cross-tissue networks to enhance functional knowledge (Greco et al., 2008). The next logical target would be primary lymphoid organs; bursa of Fabricius, thymus and bone marrow. Humoral responses and B-cell activity could be better characterized by the bursa of Fabricius and bone marrow while T-cell activity could be better understood by profiling the thymus.

Work on understanding APEC, as a bacterium, has shown that many similarities and differences exist among strains (Johnson et al., 2008; Maturana et al., 2011; Rodriguez-Siek et al., 2005). How these differences in APEC impact host response should be determined to avoid APEC strain-specific improvement of the host, rather than increased resistance to all APEC. Understanding differences between strains may help in utilization of the chicken as a model organism for different strains of human ExPEC. Expression responses are valuable tools for understanding host response. Discovering how gene expression changes in infected chickens and at different pathological states will aid in the development of resistant chicken lines for healthier poultry and people.

## References

- Ask, B., E. H. van der Waaij, A. Stegeman, and J. A. M. van Arendonk. 2006. Genetic variation among broiler genotypes in susceptibility to colibacillosis. *Poult. Sci.* 85:415-421.

- Bliss, T. W., J. E. Dohms, M. G. Emara, and C. L. Keeler Jr. 2005. Gene expression profiling of avian macrophage activation. *Vet. Immunol. Immunopathol.* 105:289-299.
- Ciraci, C., C. K. Tuggle, M. J. Wannemuehler, D. Nettleton, and S. J. Lamont. 2010. Unique genome-wide transcriptome profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin. *BMC Genomics.* 11:545.
- Derache, C., E. Esnault, C. Bonsergent, Y. Le Vern, P. Quéré, and A. C. Lalmanach. 2009. Differential modulation of  $\beta$ -defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chicken inbred lines. *Dev. Comp. Immunol.* 33:959-966.
- Dwars, R. M., M. G. R. Matthijs, A. J. J. M. Daemen, J. H. H. van Eck, L. Vervelde, and W. J. M. Landman. 2009. Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus. *Vet. Immunol. Immunopathol.* 127:65-76.
- Gheyas, A. A., C. Boschiero, F. Turner, A. Kranis, K. Watson, L. Yu, J. Smith, R. Talbot, P. Kaiser, P. Hocking, J. E. Fulton, T. M. Strom, G. Haberer, S. Weigend, R. Preisinger, R. Fries, S. Qanbari, H. Simianer, F. Brew, J. A. Woolliams, and D. W. Burt. 2011. Detection and selection of SNPs from chicken whole genome sequencing for the design of high throughput genotyping arrays [Abstract]. In: Proceedings of the 6<sup>th</sup> International Chick Meeting. Sept. 17-20. Edinburgh, Scotland. p.38. Abstract number 30.
- Gimeno, I. M., and A. L. Cortes. 2011. Chronological study of cytokine transcription in the spleen and lung of chickens after vaccination with serotype 1 Marek's disease vaccines. *Vaccine.* 29:1583-1594.

- Greco, D., P. Somervuo, A. Di Lieto, T. Raitila, L. Nitsch, E. Castrén, and P. Auvinen. 2008. Physiology, pathology and relatedness of human tissues from gene expression meta-analysis. PLoS One. 3:e1880.
- Groenen, M. A., H. J. Megens, Y. Zare, W. C. Warren, L. W. Hillier, R. P. Crooijmans, A. Vereijken, R. Okimoto, W. M. Muir, and H. H. Cheng. 2011. The development and characterization of a 60K SNP chip for chicken. BMC Genomics. 12:274.
- Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger and L. K. Nolan. 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46:3987-3996.
- Li, X., C. L. Swaggerty, M. H. Kogut, H. I. Chiang, Y. Wang, K. J. Genovese, H. He, F. M. McCarthy, S. C. Burgess, I. Y. Pevzner, and H. Zhou. 2011. Systemic response to *Campylobacter jejuni* infection by profiling gene transcription in the spleen of two genetic lines of chickens. Immunogenetics. Doi: 10.1007/s00251-011-057-1. [Epub ahead of print]
- Lu, Y., A. J. Sarson, J. Gong, H. Zhou, W. Zhu, Z. Kang, H. Yu, S. Sharif, and Y. Han. 2009. Expression profiles of genes in Toll-like receptor-mediated signaling of broiler infected with *Clostridium perfringens*. Clin. Vaccine Immunol. 16:1639-1647.
- Malone, J. H., and B. Oliver. 2011. Microarrays, deep sequencing and the true measure of the transcriptome. BMC Biol. 9:34.
- Maturana, V. G., F. de Pace, C. Carlos, M. Mistretta Pires, T. Amabile de Campos, G. Nakazato, E. Guedes Stheling, C. M. Logue, L. K. Nolan, and W. Dias da Silveira. 2011. Subpathotypes of avian pathogenic *Escherichia coli* (APEC) exist as defined by their syndromes and virulence traits. Open Microbiol. J. 5:55-64.



- Muir, W. M., G. K. Wong, Y. Zhang, J. Wang, M. A. Groenen, R. PP. Crooijmans, H. J. Megens, H. Zhang, R. Okimoto, A. Vereijken, A. Jungerius, G. A. Albers, C. T. Lawley, M. E. Delany, S. MacEachern, and H. H. Cheng. 2008. Genome-wide assessment of worldwide chicken SNP genetic diversity indicates significant absence of rare alleles in commercial breeds. *Proc. Natl. Acad. Sci. USA*. 105:17312-17317.
- Pedotti, P., P. A. Hoen, E. Vreugdenhil, G. J. Schenk, R. H. Vossen, Y. Ariyurek, M. de Hollander, R. Kuiper, G. J. van Ommen, J. T. den Dunnen, J. M. Boer, and R. X. de Menezes. 2008. Can subtle changes in gene expression be consistently detected with different microarray platforms? *BMC Genomics*. 9:124.
- Redmond, S. B., P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2009. Chicken heterophils from commercially selected and non-selected genetic lines express cytokines differently after *in vitro* exposure to *Salmonella enteritidis*. *Vet. Immunol. Immunopathol.* 132:129-134.
- Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan. 2005. Characterizing the APEC pathotype. *Vet. Res.* 36:241-256.
- van den Berg, B. H., F. M McCarthy, S. J. Lamont, and S. C. Burgess. 2010. Re-annotation is an essential step in systems biology modeling of functional genomics data. *PLoS One*. 5:e10642.
- Zhou, H., and S. J. Lamont. 2007. Global gene expression profile after *Salmonella enterica* Serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. *Cytogenet. Genome Res.* 117:131-138.

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