Cellular Events During Coccidial Infection in Chickens

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ABSTRACT

Avian coccidiosis is caused by the intestinal protozoa *Eimeria*. The parasite's site of infection in the intestine is site specific. *Eimeria acervulina* mainly affects the duodenum, *E. maxima* the jejunum, and *E. tenella* the ceca. Lesions in the intestinal mucosa cause reduced feed efficiency and body weight gain in *Eimeria*-challenged chickens. My previous studies showed that the growth reduction may be due to changes in expression of digestive enzymes and nutrient transporters in the intestine. This can also lead to diminished intracellular pools of nutrients and inhibit pathogen replication. In this dissertation, further analysis of cellular events was performed. Expression of host defense peptides (HDPs), apoptosis and autophagy related genes were examined in *Eimeria* challenged broilers. The results showed that upon *Eimeria* infection, LEAP2 was consistently downregulated in the target tissues, while the avian beta-defensins (AvBDs) showed many variations in expression patterns. Downregulation of LEAP2 may be a mechanism for *Eimeria* to combat the host defense system, and to promote its survival inside the host cell. The in situ hybridization results showed that LEAP2 was expressed only along the villus in the small intestine and not in the crypt. This is the first time LEAP2 has been localized to epithelial cells of the chicken intestine. *Eimeria* infection can also induce an anti-apoptotic and anti-autophagy state in the host cells. This condition can be both favorable and unfavorable to parasite survival and replication inside the host cell.

A comparison of gene expression between Ross and *Eimeria* resistant Fayoumi (line M5.1 and M15.2) chickens challenged with *Eimeria maxima* was conducted. The comparison among different lines of chickens showed differential gene expression patterns in lines with different resistance to *Eimeria*. The similar body weight reduction indicated that there may not be a significant *Eimeria* resistant line among the Ross, Fayoumi M5.1 and M15.2 birds. The interaction between *Eimeria* and the host cell is very complex. Studying the mechanisms behind the changes of gene expression during *Eimeria* infection may give rise to potential therapeutic targets of coccidiosis.

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AA: Amino Acid

AvBD: Avian beta-defensin

AMP: Antimicrobial peptide

BW: Body weight

BWG: Body weight gain

dpi: Days post infection

EAAT3: Excitatory amino acid transporter 3

HDP: Host defense peptide

LC3: Microtubule-associated protein light chain 3

LEAP2: Liver expressed antimicrobial peptide 2

PCR: Polymerase chain reaction

Chapter 1. Introduction

Commercial poultry production

Poultry meat and eggs are one of the major food sources for the world's rapidly expanding population (FAO statistical yearbook, 2013). The production cost for poultry products are relatively low compared to pork and beef, as poultry requires much smaller amount of feed per kilogram of meat or egg production (Landoni and Albarellos, 2015; FAO statistical yearbook, 2013). The absence of religious restriction on the consumption of poultry products may be one of the reasons that the poultry industry is the most widespread food production industry worldwide (Landoni and Albarellos, 2015). Global poultry production has tripled in the past 20 years and the world's chicken flock is estimated at approximately 21 billion, which produced 1.1 trillion eggs and 90 million tons of meat each year (Blake and Tomley, 2014). The global market of poultry meat and egg has grown 19% and 9.52%, respectively, from year 2006 to 2010 (FAO statistical yearbook, 2013; Landoni and Albarellos, 2015). Commercial poultry production is a very intensive animal agricultural system. One poultry house can contain as many as 100,000 commercial layers (Landoni and Albarellos, 2015). The intensive selection for production traits in the commercial chicken has indeed compromised their immune function (van der Most et al., 2011). This means that disease control and prevention remains very important in the poultry industry. Common economically important poultry diseases are: Newcastle disease, avian influenza, Marek's disease, necrotic enteritis and coccidiosis.

Avian coccidiosis and *Eimeria*

Coccidiosis is a major disease of poultry caused by the intestinal protozoa *Eimeria* (Conway, 2007). Lesions in the intestinal mucosa reduce feed efficiency and body weight gain. A damaged intestinal barrier leads to bacterial infection, which can increase mortality in birds. Coccidiosis is responsible for the loss of approximately $3 billion in the poultry industry worldwide (Dalloul et al., 2007). The common treatment for coccidiosis is the use of anticoccidial drugs, but large-scale and long-term use of these drugs has led to the worldwide development of resistance against most of these drugs. Live attenuated and non-attenuated anticoccidial vaccines have shown positive results in preventing coccidiosis. Highly efficient and low-cost anticoccidial vaccines could potentially replace anticoccidial drugs in the future (Peek and Landman, 2011). The use of feed additives like prebiotics and probiotics increase body weight gain and feed conversion during *Eimeria* challenge (Bozkurt et al., 2014). The current high cost of production of natural products may limit their usefulness as feed additives in large commercial settings (Quiroz-Castaneda and Dantan-Gonzalez, 2015). An alternative approach to disease control involves enhancement of the immune system.

Life cycle of *Eimeria*

The life cycle of *Eimeria* takes about 4 to 7d to complete (Figure I-1). The bird can pick up oocysts from the environment by swallowing infected litter. An oocyst contains 4 sporocysts, which each contains 2 sporozoites. Oocysts are generally ovoid to ellipsoid in shape, and range from 10-40 μm in length by 10-30 μm in width. The wall of the oocyst contains peptide, lipid and carbohydrate. The likely physical arrangement of the components places the lipid in a 10 nm thick outer layer, covering a 90 nm thick layer of glycoprotein (Stotish et al., 1978). After the bird consumes the oocyst, 8 sporozoites are released into the digestive system. The sporozoites

then invade the intestinal epithelial cells, and use the host cell as a nutrient supply for replication. After several generations of asexual multiplication, a sexual stage occurs in which male and female gametes unite and form new oocysts that are protected by a thick wall. These oocysts are shed in the feces, to be picked up by other animals (Allen and Fetterer, 2002).

Eimeria infection in chicken

Eimeria infection is species- and site-specific. The species of *Eimeria* that infect chickens are different from those that infect turkeys or other species. In the U.S., the three species of *Eimeria* that most impact the poultry industry are *E. acervulina*, *E. maxima* and *E. tenella*. *E. acervulina* preferentially affects the duodenum, *E. maxima* the jejunum, and *E. tenella* the ceca (Lillehoj and Trout, 1996). Lesions in the intestinal mucosa can be measured on a scale of 0 to 4. A score of 0 shows no lesions, and a score of 4 shows many lesions (Johnson and Reid, 1970). Chickens challenged with *E. maxima* oocysts yield the same lesion score as chickens challenged with a higher dose of *E. acervulina* or *E. tenella* oocysts. Jenkins et al. (2013) compared the relative sensitivities of *E. acervulina*, *E. maxima*, and *E. tenella* oocysts to dessication and showed that *E. maxima* oocysts have greater resistance to drying compared to *E. acervulina* and *E. tenella*.

Immune response to *Eimeria* challenge in chicken

The mucosal immune system is composed of the mucosal associated lymphoid tissues (MALT) that attacks the pathogen at its site of entry (Yun et al., 2000a). Gut associated lymphoid tissues (GALT) are the largest component of MALT. Unlike mammals, chickens do not have lymph nodes, they have lymphoid structures such as the bursa of Fabricius, cecal tonsils, Meckel's diverticulum and Peyer's patches (PP) (Lillehoj and Trout, 1996). Microfold cells at the PP

present antigens to T lymphocytes at the epithelial layer and antibody-producing B lymphocytes at the lamina propria. Immunoglobulin A (IgA) is produced by B lymphocytes (Lillehoj and Trout, 1996). Following *E. maxima* infection, intestinal IgA and cytokine interferon-gamma (IFN-gamma) levels were increased (Yun et al., 2000b). Analysis of *E. acervulina*, *E. maxima* and *E. tenella* treated chicken macrophages showed common regulation of interleukins (IL) and chemokines. There was induced expression of IL-1β, IL-6, and IL-18 and repressed expression of IL-16. Expression of macrophage inflammatory protein (MIP)-1β (CCLi1), K203 (CCLi3), and ah221 (CCLi7) were commonly increased but CXCL chemokine K60 (CXCLi1) was found to be induced by macrophage exposure to *E. tenella* only (Dalloul et al., 2007).

Fayoumi chickens

The Fayoumi chickens are a native Egyptian breed, which can be used for meat and table egg production (Hassan et al., 2002). These chickens were imported into the US in 1954, primarily because of their resistance to avian leucosis (Kim et al., 2008). Fayoumi chickens are more coccidiosis resistant compared to commercial breeds in the US, such as Rhode Island Red and white Leghorn (Pinard-Van Der Laan et al., 1998). But they may be more susceptible to other infections when compared to other Egyptian breeds, such as Mandarah (Hassan, et al., 2002). The two lines of Fayoumi chickens used in this dissertation are congenic paired M5.1 and M15.2. These two lines are both highly inbred, and share an identical genetic background and only differ at the Major histocompatibility complex (MHC). Both of these lines are genetically distant from the commercial broiler and Leghorn lines (Kim, et al., 2008; Zhou and Lamont, 1999). The M5.1 line showed increased resistance to *E. maxima* infection compared to the M15.2 line (Kim, et al., 2008).

The MHC is a gene region that contains important immune genes (Miller and Taylor, 2016). Chicken MHC genes are located on chromosome 16 in two separate regions: MHC-Y and MHC-B (Delany et al., 2009; Miller and Taylor, 2016). The major function of MHC molecules are antigen presentation to T cells, thus they bind to fragments from pathogens and display them on the cell surface (Janeway, 2001). MHC class I and class II molecules are highly polymorphic and polygenic, and are able to respond to different and evolving pathogens. Other MHC genes, some of which are also polymorphic and polygenic, contribute to immunity in additional ways (Miller and Taylor, 2016). The structures of class I and class II molecules are very similar, but the domains are connected differently. Class I molecules bind to peptides in the cytoplasm, while class II molecules bind to proteins in intracellular vesicles (Kaufman, 2008).

Morphology of the intestine

The majority of nutrient absorption occurs in the small intestine (Leeson et al., 2001). Structures such as plicae circularis (mucosal folds), villi and microvilli present in the small intestine increase the surface for absorption. In an adult chicken, the small intestine is about 1.3m in length and can be divided into three sections: duodenum, jejunum and ileum.

The duodenum is the first section of the small intestine, and loops around the pancreas, which is called the duodenal loop. The primary function of the duodenum is to mix food chyme with digestive enzymes secreted from the liver, pancreas and the duodenal wall, this process also results in neutralizing the acid in the food chyme from the stomach (Smith and Morton, 2010).

The jejunum has a very similar anatomical structure to the duodenum, but there is a reduction of the mucosal folds compared to the duodenum (Smith and Morton, 2010). In many species there is no distinct anatomical feature that separates the jejunum and the ileum. In chicken, Meckel's diverticulum marks the end of the jejunum and the start of the ileum (Noy and Sklan, 2001). Right before hatch, the yolk sac is absorbed into the navel cavity of the chicken embryo, and the residual tiny yolk sac stalk is Meckel's diverticulum.

There is further reduction of the mucosal folds in the ileum, the distal part of the small intestine. The mucosal folds are absent at the end of the ileum. The ileum has abundant lymph node like structures, called Peyer's patches, which are located in the mucosa and submucosal layer of the ileum (Smith and Morton, 2010). The ileocecal sphincter separates the ileum and the large intestine, which functions in reduction of reflux from the colon (Smith and Morton, 2010).

Birds have two ceca located below the junction of the small intestine and the large intestine (Moreto and Planas, 1989; Smith and Morton, 2010). The wall of the ceca has mucosal folds but not villus structures. The main function of the ceca is fermentation of dietary fiber, absorption of water, sugar and amino acids (Salanitro et al., 1976; Wilkinson et al., 2003)

A single simple columnar epithelium layer covers the villi (Smith and Morton, 2010). Most of these cells are enterocytes, which are highly specialized and polarized cells. The specialized brush border membrane contains many cytoplasmic extensions, which characterize the brush border membrane and is essential for nutrient digestion and absorption. The tight junction formed between adjacent enterocytes separates these two types of membranes (Van Beers et al.,

1995). Membrane bound glycoproteins like mucins at the brush border membrane form a barrier to protect the host against intestinal pathogens (Belley et al., 1999).

Nutrient digestion and absorption at enterocytes

The process of digestion, chemical breakdown of food by digestive enzymes, begins in the mouth. The final stage of digestion and nutrient absorption takes place in the intestinal lumen at the enterocyte surface (Johnson, 2007). At the brush border membrane, disaccharides are degraded into monosaccharides by saccharidases, and small peptides are further broken down to di- and tri-peptides or amino acids by peptidases (Van Beers et al., 1995). Digestion is accomplished by hydrolysis by enzymes produced by enterocytes. Transporters located at the brush border mediate absorption of monosaccharides, amino acids and di- and tri-peptides (Johnson, 2007). Only a small fraction of the absorbed nutrients is used within the enterocyte. Most of the nutrients exit the cell via transporters located at the basolateral membrane. Once the nutrients are passed into the blood, they are exported via the portal vein to the liver and the rest of the body (Van Beers et al., 1995).

Solute carrier (SLC) transporters

Transporters are membrane-bound proteins that mediate the translocation of substrates across biological membranes. Transporter proteins are widely expressed in the epithelial tissues throughout the body, such as the liver, intestine, kidney, placenta and brain. Expression patterns of transporters are unique for each tissue at different developmental stages. The two main transporter superfamilies are the ATP-binding cassette (ABC) superfamily and the solute carrier (SLC) superfamily. ABC transporters are powered directly by ATP hydrolysis and function as

efflux transporters, whereas SLC transporters are primarily involved in the uptake of small molecules into cells. There are more than 300 proteins in the SLC transporter superfamily, which are important in physiological processes ranging from the cellular uptake of nutrients to the absorption of drugs and other xenobiotics (Lin et al., 2015). All transporters examined in this dissertation belong to the SLC superfamily (Table I-1).

Protein digestion and absorption

Dietary protein is required to supply the essential amino acids, which the body cannot produce or cannot synthesize rapidly enough, and replace nitrogen lost in the urine (Smith and Morton, 2010). Degradation of protein into di- and tri-peptides and free amino acids is accomplished by two kinds of proteolytic enzymes: endopeptidases and exopeptidases. Endopeptidases are digestive enzymes like pepsin produced by the stomach and trypsin, chymotrypsin and elastase secreted by the pancreas. These pancreatic proteases cleave peptide bonds in the center of the peptides. Exopeptidases cleave peptide bonds at the ends of the peptides. Carboxypeptidases break the peptide bond at the C-terminus and aminopeptidases at the N-terminus.

Aminopeptidase N (APN). The APN/CD13 is a type II metalloprotease that belongs to the M1 family of the Metallopeptidase, clan MA(E). There are two types of APN: the membrane bound aminopeptidase N and the soluble aminopeptidase N, both of which have many isoforms with different functions (Luan and Xu, 2007). APN cleaves neutral amino acids from the N-terminus of oligopeptides (Danziger, 2008). Besides its function as a digestive enzyme, APN is also involved in the trimming of antigens and in the process of antigen presentation (Luan and Xu, 2007).

Peptide transporter 1 (PepT1). Di- and tri-peptides are transported across the brush border membrane via peptide transporters, which are part of the SLC15 family (Daniel and Kottra, 2004). PepT1 (SLC15A1) is a high capacity, low affinity peptide transporter located at the brush border membrane of intestinal enterocytes. The uptake of peptides is mediated by the cotransport of a H^+ ion (Daniel and Kottra, 2004). Most of the small peptides that enter the enterocyte are hydrolyzed by intracellular peptidases, and transported out of the cell via amino acid transporters at the basolateral membrane. Free amino acids cross the brush border membrane via different types of transporters depending on the size and the electrical property of the amino acids (Smith and Morton, 2010).

$b^{o,+}$AT and rBAT transporter complex. The $b^{o,+}$AT (SLC7A9) and rBAT (SLC3A1) form a heteromeric amino acid transporter by a disulfide bridge (Fotiadis et al., 2013; Palacin and Kanai, 2004; Verrey et al., 2004; Wagner et al., 2001). The $b^{o,+}$AT is the light subunit, which has transporter function. rBAT is the heavy subunit, which is essential for trafficking the complex to the cell membrane (Palacin and Kanai, 2004; Verrey et al., 2004). Transport activity of this complex is Na+-independent and exchanges extracellular cationic amino acids and cystine for intracellular neutral amino acids (Fotiadis et al., 2013). $b^{o,+}$AT has high-affinity transport of L-cysteine and cationic amino acids and lower affinity transport of neutral amino acids (Verrey et al., 2004).

Na+-dependent neutral amino acid transporter (B^0AT). The B^0AT (SLC6A19) transports a broad range of neutral amino acids into the cell (Broer et al., 2004; Romeo et al., 2006). Some of

these neutral amino acids are used by $b^{0,+}$AT in exchange for cationic amino acids and cysteine (Fotiadis et al., 2013). Transport of amino acids via B^0AT is driven by the membrane potential. The most preferred substrate for B^0AT is leucine in a pH-dependent manner, which strongly increases with alkaline pH (Broer et al., 2004). B^0AT is highly expressed in the brush border membrane in the small intestine (Broer et al., 2004; Terada et al., 2005).

Excitatory amino acid transporter 3 (EAAT3). EAAT3 (SLC1A1), also known as EAAC1, is a Na+-dependent high-affinity transporter of anionic amino acids such as aspartate and glutamate and is located at the brush border membrane of enterocytes (Gilbert et al., 2007; Kanai et al., 2013). Glutamate is one of the most abundant amino acids in dietary protein, but the blood concentration is quite low. This is because in the small intestine, glutamate is the energy source of the enterocytes (Fan et al., 2004; Iwanaga et al., 2005), and is also used by the enterocytes to synthesize other amino acids (Blachier et al., 2009). Fan et al. (2004) showed that EAAT3 is the major L-glutamate transporter. Expression of EAAT3 can be detected along the crypt-villus axis, but there is higher capacity and lower affinity transport activity in crypt than in villus cells (Fan et al., 2004). In chickens, expression of EAAT3 is greatest in the ileum, which indicates higher uptake of glutamate in the lower part of the small intestine (Gilbert et al., 2007).

Carbohydrate digestion and absorption

In chicken, the major source of carbohydrates is starch in grains. Digestion of carbohydrates provides an energy source for the body. There are several enzymes in the gastrointestinal tract that degrade starch and glycogen. Sucrase isomaltase secreted by the intestinal cells are responsible for the major part of the final digestion of polysaccharides. Hydrolyzed

monosaccharides such as glucose, galactose, mannose and fructose are transported into the enterocyte by the Na+-dependent glucose transporter 1 (SGLT1) and glucose transporter 5 (GLUT5), and exit the cell via glucose transporter 2 (GLUT2) and glucose transporter 1 (GLUT1) (Smith and Morton, 2010). SGLT1, GLUT5 and GLUT2 are the most abundant monosaccharide transporters in the small intestine compared with other hexose carriers (Yoshikawa et al., 2011).

Sucrase isomaltase (SI). SI is an enzyme complex that is responsible for 80% of the maltase activity in the small intestine (Van Beers et al., 1995). The sucrase subunit hydrolyzes sucrose, but not α (1-6) glucosidic bonds. The isomaltase subunit hydrolyzes α (1-6) glucosidic bonds but not sucrose. Both subunits hydrolyze maltose, maltotriose and hydrophobic aryl- α-glucopyranosides. The complex has no activity towards polysaccharides like starch (Van Beers et al., 1995). Sucrase isomaltase is highly expressed in the small intestine, which accounts for 10% of the brush border membrane protein. In chicken, expression of SI has been reported in embryonic and post-hatch intestine (Sklan et al., 2003). Very low expression of SI has been detected in the embryonic yolk sac (Yadgary et al., 2011).

Sodium-dependent glucose transporter-1 (SGLT1). SGLT1 (SLC5A1) is a Na+-dependent glucose cotransporter at the brush border membrane, which was the first cotransporter protein identified using rabbit intestine (Wright and Turk, 2004). SGLT1 is a uniporter, i.e., it pumps one glucose molecule into the cell along with 2 Na+ ions (Hediger and Rhoads, 1994). Expression of SGLT1 can be found at the plasma membrane of cells located at the small intestine, trachea, kidney and heart (Wright and Turk, 2004). SGLT1 is highly expressed in the

duodenum, and the expression level decreases in the distal part of the small intestine in mice (Yoshikawa et al., 2011). In chickens, SGLT1 expression level is greater in the jejunum and ileum than duodenum (Gilbert et al., 2007).

Glucose transporter-2 (GLUT2). The GLUT2 (SLC2A2) transporter is located at the basolateral membrane, and mediates the Na+-independent, low-affinity transport of glucose, galactose, mannose and fructose, and high-affinity transport of glucosamine (Uldry and Thorens, 2004). GLUT2 translocation to the brush border membrane in response to high glucose load has also been reported (Mithieux, 2005). In chicken, GLUT2 is highly expressed in the jejunum, and mRNA expression increased linearly with age (Gilbert et al., 2007).

Mineral absorption

Absorption of ion minerals and trace elements occurs in the jejunum and ileum. Calcium, magnesium and phosphate can be absorbed by passive diffusion, but also can be transported across the membrane by active transporters like other ions such as sodium and zinc (Leeson et al., 2001). The rate of absorption depends on pH, membrane potential, transporters and the presence of other minerals (Leeson et al., 2001). The body requires minerals in many physiological processes (Smith and Morton, 2010). Calcium is important in bone development and cellular signaling pathways. Magnesium is an important co-factor for many enzymes. Phosphate is also involved in bone formation, acid-base balance and nucleic acid synthesis. Sodium is the key element in maintaining and changing membrane potential, and is also required for many nutrient co-transporters as discussed above. Zinc is a trace mineral, which functions as a cofactor of enzymes, nuclear factors and hormones (Devergnas et al., 2004).

Zinc transporter 1 (ZnT1). The ZnT proteins are members of the cation diffusion facilitator family. They function in transporting zinc out of the cells or contained in cellular compartments (Tako et al., 2005). ZnTs are expressed in a tissue-specific manner. ZnT1 (SLC22A18) is ubiquitously expressed in the body, but is most abundant at the basolateral membrane of enterocytes in the duodenum and jejunum (McMahon and Cousins, 1998). Expression of ZnT1 in the small intestine can be induced by increasing dietary zinc, which was first found in rat (McMahon and Cousins, 1998) and later confirmed in chicken (Tako et al., 2005).

Host defense peptides in chickens

Innate immune system

Innate immunity is the first line of host defense against infections in vertebrate animals, and the only defense in plants and invertebrates (Dziarski, 2013). Surface tissues such as skin, respiratory, reproductive and gastro-intestinal tracts are constantly exposed to the external environment. Membranes that line these organs provide mechanical and chemical protection against infectious agents (Gallo and Hooper, 2012). Other components of the innate immune system include: immune cells, antimicrobial peptides (AMPs), enzymes, and pro-inflammatory factors (Dziarski, 2013).

Avian innate host defense

The components of the avian innate immune system are similar to mammalian species, which both include immune cells, enzymes, proteins and peptides. These components act as the first

line of defense against pathogens and other environmental challenges. Macrophages and dendritic cells are present in both avian and mammalian species, neutrophils are not found in birds. Instead, birds have heterophils, which infiltrate the site of infection during the early stage of inflammation (Harmon, 1998). For the detection of pathogens, avian immune cells have a variety of pattern-recognition receptors like mammals, including the Toll-like receptors (TLRs). But the chicken immune system contains a slightly different TLR repertoire (Davison et al., 2008). Lysozyme, which is mainly present in eggwhite and mature macrophages, achieves its antimicrobial function by disrupting the peptidoglycan structure of Gram-positive bacteria (Cuperus et al., 2013). Collectins bind to the carbohydrate ligands on the surface of microorganisms, which play an important role in defending against invading pathogens (van de Wetering et al., 2004). Sequence analysis showed that chicken collectins have very similar structure to their human and murine counterparts (Hogenkamp et al., 2006).

Host defense peptides

Host defense peptides (HDPs) were first discovered in 1962 from the frog *Bombina variegata*. During the late 1970s, more groups of defensins were discovered from humans and rabbits (Piyadasa et al., 2015). HDPs were originally called AMPs because of their antibacterial function. Later on many research groups reported that HDPs are involved in fertility, development, activation of leukocytes, DNA uptake and wound healing (Semple and Dorin, 2012; Cuperus, et al., 2013). HDPs are produced by almost all plants and animals and have broad-spectrum activity against a range of bacteria, fungi and enveloped viruses (Townes et al., 2009; Gallo and Hooper, 2012). They are essential for epithelial surfaces to confront everyday microbial challenges (Gallo and Hooper, 2012). There are several distinct protein families of

HDPs, which include defensins, cathelicidins, C-type lectins, ribonucleases and S100 proteins (Gallo and Hooper, 2012).

Avian HDPs

Avian HDPs were first described in the mid 1990's. Five avian defensins were isolated from chicken and turkey leukocytes (Evans et al., 1994.) Currently, 14 avian beta-defensins, 4 cathelicidins, 4 ovodefensins, 4 collectins and liver expressed antimicrobial peptide 2 (LEAP2) have been identified in chickens. With increased antibiotic resistance of avian pathogens, intensive research is ongoing to discover alternative methods against infectious diseases. Use of HDPs as treatment is one of the alternatives to antibiotics (Cuperus et al., 2013).

Defensins

Defensins are short cysteine-rich, cationic peptides, which can be subdivided into three main groups: the α-, β- and θ-defensins. α-defensins are only present in mammalian species, β-defensins can be found in all vertebrate species, and θ-defensins are found in some species of non-human primates. α- and β-defensins are very different in their secondary structures, due to the position of disulfide bonds. But the tertiary structures of α- and β-defensins are very similar. θ-defensins are the only known cyclic polypeptide motif in animals (Selsted and Ouellette, 2005). Defensins have broad-spectrum activity against bacteria, viruses, and fungi as well as showing activity against protozoan parasites (Hellgren and Ekblom, 2010). The most common mode of action of defensins is to bind and disrupt the membrane of invading pathogens, and change membrane permeability and homeostasis of the microorganisms (Hellgren and Ekblom,

2010; Selsted and Ouellette, 2005). Many defensins achieve their antiviral function by acting like lectins, because they bind to the glycoprotein on the viral envelope, thus blocking the attachment of viruses to host cells (Bomminei, 2008).

Avian beta-defensins. β-defensin was first found in bovine tracheal mucosa (Diamond et al., 1991). They are the only defensins found in avian species, with more than 25 family members identified. In chicken, there are 14 AvBDs located on a cluster on chromosome 3 (van Dijk et al., 2008). Each AvBD gene contains 4 exons (E1-4), with E1 coding for 5' untranslated reagion (UTR), E2 the signal peptide and a part of the prepeptide, E3 for the rest of the prepeptide and the mature peptide and E4 for the 3'-UTR (Hellgren and Ekblom, 2010). β-defensins are expressed and secreted by neutrophils and epithelial cells in various organs. In chickens, the tissue-specific patterns seem to vary across the different defensin genes with some showing expression in a wide array of tissues. For example, AvBD9 was found expressed in 22 different tissues, including brain, intestine and testis. Other AvBDs seem to have more limited expression patterns. For example, AvBD8 is expressed only in the liver and gall-bladder (Hellgren and Ekblom, 2010). Hong et al. (2012) examined the expression of AvBDs in the jejunum of Cobb and Ross broilers following an *E. maxima* and *Clostridium perfringens* challenge to induce necrotic enteritis. In uninfected chickens, abundance of AvBD8, AvBD10 and AvBD13 mRNA was high, AvBD1, AvBD6, AvBD9, AvBD11 and AvBD12 mRNA was moderate, and AvBD3 and AvBD4 mRNA was very low. Infected Cobb and Ross chickens showed different patterns of upregulation and downregulation of AvBDs.

Liver-expressed antimicrobial peptide-2 (LEAP2). The chicken LEAP2 gene was first discovered by bioinformatics screening of the chicken genome (Lynn et al., 2004). Based on the in silico sequence, LEAP2 expression was detected in a number of tissues including the small intestine, liver, lung and kidney (Townes et al., 2004). Birds orally challenged with *Salmonella enterica* showed upregulation of LEAP2 expression in both small intestine and liver. An in vitro assay showed that LEAP2 has antimicrobial activity against *Salmonella* (Townes et al., 2004). Later, the same research group discovered that LEAP2 could interact with the outer membrane of bacteria and change its permeability. LEAP2 also has broad-spectrum antimicrobial activity and plays an important role in the chicken innate host defense (Townes et al., 2009). In contrast, in *E. maxima*-challenged chickens, LEAP2 showed up to 71-fold downregulation in the jejunum, and chickens with higher lesion scores showing greater downregulation of LEAP2. The mechanism behind this expression pattern is to be further investigated, but it was hypothesized that *E. maxima* causes a downregulation of LEAP2 in the intestinal epithelia (Casterlow et al., 2011).

Expression of Digestive Enzymes and Nutrient Transporters in *Eimeria* Infected Chickens

The small intestine is the major site for nutrient absorption in birds and mammals. Free amino acids, short peptides, and monosaccharides are absorbed by the enterocytes by specific transporters located at the brush border membrane and exported from the cell via transporters at the basolateral membrane (Broer, 2008; Fotiadis, et al., 2013). The growth reduction in *Eimeria* infected chickens may be due to changes in expression of digestive enzymes and nutrient transporters in the intestine (Fetterer et al., 2014; Paris and Wong, 2013; Su et al., 2014; Su et al., 2015). At the respective target tissue, *E. acervulina*, *E. maxima* and *E. tenella* infection caused

common downregulation of digestive enzymes APN and SI, amino acid transporters $b^{0,+}AT$, rBAT and EAAT3, monosaccharide transporters GLUT2 and GLUT5, zinc transporter ZnT1 and antimicrobial peptide LEAP2. The downregulation of nutrient transporters would result in a decrease in the efficiency of protein and polysaccharide digestion and uptake, which may partially explain the weight loss. The downregulation of nutrient transporters may also be a cellular response to reduced expression of the host defense protein LEAP2, which would diminish intracellular pools of nutrients and inhibit pathogen replication.

Apoptosis and autophagy

Apoptosis

The survival or death of a cell is controlled by proliferative status, repair enzyme capacity, and the ability to deduce proteins that can either promote or inhibit cell death. Abnormal regulation of these processes can lead to pathological conditions of cell over growth or tissue degeneration, such as cancer or muscular dystrophy, respectively. (Robertson and Orrenius, 2000; Goldstein and McNally, 2010). Apoptosis is programed cell death, which is tightly regulated by many genes, and is biochemically and morphologically different from necrosis (Leist et al., 1997; Robertson and Orrenius, 2000). The key steps of apoptosis involve: nuclear condensation, cell shrinkage, membrane blebbing and DNA fragmentation (Elmore, 2007; Robertson and Orrenius, 2000). Necrosis is a passive form of cell death, which causes the cell to swell and break down the plasma membrane, releasing the cell contents to the extracellular space. This process is often associated with inflammation. Intracellular ATP level is important in the determination of mode of cell death (Leist, et al., 1997; Robertson and Orrenius, 2000). Cytochrome c is a component of the electron transport chain and is indirectly involved in ATP production. The release of

cytochrome c from mitochondria may activate apoptosis by decreasing ATP production (Rivoira et al., 2012). Other regulators of apoptosis include the caspases and Bcl-2 family proteins, which will be described in more detail in the following sections.

Cytochrome C. Cytochrome c may regulate apoptosis by altering the ATP concentration in the cell, but the release of cytochrome c from the mitochondria can activate the apoptosis pathway. Cytosolic cytochrome c interacts with Apaf-1 and procaspase-9 in the presence of dATP, and form the complex called an apoptosome, which will activate caspase-3 and ultimately lead to cell death (Wilkinson, et al., 2003). *E. tenella* infection causes the release of cytochrome c from the mitochondria, increasing intracellular Ca^{2+} concentration. This effect can be reversed by adding Ca^{2+} chelators to restore Ca^{2+} homeostasis (Cui et al., 2016).

Caspase. The name "caspase" comes from cysteinyl aspartate-specific protease (Robertson and Orrenius, 2000; Qin et al., 2004). Based on their role in apoptosis, caspases can be subclassified as initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7) (McIlwain, et al., 2013). Caspase-18, which is most similar to caspase-8, has been conserved among chicken, platypus, and opossum but is absent from placental mammals (Eckhart, et al., 2008). Within the apoptosome complex, an autocatalytic reaction causes the activation of caspase-9, which subsequently cleaves and activates caspase-3. The activation of caspase-3 results in the proteolytic cleavage of a range of cellular targets that ultimately leads to cell death (Wilkinson, et al., 2003). Inhibition of caspase-9 in chicken embryonic ceca cells promotes the infection of *E. tenella* (Zhang et al., 2015). Heat stress downregulated expression of caspase-6 and upregulated catalase in the liver of chickens (Rimoldi et al., 2015).

Autophagy

Autophagy, often called programmed cell survival, is a cellular recycling system that degrades less important cytoplasmic contents to protect vital cell functions in response to stress (Aburto et al., 2012). Autophagy also has a housekeeping role of preventing accumulation of proteins and recycling damaged organelles. Key steps of autophagy involve: autophagy induction, autophagosome formation, autophagosome lysosome fusion, and degradation of cellular components (Eskelinen and Saftig, 2009). Because intracellular pathogens have the ability to enter and replicate inside the host cell, the host cell needs to have proper mechanisms to respond to the invasion of intracellular pathogens. Autophagy may have an antimicrobial function that can degrade intracellular parasites (Deretic and Levine, 2009). The cellular machinery of autophagy was first identified in yeast *S. cerevisiae*, which is controlled by the autophagy-related (Atg) gene family (Deretic and Levine, 2009; Eskelinen and Saftig, 2009).

Beclin 1. Beclin 1 is a homolog to the yeast protein Atg6 (Eskelinen and Saftig, 2009), which was originally isolated as a Bcl-2 (B-cell lymphoma 2)-interacting protein (Yang and Klionsky, 2010). In the early stage of autophagy, Bcl-2 is part of the class III PI3K (Phosphoinositide 3-kinase) complex. Further, a protein called Ambra-1 binds to Beclin 1 and activates Vps34 kinase function and autophagosome formation. The binding of Bcl-2 protein can decrease PI3K activity associated with Beclin 1 and inhibit autophagosome formation (Eskelinen and Saftig, 2009).

Microtubule-associated protein light chain 3 (LC3). LC3 is a homolog of yeast Atg8, which is important in membrane translocation. The cleavage of LC3 yields two cytosolic forms, the

soluble LC3-I and the lipidated, autophagosome-specific form called LC3-II (Eskelinen and Saftig, 2009). LC3-I associates with phosphatidylethanolamine to form the membrane-bound form LC3-II. This protein–lipid complex is part of the autophagosome (Aburto, et al., 2012). In the infection of intracellular pathogen *Toxoplasma gondii*, autophagic inducers increased the level of LC3 protein and decreased the infection and parasite proliferation (Souto et al., 2016).

By examining the expression of digestive enzymes, nutrient transporters, HDPs, apoptosis and autophagy related genes in *Eimeria* challenged chickens, the goal is to gain a better understanding of the cellular events during *Eimeria* infection and to identify potential prevention and/or treatments to avian coccidiosis.

Objectives

Our previous studies showed changes in expression of digestive enzymes, nutrient transporters and LEAP2 in *Eimeria*-challenged chickens. In this dissertation, I expanded that analysis to AvBDs, apoptosis and autophagy related genes. The first objective was to profile the expression of avian beta-defensins (AvBD1, 6, 8, 10, 11, 12, 13) in the intestine of *E. acervulina-, E. maxima-* and *E. tenella*-challenged broilers. The second objective was to profile the expression of apoptosis (BAX, Bcl-2, caspase 3, 6, 7, 8, 18, CARD11 and cytochrome c) and autophagy (Beclin 1, LC3a and LC3b) related genes in the same chickens. The third objective was to compare temporal changes of AvBDs, apoptosis and autophagy related gene expression in *E. acervulina*-challenged chickens. The fourth objective was to examine the expression of digestive enzymes (APN and SI), amino acid transporters ($b^{o,+}$AT/rBAT, B^0AT and EAAT3), sugar transporters (GLUT2 and SGLT1), a mineral transporter (ZnT1), avian HDPs (AvBD1, 6, 8, 10,

11, 12, 13 and LEAP2) apoptosis related genes (cytochrome c, caspase 6, 7 and 18) and autophagy related genes (Beclin, LC3a and LC3b) in the duodenum, jejunum and ileum of *E. maxima*-challenged broilers and Fayoumi chickens. The fifth objective was to examine the location of LEAP2 and EAAT3 mRNAs in chicken small intestine following *Eimeria* infection.

References

Aburto, M. R., H. Sanchez-Calderon, J. M. Hurle, I. Varela-Nieto, and M. Magarinos. 2012. Early otic development depends on autophagy for apoptotic cell clearance and neural differentiation. Cell Death Dis 3:e394. doi 10.1038/cddis.2012.132

Allen, P. C., and R. H. Fetterer. 2002. Recent advances in biology and immunobiology of Eimeria species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin Microbiol Rev 15:58-65.

Belley, A., K. Keller, M. Gottke, and K. Chadee. 1999. Intestinal mucins in colonization and host defense against pathogens. Am J Trop Med Hyg 60:10-15.

Blachier, F., C. Boutry, C. Bos, and D. Tome. 2009. Metabolism and functions of L-glutamate in the epithelial cells of the small and large intestines. Am J Clin Nutr 90:814S-821S. doi 10.3945/ajcn.2009.27462S

Blake, D. P., and F. M. Tomley. 2014. Securing poultry production from the ever-present Eimeria challenge. Trends Parasitol 30:12-19. doi 10.1016/j.pt.2013.10.003

Bomminei, Y. R. 2008. Evaluation of Chicken Cathelicidins as Novel Antimicrobials. Doctor of Phillosophy. Oklahoma State Univerisy.

Bozkurt, M., N. Aysul, K. Kucukyilmaz, S. Aypak, G. Ege, A. U. Catli, H. Aksit, F. Coven, K. Seyrek, and M. Cinar. 2014. Efficacy of in-feed preparations of an anticoccidial, multienzyme, prebiotic, probiotic, and herbal essential oil mixture in healthy and Eimeria spp.-infected broilers. Poult Sci 93:389-399. doi 10.3382/ps.2013-03368

Broer, A., K. Klingel, S. Kowalczuk, J. E. Rasko, J. Cavanaugh, and S. Broer. 2004. Molecular cloning of mouse amino acid transport system B^0, a neutral amino acid transporter related to Hartnup disorder. J Biol Chem 279:24467-24476. doi 10.1074/jbc.M400904200

Broer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 88:249-286. doi 10.1152/physrev.00018.2006

Casterlow, S., H. Li, E. R. Gilbert, R. A. Dalloul, A. P. McElroy, D. A. Emmerson, and E. A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of Eimeria maxima-infected chickens. Poult Sci 90:1212-1219. doi 10.3382/ps.2010-01110

Conway, D. P., and M. E. McKenzie. 2007. Poultry coccidiosis: diagnostic and testing procedure. 3rd ed. Blackwell Pub., Ames, Iowa.

Cui, X. Z., M. X. Zheng, Y. Zhang, R. L. Liu, S. S. Yang, S. Li, Z. Y. Xu, R. Bai, Q. H. Lv, and W. L. Zhao. 2016. Calcium homeostasis in mitochondrion-mediated apoptosis of chick embryo cecal epithelial cells induced by Eimeria tenella infection. Res Vet Sci 104:166-173. doi 10.1016/j.rvsc.2015.12.015

Cuperus, T., M. Coorens, A. van Dijk, and H. P. Haagsman. 2013. Avian host defense peptides. Dev Comp Immunol 41:352-369. doi 10.1016/j.dci.2013.04.019

Dalloul, R. A., T. W. Bliss, Y. H. Hong, I. Ben-Chouikha, D. W. Park, C. L. Keeler, and H. S. Lillehoj. 2007. Unique responses of the avian macrophage to different species of Eimeria. Mol Immunol 44:558-566. doi 10.1016/j.molimm.2006.02.004

Daniel, H., and G. Kottra. 2004. The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch 447:610-618. doi 10.1007/s00424-003-1101-4

Danziger, R. S. 2008. Aminopeptidase N in arterial hypertension. Heart Fail Rev 13:293-298. doi 10.1007/s10741-007-9061-y

Davison, F., B. Kaspers, and K. A. Schat. 2008. Avian Immunology. first ed. Elsevier, London.

Delany, M. E., C. M. Robinson, R. M. Goto, and M. M. Miller. 2009. Architecture and organization of chicken microchromosome 16: order of the NOR, MHC-Y, and MHC-B subregions. J Hered 100:507-514. doi 10.1093/jhered/esp044

Deretic, V., and B. Levine. 2009. Autophagy, immunity, and microbial adaptations. Cell Host Microbe 5:527-549. doi 10.1016/j.chom.2009.05.016

Devergnas, S., F. Chimienti, N. Naud, A. Pennequin, Y. Coquerel, J. Chantegrel, A. Favier, and M. Seve. 2004. Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT-PCR study. Biochem Pharmacol 68:699-709. doi 10.1016/j.bcp.2004.05.024

Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W. L. Maloy, and C. L. Bevins. 1991. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proc Natl Acad Sci U S A 88:3952-3956.

Dziarski, R. 2013. Chapter 6: Innate Immunity. Pages xvii, 826 p. in Schaechter's mechanisms of microbial disease. M. Schaechter, N. C. Engleberg, V. J. DiRita, and T. Dermody eds. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516. doi 10.1080/01926230701320337

Eskelinen, E. L., and P. Saftig. 2009. Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochim Biophys Acta 1793:664-673. doi 10.1016/j.bbamcr.2008.07.014

Fan, M. Z., J. C. Matthews, N. M. Etienne, B. Stoll, D. Lackeyram, and D. G. Burrin. 2004. Expression of apical membrane L-glutamate transporters in neonatal porcine epithelial cells along the small intestinal crypt-villus axis. Am J Physiol Gastrointest Liver Physiol 287:G385-398. doi 10.1152/ajpgi.00232.2003

FAO statistical yearbook. 2013. Pages v. Food and Agriculture Organization of the United Nations, Rome.

Fetterer, R. H., K. B. Miska, M. C. Jenkins, and E. A. Wong. 2014. Expression of nutrient transporters in duodenum, jejunum, and ileum of Eimeria maxima-infected broiler chickens. Parasitol Res 113:3891-3894. doi 10.1007/s00436-014-4114-3

Fotiadis, D., Y. Kanai, and M. Palacin. 2013. The SLC3 and SLC7 families of amino acid transporters. Mol Aspects Med 34:139-158. doi 10.1016/j.mam.2012.10.007

Gallo, R. L., and L. V. Hooper. 2012. Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 12:503-516. doi 10.1038/nri3228

Goldstein, J. A., and E. M. McNally. 2010. Mechanisms of muscle weakness in muscular dystrophy. J Gen Physiol 136:29-34. doi 10.1085/jgp.201010436

Harmon, B. G. 1998. Avian heterophils in inflammation and disease resistance. Poult Sci 77:972-977.

Hassan, M. K., M. Afify, and M. M. Aly. 2002. Susceptibility of vaccinated and unvaccinated Egyptian chickens to very virulent infectious bursal disease virus. Avian Pathol 31:149-156. doi 10.1080/03079450120118630

Hellgren, O., and R. Ekblom. 2010. Evolution of a cluster of innate immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. Immunome Res 6:3. doi 10.1186/1745-7580-6-3

Hogenkamp, A., M. van Eijk, A. van Dijk, A. J. van Asten, E. J. Veldhuizen, and H. P. Haagsman. 2006. Characterization and expression sites of newly identified chicken collectins. Mol Immunol 43:1604-1616. doi 10.1016/j.molimm.2005.09.015

Hong, Y. H., W. Song, S. H. Lee, and H. S. Lillehoj. 2012. Differential gene expression profiles of beta-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult. Sci. 91:1081-1088. doi 10.3382/ps.2011-01948

Iwanaga, T., M. Goto, and M. Watanabe. 2005. Cellular distribution of glutamate transporters in the gastrointestinal tract of mice: an immunohistochemical and in situ hybridization approach. Biomed Res 26:271-278.

Janeway, C. J., et al. . 2001. The major histocompatibility complex and its functions in Immunobiology: The Immune System in Health and DiseaseNew York Garland Science, New York.

Jenkins, M. C., C. Parker, C. O'Brien, K. Miska, and R. Fetterer. 2013. Differing susceptibilities of Eimeria acervulina, Eimeria maxima, and Eimeria tenella oocysts to desiccation. J Parasitol 99:899-902. doi 10.1645/13-192.1

Johnson, J., and W. M. Reid. 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp Parasitol 28:30-36.

Johnson, L. R. 2007. Gastrointestinal physiology. 7th ed. Mosby Elsevier, Philadelphia.

Kanai, Y., B. Clemencon, A. Simonin, M. Leuenberger, M. Lochner, M. Weisstanner, and M. A. Hediger. 2013. The SLC1 high-affinity glutamate and neutral amino acid transporter family. Mol Aspects Med 34:108-120. doi 10.1016/j.mam.2013.01.001

Kaufman, J. 2008. The Avian MHC. Pages xiv, 481 p. in Avian immunology. T. F. Davison, B. Kaspers, and K. A. Schat eds. Academic Press, Amsterdam ; Boston.

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. doi 10.3382/ps.2007-00383

Landoni, M. F., and G. Albarellos. 2015. The use of antimicrobial agents in broiler chickens. Vet J 205:21-27. doi 10.1016/j.tvjl.2015.04.016

Leeson, S., M. L. Scott, and J. D. Summers. 2001. Nutrition of the chicken. 4th ed. University Books, Guelph, Ontario.

Leist, M., B. Single, A. F. Castoldi, S. Kuhnle, and P. Nicotera. 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 185:1481-1486.

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin Microbiol Rev 9:349-360.

Lin, L., S. W. Yee, R. B. Kim, and K. M. Giacomini. 2015. SLC transporters as therapeutic targets: emerging opportunities. Nat Rev Drug Discov 14:543-560. doi 10.1038/nrd4626

Luan, Y., and W. Xu. 2007. The structure and main functions of aminopeptidase N. Curr Med Chem 14:639-647.

Lynn, D. J., R. Higgs, S. Gaines, J. Tierney, T. James, A. T. Lloyd, M. A. Fares, G. Mulcahy, and C. O'Farrelly. 2004. Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. Immunogenetics 56:170-177. doi 10.1007/s00251-004-0675-0

McMahon, R. J., and R. J. Cousins. 1998. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc Natl Acad Sci U S A 95:4841-4846.

Miller, M. M., and R. L. Taylor, Jr. 2016. Brief review of the chicken Major Histocompatibility Complex: the genes, their distribution on chromosome 16, and their contributions to disease resistance. Poult Sci 95:375-392. doi 10.3382/ps/pev379

Mithieux, G. 2005. The new functions of the gut in the control of glucose homeostasis. Curr Opin Clin Nutr Metab Care 8:445-449.

Moreto, M., and J. M. Planas. 1989. Sugar and amino acid transport properties of the chicken ceca. J Exp Zool Suppl 3:111-116.

Noy, Y., and D. Sklan. 2001. Yolk and exogenous feed utilization in the posthatch chick. Poult Sci 80:1490-1495.

Palacin, M., and Y. Kanai. 2004. The ancillary proteins of HATs: SLC3 family of amino acid transporters. Pflugers Arch 447:490-494. doi 10.1007/s00424-003-1062-7

Paris, N. E., and E. A. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult Sci 92:1331-1335. doi 10.3382/ps.2012-02966

Peek, H. W., and W. J. Landman. 2011. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Vet Q 31:143-161. doi 10.1080/01652176.2011.605247

Pinard-Van Der Laan, M. H., J. L. Monvoisin, P. Pery, N. Hamet, and M. Thomas. 1998. Comparison of outbred lines of chickens for resistance to experimental infection with coccidiosis (Eimeria tenella). Poult Sci 77:185-191.

Piyadasa, H., K.-Y. G. Choi, and N. Mookherjee. 2015. Antibacterial Host Defense Peptides. Pages 1-9 in Encyclopedia of Inflammatory Diseases. M. Parnham ed. Springer Basel, Basel.

Qin, Y., T. L. Vanden Hoek, K. Wojcik, T. Anderson, C. Q. Li, Z. H. Shao, L. B. Becker, and K. J. Hamann. 2004. Caspase-dependent cytochrome c release and cell death in chick cardiomyocytes after simulated ischemia-reperfusion. Am J Physiol Heart Circ Physiol 286:H2280-2286. doi 10.1152/ajpheart.01063.2003

Quiroz-Castaneda, R. E., and E. Dantan-Gonzalez. 2015. Control of avian coccidiosis: future and present natural alternatives. Biomed Res Int 2015:430610. doi 10.1155/2015/430610

Rimoldi, S., E. Lasagna, F. M. Sarti, S. P. Marelli, M. C. Cozzi, G. Bernardini, and G. Terova. 2015. Expression profile of six stress-related genes and productive performances of fast and slow

growing broiler strains reared under heat stress conditions. Meta Gene 6:17-25. doi 10.1016/j.mgene.2015.08.003

Rivoira, M. A., A. M. Marchionatti, V. A. Centeno, G. E. Diaz de Barboza, M. E. Peralta Lopez, and N. G. Tolosa de Talamoni. 2012. Sodium deoxycholate inhibits chick duodenal calcium absorption through oxidative stress and apoptosis. Comp Biochem Physiol A Mol Integr Physiol 162:397-405. doi 10.1016/j.cbpa.2012.04.016

Robertson, J. D., and S. Orrenius. 2000. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Crit Rev Toxicol 30:609-627. doi 10.1080/10408440008951122

Romeo, E., M. H. Dave, D. Bacic, Z. Ristic, S. M. Camargo, J. Loffing, C. A. Wagner, and F. Verrey. 2006. Luminal kidney and intestine SLC6 amino acid transporters of B0AT-cluster and their tissue distribution in Mus musculus. Am J Physiol Renal Physiol 290:F376-383. doi 10.1152/ajprenal.00286.2005

Salanitro, J. P., P. A. Muirhead, and J. R. Goodman. 1976. Morphological and physiological characteristics of Gemmiger formicilis isolated from chicken ceca. Appl Environ Microbiol 32:623-632.

Selsted, M. E., and A. J. Ouellette. 2005. Mammalian defensins in the antimicrobial immune response. Nat Immunol 6:551-557. doi 10.1038/ni1206

Semple, F., and J. R. Dorin. 2012. beta-Defensins: multifunctional modulators of infection, inflammation and more? J Innate Immun 4:337-348. doi 10.1159/000336619

Sklan, D., A. Geyra, E. Tako, O. Gal-Gerber, and Z. Uni. 2003. Ontogeny of brush border carbohydrate digestion and uptake in the chick. Br J Nutr 89:747-753. doi 10.1079/BJN2003853

Smith, M. E., and D. G. Morton. 2010. The digestive system: basic science and clinical. 2nd ed. Churchill Livingstone, Edinburgh ; New York.

Souto, X. M., H. S. Barbosa, and R. F. Menna-Barreto. 2016. The morphological analysis of autophagy in primary skeletal muscle cells infected with Toxoplasma gondii. Parasitol Res 115:2853-2861. doi 10.1007/s00436-016-5040-3

Stotish, R. L., C. C. Wang, and M. Meyenhofer. 1978. Structure and composition of the oocyst wall of Eimeria tenella. J Parasitol 64:1074-1081.

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult Sci 93:1217-1226. doi 10.3382/ps.2013-03807

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp Parasitol 150:13-21. doi 10.1016/j.exppara.2015.01.003

Tako, E., P. R. Ferket, and Z. Uni. 2005. Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration. J Nutr Biochem 16:339-346. doi 10.1016/j.jnutbio.2005.01.002

Terada, T., Y. Shimada, X. Pan, K. Kishimoto, T. Sakurai, R. Doi, H. Onodera, T. Katsura, M. Imamura, and K. Inui. 2005. Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract. Biochem Pharmacol 70:1756-1763. doi 10.1016/j.bcp.2005.09.027

Townes, C. L., G. Michailidis, and J. Hall. 2009. The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane. Biochem Biophys Res Commun 387:500-503. doi 10.1016/j.bbrc.2009.07.046

Townes, C. L., G. Michailidis, C. J. Nile, and J. Hall. 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect Immun 72:6987-6993. doi 10.1128/IAI.72.12.6987-6993.2004

Uldry, M., and B. Thorens. 2004. The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch 447:480-489. doi 10.1007/s00424-003-1085-0

Van Beers, E. H., H. A. Buller, R. J. Grand, A. W. Einerhand, and J. Dekker. 1995. Intestinal brush border glycohydrolases: structure, function, and development. Crit Rev Biochem Mol Biol 30:197-262. doi 10.3109/10409239509085143

van de Wetering, J. K., L. M. van Golde, and J. J. Batenburg. 2004. Collectins: players of the innate immune system. Eur J Biochem 271:1229-1249. doi 10.1111/j.1432-1033.2004.04040.x

van der Most, P. J., B. de Jong, H. K. Parmentier, and S. Verhulst. 2011. Trade-off between growth and immune function: a meta-analysis of selection experiments. Functional Ecology 25:74-80. doi 10.1111/j.1365-2435.2010.01800.x

van Dijk, A., E. J. Veldhuizen, and H. P. Haagsman. 2008. Avian defensins. Vet Immunol Immunopathol 124:1-18. doi 10.1016/j.vetimm.2007.12.006

Verrey, F., E. I. Closs, C. A. Wagner, M. Palacin, H. Endou, and Y. Kanai. 2004. CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch 447:532-542. doi 10.1007/s00424-003-1086-z

Wagner, C. A., F. Lang, and S. Broer. 2001. Function and structure of heterodimeric amino acid transporters. Am J Physiol Cell Physiol 281:C1077-1093.

Wilkinson, B. L., J. S. Elam, D. A. Fadool, and R. L. Hyson. 2003. Afferent regulation of cytochrome-c and active caspase-9 in the avian cochlear nucleus. Neuroscience 120:1071-1079.

Wright, E. M., and E. Turk. 2004. The sodium/glucose cotransport family SLC5. Pflugers Arch 447:510-518. doi 10.1007/s00424-003-1063-6

Yadgary, L., R. Yair, and Z. Uni. 2011. The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes. Poult Sci 90:410-416. doi 10.3382/ps.2010-01075

Yoshikawa, T., R. Inoue, M. Matsumoto, T. Yajima, K. Ushida, and T. Iwanaga. 2011. Comparative expression of hexose transporters (SGLT1, GLUT1, GLUT2 and GLUT5) throughout the mouse gastrointestinal tract. Histochem Cell Biol 135:183-194. doi 10.1007/s00418-011-0779-1

Yun, C. H., H. S. Lillehoj, and E. P. Lillehoj. 2000a. Intestinal immune responses to coccidiosis. Dev Comp Immunol 24:303-324.

Yun, C. H., H. S. Lillehoj, J. Zhu, and W. Min. 2000b. Kinetic differences in intestinal and systemic interferon-gamma and antigen-specific antibodies in chickens experimentally infected with Eimeria maxima. Avian Dis 44:305-312.

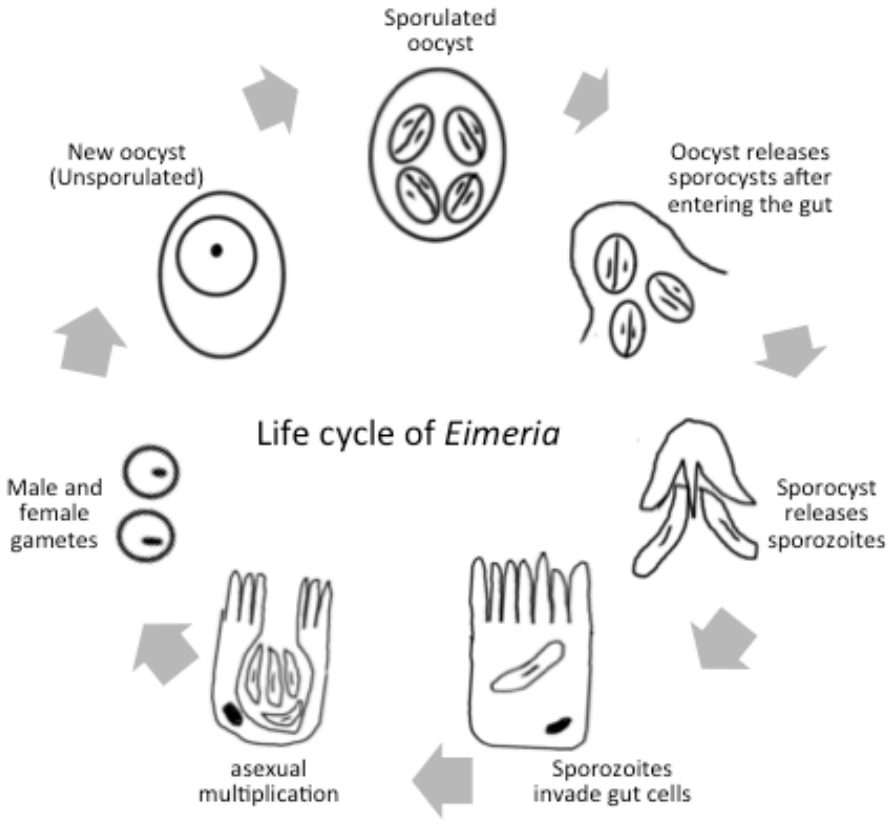
Zhang, Y., M. X. Zheng, Z. Y. Xu, H. C. Xu, X. Z. Cui, S. S. Yang, W. L. Zhao, S. Li, Q. H. Lv, and R. Bai. 2015. Relationship between Eimeria tenella development and host cell apoptosis in chickens. Poult Sci 94:2970-2979. doi 10.3382/ps/pev293

Zhou, H., and S. J. Lamont. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim Genet 30:256-264.

Table 1-1. Summary of SLC transporters.

Gene	Gene full name	Location	Function
$b^{0,+}AT$	Solute carrier family 7, member 9 (SLC7A9)	Brush Border	Na^+-independent neutral/cysteine, cationic amino acid exchanger
B^0AT	Solute carrier family 6, member 19 (SLC6A19)	Brush Border	Na^+-dependent neutral amino acid transporter
EAAT3	Excitatory amino acid transporter 3 (SLC1A1)	Brush Border	Transports aspartate, glutamate and cysteine
GLUT2	Glucose transporter-2 (SLC2A2)	Basolateral	Transports fructose, mannose, galactose, glucose and glucosamine
rBAT	Solute carrier family 3, member1 (SLC3A1)	Brush Border	Dimerizes with $b^{o,+}AT$
SGLT1	Sodium glucose transporter-1 (SLC5A1)	Brush Border	Transports low concentrations of D-glucose
ZnT1	Zinc transporter-1 (SLC22A18)	Basolateral	Efflux of Zn^{2+}

Figure 1-1. Life cycle of *Eimeria*. (Su, 2013)



Chapter 2. Expression of avian beta-defensins in the intestine of *Eimeria*-challenged chickens

Introduction

Coccidiosis is a common disease of poultry caused by the intestinal protozoa *Eimeria* (Conway and McKenzie, 2007). Infected birds usually show reduced feed efficiency and body weight gain due to lesions in the intestinal mucosa (Kipper et al., 2013). Damage to the intestinal barrier also increases the mortality of the birds. *Eimeria* infection is responsible for more than 3 billion dollars of loss in the poultry industry (Dalloul et al., 2007). *Eimeria* infection causes tissue-specific lesions, for example: *E. acervulina* mainly affects the duodenum, *E. maxima* the jejunum, and *E. tenella* the ceca (Lillehoj and Trout, 1996).

The current prevention and treatment of coccidiosis is through vaccination, prebiotic, probiotic and anticoccidial drugs (Quiroz-Castaneda and Dantan-Gonzalez, 2015). Live, wild-type coccidial vaccines can induce a strong immune response in chickens, which results in reduced feed efficiency and body weight gain. One alternative is to use precocious stains that are able to complete the life cycle faster than others from the same parental strain (Sharman et al., 2010). Precocious strains can stimulate immunity and not interfere with production, but require additional labor to isolate the virulent strains that need to be continually passed through naïve birds (Fetterer et al., 2014a; Peek and Landman, 2011). The use of feed additives like prebiotics and probiotics increase body weight gain and feed conversion during *Eimeria* challenge (Bozkurt et al., 2014). The current high cost of production of natural products may limit it usefulness as

feed additives in large commercial settings (Quiroz-Castaneda and Dantan-Gonzalez, 2015). There are two major types of anticoccidial drugs: coccidiostats and coccidiocidals. Coccidiostats act by inhibiting the development of *Eimeria* while coccidiocidals destroy the structural integrity of *Eimeria*. Although these are effective, the development of drug resistance and consumer concerns about chemical residues in poultry products have caused a decrease in the use of anticoccidial drugs (Quiroz-Castaneda and Dantan-Gonzalez, 2015).

An alternative approach to disease control involves enhancement of the immune system. Innate immunity is the first line of host defense against infections in vertebrate animals (Dziarski, 2013). Components of the innate immune system include: immune cells, host defense peptides (HDPs), enzymes, and pro-inflammatory factors (Dziarski, 2013). Host defense peptides have antimicrobial and immunomodulatory properties and show broad-spectrum activity against a range of bacteria, fungi and enveloped viruses (Robinson et al., 2015). Because HDPs attach to and insert into membranes of microbes, there is a low risk of triggering resistance (Brogden, 2005; Melo et al., 2009). Thus HDPs are attractive candidates for use in antimicrobial therapies.

In avians, 14 avian beta-defensins (AvBDs), four cathelicidins (CATHs) and liver expressed antimicrobial peptide-2 (LEAP2) have been identified as HDPs (Cuperus et al., 2013; Zhang and Sunkara, 2014). In vitro studies showed some of these HDPs have a direct negative effect on *Campylobacter* and *Salmonella* (Milona et al., 2007; Townes et al., 2009; van Dijk et al., 2012). Hong et al. (2012) examined the expression of AvBDs in the jejunum of Cobb and Ross broilers following an *E. maxima* and *Clostridium perfringens* challenge to induce necrotic enteritis. In uninfected chickens, abundance of AvBD8, AvBD10 and AvBD13 mRNA was high, AvBD1,

AvBD6, AvBD9, AvBD11 and AvBD12 mRNA was moderate, and AvBD3 and AvBD4 mRNA was very low. Infected Cobb and Ross chickens showed different patterns of upregulation and downregulation of AvBDs. In this study, we compared expression of AvBDs in the duodenum, jejunum, ileum and ceca of chickens following challenge with three different *Eimeria* species.

Materials and Methods

Birds and *Eimeria* infection

Chickens used in this study were Ross Heritage broiler males (Longenecker's Hatchery, Elizabethtown, PA). *Eimeria* were all USDA strains: *E. acervulina* (USDA #12 isolate), *E. maxima* (USDA APU1 isolate) and *E. tenella* (Wampler isolate). Oocysts were maintained and isolated as previously described (Fetterer and Barfield, 2003). Broiler chickens were housed at the USDA-ARS facility (Beltsville, MD) from hatch and maintained coccidia-free in suspended wire cages. Chickens were given water and a standard starter-type corn-soybean meal, ad libitum. At 21 d of age, chicks were inoculated with *Eimeria* via oral gavage. The tissue samples were the same as those from the study reported by Su et al. (2015). In the first experiment, chickens were inoculated with *E. acervulina* (200,000 oocysts/chicken, n = 6) or *E. maxima* (10,000 oocysts/chicken, n = 6). Control 1 (n = 6) received no *Eimeria* oocysts. In the second experiment chickens were inoculated with *E. tenella* (150,000 oocysts/chicken, n = 6). Control 2 (n = 6) received no *Eimeria* oocysts. Body weights of chickens were obtained on day of challenge (d21) and 7 days post infection (dpi). This study was carried out under a protocol approved by the Beltsville Research Center Animal Care and Use Committee and conducted at the Animal Parasitic Disease Laboratory (USDA Agricultural Research Service, Beltsville, MD).

For both studies, chickens were euthanized by cervical dislocation and intestinal segments were collected on d28 (controls and 7 dpi). Duodenum, jejunum, ileum and ceca were collected (n = 6). The contents of the intestine were removed and the tissue segments were immediately stored individually in RNAlater (Invitrogen, Grand Island, NY).

RNA extraction and quantitative real-time PCR

The tissue samples were removed from RNAlater and minced. A 20 to 30 mg sample of tissue was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and total RNA was extracted following the manufacturer's instruction of Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) and RNA quality was assessed by agarose-formaldehyde gel electrophoresis. The cDNA was synthesized from total RNA (500 ng) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific,Waltham, MA) and then diluted 1:30 for real time PCR analysis. The seven AvBDs (AvBD1, AvBD6, AvBD8, AvBD10, AvBD11, AvBD12, and AvBD13) that showed the greatest expression in the intestine (Hong et al., 2012) were analyzed by quantitative real time PCR. β-actin was chosen as the reference gene, because the Ct values for β-actin were the same for control and infected samples. The forward and reverse primers for the seven AvBDs and β-actin are shown in Table II-1. qPCR was performed on an Applied Biosystems 7500 system (Thermo Fisher Scientific,Waltham, MA) with Fast SYBR green (Thermo Fisher Scientific,Waltham, MA) using the following conditions for all genes: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Samples were run in duplicate and relative gene expression data were analyzed

using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), as described in Su et al. (2015). The mean ΔCt of the control samples was used to calculate the ΔΔCt value, which was performed separately for each intestinal segment. *Eimeria* treatment and each gene are a group.

Statistical analysis

All data were analyzed by ANOVA using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). Control 1, *E. acervulina*- and *E. maxima*-challenged chickens were analyzed separately from control 2 and *E. tenella*-challenged chickens. For gene expression of each *Eimeria* challenge, the model included the main effects of treatment, sorted by genes. Significance level was set at $P < 0.05$ when compared with the control.

Results

The expression of 7 AvBDs were profiled in the small intestine and ceca of broilers challenged with *E. acervulina*, *E. maxima*, or *E. tenella.* In the duodenum (Figure II-1), *E. acervulina* challenge resulted in the downregulation of AvBD1, AvBD6, AvBD10, AvBD11, AvBD12 and AvBD13 to 15% to 42% of control. *E. maxima* challenge caused downregulation of AvBD6, AvBD10, and AvBD11 to 37% to 51% of control, while *E. tenella* challenge had no effect on expression of any AvBDs. In the jejunum (Figure II-2), *E. maxima* caused downregulation of AvBD10 to 34% of control. Both *E. acervulina* and *E. tenella* showed no changes in expression of AvBDs. In the ileum (Figure II-3), none of the three *Eimeria* species caused changes in AvBD expression. In the ceca (Figure II-4), *E. maxima* challenge resulted in an increase of 242% and

201% above control for AvBD8 and AvBD13, respectively. There were no changes in gene expression for *E. acervulina* or *E. tenella* challenge.

Discussion

Host defense peptides not only show direct antimicrobial activity by disrupting membrane integrity but also enhance mucosal barrier function by inducing expression of mucins and tight junction proteins (Robinson et al., 2015). As a component of the innate immune system, HDP expression would likely show a different intracellular gene expression response to various pathogens. Thus in chickens the response to bacteria like *Salmonella* and *Campylobacter* may be different from that of protozoa such as *Eimeria*.

Chickens are carriers to both *Campylobacter jejuni* and *Salmonella enterica* (Meade et al., 2009; van Dijk et al., 2012; Awad et al., 2014). These two gram negative bacteria are both leading causes of food borne diseases and can cause diarrhea in humans, but chickens are able to carry these bacteria in their intestinal tract without showing any clinical signs (Rosenthal et al., 2011; McCormick and DiRita, 2013). In *C. jejuni* infected chickens, AvBD3, AvBD4, AvBD8, AvBD13 and AvBD14 mRNA abundance was decreased in peripheral blood leukocytes (PBL) 6 hours post infection (hpi) (Meade et al., 2009). Broilers challenged with *C. jejuni* also showed a downregulation of intestinal CATH-2 expression at 48, but not at 8 hpi (van Dijk et al., 2012). Both up- and downregulation of HDP expression were observed during salmonellosis. Challenge of broilers with *Salmonella enterica* serovar Pullorum resulted in upregulation of AvBD3, AvBD4, AvBD5 and AvBD12 and downregulation of AvBD6, AvBD10, AvBD11, AvBD13 and

AvBD14 in the small intestine (Ramasamy et al., 2012). Broilers challenged with *Salmonella enterica* serovar Typhimurium caused upregulation of LEAP2 in the intestine (Townes et al., 2004) and upregulation of AvBD3, AvBD10 and AvBD12 in PBL (Meade et al., 2009). *Salmonella enteriditis* challenge of broilers led to upregulation of LEAP2 in the small intestine and liver (Townes et al., 2004) but no change in CATH-2 expression in the small intestine (Van Dijk et al., 2012).

Eimeria is a protozoa that invades intestinal epithelial cells and induces an immune response. LEAP2 has been found to be downregulated in the intestine following challenge with *E. acervulina*, *E. maxima*, *E. tenella* and *E. praecox* (Casterlow et al., 2011; Sumners et al., 2011; Paris and Wong, 2013; Fetterer et al., 2014b; Su et al., 2014, 2015, Yin et al., 2015). In a necrotic enteritis model induced by co-infection with *E. maxima* and *Clostridium perfringens*, Hong et al. (2012) profiled the expression of HDPs in the jejunum of both Cobb and Ross broilers. In Cobb broilers showing necrotic enteritis, AvBD3, AvBD4, and AvBD12 were downregulated, while AvBD8, AvBD11 and AvBD13 were upregulated. Ross broilers showed a different pattern with only AvBD12 downregulated and AvBD1, AvBD6 AvBD8 and AvBD10 upregulated. We observed downregulation of AvBD10 in the jejunum of *E. maxima* challenged broilers, which differs from the upregulation of AvBD10 observed by Hong et al. (2012) in their necrotic enteritis model. It is likely that the co-infection with *C. perfringens* reversed the *Eimeria*-mediated downregulation of AvBD10.

In the current study we compared the expression of AvBDs in chickens infected with three different *Eimeria* species. Our results showed that AvBDs are downregulated in *E. acervulina*

and *E. maxima* infected chickens in different intestinal segments, similar to the downregulation reported for LEAP2 (Su et al., 2015). There was common downregulation of LEAP2, AvBD6, AvBD10 and AvBD11 in the duodenum of *E. acervulina* and *E. maxima* infected chickens. *E. acervulina* also showed downregulation of AvBD1, AvBD12 and AvBD13 in the duodenum and no effect in jejunum, ileum or ceca. This is consistent with *E. acervulina* causing lesions in the duodenum. *E. maxima* also caused downregulation of AvBD10 and LEAP2 (Su et al., 2015) in the jejunum, which is the site for *E. maxima* lesions. Interestingly in the ceca of *E. maxima* challenged broilers, there was upregulation of AvBD8 and AvBD13. *E. tenella* challenged broilers had no effect on AvBD expression in any intestinal segment and only caused downregulation of LEAP2 in the ceca (Su et al., 2015).

We have previously reported the profiling of the expression of nutrient transporters in these *Eimeria* challenged broilers (Su et al., 2015). These results show that there are associations between *Eimeria* species, AvBD and nutrient transporter gene expression. For *E. acervulina*, there was downregulation of 6 AvBDs, LEAP2 and numerous nutrient transporters in the duodenum, which is the primary site for *E. acervulina* lesions. There were no changes in AvBD and LEAP2 expression in the jejunum, ileum or ceca; however, there was downregulation of nutrient transporters in the ceca but not the jejunum and ileum. In contrast, for *E. maxima*, which primarily causes lesions in the jejunum, there were changes in nutrient transporter gene expression in the duodenum, jejunum, ileum and ceca and changes in AvBD and LEAP2 expression in the duodenum, jejunum and ceca. For *E. tenella*, there were no changes in AvBD expression in any intestinal segment and downregulation of LEAP2 in only the ceca; however, there were changes in nutrient transporter gene expression in the jejunum, ileum and ceca.

Infection with *Eimeria* promotes both antibody and cell-mediated immune responses (reviewed in Chapman, 2014). Although antibodies can be abundantly produced locally, they cannot access and act on these intracellular pathogens (Lillehoj et al., 2004). Therefore, antibody-mediated responses play a minor role in protective immunity against coccidiosis. Cell-mediated immune responses are the major host immune response during *Eimeria* infection, and they also can provide protection against reinfection (Lillehoj and Trout, 1996; Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). T- lymphocytes, natural killer cells, and macrophages are involved in the avian cellular immune response to *Eimeria* infection (Lillehoj and Trout, 1996; Dalloul et al., 2007). In the current study, *Eimeria* downregulated the expression of AvBDs, which is consistent with the downregulation of LEAP2 reported by Su et al. (2015). Only in the ceca was there upregulation of AvBD8 and AvBD12 following *E. maxima* infection.

An in vitro study showed that LEAP-2 has a negative effect on *Eimeria*. The sporozoites were incubated with different concentration of synthesized LEAP-2 for different periods of time and then the number of viable sporozoites was determined 6 and 24 hours after treatment. The results showed that there was a dose-dependent effect of LEAP-2 on *Eimeria* viability (Valdez and Wong, unpublished). Downregulation of HDPs would allow *Eimeria* to evade the host defense system.

The intestinal epithelial cells also downregulate a number of nutrient transporters in response to *Eimeria* infection (Paris and Wong, 2013; Fetterer et al, 2014b; Su et al., 2014, 2015; Yin et al., 2015). The anionic amino acid transporter EAAT3 was found to be consistently downregulated following *Eimeria* infection. EAAT3 transports aspartate and glutamate, of which the latter is the

main energy source for intestinal epithelial cells (Iwanaga et al., 2005; Kanai et al., 2013). Decreased influx of glutamate would result in energy depletion that can lead to altered cellular metabolism that impairs *Eimeria* replication or induces cellular apoptosis. This may represent a mechanism for the host cell to combat the infection following repression of the HDPs. Consistent with the apoptosis hypothesis, Zhang et al. (2015) showed that *E. tenella* infection of cecal epithelial cells promoted apoptosis during the middle to late stages of *Eimeria* development.

In summary, intestinal cells mount a multifaceted immunological and cellular response to invasion by a pathogen, such as *Eimeria*. The pathogen induces a cellular environment that is favorable to its replication by repressing HDP expression. This is countered by changes in host cell nutrient transporter gene expression that alters nutrient uptake to inhibit pathogen replication or promote cellular apoptosis, both of which can attenuate the infection.

References

Awad, W. A., J. R. Aschenbach, K. Ghareeb, B. Khayal, C. Hess, and M. Hess. 2014. Campylobacter jejuni influences the expression of nutrient transporter genes in the intestine of chickens. Vet. Microbiol. 172:195-201. doi 10.1016/j.vetmic.2014.04.001

Bozkurt, M., N. Aysul, K. Kucukyilmaz, S. Aypak, G. Ege, A. U. Catli, H. Aksit, F. Coven, K. Seyrek, and M. Cinar. 2014. Efficacy of in-feed preparations of an anticoccidial, multienzyme, prebiotic, probiotic, and herbal essential oil mixture in healthy and Eimeria spp.-infected broilers. Poult. Sci. 93:389-399. doi 10.3382/ps.2013-03368

Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3:238-250. doi 10.1038/nrmicro1098

Casterlow, S., H. Li, E. R. Gilbert, R. A. Dalloul, A. P. McElroy, D. A. Emmerson, and E. A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of Eimeria maxima-infected chickens. Poult. Sci. 90:1212-1219. doi 10.3382/ps.2010-01110

Chapman, H. D. 2014. Milestones in avian coccidiosis research: a review. Poult. Sci . 93:501-511. doi 10.3382/ps.2013-03634

Conway, D. P., and M. E. McKenzie. 2007. Poultry coccidiosis: diagnostic and testing procedure. 3rd ed. Blackwell Pub., Ames, Iowa.

Cuperus, T., M. Coorens, A. van Dijk, and H. P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immunol. 41:352-369. doi 10.1016/j.dci.2013.04.019

Dalloul, R. A., T. W. Bliss, Y. H. Hong, I. Ben-Chouikha, D. W. Park, C. L. Keeler, and H. S. Lillehoj. 2007. Unique responses of the avian macrophage to different species of Eimeria. Mol. Immunol. 44:558-566. doi 10.1016/j.molimm.2006.02.004

Dziarski, R. 2013. Chapter 6: Innate Immunity. Pages xvii, 826 p. in Schaechter's mechanisms of microbial disease. M. Schaechter, N. C. Engleberg, V. J. DiRita, and T. Dermody eds. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J. Parasitol. 89:553-564. doi 10.1645/GE-3159

Fetterer, R. H., M. C. Jenkins, K. B. Miska, and R. C. Barfield. 2014a. Evaluation of an experimental irradiated oocyst vaccine to protect broiler chicks against avian coccidiosis. Avian. Dis. 58:391-397. doi 10.1637/10679-092613-Reg.1

Fetterer, R. H., K. B. Miska, M. C. Jenkins, and E. A. Wong. 2014b. Expression of nutrient transporters in duodenum, jejunum, and ileum of Eimeria maxima-infected broiler chickens. Parasitol. Res. 113:3891-3894. doi 10.1007/s00436-014-4114-3

Hong, Y. H., W. Song, S. H. Lee, and H. S. Lillehoj. 2012. Differential gene expression profiles of beta-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult. Sci. 91:1081-1088. doi 10.3382/ps.2011-01948

Iwanaga, T., M. Goto, and M. Watanabe. 2005. Cellular distribution of glutamate transporters in the gastrointestinal tract of mice: an immunohistochemical and in situ hybridization approach. Biomed. Res. 26:271-278.

Kanai, Y., B. Clemencon, A. Simonin, M. Leuenberger, M. Lochner, M. Weisstanner, and M. A. Hediger. 2013. The SLC1 high-affinity glutamate and neutral amino acid transporter family. Mol. Aspects. Med. 34:108-120. doi 10.1016/j.mam.2013.01.001

Kipper, M., I. Andretta, C. R. Lehnen, P. A. Lovatto, and S. G. Monteiro. 2013. Meta-analysis of the performance variation in broilers experimentally challenged by Eimeria spp. Vet. Parasitol. 196:77-84. doi 10.1016/j.vetpar.2013.01.013

Lillehoj, H. S., and E. P. Lillehoj. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian. Dis. 44:408-425.

Lillehoj, H. S., W. Min, and R. A. Dalloul. 2004. Recent progress on the cytokine regulation of intestinal immune responses to Eimeria. Poult. Sci. 83:611-623.

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin. Microbiol. Rev. 9:349-360.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408. doi 10.1006/meth.2001.1262

McCormick, B. A., and V. J. DiRita. 2013. Chapter 17: Invasive and Tissue-Damaging Enteric Bacterial Pathogens: Bloody Diarrhea and Dysentery. Pages xvii, 826 p. in Schaechter's mechanisms of microbial disease. M. Schaechter, N. C. Engleberg, V. J. DiRita, and T. Dermody eds. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

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. doi 10.1007/s00251-008-0346-7

Melo, M. N., R. Ferre, and M. A. Castanho. 2009. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nat. Rev. Microbiol. 7:245-250. doi 10.1038/nrmicro2095

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. doi 10.1016/j.bbrc.2007.02.098

Paris, N. E., and E. A. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult. Sci. 92:1331-1335. doi 10.3382/ps.2012-02966

Peek, H. W., and W. J. Landman. 2011. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Vet. Q. 31:143-161. doi 10.1080/01652176.2011.605247

Quiroz-Castaneda, R. E., and E. Dantan-Gonzalez. 2015. Control of avian coccidiosis: future and present natural alternatives. Biomed. Res. Int. 2015:430610. doi 10.1155/2015/430610

Ramasamy, K. T., P. Verma, and M. R. Reddy. 2012. Differential gene expression of antimicrobial peptides beta-defensins in the gastrointestinal tract of Salmonella serovar Pullorum infected broiler chickens. Vet. Res. Commun. 36:57-62. doi 10.1007/s11259-011-9512-8

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Front. Vet. Sci. 2:57. doi 10.3389/fvets.2015.00057

Rosenthal, K. S., M. J. Tan, and K. S. Rosenthal. 2011. Rapid review microbiology and immunology. 3rd ed. Mosby/Elsevier, Philadelphia, PA.

Sharman, P. A., N. C. Smith, M. G. Wallach, and M. Katrib. 2010. Chasing the golden egg: vaccination against poultry coccidiosis. Parasite Immunol 32:590-598. doi 10.1111/j.1365-3024.2010.01209.x

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult. Sci. 93:1217-1226. doi 10.3382/ps.2013-03807

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp. Parasitol. 150:13-21. doi 10.1016/j.exppara.2015.01.003

Sumners, L. H., K. B. Miska, M. C. Jenkins, R. H. Fetterer, C. M. Cox, S. Kim, and R. A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during Eimeria praecox infection in chickens. Exp. Parasitol. 127:714-718. doi 10.1016/j.exppara.2010.12.002

Townes, C. L., G. Michailidis, and J. Hall. 2009. The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane. Biochem. Biophys. Res. Commun. 387:500-503. doi 10.1016/j.bbrc.2009.07.046

Townes, C. L., G. Michailidis, C. J. Nile, and J. Hall. 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect. Immun. 72:6987-6993. doi 10.1128/IAI.72.12.6987-6993.2004

van Dijk, A., M. Herrebout, M. H. Tersteeg-Zijderveld, J. L. Tjeerdsma-van Bokhoven, N. Bleumink-Pluym, A. J. Jansman, E. J. Veldhuizen, and H. P. Haagsman. 2012. Campylobacter jejuni is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. Vet. Microbiol. 160:347-354. doi 10.1016/j.vetmic.2012.05.034

Yin, H., L. H. Sumners, R. A. Dalloul, K. B. Miska, R. H. Fetterer, M. C. Jenkins, Q. Zhu, and E. A. Wong. 2015. Changes in expression of an antimicrobial peptide, digestive enzymes, and nutrient transporters in the intestine of E. praecox-infected chickens. Poult Sci 94:1521-1526. doi 10.3382/ps/pev133

Zhang, G., and L. T. Sunkara. 2014. Avian antimicrobial host defense peptides: from biology to therapeutic applications. Pharmaceuticals (Basel) 7:220-247. doi 10.3390/ph7030220

Zhang, Y., M. X. Zheng, Z. Y. Xu, H. C. Xu, X. Z. Cui, S. S. Yang, W. L. Zhao, S. Li, Q. H. Lv, and R. Bai. 2015. Relationship between Eimeria tenella development and host cell apoptosis in chickens. Poult Sci 94:2970-2979. doi 10.3382/ps/pev293

Table 2-1. Primer sequences for AvBDs.

Gene	Forward primer	Reverse primer
AvBd1	GAGTGGCTTCTGTGCATTTCTG	TTGAGCATTTCCCACTGATGAG
AvBd6	GCCCTACTTTTCCAGCCCTATT	GGCCCAGGAATGCAGACA
AvBd8	ATGCGCGTACCTAACAACGA	TGCCCAAAGGCTCTGGTATG
AvBd10	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT
AvBd11	GGTACTGCATCCGTTCCAAAG	GCATGTTCCAAATGCAGCAA
AvBd12	TGTAACCACGACAGGGGATTG	GGGAGTTGGTGACAGAGGTTT
AvBd13	CAGCTGTGCAGGAACAACCA	CAGCTCTCCATGTGGAAGCA
β-actin	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT

Figure 2-1. Expression of AvBDs in the duodenum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each AvBD. * indicates statistical significance from control at $P < 0.05$.

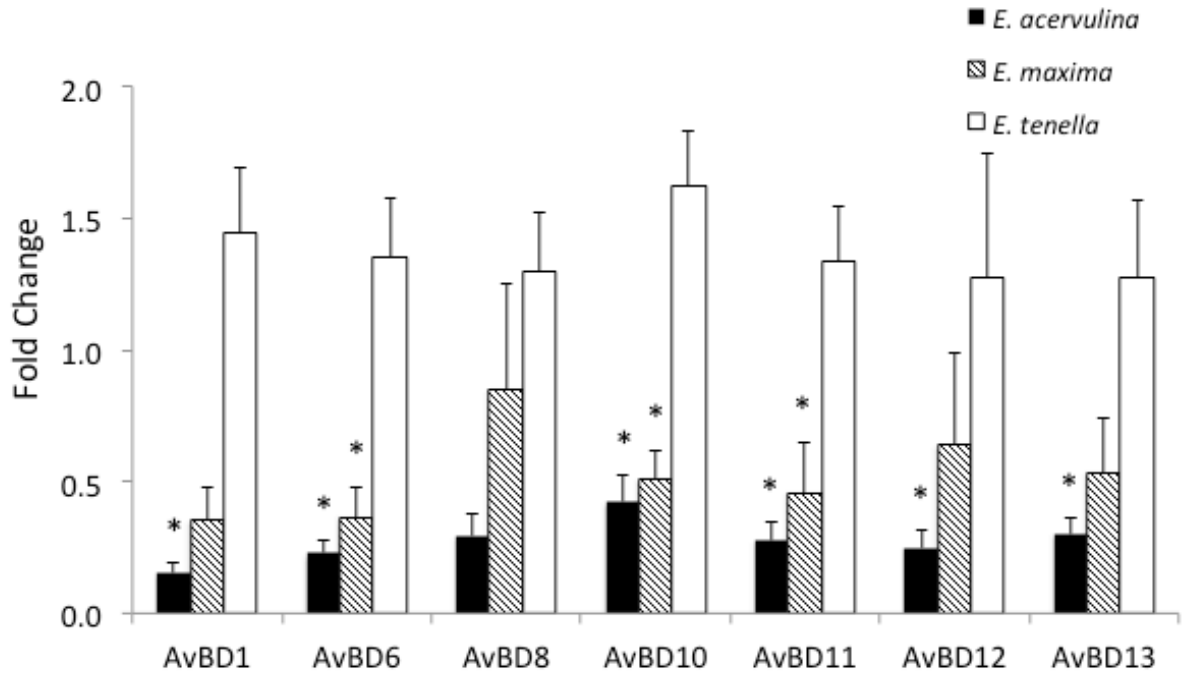


Figure 2-2. Expression of AvBDs in the jejunum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each AvBD. * indicates statistical significance from control at $P < 0.05$.

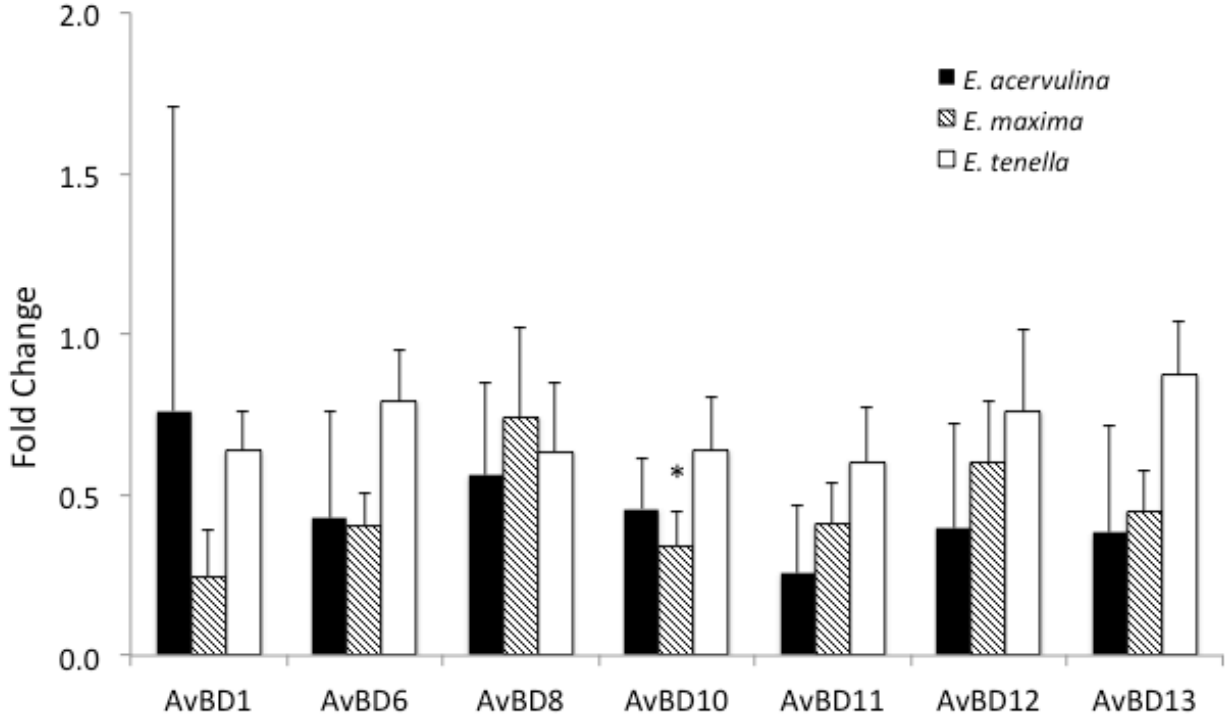


Figure 2-3. Expression of AvBDs in the ileum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each AvBD.

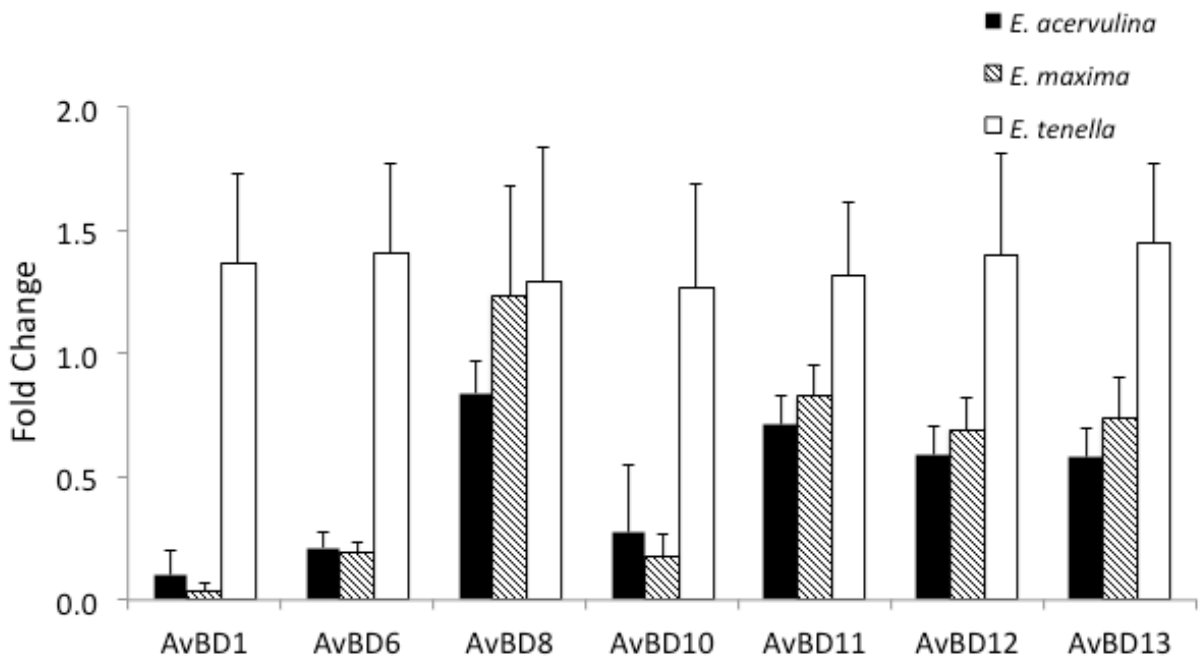
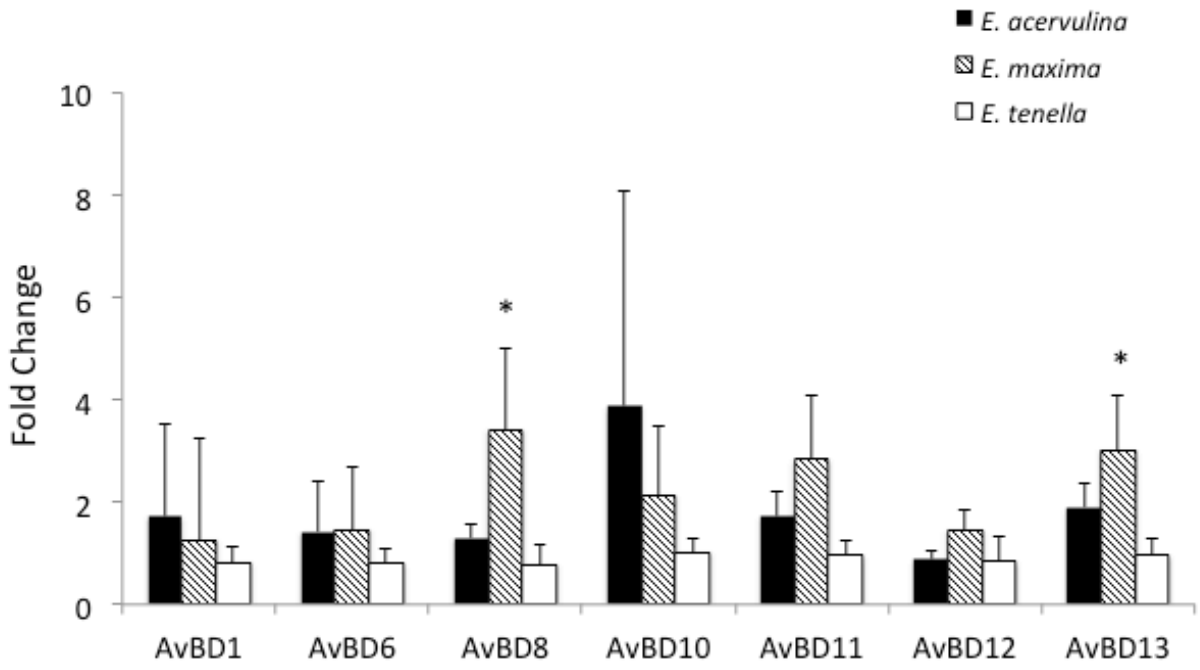


Figure 2-4. Expression of AvBDs in the ceca of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each AvBD. * indicates statistical significance from control at $P < 0.05$.



Chapter 3. Expression of apoptosis and autophagy related genes in the intestine of *Eimeria*-challenged chickens

Introduction

Avian coccidiosis is caused by the intracellular protozoa *Eimeria*, and can result in great economical losses in the poultry industry. There are three species of *Eimeria* that are important in the US and cause tissue-specific lesions: *E. acervulina* preferentially affects the duodenum, *E. maxima* the jejunum, and *E. tenella* the ceca (Lillehoj and Trout, 1996). As an obligate intracellular pathogen, *Eimeria* needs to be able to adapt to the intracellular environment (Del Cacho et al., 2014).

Previous studies showed that *Eimeria* infected intestinal cells downregulate nutrient transporters, which may be a host defense mechanism for eliminating infected cells and inhibiting pathogen replication (Fetterer et al., 2014; Paris and Wong, 2013; Su et al., 2014; Su et al., 2015). On the contrary, *Eimeria* infected chickens showed downregulation of host defense peptides, which may represent repression of the host innate immune system to promote pathogen invasion and replication (Su unpublished). *Toxoplasma gondii* is a parasite that is in the same order as *Eimeria* (Hughes et al., 1987). *T. gondii* infection decreased protein level of several pro-apoptotic genes, which may have promoted pathogen survival in the host cell (Carmen and Sinai, 2011).

Apoptosis is a controlled process of cell death characterized by nuclear condensation, cell shrinkage, membrane blebbing and DNA fragmentation (Elmore, 2007). Important regulators of

this process are the BCL family members, which include the pro-apoptotic gene (BAX) and anti-apoptotic member (Bcl-2) (Carmen and Sinai, 2011). Caspases are a family of endoproteases that regulate inflammation and apoptosis. Based on their role in apoptosis, caspases can be subclassified as initiator caspases (caspase 8 and 9) or executioner caspases (caspase 3, 6, and 7) (McIlwain et al., 2013). Caspase 18, which is most similar to caspase-8, has been conserved among chicken, platypus, and opossum but is absent from placental mammals (Eckhart et al., 2008). Activation of the caspase recruitment domain-containing protein 11 (CADR 11) allows it to coil and to recruit multiple signaling proteins, including Bcl-1 and caspase 8 (Jattani et al., 2016). The recruitment of caspases activates the systematic dismantling of the cell. Mitochondrial released cytochrome c is another important regulator of apoptosis.

Autophagy is often called programmed cell survival, which is a cellular recycling system that degrades less important cytoplasmic contents to protect vital cell functions. This process is controlled by Beclin 1, LC3s and other autophagy-related genes. Beclin 1 was originally isolated as a Bcl-2 (B-cell lymphoma 2)-interacting protein (Yang and Klionsky, 2010). In the early stage of autophagy, Beclin 1 is part of the class III PI3K (Phosphoinositide 3-kinase) complex. This complex is crucial for the formation of autophagosomes (Ferraro and Cecconi, 2007), and the recruitment of microtubule-associated protein light chain 3 (LC3). LC3 is a homolog of yeast Atg8 and has three isoforms, which have different localizations and expression patterns in cancer cell lines (Koukourakis et al., 2015) and cells under stress (Maier et al., 2013).

Because intracellular parasites replicate within the host cell, we hypothesized that *Eimeria* may downregulate expression of genes involved in apoptosis and autophagy to promote replication.

The objective of this study was to investigate the effect of challenge by different species of *Eimeria* in broilers on the expression of apoptosis and autophagy related genes in duodenum, jejunum, ileum and ceca.

Materials and Methods

Birds and *Eimeria* infection

Chickens used in this study were Ross Heritage broiler males (Longenecker's Hatchery, Elizabethtown, PA). *Eimeria* were all USDA strains: *E. acervulina* (USDA #12 isolate), *E. maxima* (USDA APU1 isolate) and *E. tenella* (Wampler isolate). Oocysts were maintained and isolated as previously described (Fetterer and Barfield, 2003). Broiler chickens were housed at the USDA-ARS facility (Beltsville, MD) from hatch and maintained coccidia-free in suspended wire cages. Chickens were given water and a standard starter-type corn-soybean meal, ad libitum. At 21 d of age, chicks were inoculated with *Eimeria* via gavage. The tissue samples were the same as those from the study reported by Su et al. (2015). In the first experiment, chickens were inoculated with *E. acervulina* (200,000 oocysts/chicken, n = 6) or *E. maxima* (10,000 oocysts/chicken, n = 6). Control 1 (n = 6) received no *Eimeria* oocysts. In the second experiment chickens were inoculated with *E. tenella* (150,000 oocysts/chicken, n = 6). Control 2 (n = 6) received no *Eimeria* oocysts. Body weights of chickens were obtained on day of challenge (d21) and 7 day post infection (dpi). This study was carried out under a protocol approved by the Beltsville Research Center Animal Care and Use Committee and conducted at the Animal Parasitic Disease Laboratory (USDA Agricultural Research Service, Beltsville, MD).

For both studies, chickens were euthanized by cervical dislocation and intestinal segments were collected on d28 (controls and 7 dpi). Duodenum, jejunum, ileum and ceca were collected (n = 6). The contents of the intestine were removed and the tissue segments were immediately stored individually in RNAlater (Invitrogen, Grand Island, NY).

RNA extraction and quantitative real-time PCR

The tissue samples were removed from RNAlater and minced. A 20–30 mg sample of tissue was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and total RNA was extracted following the manufacturer's instruction of Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) and RNA quality was assessed by agarose-formaldehyde gel electrophoresis. The cDNA was synthesized from total RNA (500 ng) using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and then diluted 1:30 for real time PCR analysis. Apoptosis related genes Bax, Bcl-2, Caspase 3, 6, 7, 8, 18, CARD 11 and cytochrome c, and the autophagy regulators Beclin 1, LC3a and LC3b were analyzed by quantitative real time PCR. β-actin was chosen as the reference gene, because the Ct values for β-actin were the same for control and infected samples. The forward and reverse primers for all genes studied are shown in Table III-1. qPCR was performed on an Applied Biosystems 7500 system with Fast SYBR green using the following conditions for all genes: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Samples were run in duplicate and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), as described in Su et al. (2015). The mean ΔCt of the control samples was

used to calculate the ΔΔCt value, which was performed separately for each intestinal segment. *Eimeria* treatment and each gene are a group.

Statistical analysis

All data were analyzed by ANOVA using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). Control 1, *E. acervulina*- and *E. maxima*-challenged chickens were analyzed separately from control 2 and *E. tenella*-challenged chickens. For gene expression of each *Eimeria* challenge, the model included the main effects of treatment, sorted by genes. Significance level was set at $P < 0.05$ when compared with the control.

Results

The expression of 9 apoptosis and 3 autophagy related genes were profiled in the small intestine and ceca of broilers challenged with *E. acervulina*, *E. maxima*, or *E. tenella.* In the duodenum (Figure III-1), *E. acervulina* challenge resulted in the downregulation of LC3a to 38% of control. There were no changes in gene expression for *E. maxima* or *E. tenella* challenge. In the jejunum (Figure III-2), *E. maxima* caused downregulation of Caspase 6, 7 and 18 to 63%, 69% and 75% of control, respectively. There were no changes in gene expression for *E. acervulina* or *E. tenella* challenge. None of the three species of *Eimeria* showed any changes in gene expression in the ileum (Figure III-3). In the ceca (Figure III-4), *E. tenella* challenge resulted in decrease of Bax, Caspase 7 and 18, LC3a and LC3b, to 55%, 35%, 55%, 56% and 74% of control, respectively. There were no changes in gene expression for *E. acervulina* or *E. maxima* challenge in the ceca.

Discussion

Changes in expression of apoptosis and autophagy related genes in *Eimeria* infected chickens were species- and tissue-specific. The downregulation of LC3a was observed in the duodenum of *E. acervulina* challenged birds, and no changes in the non-target tissues. All downregulation of genes in *E. maxima* and *E. tenella* challenged chickens were limited to the respective target tissues as well.

Reports on apoptosis and autophagy events during *Eimeria* infection are limited (del Cacho et al., 2004; Zhang et al., 2015; Cui et al., 2016). *Toxoplasma* and *Eimeria* are protozoan parasites, which have very similar structure, life cycle and invasion mechanism. Studies investigating apoptosis and autophagy events during *Toxoplasma* infection, showed that the pathogen can both promote and inhibit apoptosis and autophagy in the host cell. In some cases, *Toxoplasma* infection was able to protect the host from environmental factor induced apoptosis. For example, chemical induced DNA fragmentation was inhibited by *T. gondii* tachyzoites infection in mouse spleen cells by caspase inactivation and NF-κB activation (Kim et al., 2006). *T. gondii* inactivated the host cell apoptosis response to promote its survival inside the host cell.

E. tenella infection induced apoptosis and the release of cytochrome c from the mitochondria in chicken embryo ceca cells (CECC). Infected cells showed an increase of cytochrome c at 24h and 72 to 120 hours post infection (hpi), and the effect was blocked by restoring cellular Ca^{2+} concentration (Cui et al., 2016). Zhang et al. (2015) reported that *E. tenella* infected CECC had less apoptosis at 4 h and more apoptosis at 24 to 120h compared to control. These results suggest

that *E. tenella* can protect host cells from apoptosis at early stages of development but can promote apoptosis during the middle to late stages. In addition, when cells are treated with caspase-9 inhibitor, the *E. tenella* infection rate was enhanced (Zhang et al., 2015). These results suggest that if apoptosis is induced in the host cell, it may be able to arrest *Eimeria* infection.

There are other conditions that contribute to changes in apoptotic gene expression in chickens, including chemical toxicity or heat stress. Broilers fed a high nickel (Ni) diet showed increased DNA damage in the kidney (Guo et al., 2015) and small intestine (Wu et al., 2016); and decreased Bcl-2 and increased Bax mRNA and protein expression. The ratio of Bax/Bcl-2 RNA and protein expression increased with the Ni level in the diet. Wu et al. (2016) also showed increased Caspase-3 expression associated with dietary Ni. Aflatoxin B1 affected apoptosis and increased expression of Bax and Caspase-3, and decreased expression of Bcl-2 in thymus and bursa in broiler chickens (Peng et al., 2015). These studies led to the conclusion that decreased expression of Bcl-2 and Caspase-3, and increased Bax and Bax/Bcl-2 ratio can be indicators of apoptosis.

Our results showed that *E. maxima* challenge resulted in downregulation of Caspase 6, 7 and 18 in the jejunum. *E. tenella* challenge resulted in the decrease of Bax, Caspase 7 and 18 in the ceca at 7 dpi. These results indicated that *Eimeria* infection induced an anti-apoptotic state in the host cell. But according to Zhang et al. (2015), *E. tenella* infected CECC showed more apoptosis from 1 to 5 dpi. The differences between these two experiments may be due to the nature of the study, in vivo vs. in vitro. Because it usually takes about 4-6 days for *Eimera* to complete its life

cycle, induced host cell apoptosis can be beneficial to *Eimeria* to escape the host cell (Quiroz-Castaneda and Dantan-Gonzalez, 2015).

Autophagy plays an important role in the defense against intracellular pathogens. For example, induced autophagy in primary skeletal muscle cells (SkMC) would lead to increased LC3 expression and promote autophagosome fusion with parasitophorous vacuoles containing degraded *T.gondii* (Souto et al., 2016). But others found evidence of *T. gondii* infection inducing host cell autophagy in HeLa cells and primary fibroblasts up to 24 hpi, which can allow the parasite to capture the autophagy products to promote its growth (Wang et al., 2009). Host cell autophagy can also be inhibited by *T. gondii* proliferation. In Hela cells treated with autophagy enhancer and *T. gondii*, there was increased LC3 II and Beclin 1 expression at 18 hr and 24 hr, which decreased by 36 hr (Lee et al., 2013). In my study, *E. acervulina* challenge produced decreased LC3a in the duodenum. *E. tenella* challenge resulted in a decrease of LC3a and LC3b in the ceca. These results indicated that *Eimeria* infection could also induce an anti-autophagic state at 7 dpi.

Our previous results showed that during *Eimeria* infection, host cell digestive enzymes, nutrient transporters and antimicrobial peptide expression were decreased (Su et al., 2015). These changes may result in amino acid and sugar depletion, and zinc balance disruption. This condition can be unfavorable for *Eimeria*, and could be a host defense mechanism to counter the *Eimeria*-induced downregulation of the host defense peptide LEAP2. The anti-apoptotic and anti-autophagic state during *Eimeria* infection can be beneficial and/or unfavorable for *Emieria* survival in the host cell. Under the anti-apoptotic state, the probability of *Eimeria* being

destroyed with the death of the host cell is lowered, which favors its survival. But as *Eimeria* may need to activate the apoptosis pathway to escape the host cell, decreasing expression of caspases may inhibit parasite replication. The anti-autophagic state can protect the parasitophorous vacuole, which would promote parasite replication. However, it can also lead to deprivation of intracellular nutrient levels, thus being unfavorable for the intracellular pathogen.

References

Carmen, J. C., and A. P. Sinai. 2011. The differential effect of toxoplasma gondii infection on the stability of bcl2-family members involves multiple activities. Front Microbiol 2:1. doi 10.3389/fmicb.2011.00001

Cui, X. Z., M. X. Zheng, Y. Zhang, R. L. Liu, S. S. Yang, S. Li, Z. Y. Xu, R. Bai, Q. H. Lv, and W. L. Zhao. 2016. Calcium homeostasis in mitochondrion-mediated apoptosis of chick embryo cecal epithelial cells induced by Eimeria tenella infection. Res Vet Sci 104:166-173. doi 10.1016/j.rvsc.2015.12.015

Del Cacho, E., M. Gallego, H. S. Lillehoj, J. Quilez, E. P. Lillehoj, A. Ramo, and C. Sanchez-Acedo. 2014. IL-17A regulates Eimeria tenella schizont maturation and migration in avian coccidiosis. Vet Res 45:25. doi 10.1186/1297-9716-45-25

del Cacho, E., M. Gallego, F. Lopez-Bernad, J. Quilez, and C. Sanchez-Acedo. 2004. Expression of anti-apoptotic factors in cells parasitized by second-generation schizonts of Eimeria tenella and Eimeria necatrix. Vet Parasitol 125:287-300. doi 10.1016/j.vetpar.2004.07.017

Eckhart, L., C. Ballaun, M. Hermann, J. L. VandeBerg, W. Sipos, A. Uthman, H. Fischer, and E. Tschachler. 2008. Identification of novel mammalian caspases reveals an important role of gene loss in shaping the human caspase repertoire. Mol Biol Evol 25:831-841. doi 10.1093/molbev/msn012

Elmore, S. 2007. Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516. doi 10.1080/01926230701320337

Ferraro, E., and F. Cecconi. 2007. Autophagic and apoptotic response to stress signals in mammalian cells. Arch Biochem Biophys 462:210-219. doi 10.1016/j.abb.2007.02.006

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J Parasitol 89:553-564. doi 10.1645/GE-3159

Fetterer, R. H., K. B. Miska, M. C. Jenkins, and E. A. Wong. 2014. Expression of nutrient transporters in duodenum, jejunum, and ileum of Eimeria maxima-infected broiler chickens. Parasitol Res 113:3891-3894. doi 10.1007/s00436-014-4114-3

Guo, H., H. Cui, X. Peng, J. Fang, Z. Zuo, J. Deng, X. Wang, B. Wu, K. Chen, and J. Deng. 2015. Modulation of the PI3K/Akt pathway and Bcl-2 family proteins involved in chicken's tubular apoptosis induced by Nickel Chloride (NiCl(2)). Int J Mol Sci 16:22989-23011. doi 10.3390/ijms160922989

Hughes, H. P., C. A. Speer, J. E. Kyle, and J. P. Dubey. 1987. Activation of murine macrophages and a bovine monocyte cell line by bovine lymphokines to kill the intracellular pathogens Eimeria bovis and Toxoplasma gondii. Infect Immun 55:784-791.

Jattani, R. P., J. M. Tritapoe, and J. L. Pomerantz. 2016. Intramolecular interactions and regulation of cofactor binding by the four repressive elements in the caspase recruitment domain-containing protein 11 (CARD11) Inhibitory Domain. J Biol Chem 291:8338-8348. doi 10.1074/jbc.M116.717322

Kim, J. Y., M. H. Ahn, H. S. Jun, J. W. Jung, J. S. Ryu, and D. Y. Min. 2006. Toxoplasma gondii inhibits apoptosis in infected cells by caspase inactivation and NF-kappaB activation. Yonsei Med J 47:862-869. doi 10.3349/ymj.2006.47.6.862

Koukourakis, M. I., D. Kalamida, A. Giatromanolaki, C. E. Zois, E. Sivridis, S. Pouliliou, A. Mitrakas, K. C. Gatter, and A. L. Harris. 2015. Autophagosome proteins LC3A, LC3B and LC3C have distinct subcellular distribution kinetics and expression in cancer cell lines. PLoS One 10:e0137675. doi 10.1371/journal.pone.0137675

Lee, Y. J., H. O. Song, Y. H. Lee, J. S. Ryu, and M. H. Ahn. 2013. Proliferation of Toxoplasma gondii suppresses host cell autophagy. Korean J Parasitol 51:279-287. doi 10.3347/kjp.2013.51.3.279

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin Microbiol Rev 9:349-360.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408. doi 10.1006/meth.2001.1262

Maier, H. J., E. M. Cottam, P. Stevenson-Leggett, J. A. Wilkinson, C. J. Harte, T. Wileman, and P. Britton. 2013. Visualizing the autophagy pathway in avian cells and its application to studying infectious bronchitis virus. Autophagy 9:496-509. doi 10.4161/auto.23465

McIlwain, D. R., T. Berger, and T. W. Mak. 2013. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5:a008656. doi 10.1101/cshperspect.a008656

Paris, N. E., and E. A. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult Sci 92:1331-1335. doi 10.3382/ps.2012-02966

Peng, X., K. Chen, J. Chen, J. Fang, H. Cui, Z. Zuo, J. Deng, Z. Chen, Y. Geng, and W. Lai. 2015. Aflatoxin B1 affects apoptosis and expression of Bax, Bcl-2, and Caspase-3 in thymus and bursa of fabricius in broiler chickens. Environ Toxicol. doi 10.1002/tox.22120

Quiroz-Castaneda, R. E., and E. Dantan-Gonzalez. 2015. Control of avian coccidiosis: future and present natural alternatives. Biomed Res Int 2015:430610. doi 10.1155/2015/430610

Souto, X. M., H. S. Barbosa, and R. F. Menna-Barreto. 2016. The morphological analysis of autophagy in primary skeletal muscle cells infected with Toxoplasma gondii. Parasitol Res. doi 10.1007/s00436-016-5040-3

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult Sci 93:1217-1226. doi 10.3382/ps.2013-03807

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp Parasitol 150:13-21. doi 10.1016/j.exppara.2015.01.003

Wang, Y., L. M. Weiss, and A. Orlofsky. 2009. Host cell autophagy is induced by Toxoplasma gondii and contributes to parasite growth. J Biol Chem 284:1694-1701. doi 10.1074/jbc.M807890200

Wu, B., H. Guo, H. Cui, X. Peng, J. Fang, Z. Zuo, J. Deng, X. Wang, and J. Huang. 2016. Pathway underlying small intestine apoptosis by dietary nickel chloride in broiler chickens. Chem Biol Interact 243:91-106. doi 10.1016/j.cbi.2015.11.010

Yang, Z., and D. J. Klionsky. 2010. Eaten alive: a history of macroautophagy. Nat Cell Biol 12:814-822. doi 10.1038/ncb0910-814

Zhang, Y., M. X. Zheng, Z. Y. Xu, H. C. Xu, X. Z. Cui, S. S. Yang, W. L. Zhao, S. Li, Q. H. Lv, and R. Bai. 2015. Relationship between Eimeria tenella development and host cell apoptosis in chickens. Poult Sci 94:2970-2979. doi 10.3382/ps/pev293

Table 3-1. Primer sequence for apoptosis and autophagy related genes.

Gene	Forward primer	Reverse primer
BAX	GGTGACAGGGATCGTCACAG	ACCAACTGTGTGTCGTAGGC
Bcl-2	TCGTCGCCTTCTTCGAGTTC	CATCCCATCCTCCGTTGTCC
Caspase 3	AAAGATGGACCACGCTCAGG	TCCGGTATCTCGGTGGAAGT
Caspase 6	TGCCAGATAGACGTGGGACT	AGTCATCCCGAGAGGCTTCA
Caspase 7	TGGGTACACGCAATGGAACT	TCCTCACAGCTTCGGTCATT
Caspase 8	CTCCTACAGAAGCCCAAGCC	GGCATTTGCTTCCCTGCATT
Caspase 18	GACCAGGCTGATGTTCTGGT	CCATCTTGTCACAGAGGCACT
CARD 11	GCATCGCTTGAACAAGGTGG	CCTCCTTACGGTCGTGTTCC
Cytochrome c	TTCCCAGTGCCATACGGTTG	GCTTGTCCTGTTTTGCGTCC
Beclin 1	TTGAGAGAGTCAGGGCAGAG	TGCATCTGAGCATAACGCAT
LC3a	CAGGAGAAGGATGAGGATGGC	TCTCGGCAGGTCTCAGTAGC
LC3b	TCTTCCTCCTGGTGAACGGA	TCGTCCTTCTCGCTCTCGTA
β-actin	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT

Figure 3-1. Expression of apoptosis and autophagy related genes in the duodenum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

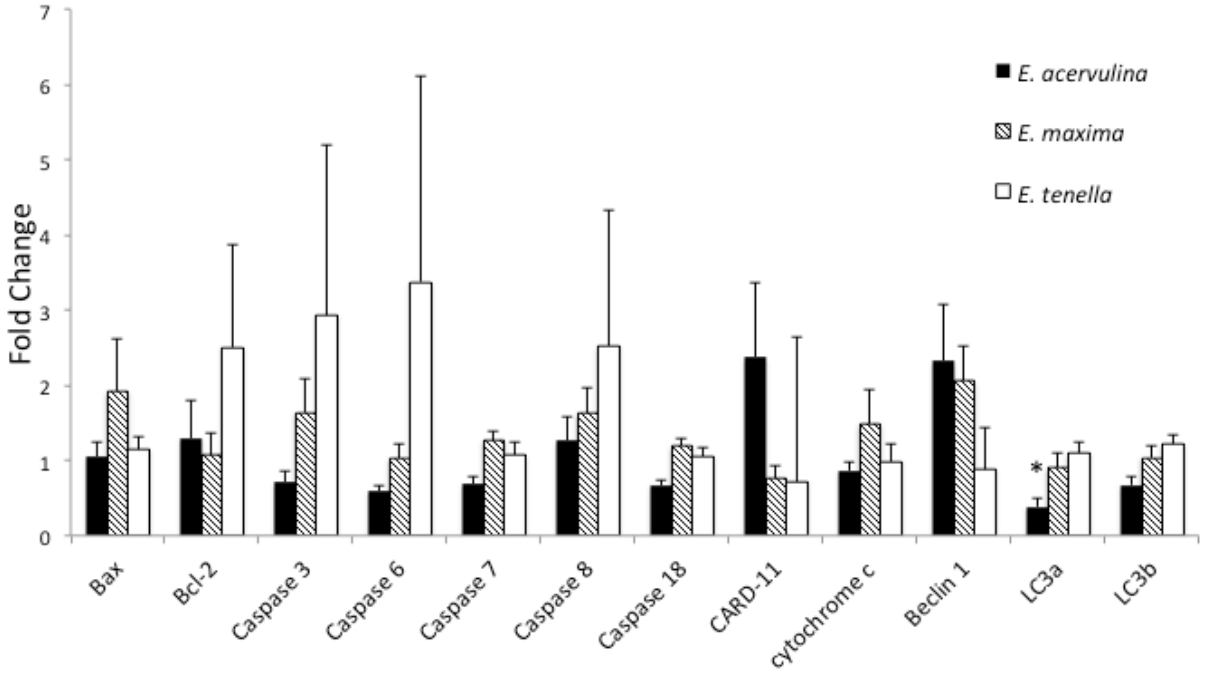


Figure 3-2. Expression of apoptosis and autophagy related genes in the jejunum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

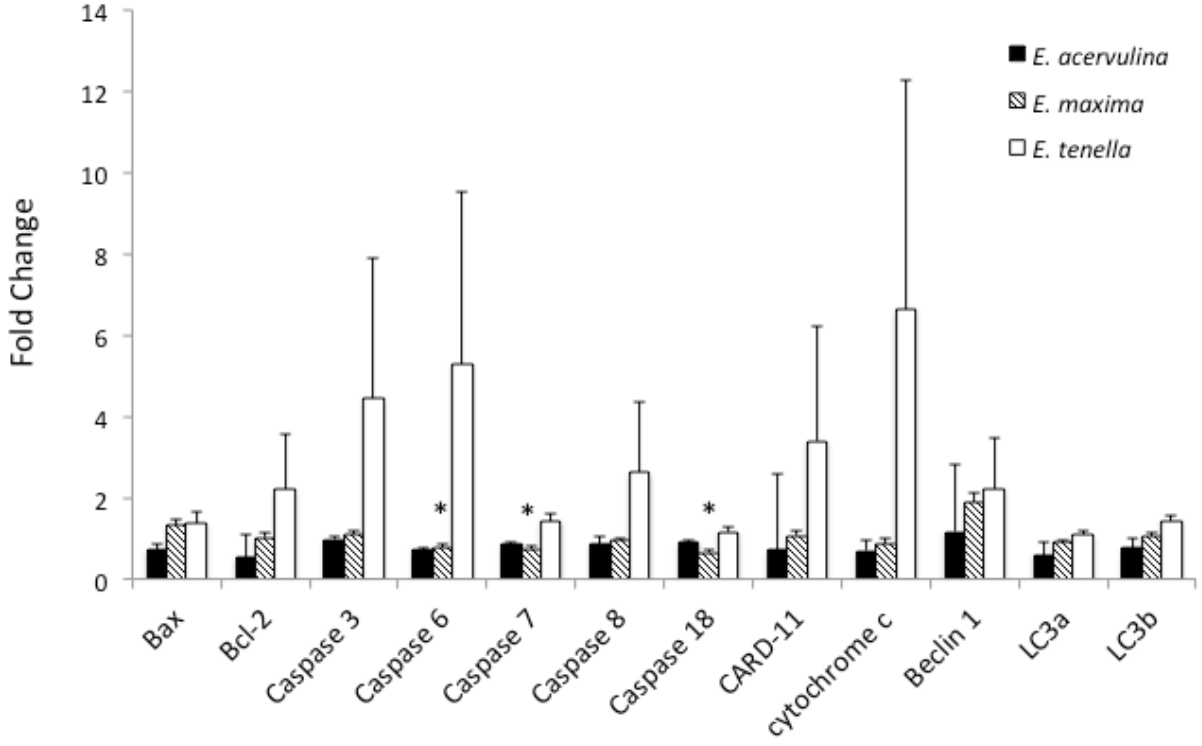


Figure 3-3. Expression of apoptosis and autophagy related genes in the ileum of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene.

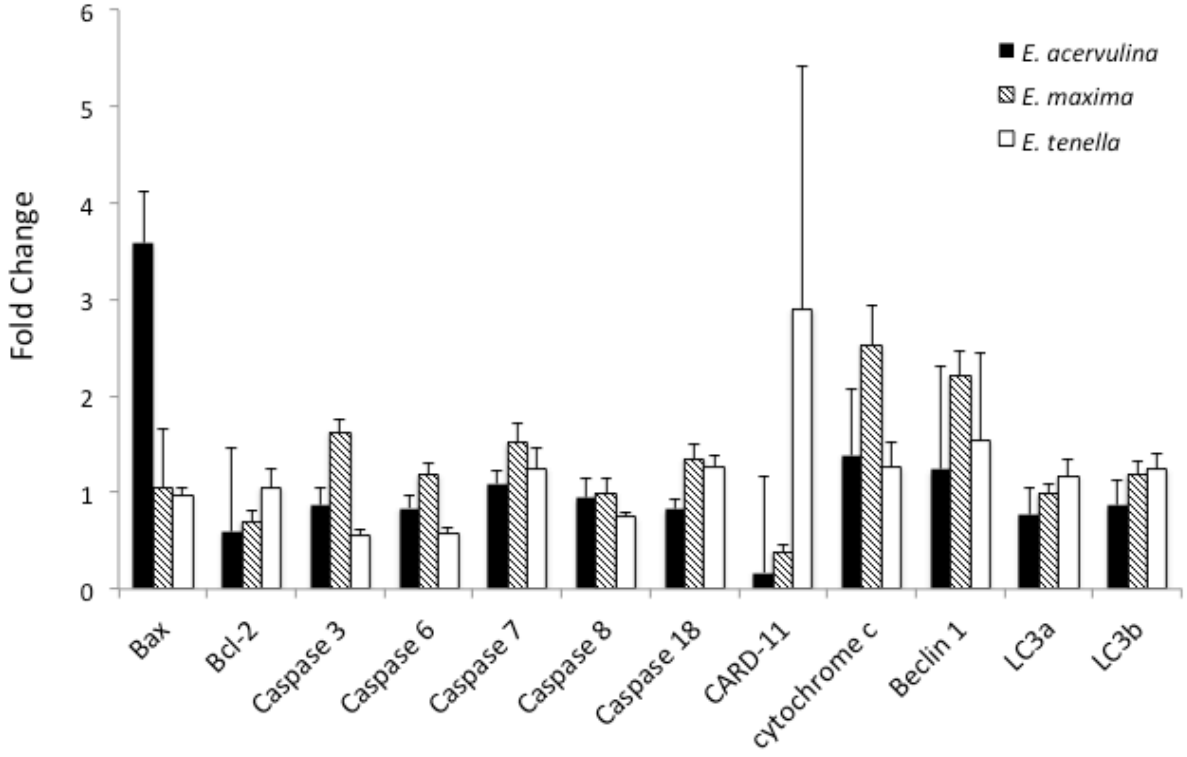
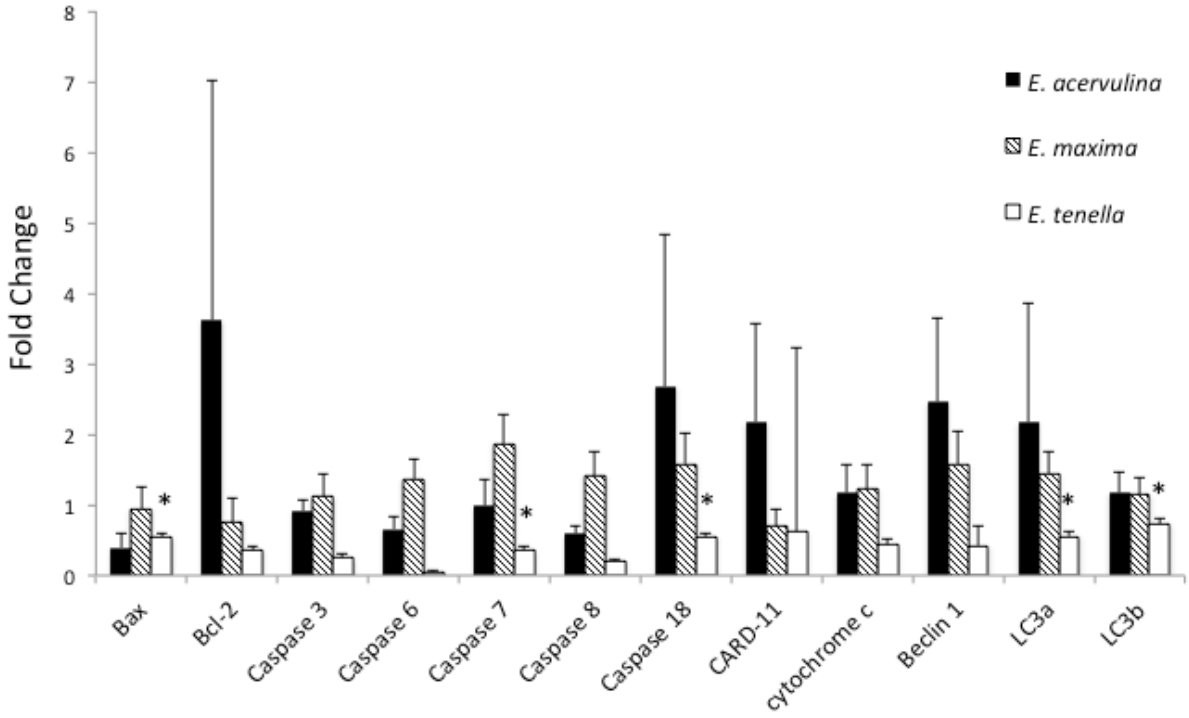


Figure 3-4. Expression of apoptosis and autophagy related genes in the ceca of *E. acervulina*-, *E. maxima*- and *E. tenella*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.



Chapter 4. Temporal expression of avian host defense peptides, apoptosis and autophagy related genes during *Eimeria acervulina* infection in chickens.

Introduction

The life cycle of *Eimeria* takes about 7 days. In *E. acervulina* infected chickens, sporozoites can be observed in the crypts 1 day post infection (dpi). On 2 to 3 dpi, schizonts appear in the epithelial cells, and merozoites develop. The formation of mature gametocytes is on the fifth day, and new oocysts are developed later (Warren and Ball, 1967). In previous studies, I examined the expression of digestive enzymes, nutrient transporters, host defense peptides (HDPs), apoptosis and autophagy related genes in different *Eimeria* challenged broilers. All samples, however were collected at the same time point on 7 dpi. Transporters, HDPs, apoptosis and autophagy related genes have different expression patterns, regulatory mechanisms and responses to cellular stimuli. It is very likely that these genes show differential expression at various stages of *Eimeria* infection. In *E. praecox*-challenged chickens, expression of Toll-like receptor 3, 4, 15, LEAP2 and CATHL3 were dependent on challenge dose and time (Sumners et al., 2011), most of the downregulation of LEAP2 and CATHL3 was observed on 3 to 5 dpi. In contrast, my studies showed that LEAP2 was downregulated on 7 dpi in *E. acervulina*, *E. maxima* and *E. tenella* infected broilers in their respective target tissue (Su et al., 2015). The objective of this study was to examine the expression of avian HDPs, apoptosis and autophagy related genes during *E. acervulina* infection in chickens at different days post infection.

Material and Methods

Chicken and *Eimeria* infection

Chickens used in this study were Ross Heritage broiler males (Longenecker's Hatchery, Elizabethtown, PA). *E. acervulina* (USDA #12 isolate) was used for the infection. Oocysts were maintained and isolated as previously described (Fetterer and Barfield, 2003). Broiler chickens were housed at the USDA-ARS facility (Beltsville, MD) from hatch and maintained coccidia-free in suspended wire cages. Chickens were given water and a standard starter-type corn-soybean meal, ad libitum. At 21 d of age, chicks were inoculated with 300,000 *E. acervulina* oocysts via oral gavage. On 3, 5 and 7 dpi (n=6), mucosal scrapings from duodenum were collected from control and challenged birds, and were stored at -80 °C. This study was carried out under a protocol approved by the Beltsville Research Center Animal Care and Use Committee and conducted at the Animal Parasitic Disease Laboratory (USDA Agricultural Research Service, Beltsville, MD).

RNA extraction and quantitative real-time PCR

A 20 to 30 mg piece of frozen sample was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and total RNA was extracted following the manufacturer's instruction of Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and RNA quality was assessed by agarose-formaldehyde gel electrophoresis. The cDNA was synthesized from total RNA (500 ng) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) and then diluted 1:30 for real time

PCR analysis. The seven avian beta-defensins (AvBDs) (AvBD1, AvBD6, AvBD8, AvBD10, AvBD11, AvBD12, and AvBD13) were analyzed by quantitative real time PCR. The apoptosis related genes included: cytochrome c, caspase 6, 7 and 18. Autophagy related genes examined in this study were Beclin 1, LC3a and LC3b. β-actin was chosen as the reference gene, because the Ct values for β-actin were the same for control and infected samples. The forward and reverse primers for the seven AvBDs and β-actin are shown in Table IV-1. qPCR was performed on an Applied Biosystems 7500 system (Thermo Fisher Scientific, Waltham, MA) with Fast SYBR green (Thermo Fisher Scientific, Waltham, MA) using the following conditions for all genes: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Samples were run in duplicate and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), as described in Su et al. (2015). The mean ΔCt of the control samples was used to calculate the ΔΔCt value, which was performed separately for each sampling day.

Statistical analysis

All data were analyzed by ANOVA using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). For gene expression of each *Eimeria* challenge, the model included the main effects of treatment, sorted by genes. Significance level was set at $P < 0.05$ when compared with the control.

Results

The expression of HDPs, apoptosis and autophagy related genes were profiled in the duodenum of broilers challenged with *E. acervulina.* For the expression of AvBDs and LEAP2 (Figure IV-

1), *Eimera* challenge resulted in the downregulation of LEAP2 to 37%, 2% and 9% of control on 3, 5 and 7 dpi, respectively. There was no change in expression of any of the 7 AvBDs at any time point. Challenged birds showed Caspase 6 downregulation to 26% and 39% of control on 5 and 7 dpi, respectively (Figure IV-2). Caspase 7 was decreased to 73%, 29% and 61% of control on 3, 5 and 7 dpi, respectively. Caspase 18 only showed downregulation on 5 dpi, to 47% of control. All of the three autophagy related genes were downregulated in the challenged group, with the exception of LC3b on 7 dpi. Beclin 1 was downregulated to 53%, 52% and 55% of control on 3, 5 and 7 dpi, respectively. LC3a was decreased to 60%, 34% and 34% of control on 3, 5 and 7 dpi, respectively. LC3b was decreased to 58% and 36% of control on 3 and 5 dpi, respectively.

Discussion

The objective of this time course study was to examine the expression of avian HDPs, apoptosis and autophagy related genes during *E. acervulina* infection in chickens at different times post infection. I expected to be able to confirm the previous changes of gene expression shown in chapter II. In that chapter, AvBD1, 6, 10, 11, 12 and 13 were downregulated in the duodenum of *E. acervulina* infected broilers. In this time course experiment, however, no changes in the AvBD expression were seen. On the contrary, the expression of apoptosis and autophagy related genes showed much more downregulation than expected. As described in chapter III, the only change seen for *E. acervulina* infected chickens was on 7 dpi, which was the downregulation of LC3a in the duodenum. But in the time course study, all apoptosis and autophagy related genes were downregulated during at least two time points, with the exception of cytochrome c.

The differences between the two studies were *Eimeria* treatment and sample collection method. In chickens infected with three different species of *Eimera* (Su et al., 2015), intestinal segments were collected and stored in RNAlater for gene expression analysis. In the time course study, chickens were infected with a higher dose of *E. acervulina* at 300,000 oocysts/bird, which was 100,000 more oocysts per bird. Mucosal scrapings were collected and were stored at -80 °C without RNAlater. The differences in results may be largely due to the dosage difference and the presence of non-mucosal tissue of the intestine. Also birds can vary with each hatch.

Many AvBDs were downregulated in whole intestine tissue samples, and not in mucosal scrapings. These results may indicate that most changes of expression in AvBDs were in the non-mucosal tissue, possibly the immune cells located in the lymph vessel. In birds infected with *E. maxima* on D14 and followed with *C. perfringens* on D18, many changes in AvBDs were observed in the mucosal samples 2 days after *C. perfringens* infection (Hong et al., 2012). This dual infection led to necrotic enteritis, and may have induced changes in expression of AvBDs in the mucosal tissue.

In this time course study, chickens were infected with 300,000 oocysts/bird, which was 150% of the oocysts in the previous experiments. The differences in expression of apoptosis and autophagy related genes may be due to increasing the *E. acervulina* dosage. However, 300,000 oocysts may be equal to 200,000 oocysts, and changes of gene expression may not be a dose dependent effect due to virulence difference between experiments. The epithelial cell turnover in the chicken intestine usually takes about 3 to 4 days (Takeuchi et al., 1998). *Eimeria* needs about

3 days to reach the merozoite stage (Warren and Ball, 1967), when it is able to invade the neighboring host cells. Decrease of caspases during *Eimeria* infection indicated that the parasites might repress host cell death to prolong its survival. The downregulation of autophagy related genes indicated a possible defense mechanism of *Eimeria*, which protects its parasitic vacuole from being destroyed by the host autophagic pathway. Apart from cytochrome c, all apoptosis and autophagy related genes were downregulated at 5 dpi, which is around the time for *Eimeria* gametocytes formation. These results suggest that the gametocyte stage is critical in *Eimeria* development. If the apoptosis and/or autophagy pathways in chicken intestinal epithelial cells can be activated during *Eimeria* infection, there may be a potential attenuation of the infection. Because the intestinal epithelial cells are constantly being renewed, accelerated loss of these cells would not be expected to have a long lasting negative impact on the chicken.

References

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J Parasitol 89:553-564. doi 10.1645/GE-3159

Hong, Y. H., W. Song, S. H. Lee, and H. S. Lillehoj. 2012. Differential gene expression profiles of beta-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult Sci 91:1081-1088. doi 10.3382/ps.2011-01948

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408. doi 10.1006/meth.2001.1262

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp Parasitol 150:13-21. doi 10.1016/j.exppara.2015.01.003

Sumners, L. H., K. B. Miska, M. C. Jenkins, R. H. Fetterer, C. M. Cox, S. Kim, and R. A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during Eimeria praecox infection in chickens. Exp Parasitol 127:714-718. doi 10.1016/j.exppara.2010.12.002

Takeuchi, T., H. Kitagawa, T. Imagawa, and M. Uehara. 1998. Proliferation and cellular kinetics of villous epithelial cells and M cells in the chicken caecum. J Anat 193 (Pt 2):233-239.

Warren, E. W., and S. J. Ball. 1967. Schizogonous stages of Eimeria acervulina Tyzzer, 1929. Nature 214:829-830.

Table 4-1. Primer sequences in time course study.

Gene	Forward primer	Reverse primer
AvBd1	GAGTGGCTTCTGTGCATTTCTG	TTGAGCATTTCCCACTGATGAG
AvBd6	GCCCTACTTTTCCAGCCCTATT	GGCCCAGGAATGCAGACA
AvBd8	ATGCGCGTACCTAACAACGA	TGCCCAAAGGCTCTGGTATG
AvBd10	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT
AvBd11	GGTACTGCATCCGTTCCAAAG	GCATGTTCCAAATGCAGCAA
AvBd12	TGTAACCACGACAGGGGATTG	GGGAGTTGGTGACAGAGGTTT
AvBd13	CAGCTGTGCAGGAACAACCA	CAGCTCTCCATGTGGAAGCA
LEAP2	CTCAGCCAGGTGTACTGTGCTT	CGTCATCCGCTTCAGTCTCA
BAX	GGTGACAGGGATCGTCACAG	ACCAACTGTGTGTCGTAGGC
Bcl-2	TCGTCGCCTTCTTCGAGTTC	CATCCCATCCTCCGTTGTCC
Caspase 6	TGCCAGATAGACGTGGGACT	AGTCATCCCGAGAGGCTTCA
Caspase 8	CTCCTACAGAAGCCCAAGCC	GGCATTTGCTTCCCTGCATT
Caspase 18	GACCAGGCTGATGTTCTGGT	CCATCTTGTCACAGAGGCACT
Cytochrome c	TTCCCAGTGCCATACGGTTG	GCTTGTCCTGTTTTGCGTCC
Beclin 1	TTGAGAGAGTCAGGGCAGAG	TGCATCTGAGCATAACGCAT
LC3a	CAGGAGAAGGATGAGGATGGC	TCTCGGCAGGTCTCAGTAGC
LC3b	TCTTCCTCCTGGTGAACGGA	TCGTCCTTCTCGCTCTCGTA
β-actin	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT

Figure 4-1. Expression of HDPs in the duodenum of *E. acervulina*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

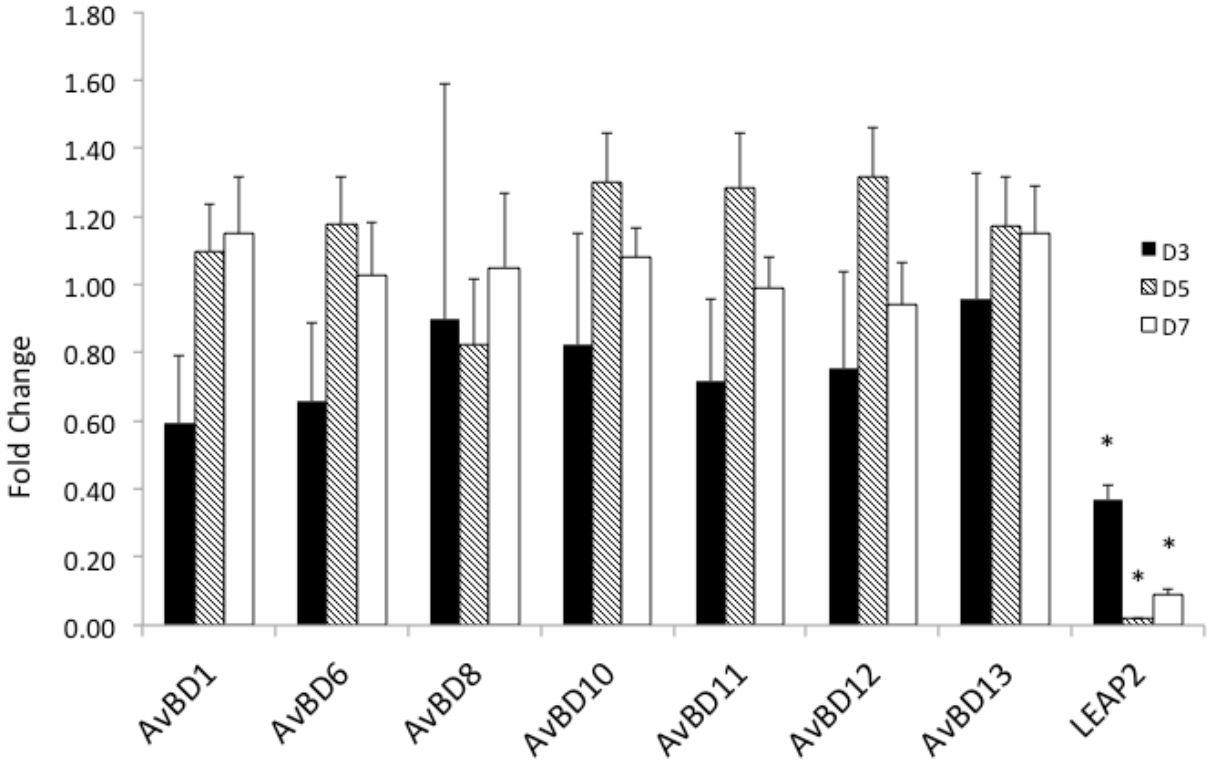
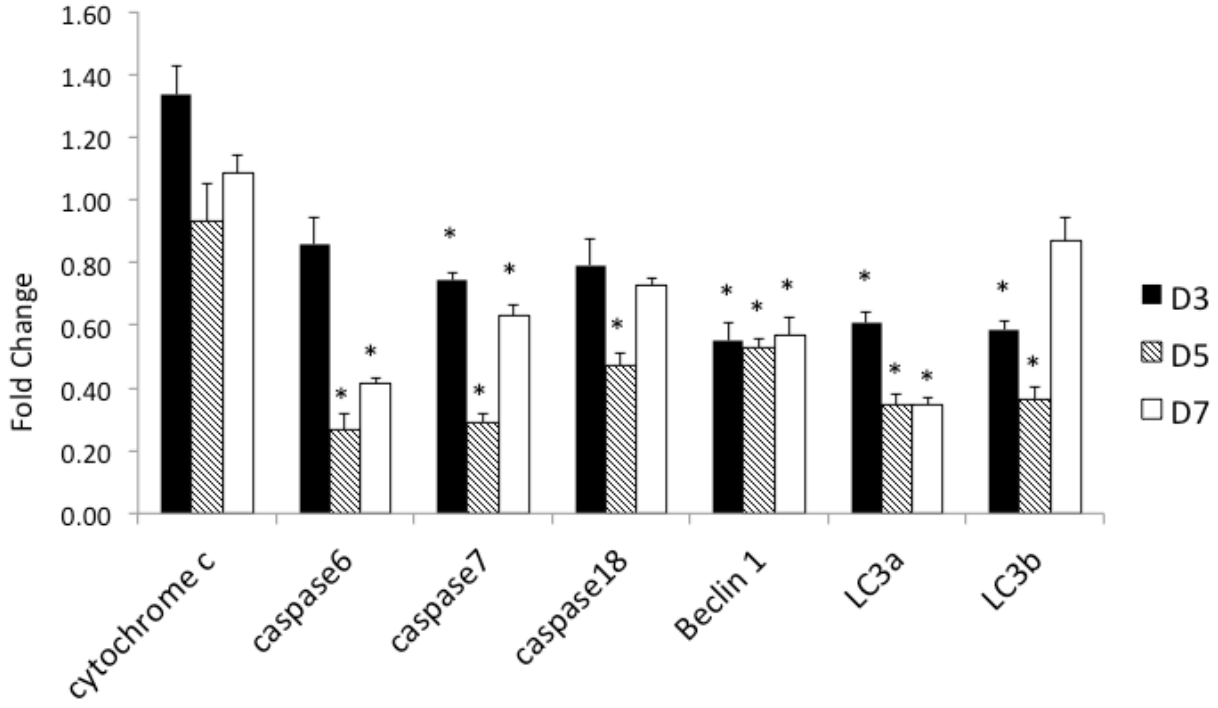


Figure 4-2. Expression of apoptosis and autophagy related genes in the duodenum of *E. acervulina*-challenged broilers. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.



Chapter 5. Expression of digestive enzymes, nutrient transporters, antimicrobial peptides, apoptosis and autophagy related genes in *E. maxima* infected Fayoumi and broilers chickens

Introduction

The Fayoumi chickens originated in Egypt, and are used for both meat and table egg production (Hassan et al., 2002). These chickens were imported into the US in 1954, primarily because of their resistance to avian leucosis (Kim et al., 2008). They have been shown to be more coccidiosis resistant compared to commercial breeds in the US (Pinard-Van Der Laan et al., 1998). The two lines of Fayoumi chickens used in this dissertation are congenic paired M5.1 and M15.2. These two lines are both highly inbred, and share an identical genetic background and only differ at the Major histocompatibility complex (MHC). These two lines are genetically distant from the commercial broiler and Leghorn lines (Zhou and Lamont, 1999; Kim et al., 2008). The M5.1 line showed more resistance to *E. maxima* infection compared to the M15.2 line (Kim et al., 2008).

Our previous results showed reduced body weight gain in *E. acervulina*, *E. maxima* and *E. tenella* infected chickens. This can be caused by damage to the intestinal epithelial layer, or by changes in expression of digestive enzymes and nutrient transporters in the intestine or both. In *Eimeria* challenged chickens, there was downregulation of APN, $b^{0,+}$AT/rBAT, EAAT3, SI, GLUT2, GLUT5, and ZnT1, which may result in a decrease in amino acid and sugar pools and zinc balance disruption. These changes may be part of the host defense mechanism to inhibit

Eimeria replication (Su et al., 2015). In chapters II and III, there was downregulation of HDPs, apoptosis and autophagy related genes, which may be *Eimeria*-induced regulation to promote its survival inside the host cell. The objective of this study was to compare the expression of digestive enzymes, nutrient transporters, antimicrobial peptides, apoptosis and autophagy related genes in *E. maxima* infected Fayoumi and broiler chickens.

Material and Methods

Chicken and *Eimeria* infection

Chickens used in this study were male Ross Heritage broilers (Longenecker's Hatchery, Elizabethtown, PA), and Fayoumi line M5.1 and M15.2. the Fayoumi lines were shipped on day of hatch from Iowa State to USDA (Beltsville, MD). *E. maxima* (USDA APU1 isolate) was used for infection. Oocysts were maintained and isolated as previously described (Fetterer and Barfield, 2003). Chickens were housed at the USDA-ARS facility (Beltsville, MD) from hatch and maintained in coccidia-free housing. Chickens were given water and a standard starter-type corn-soybean meal, ad libitum. At 21 d of age, chicks were inoculated with 1,000 *E. maxima* oocysts via oral gavage. 7 days post infection (dpi), duodenum, jejunum and ileum were collected from control and challenged birds. The contents of the intestine were removed and the tissue segments were immediately stored individually in RNAlater (Invitrogen, Grand Island, NY). Body weight was recorded at 21d and 28d (7 dpi) for weight gain calculation. This study was carried out under a protocol approved by the Beltsville Research Center Animal Care and Use Committee and conducted at the Animal Parasitic Disease Laboratory (USDA Agricultural Research Service, Beltsville, MD).

RNA extraction and quantitative real-time PCR

A 20–30 mg of frozen sample was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and total RNA was extracted following the manufacturer's instruction of Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and RNA quality was assessed by agarose-formaldehyde gel electrophoresis. The cDNA was synthesized from total RNA (500 ng) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) and then diluted 1:30 for real time PCR analysis. β-actin was chosen as the reference gene, because the Ct values for β-actin were the same for control and infected samples. The forward and reverse primers for all of the genes analyzed in this study are shown in Table V-1. qPCR was performed on an Applied Biosystems 7500 system (Thermo Fisher Scientific, Waltham, MA) with Fast SYBR green (Thermo Fisher Scientific, Waltham, MA) using the following conditions for all genes: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Samples were run in duplicate and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), as described in Su et al. (2015). The mean ΔCt of the uninfected samples was used to calculate the ΔΔCt value, which was performed separately for each line.

Statistical analysis

All data were analyzed by ANOVA using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). For gene expression of each line, the model included the main effects of

infection, sorted by tissue and genes. Significance level was set at $P < 0.05$ when compared with the control.

Results

Body weight gain for Eimeria challenged broilers

During the 7 d challenge, the non-infected Ross, Fayoumi M5.1 and Fayoumi M15.2 chickens gained 494 ± 28 g (mean ± SE), 70 ± 3 g and 72 ± 2 g, respectively. The infected Ross, Fayoumi M5.1 and Fayoumi M15.2 lines gained 418 ± 24 g, 55 ± 3 g and 56 ± 2 g, respectively. The challenge resulted in a 15% weight gain depression in Ross chickens ($P < 0.05$), a 21% weight gain depression in the M5.1 line ($P < 0.01$) and a 22% weight gain depression in the M15.2 line ($P < 0.001$) (Table V-2).

Gene expression in *E. maxima* infected chickens

The expression of digestive enzymes, nutrient transporters, antimicrobial peptides, apoptosis and autophagy related genes during *E. maxima* infection in the small intestine was assessed in Ross and Fayoumi lines M5.1 and M15.2. Changes of gene expression in the duodenum are shown in Figure V-1. For the digestive enzymes and nutrient transporter (Figure V-1.A, Table V-1), Ross broilers showed downregulation of $b^{0,+}$AT, EAAT3, SI, GLUT2 and ZnT1; Fayoumi line M5.1 showed decreased expression in $b^{0,+}$AT, B^0AT, EAAT3, SI, GLUT2, SGLT1and ZnT1; and Fayoumi line M15.2 showed downregulation of $b^{0,+}$AT, rBAT, B^0AT, EAAT3, GLUT2 and SGLT1. For the HDPs (Figure V-1.B, Table V-2), Ross and line M5.1 showed downregulation of LEAP2, line M5.1 showed upregulation of AvBD10; and line M15.2 showed upregulation of

AvBD6, 10, 11, 12 and 13. For the apoptosis and autophagy related genes (Figure V-1.C, Table V-3), Ross birds showed increased expression of cytochrome c and decreased LC3b; line M5.1 showed decreased Beclin 1 and LC3b; line M15.2 showed downregulation of caspase 18.

In the jejunum, for the digestive enzymes and nutrient transporters (Figure V-2.A, Table V-1), Ross broilers showed decreased APN, $b^{0,+}$AT, rBAT, B^0AT, EAAT3, SGLT1 and ZnT1; Fayoumi line M5.1 showed downregulation of APN, $b^{0,+}$AT, rBAT, B^0AT, EAAT3, SI, GLUT2, SGLT1 and ZnT1; and Fayoumi line M15.2 showed decreased APN, $b^{0,+}$AT, rBAT, B^0AT and EAAT3. For the HDPs (Figure V-2.B, Table V-2), Ross broilers showed decreased AvBD11 and LEAP2; line M5.1 showed downregulation of AvBD1, 6, 10, 11, 12, 13 and LEAP2; line M15.2 showed decreased AvBD1, 6, 10, 11, 12 and13. For the apoptosis and autophagy related genes (Figure V-2.C, Table V-3), Ross birds showed downregulation of caspase 6, 18, LC3a and LC3b; line M5.1 showed increased Beclin 1; line M15.2 showed decreased caspase 18, Beclin 1, LC3a and LC3b.

In the ileum, for the digestive enzymes and nutrient transporters (Figure V-3.A, Table V-1), Ross broilers showed downregulation of APN, $b^{0,+}$AT, rBAT, B^0AT, EAAT3 and ZnT1; Fayoumi line M5.1 showed decreased $b^{0,+}$AT and EAAT3; and Fyoumi line M15.2 showed downregulation of APN, $b^{0,+}$AT, rBAT, B^0AT, EAAT3, SI, GLUT2, SGLT1 and ZnT1. For the HDPs (Figure V-3.B, Table V-2), Ross broilers showed decreased AvBD1, 11 and LEAP2; line M5.1showed decreased AvBD10 and 11; line M15.2 showed downregulation of LEAP2. For the apoptosis and autophagy related genes (Figure V-3.C, Table V-3), Ross birds showed decreased

caspase 6, LC3a and LC3b; line M5.1 showed upregulation of caspase 7 and downregulation of LC3a; line M15.2 showed decreased caspase 6, 7 18, LC3a and LC3b.

Discussion

Fayoumi chickens are more resistant to *Eimeria* infection when compared to other chicken breeds. Pinard-Van Der Laan et al. (1998) used five lines of chickens to compare the resistance to *Eimeria* infection. Among the Mandarah, Fayoumi, Rhode Island Red and two white leghorn (WLB21 and WLDW) lines, the Fayoumi line was the most resistant, with only about 30% weight gain reduction 10 days after infection. The WLDW line was the most susceptible, which showed about 85% weight gain reduction. It is unclear how the Fayoumi chickens used in this experiment are related to the two lines used in this dissertation.

Kim et al. (2008) compared Fayoumi lines M5.1 and M15.2 following *E. maxima* infection. The M5.1 line was more resistant to *Eimeria* infection because it showed no significant body weight reduction 9 dpi, whereas line M15.2 showed about a 20% weight gain depression. Lee et al. (2016) showed similar repression of bodyweight in *E. tenella* infected Fayoumi chickens, with line M15.2 significantly decreased, whereas line M5.1 was not. But in the secondary *E. tenella* infection, both lines showed about 30% weight gain reduction compared to the noninfected birds.

In this dissertation, none of the three lines showed enhanced resistance to *Eimeria* infection based on body weight gain. All lines showed about 20% weight gain depression, but there were many differences in gene expression patterns.

In the duodenum, all three lines showed downregulation of many nutrient transporters and some changes in autophagy related genes. But the most interesting part is the upregulation of AvBDs in the M15.2 line, whereas all of these genes were downregulated in the jejunum. These results suggested that because defensins are secretory peptides, upregulation in the duodenum may compensate for the lack of expression in the jejunum.

In the jejunum, all three lines showed downregulation of APN and many other nutrient transporters, but only line M5.1 showed decreased expression of all digestive enzymes and nutrient transporters examined. The Ross birds only showed downregulation of AvBD11 and LEAP2 in the jejunum, which is different from the results of *E. maxima* infected chickens in Chapter II. These differences may be due to the dosage difference between these two experiments. The chickens in Chapter II were infected with a 10-fold higher dose of *E. maxima*. The infection however, did induce a strong response in both of the Fayoumi lines, as the results showed that all HDPs were downregulated, with the exception of LEAP2 in line M15.2. The different response in HDP expression between Ross and Fayoumi lines is likely due to the body weight difference. Since the Ross chickens are about 5 times heavier than the Fayoumi chickens, a 1,000 oocysts/bird infection may not be able to induce HDP expression changes in Ross, but it can cause great changes in Fayoumi chickens. In the expression of apoptosis and autophagy related genes, both Ross and line M15.2 showed some downregulation. But in line M5.1, there is upregulation of Beclin 1 and no changes in other genes. As discussed in previous chapters, inhibition of apoptosis and autophagy is favorable to *Eimeria* survival. Upregulation of Beclin 1 may induce autophagosome formation (Eskelinen and Saftig, 2009), and promote host cell

defense against *Eimeria* infection. Although the body weight gain data did not support the theory that the M5.1 line is more resistant to *Eimeria* infection, the gene expression results suggested that the resistance of M5.1 may be at the cellular level.

In the ileum, Ross chickens continued to show downregulation of some nutrient transporters. But the expression pattern in the M5.1 and M15.2 line were switched, when compared with jejunum samples. The M15.2 line showed downregulation of all digestive enzymes and nutrient transporters, while line M5.1 only had decreased $b^{o,+}AT$ and EAAT3. In the expression of HDPs, Ross chickens continue to show downregulation of AvBD11 and LEAP2 as in jejunum. Both of the Fayoumi lines did not show the downregulation pattern, with the exception of decreased expression of AvBD10 and 11 in line M5.1. Line M15.2 showed more downregulation of apoptosis and autophagy related genes than the other two lines. The M5.1 showed upregulation of Caspase 7, which is one of the executioner caspases (McIlwain et al., 2013), functioning as a protease during apoptosis (Walsh et al., 2008). Increasing expression of Caspase 7 may accelerate host cell apoptosis, and destroy the intracellular parasites.

To compare with our previous results, there was common downregulation of APN, $b^{o,+}AT$, rBAT, B^0AT and EAAT3 in the jejunum of all three lines of chickens during *E. maxima* infection. These results agree with the model of host cell inhibiting *Eimeria* replication by decreasing the uptake of some essential amino acids and glutamate, the energy source for intestinal cells (Su et al., 2015). In this study, all three lines of chickens showed common downregulation of AvBD11 in the jejunum. While in previous experiments, AvBD10 was downregulated in the jejunum of *E. maxima* challenged broilers. When Ross birds were

challenged with 10,000 oocysts, downregulation of Caspase 6, 7 and 18 was observed. When the challenge dose was decreased to 1,000 oocysts/bird, there was downregulation of Caspase 6 and 7, LC3a and LC3b, which indicated inhibition of both apoptosis and autophagy of the host cell. Since there are more cellular responses in lower dose challenge than the higher one, the host cells may be overwhelmed by the higher dose challenge, which leads to a poorer response to the infection. Thus both anti-apoptotic and anti-autophagic effects should be unfavorable for *Eimeria* survival inside the host cell. The anti-apoptotic state may inhibit *Eimeria* escaping the host cell after replication, and the anti-autophagic state can lead to diminished intracellular pools of nutrients and inhibit pathogen replication.

References

Eskelinen, E. L., and P. Saftig. 2009. Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochim Biophys Acta 1793:664-673. doi 10.1016/j.bbamcr.2008.07.014

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J Parasitol 89:553-564. doi 10.1645/GE-3159

Hassan, M. K., M. Afify, and M. M. Aly. 2002. Susceptibility of vaccinated and unvaccinated Egyptian chickens to very virulent infectious bursal disease virus. Avian Pathol 31:149-156. doi 10.1080/03079450120118630

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. doi 10.3382/ps.2007-00383

Lee, S. H., X. Dong, H. S. Lillehoj, S. J. Lamont, X. Suo, D. K. Kim, K. W. Lee, and Y. H. Hong. 2016. Comparing the immune responses of two genetically B-complex disparate Fayoumi chicken lines to Eimeria tenella. Br Poult Sci 57:165-171. doi 10.1080/00071668.2016.1141172

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408. doi 10.1006/meth.2001.1262

McIlwain, D. R., T. Berger, and T. W. Mak. 2013. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5:a008656. doi 10.1101/cshperspect.a008656

Pinard-Van Der Laan, M. H., J. L. Monvoisin, P. Pery, N. Hamet, and M. Thomas. 1998. Comparison of outbred lines of chickens for resistance to experimental infection with coccidiosis (Eimeria tenella). Poult Sci 77:185-191.

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp Parasitol 150:13-21. doi 10.1016/j.exppara.2015.01.003

Walsh, J. G., S. P. Cullen, C. Sheridan, A. U. Luthi, C. Gerner, and S. J. Martin. 2008. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci U S A 105:12815-12819. doi 10.1073/pnas.0707715105

Zhou, H., and S. J. Lamont. 1999. Genetic characterization of biodiversity in highly inbred chicken lines by microsatellite markers. Anim Genet 30:256-264.

Table 5-1. Primer sequence in Fayoumi study.

Gene	Forward primer	Reverse primer
APN	AATACGCGCTCGAGAAAACC	AGCGGGTACGCCGTGTT
$b^{0,+}AT$	CAGTAGTGAATTCTCTGAGTGTGAAGCT	GCAATGATTGCCACAACTACCA
rBAT	CCCGCCGTTCAACAAGAG	AATTAAATCCATCGACTCCTTTGC
B^0AT	GGGTTTTGTGTTGGCTTAGGAA	TCCATGGCTCTGGCAGAGAT
EAAT3	TGCTGCTTTGGATTCCAGTGT	AGCAATGACTGTAGTGCAGAAGT
SI	CGCAAAAGCACAGGGACAGT	TCGATACGTGGTGTGCTCAGTT
GLUT2	CACACTATGGGCGCATGCT	ATTGTGCCTGGAGGTGTTGGT
SGLT1	GCCATGGCCAGGGCTTA	CAATAACCTGATCTGTGCACCAG
ZnT1	TCCGGGAGTAATGGAAATCTTC	AATCAGGAACAAACCTATGGGAA
AvBd1	GAGTGGCTTCTGTGCATTTCTG	TTGAGCATTTCCCACTGATGAG
AvBd6	GCCCTACTTTTCCAGCCCTATT	GGCCCAGGAATGCAGACA
AvBd10	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT
AvBd11	GGTACTGCATCCGTTCCAAAG	GCATGTTCCAAATGCAGCAA
AvBd12	TGTAACCACGACAGGGGATTG	GGGAGTTGGTGACAGAGGTTT
AvBd13	CAGCTGTGCAGGAACAACCA	CAGCTCTCCATGTGGAAGCA
LEAP2	CTCAGCCAGGTGTACTGTGCTT	CGTCATCCGCTTCAGTCTCA
Caspase 6	TGCCAGATAGACGTGGGACT	AGTCATCCCGAGAGGCTTCA
Caspase 8	CTCCTACAGAAGCCCAAGCC	GGCATTTGCTTCCCTGCATT
Caspase 18	GACCAGGCTGATGTTCTGGT	CCATCTTGTCACAGAGGCACT
Cytochrome c	TTCCCAGTGCCATACGGTTG	GCTTGTCCTGTTTTGCGTCC
Beclin 1	TTGAGAGAGTCAGGGCAGAG	TGCATCTGAGCATAACGCAT
LC3a	CAGGAGAAGGATGAGGATGGC	TCTCGGCAGGTCTCAGTAGC
LC3b	TCTTCCTCCTGGTGAACGGA	TCGTCCTTCTCGCTCTCGTA
β-actin	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT

Table 5-2. Body weight gain of *Eimeria* challenged broilers. BW=Body weight; BWG=Body weight gain; all body weight measurements were expressed as mean±SE; BW and BWG were measured in grams (g).

Breed			Ross			Fayoumi M5.1		Fa
Measurement	BW d21	BW d28	BWG	BW d21	BW d28	BWG	BW d21	
Non-infected	817±24	1311±47	494 ± 28	138±7	208±7	70 ± 3	161±6	
Infected	725±58	1144±79	418 ± 24	127±11	182±14	55 ± 3	157±4	
Weight gain depression			15%			21%		
p value			<0.05			<0.01		

Figure 5-1. Expression of digestive enzymes and nutrient transporters (A), HDPs (B), apoptosis and autophagy related genes (C) in the duodenum of *E. maxima*-challenged Ross and Fayoumi line M5.1 and line M15.2 chickens. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

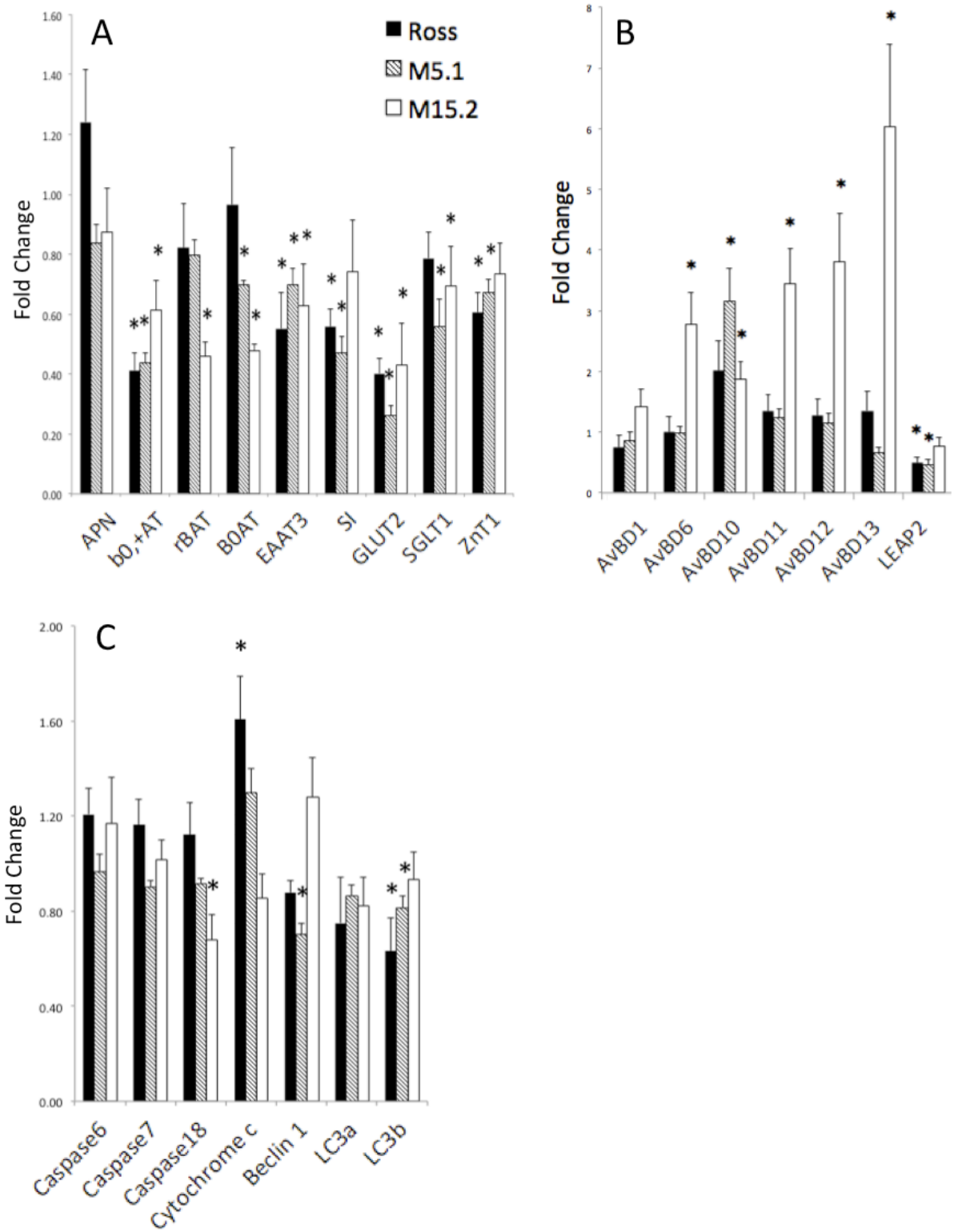


Figure 5-2. Expression of digestive enzymes and nutrient transporters (A), HDPs (B), apoptosis and autophagy related genes (C) in the jejunum of *E. maxima*-challenged Ross and Fayoumi line M5.1 and line M15.2 chickens. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

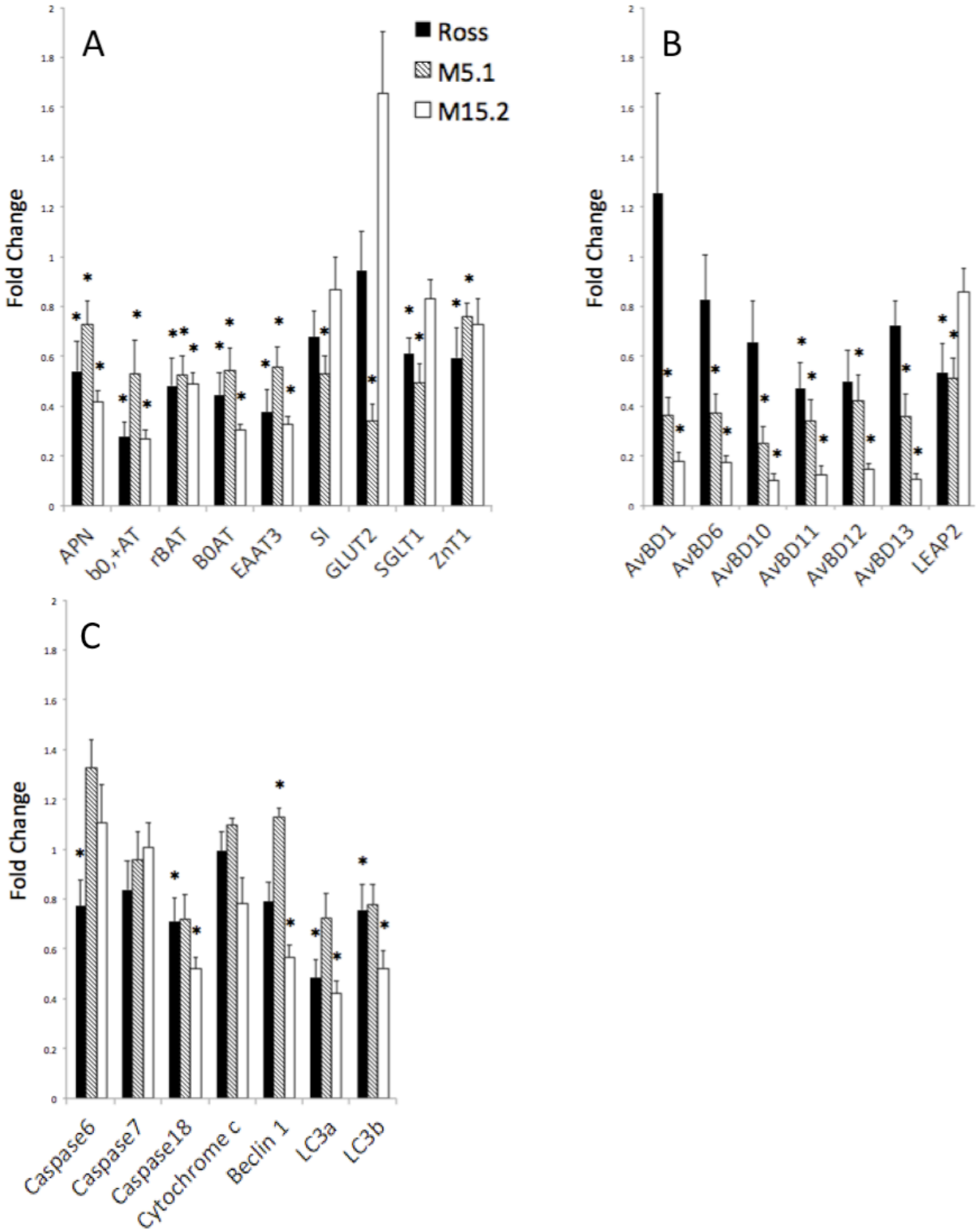


Figure 5-3. Expression of digestive enzymes and nutrient transporters (A), HDPs (B), apoptosis and autophagy related genes (C) in the ileum of *E. maxima*-challenged Ross and Fayoumi line M5.1 and line M15.2 chickens. Controls (non-challenged) are equal to a fold change of 1 for each gene. * indicates statistical significance from control at $P < 0.05$.

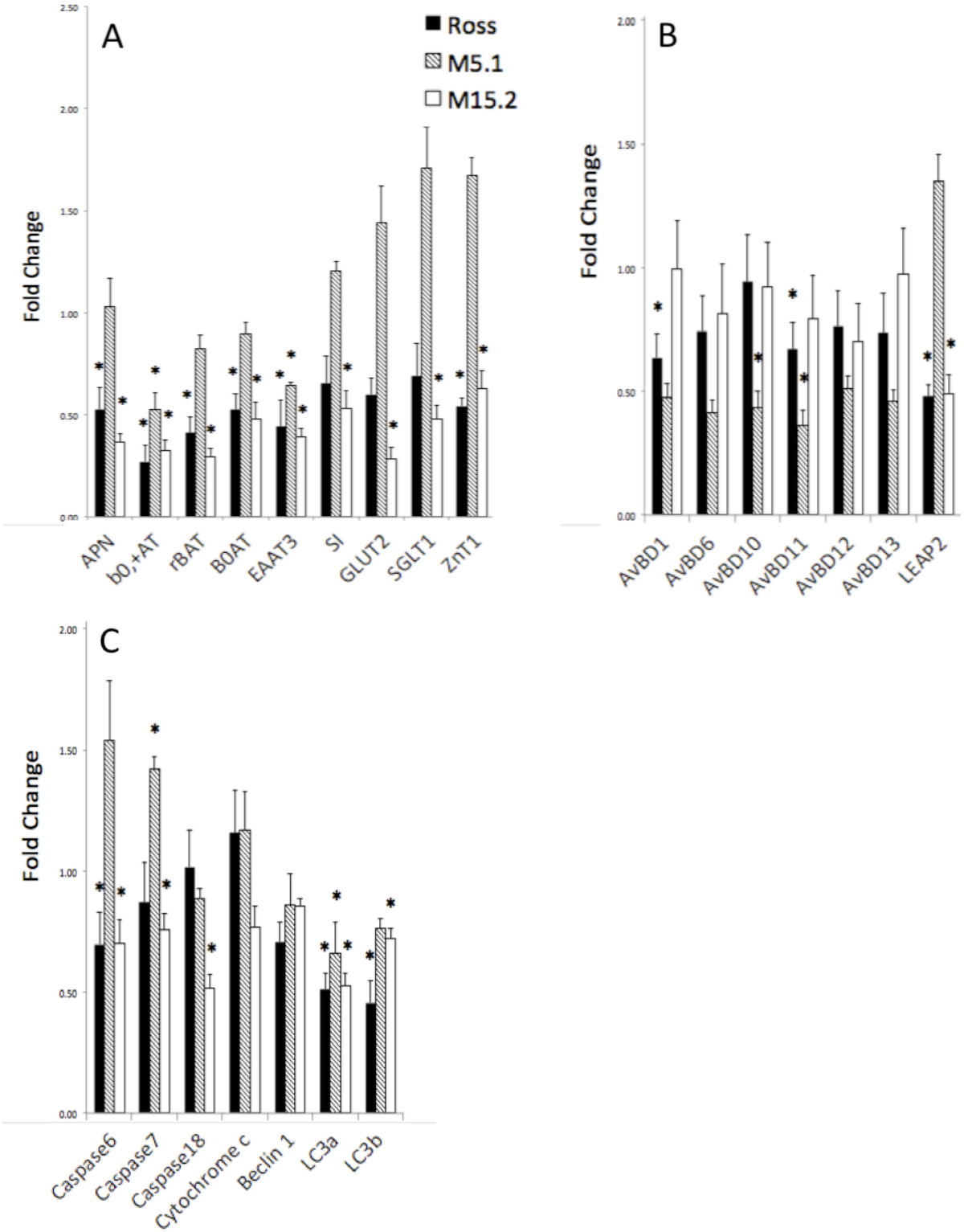


Table 5-3. Summary of digestive enzyme and nutrient transporter expression in Ross and Fayoumi chickens. ↓ indicates downregulation of the gene.

	Duodenum			Jejunum			Ileum		
	Ross	M5.1	M15.2	Ross	M5.1	M15.2	Ross	M5.1	M15.2
APN	-	-	-	↓	↓	↓	↓	-	↓
SI	↓	↓	-	-	↓	-	-	-	↓
$b^{0,+}AT$	↓	↓	↓	↓	↓	↓	↓	↓	↓
rBAT	-	-	↓	↓	↓	↓	↓	-	↓
B^0AT	-	↓	↓	↓	↓	-	↓	-	↓
EAAT3	↓	↓	↓	↓	↓	↓	↓	↓	↓
GLUT2	↓	↓	↓	-	↓	-	-	-	↓
SGLT1	-	↓	↓	↓	↓	-	-	-	↓
ZnT1	↓	↓	-	↓	↓	-	↓	-	↓

Table 5-4. Summary of HDP expression in Ross and Fayoumi chickens. ↓ indicates downregulation of the gene. ↑ indicates upregulation of the gene.

	Duodenum			Jejunum			Ileum		
	Ross	M5.1	M15.2	Ross	M5.1	M15.2	Ross	M5.1	M15.2
AvBD1	-	-	-	-	↓	↓	↓	-	-
AvBD6	-	-	↑	-	↓	↓	-	-	-
AvBD10	-	↑	↑	-	↓	↓	-	↓	-
AvBD11	-	-	↑	↓	↓	↓	↓	↓	-
AvBD12	-	-	↑	-	↓	↓	-	-	-
AvBD13	-	-	↑	-	↓	↓	-	-	-
LEAP2	↓	↓	-	↓	↓	-	↓	-	↓

Table 5-5. Summary of apoptosis and autophagy related gene expression in Ross and Fayoumi chickens. ↓ indicates downregulation of the gene. ↑ indicates upregulation of the gene.

	Duodenum			Jejunum			Ileum		
	Ross	M5.1	M15.2	Ross	M5.1	M15.2	Ross	M5.1	M15.2
Caspase 6	-	-	-	↓	-	-	↓	-	↓
Caspase 7	-	-	-	-	-	-	-	↑	↓
Caspase 18	-	-	↓	↓	-	↓	-	-	↓
Cytochrome c	↑	-	-	-	-	-	-	-	-
Beclin 1	-	↓	-	-	↑	↓	-	-	-
LC3a	-	-	-	↓	-	↓	↓	↓	↓
LC3b	↓	↓	-	↓	-	↓	↓	-	↓

Chapter 6. Localization of LEAP2 and EAAT3 mRNAs in chicken small intestine using the RNAscope technique

Introduction

In *Eimeria*-infected broilers chickens, excitatory amino acid transporter 3 (EAAT3) and liver expressed antimicrobial peptide 2 (LEAP2) were downregulated in the target tissues of each *Eimeria* species. For example, in *E. acervulina*-challenged birds, EAAT3 and LEAP2 were downregulated in the duodenum (Su et al., 2015). They were decreased in the jejunum during *E. maxima* challenge (Paris and Wong, 2013; Fetterer et al., 2014; Su et al., 2015), and in the ceca during *E. tenella* challenge (Su et al., 2015). In layers, EAAT3 and LEAP2 were downregulated in the duodenum during *E. praecox* (Sumners et al., 2011) and *E. acervulina* (Su et al., 2014) infection. LEAP2 showed up regulation in the ileum of *E. acervulina*-challenged layers (Su et al., 2014). In Chapter V, EAAT3 and LEAP2 was downregulated to 34% and 48% of control in the jejunum of *Eimeria*-infected Ross chickens.

Excitatory amino acid transporter 3 (EAAT3) is a high-affinity Na^+-dependent carrier of major importance in glutamate absorption in the small intestine (Kanai et al., 2013). EAAT3 (SLC1A1), which belongs to the SLC1A family, was the first mammalian sodium-dependent amino acid transporter cloned (Bianchi et al., 2014). In chicken, EAAT3 is located on the Z chromosome, and contains 12 exons and encodes a 647 nucleotide mRNA (Kent et al., 2002). Glutamate is one of the most abundant amino acids in dietary protein, but the blood concentration is quite low. This is because in the small intestine, glutamate is the main energy source of the enterocytes

(Fan et al., 2004; Iwanaga et al., 2005), and is also used by the enterocytes to synthesize other amino acids (Blachier et al., 2009). Fan et al, (2004) showed that EAAT3 is the major L-glutamate transporter. Expression of EAAT3 can be detected along the crypt-villus axis in neonatal porcine epithelial cells, but there is higher capacity and lower affinity transport activity in crypt than in villus cells (Fan et al., 2004).

The chicken liver expressed antimicrobial peptide 2 (LEAP2) was first discovered by bioinformatics screening of the chicken genome (Lynn et al., 2004). Based on the in silico sequence, LEAP2 expression was detected in a number of tissues including the small intestine, liver, lung and kidney (Townes et al., 2004). The chicken LEAP-2 gene is located on chromosome 13, and contains 3 exons and is translated into a prepropeptide that contains 76 amino acids. The active mature form of cLEAP2 contains 40 amino acids (37-76) An *in vitro* assay showed that LEAP2 has antimicrobial activity against *Salmonella* (Townes et al., 2004). Later, the same research group discovered that LEAP2 could interact with the outer membrane of bacteria and change membrane permeability. LEAP2 also has broad-spectrum antimicrobial activity and plays an important role in the chicken innate host defense (Townes et al., 2009) and is upregulated in the intestine of *Salmonella* infected chickens (Townes et al., 2004).

The in situ hybridization (ISH) method was first described by Pardue and Gall (1969), who used radioactive probes to detect target DNA sequences. Ever since, ISH has been widely used in research to identify gene expression in developmental processes, disease conditions and/or different treatments. New labeling methods for the probes included: digoxigenin, biotin, enzymes and fluorophores. These methods produced more sensitive detection, cell localization and less

health hazards to researchers. To improve the sensitivity of ISH, in situ PCR or in situ transcription was developed. Better results have been achieved by amplifing the signal detection using biotinylated tyramine (Grabinski et al., 2015).

RNAscope is a novel RNA in situ hybridization technique developed by Advanced Cell Diagnostics. Using a novel probe design strategy and a hybridization-based signal amplification, this technique allows the visualization of single mRNA molecules in individual cells. The RNAscope method can be used on formalin-fixed, paraffin-embedded or frozen samples on glass slides. The result can be visualized under either a standard bright-field microscope (with chromogenic labels) or an epifluorescent microscope (with fluorescent labels). A maximum of four target genes can be detected in the same sample using this technique. The company also claims that RNAscope has high sensitivity and specificity and is a promising platform for translating many RNA biomarkers into clinical use (Wang et al., 2012).

Both EAAT3 and LEAP2 genes are important for enterocytes to perform normal cell function. We hypothesized that EAAT3 and LEAP2 mRNAs were located in the intestinal enterocytes. The objective of this study was to examine the location and change of expression level of EAAT3 and LEAP2 in control and *E. maxima* infected chickens.

Material and Methods

Chicken and *Eimeria* infection

Uninfected and infected chickens used in this study were the same as described in Chapter V.

RNA in situ hybridization of EAAT3 and LEAP2

Tissue segments were fixed in 10% neutral buffered formalin and transfered to 70% ethanol after 24h. The fixed samples were sent to the Diagnostic Services Lab at the Virginia-Maryland Regional College of Veterinary Medicine for paraffin embedding. Slide preparation and staining used the RNAscope assay (Advanced Cell Diagnostics, ACD, Hayward, CA) following the manufacturer's instructions. In brief, 8 μm sections were cut with a microtome and adhered to glass slides. Tissues were deparaffinized and incubated with RNAscope Protease Plus reagents for 15 min at 40°C. Slides were hybridized with custom designed chicken EAAT3 and LEAP2 probes (ACD) at 40°C for 2 h. Hybridization signals were amplified and visualized with RNAscope 2.0 HD detection kit (brown). Images were captured with a bright-field microscope (Nikon Eclipse 80i, Nikon Instruments Inc., Melville, NY) at 40–200× magnification. Positive signals appeared as brown dots.

Results and Discussion

EAAT3 mRNA expression

Figure VI-1. showed the in situ hybridization results for EAAT3 mRNA. The expression of EAAT3 was found along the intestinal villus, from the tip to top of the crypt. This result is consistent with Fan et al. (2004), who showed that EAAT3 was expressed along the crypt-villus axis in neonatal porcine epithelial cells. The expression appeared to be lower in the cells at the crypt for both infected and non-infected birds (Figure VI-1.C and F) when compared with those

located along the villus. Since the crypt is the location for stem cells, they would not be involved in nutrient absorption, and they would not be expected to express transporter genes. EAAT3 expression was lower in the cells at the villus tip of the non-infected bird (Figure VI-1.B). Because the cells located at the villus tip are soon to sloughed off, they do not need high expression of nutrient transporters. Based on the PCR results (Chapter V) EAAT3 is 2-fold downregulated in the jejunum of the infected birds when compared to control, but there was no visible difference in EAAT3 mRNA based on the in situ hybridization results.

LEAP2 mRNA expression

Expression of LEAP2 is shown in Figure VI-2. Most of the signal is detected along the villi, with very little expression in the crypt (Figure VI-2. A, C, D and F). Because LEAP2 is an antimicrobial peptide (AMP), cells located on the villus constantly interact with environmental factors in the intestinal chyme and thus need to express AMPs to protect the cells from pathogens. The crypt contains intestinal stem cells, and less exposed to the environment, thus there was very little expression of LEAP2. In the non-infected bird, the expression of LEAP2 was lower at the tip of the villi (Figure VI-2. A and B). This may be because the cells at the villus tip are being sloughed off, and may have reduced the ability to express certain genes, including LEAP2. In previous studies, LEAP2 was up to 200-fold downregulated in the *Eimeria* infected chickens (Casterlow et al., 2011). But in this study, LEAP2 was only 2-fold downregulated in the infected birds, and there was no visible difference in LEAP2 based on the in situ hybridization results

Both EAAT3 and LEAP2 are important for intestinal cells to perform their normal cellular function. Expression of EAAT3 and LEAP2 can be found along the villus, with very little expression in the crypt. As the intestinal cells migrate up the villus to replace the extruded old cells at the villus tip, an earlier expressed gene would show expression closer to the crypt. The location of EAAT3 at the bottom of the villus (Figure VI-1. A, D, C and F) appeared at a lower region when compared to the location of LEAP2 (Figure VI-2. A, D, C and F). It is very likely that EAAT3 is expressed earlier than LEAP2. Both EAAT3 and LEAP2 appeared to have lower expression at the tip of the villus in non-infected Ross broilers. This is the first time EAAT3 and LEAP2 has been localized to epithelial cells of the chicken intestine.

Referneces

Bianchi, M. G., D. Bardelli, M. Chiu, and O. Bussolati. 2014. Changes in the expression of the glutamate transporter EAAT3/EAAC1 in health and disease. Cell Mol Life Sci 71:2001-2015. doi 10.1007/s00018-013-1484-0

Blachier, F., C. Boutry, C. Bos, and D. Tome. 2009. Metabolism and functions of L-glutamate in the epithelial cells of the small and large intestines. Am J Clin Nutr 90:814S-821S. doi 10.3945/ajcn.2009.27462S

Casterlow, S., H. Li, E. R. Gilbert, R. A. Dalloul, A. P. McElroy, D. A. Emmerson, and E. A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of Eimeria maxima-infected chickens. Poult Sci 90:1212-1219. doi 10.3382/ps.2010-01110

Fan, M. Z., J. C. Matthews, N. M. Etienne, B. Stoll, D. Lackeyram, and D. G. Burrin. 2004. Expression of apical membrane L-glutamate transporters in neonatal porcine epithelial cells along the small intestinal crypt-villus axis. Am J Physiol Gastrointest Liver Physiol 287:G385-398. doi 10.1152/ajpgi.00232.2003

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J Parasitol 89:553-564. doi 10.1645/GE-3159

Fetterer, R. H., K. B. Miska, M. C. Jenkins, and E. A. Wong. 2014. Expression of nutrient transporters in duodenum, jejunum, and ileum of Eimeria maxima-infected broiler chickens. Parasitol Res 113:3891-3894. doi 10.1007/s00436-014-4114-3

Grabinski, T. M., A. Kneynsberg, F. P. Manfredsson, and N. M. Kanaan. 2015. A method for combining RNAscope in situ hybridization with immunohistochemistry in thick free-floating brain sections and primary neuronal cultures. PLoS One 10:e0120120. doi 10.1371/journal.pone.0120120

Iwanaga, T., M. Goto, and M. Watanabe. 2005. Cellular distribution of glutamate transporters in the gastrointestinal tract of mice: an immunohistochemical and in situ hybridization approach. Biomed Res 26:271-278.

Kanai, Y., B. Clemencon, A. Simonin, M. Leuenberger, M. Lochner, M. Weisstanner, and M. A. Hediger. 2013. The SLC1 high-affinity glutamate and neutral amino acid transporter family. Mol Aspects Med 34:108-120. doi 10.1016/j.mam.2013.01.001

Kent, W. J., C. W. Sugnet, T. S. Furey, K. M. Roskin, T. H. Pringle, A. M. Zahler, and D. Haussler. 2002. The human genome browser at UCSC. Genome Res 12:996-1006. doi 10.1101/gr.229102. Article published online before print in May 2002

Lynn, D. J., R. Higgs, S. Gaines, J. Tierney, T. James, A. T. Lloyd, M. A. Fares, G. Mulcahy, and C. O'Farrelly. 2004. Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. Immunogenetics 56:170-177. doi 10.1007/s00251-004-0675-0

Pardue, M. L., and J. G. Gall. 1969. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc Natl Acad Sci U S A 64:600-604.

Paris, N. E., and E. A. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult Sci 92:1331-1335. doi 10.3382/ps.2012-02966

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult Sci 93:1217-1226. doi 10.3382/ps.2013-03807

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp Parasitol 150:13-21. doi 10.1016/j.exppara.2015.01.003

Sumners, L. H., K. B. Miska, M. C. Jenkins, R. H. Fetterer, C. M. Cox, S. Kim, and R. A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during Eimeria praecox infection in chickens. Exp Parasitol 127:714-718. doi 10.1016/j.exppara.2010.12.002

Townes, C. L., G. Michailidis, and J. Hall. 2009. The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane. Biochem Biophys Res Commun 387:500-503. doi 10.1016/j.bbrc.2009.07.046

Townes, C. L., G. Michailidis, C. J. Nile, and J. Hall. 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect Immun 72:6987-6993. doi 10.1128/IAI.72.12.6987-6993.2004

Wang, F., J. Flanagan, N. Su, L. C. Wang, S. Bui, A. Nielson, X. Wu, H. T. Vo, X. J. Ma, and Y. Luo. 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 14:22-29. doi 10.1016/j.jmoldx.2011.08.002

Figure 6-1. In situ hybridization of EAAT3 in control and *E. maxima* infected Ross broilers. **(A)** EAAT3 expression in non-infected bird (40×); **(B)** Enlarged from A to show expression at the villus tip (200×); **(C)** Enlarged from A to show expression in the crypt (200×); **(D)** EAAT3 expression in infected bird (40×); **(E)** Enlarged from D to show expression at the villus tip (200×); **(F)** Enlarged from D to show expression in the crypt (200×).

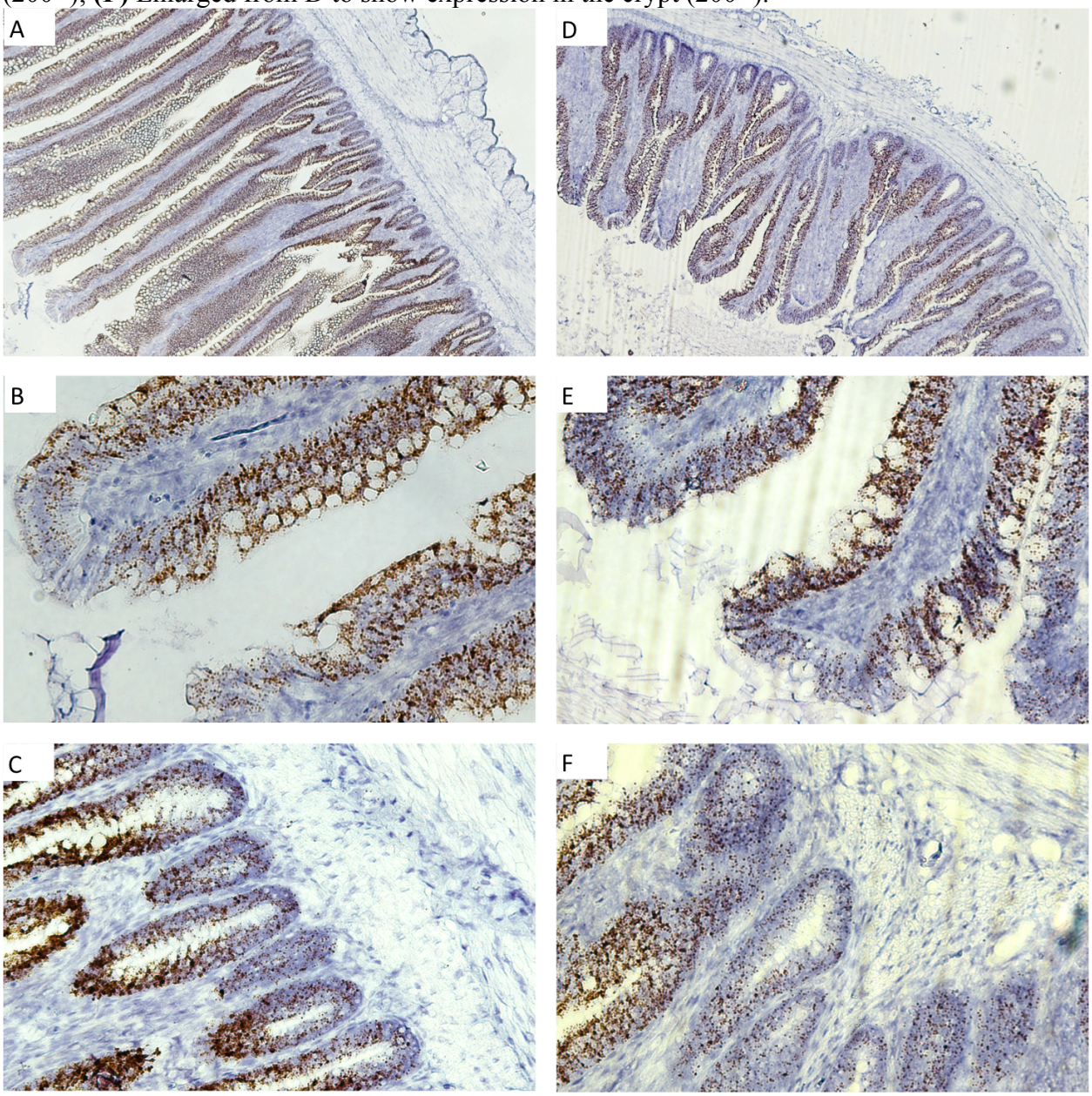
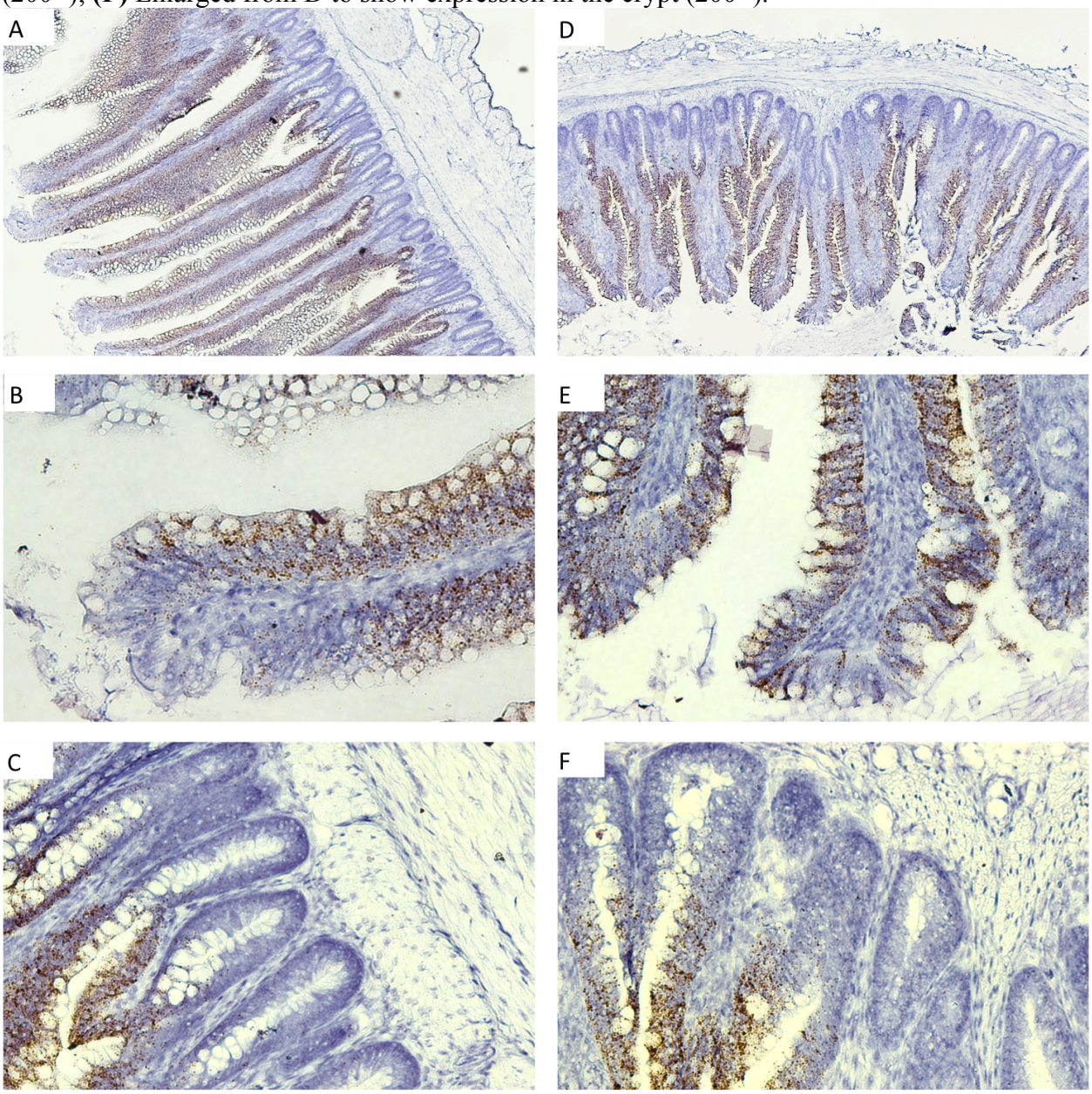


Figure 6-2. In situ hybridization of LEAP2 in control and *E. maxima* infected Ross broilers. **(A)** LEAP2 expression in non-infected bird (40×); **(B)** Enlarged from A to show expression at the villus tip (200×); **(C)** Enlarged from A to show expression in the crypt (200×); **(D)** LEAP2 expression in infected bird (40×); **(E)** Enlarged from D to show expression at the villus tip (200×); **(F)** Enlarged from D to show expression in the crypt (200×).



Chapter 7. Summary

In this dissertation I examined the expression of avian beta-defensins (AvBDs), apoptosis and autophagy related genes in *Eimeria* infected chickens (Chapter II and III), as well as the expression of these genes in *E. acevulinia* infected chickens at different times after challenge (Chapter IV). Chapter V was focused on the comparison of gene expression in Ross and Fayoumi chickens infected with *E. maxima*. The last chapter showed the localization of EAAT3 and LEAP2 mRNA in the chicken intestine.

A model for the cellular events that occur during an *Eimeria* infection has been developed. In an uninfected cell, antimicrobial peptide LEAP2 and nutrient transporters EAAT3, $b^{0,+}$AT and rBAT are expressed (Su et al., 2015) (Figure VII-1). Upon infection, *Eimeria* downregulates expression of LEAP2 in the host cells. The host then downregulates nutrient transporters, which may result in amino acid depletion and zinc balance disruption. The diminished intracellular pools of nutrients may promote cell death and inhibit pathogen replication (Figure VII-1). LEAP2 was consistently downregulated in the target tissues upon *Eimeria* infection, while AvBDs showed many variations in expression patterns (Chapters II, IV and V). Downregulation of LEAP2 may be a mechanism by which *Eimeria* combats the host defense system and promotes its survival inside the host cell.

Eimeria can also induce an anti-apoptosis and anti-autophagy state inside the host cell (Figure VII-1), which can be both favorable and unfavorable to parasite survival and replication (Chapter III). Under the anti-apoptotic state, the probability of *Eimeria* being destroyed with the death of the host cell is lowered, which favors its replication and survival. But as *Eimeria* completes the

asexual replication, it may need to activate the apoptosis and autophagy pathway to escape the host cell, decreasing expression of caspases may inhibits parasite escaping to infect the neighboring cells. The anti-autophagic state can protect the parasitophorous vacuole, which would promote parasite replication at the early stage. However, it can also lead to decreased intracellular nutrient levels, thus being unfavorable for the intracellular pathogen as it continues to replicate inside the host cell.

Downregulation of LEAP2 in the target tissue for each *Eimeria* species was consistent in all studies. These results indicated that LEAP2 may be important in host defense against intracellular pathogens, thus it was downregulated by *Eimeria*. The in situ hybridization results indicated that LEAP2 was expressed along the villus in the small intestine. This is the first time LEAP2 has been localized to epithelial cells of the chicken intestine (Chapter VI).

The comparison among different lines of chickens showed differential gene expression in lines with varying resistance to *Eimeria*. The body weight reduction, however, was similar, and there were many common downregulated genes indicating that there may not be a significant *Eimeria* resistant line among the Ross, Fayoumi M5.1 and M15.2 birds.

The interaction between *Eimeria* and the host cell is very complex, and there is more work to be done to unveil the mechanisms behind the changes of gene expression during *Eimeria* infection. It would be interesting to see how digestive enzymes and nutrient transporters are regulated at different time points during an *Eimeria* infection. The Fayoumi experiment can be expanded to examine the effects of *Eimeria* species, infective dosage and/or time on these unique birds.

Figure 7-1. Expression of transporters, HDPs, apoptosis and autophagy related genes in uninfected and *Eimeria*-infected cell. Arrow indicates the direction of nutrient transport. Decreased size of gene symbol or gene family indicates downregulation in infected cells. Eimeria infection resulted in downregulation of EAAT3, $b^{0,+}$AT/rBAT, and ZnT1, which leads to decreased intracellular glutamate and neutral amino acids, but increased intracellular Zn^{2+} level. The infection also caused downregulation of HDPs, apoptosis and autophagy related genes.