# DIFFERENTIAL GENE EXPRESSION IN INNATE IMMUNITY BETWEEN COMMERCIAL BROILERS AND LAYERS

A Dissertation

by

SHIXUE SHEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Poultry Science

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Approved by:

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# ABSTRACT

Differential Gene Expression in Innate Immunity between Commercial Broilers and Layers. (August 2006)

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Co-Chairs of Advisory Committee: Dr. James J. Zhu
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Tremendous improvements have been achieved in growth rates and feed efficiency in commercial broiler birds. However, fast growth broilers generally show weak immune competence and disease resistance. Innate immunity is the first line of defense providing immediate killing effects to a broad range of infectious pathogens and limiting infections to a minimum at an early stage before the activation of more specific adaptive immunity. Acute phase proteins (APPs), defensins and Toll-like receptors (TLRs) are all important innate immune molecules functioning from recognition to killing the foreign microbes. Tibial dyschondroplasia (TD) is one chicken disease associated with rapid growth in broilers. The objective of this research was to study the differential expression of innate immune related genes in liver and spleen tissues between commercial broilers and layers with the stimulation of lipopolysaccharide (LPS). Also, this study investigated mechanisms involved in the pathogenesis of TD at molecular levels.

This study first identified and annotated nineteen new chicken APPs genes from the chicken genome draft with bioinformatics tools. Using a relative quantitative real-

time RT-PCR method, the expression levels of all thirty-one APPs, thirteen defensins and eight TLRs genes were systemically investigated at the transcriptional level at three time points (0-, 3-, 8-hour) with the challenge of LPS. This study showed that broiler birds generally expressed significantly lower levels of all three families of innate immune related genes than layers and the inductive extent of these genes are generally smaller in broilers too. Close investigation of some important signaling transcription factors (NF-kB and IRF-3) and cytokine (IL-6) also reached the same conclusion. This study revealed that the inadequate expression of deiodinase type 2 (DIO2) contributed to the pathogenesis of TD in rapid growth broilers. All of the experimental results solidly validate the hypothesis that a compromised innate immune response or weak disease resistence is associated with fast growth broiler birds.

# DEDICATION

*My doctor of philosophy and this dissertation are dedicated with my greatest gratitude and deepest love to my parents and my elder sisters and my lovely nephews*

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# CHAPTER I

# INTRODUCTION

## Research Objective

The overall objective of this research is to identify the molecular mechanisms underlying disease susceptibility in fast growth broilers.

## Research Hypothesis

The central hypothesis is that compromised innate immunity is the reason for increased disease susceptibility. This hypothesis is based on preliminary data and research discoveries in poultry and mammals.

## Research Rationale

The rationale underlying this investigation is that the relevant molecular mechanisms are unknown, and this proposed research would lead to a better understanding of disease susceptibility in broilers and to help formulate effective strategy to protect broilers.

## Specific Objectives

1. To identify and annotate chicken acute phase protein (APP) genes and to determine the differential expression of APPs between broilers and layers.

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This dissertation follows the style and format of Immunogenetics.

2. To determine differential expression of defensins between broilers and layers.
3. To determine the differential expression of Toll-like receptors between broilers and layers.
4. To determine the mechanisms involved in the pathogenesis of tibial dyschondroplasia (TD).

# CHAPTER II

# LITERATURE REVIEW

## Innate Immunity

The immune system can be divided into the innate and adaptive systems. Both systems are complementary and highly interrelated in a host's defense system. Innate immunity is an ancient and universal mechanism utilized by many organisms. In contrast, the adaptive immune system is an evolutionarily newer system (only exists in the descendents of bony fish), and induces a delayed antigen-specific immune response, which increases with successive exposure to the same microbes (Abbas and Lichtman, 2003). The importance of the innate immune system is its immediate defensive effects on a broad range of pathogens before the establishment of a more specific adaptive immunity, which usually takes several days. Now, it is well known that the innate immune system can not only induce immediate active defense responses, but also plays important roles in initiating and instructing the adaptive immune response (Janeway and Medzhitov, 2002). Limiting infections to a minimum in the early stage is critical to the outcome: recovery from infections and restriction the spread of foreign pathogens. The recognition of innate immunity relies heavily on the limited number of receptors encoded by germ-line. These host receptors could effectively recognize conserved structures or chemicals expressed only in foreign pathogens instead of the self, and confer host the discrimination ability between the foreigner and the self (Abbas and

Lichtman, 2003). Recent discoveries of Toll and Toll-like receptors (TLRs) have led to new insights into host defense mechanisms.

The recognition of foreign bacteria is the first and critical step in immune response. The evolutionary strategy of the innate immune system has been to recognize a few highly conserved, constitutive structures such as lipopolysaccharide (LPS) or peptidoglycan (PDG), and these structures are present only in foreign microorganisms (Medzhitov, 2001). Therefore, the innate immune system does not need much flexibility to detect microorganisms. The molecular characteristics of these microbial components are named as pathogen-associated molecular patterns (PAMPs), and the host receptors that recognize these patterns are called pattern recognition receptors (PRRs). These receptors can be either secreted or bound to host cell membranes. Most secreted-PRRs are plasma proteins such as mannan-binding lectin (MBL) and C-reactive protein (CRP), which can mediate antimicrobial effects by activating the complement system, assisting phagocytosis, or facilitating the binding of surface PRRs to foreign microorganisms (Schwalbe et al., 1992; Matsushita and Fujita, 1995; Underhill and Ozinsky, 2002). Membrane-bound PRRs, such as Fc-receptors, complement-receptors and Toll-like receptors, play critical roles in phagocytosis, as well as activation of inflammatory signaling transduction pathways (Underhill and Ozinsky, 2002).

**Toll-Like receptors**

During the evolution, multi-cellular organisms developed various mechanisms to discriminate between self and non-self, and efficiently protected themselves from the invasion by infectious pathogens. Innate immunity effectively recognizing and

interacting with foreign products mainly depends on host germ-line encoded receptors. Toll receptors or Toll-like receptors (TLRs) are groups of important membrane PRRs specifically recognizing different pathogen associated molecular patterns on foreign microbes (Akira et al., 2001). As early as the 1980s, some people found that, in early *Drosophila* larvae, there was a transmembrane receptor that played a critical role in determining the embryo's dorsal-ventral polarity (Anderson et al., 1985; Hashimoto et al., 1988), and later this receptor was named as Toll receptor (Stein et al., 1991). This receptor was also found to play a significant role in other physiological functions including antifungal activities (Lemaitre et al., 1996). From the study of Toll receptor, a series of other genes (such as the Spatzle, Pelle, and Cactus) related to the Toll signaling pathway were identified (Janeway and Medzhitov, 2002).

The TLRs are homologous membrane proteins found in vertebrates, and these receptors are widely expressed in various mammals, birds and fish (Medzhitov et al., 1997; Fukui et al., 2001; Oshiumi et al., 2003). The first vertebrate TLR was identified in humans one year after the discovery of Toll receptor in *Drosophila*. This human TLR played an important role in the inducible expression of pro-inflammatory cytokines (IL-1 and IL-6) by activating NF-kB. Also, this TLR was critical in activating the naïve T cell of adaptive immunity. Close investigation revealed that this mammalian TLR contained similar structures to that of *Drosophila*.

In mammals, especially in humans, TLRs have been widely and intensively studied in recent years. Since the 1990s, there are at least eleven different human TLRs (hTLR-1 to -11) identified from various tissue/cell types, and more have been found in

other mammals such as mice, rats, rabbits and pigs (Roach et al., 2005). In human, TLR-4 was the first identified human TLR (hTLR) (Medzhitov et al., 1997). Later, hTLR-1, -2, -3, and -5 were cloned and characterized by Bazan's group in 1998 (Rock et al., 1998). hTLR-6 was isolated in 1999 (Takeuchi et al., 1999) and hTLR-7, -8 and -9 were found from the human genomic database in 2000 (Du et al., 2000; Chuang and Ulevitch, 2000). hTLR-10 was identified in 2001 and this TLR was highly expressed in immune related tissues/cells such as spleen, lymph node, and thymus (Chuang and Ulevitch, 2001). TLR-11 was found in some animals such as rats, mice, dogs, and fugu fish. However, in human, only a pseudogene of TLR-11 was obtained, and it was nonfunctional due to the pre-presence of a stop codon in the coding region (Lauw et al., 2005). Further study suggests a less selective pressure for hTLR-11, and there may be alternative innate recognition mechanisms utilized by human for TLR-11 (Yarovinsky and Sher, 2006). In mouse, the functional mTLR (mouse TLR) -11 was identified, and its expression pattern seems to be distinct from all other TLRs (Zhang et al., 2004; Lauw et al., 2005; Roach et al., 2005). From experimental results, the mTLR-11 could play important roles in preventing the infection of uropathogenic bacteria in internal organs of urogenital system (Zhang et al., 2004). There is no doubt that there may be more hTLRs, but further experiments are needed to confirm this hypothesis.

The Toll or Toll-like receptor family shares a similar structure containing leucine-rich repeats (LRRs) in the extra-cellular region, and a Toll/Interleukin-1 receptor (TIR) homology domain in intra-cytoplasma. Based on amino acid sequence and genomic structure, these mammalian TLRs could be divided into five subfamilies: TLR-

2, -3, -4, -5, and -9 (Takeda et al., 2003). The TLR-2 subfamily contains TLR-1, -2, -6, and -10, whereas the TLR-9 subfamily includes TLR-7, -8, and -9. The cytoplasmic domains of these TLRs are relatively conserved, whereas the extra-cellular parts vary among different TLRs, which confer specific binding abilities to different compounds or chemicals of foreign pathogens. The immuno-staining method was utilized to locate the exact positions of these TLRs on the host cells. Using specific TLR antibodies, the positive staining signals revealed that hTLR-1, -2, -4, -5 and -6 are preferentially expressed on the plasma membrane; whereas TLR-3, -7, -8, and -9 are usually localized to intra-cellular compartments (Matsumoto et al., 2003; Takeda and Akira, 2005). These positional differential expressions of TLRs are consistent with the specific ligands binding abilities among them. Generally, the surface-expressed TLRs mainly respond to the cell wall components of foreign pathogens, whereas the intracellular membrane-expressed TLRs recognize nucleic acids such as RNA or DNA (Dunne and O'Neill, 2005). In detail, TLR-1, -2 and -6 specifically respond to various bacterial components including lipopeptide or peptidoglycan from gram-positive bacteria, TLR-3 recognizes double-stranded RNA (dsRNA) from viruses during their replication, TLR-4 mainly recognizes LPS from gram-negative bacteria, TLR-5 responds to bacterial flagellin, TLR-7 and -8 can recognize single-stranded RNA (ssRNA) as well as imidazoquinolines, and TLR-9 responds to un-methylated CpG DNA motif from bacterium or virus and hemozoin from malaria (Iqbal et al., 2005; Kawai and Akira, 2006a). The exact ligands for TLR-10 are still unknown, but TLR-10 shares a similar structure with TLR-1 and -6, and can heterodimerize with TLR-1 or -2 (Hasan et al.,

2005). The mouse TLR-11 was thought to recognize profilin-like ligands from some parasites (Yarovinsky et al., 2005).

The specific repertoire of TLRs can be further extended by the heterodimerization or homodimerization capability of these receptors. For example, the heterodimser of TLR-2 and TLR-1 can specifically recognize bacterial lipopeptides (Wyllie et al., 2000), whereas TLR-2 and TLR-6 heterodimers can respond to mycoplasma lipoproteins and peptidoglycan (Wetzler, 2003). Interestingly, the different dimers, TLR-2/-1 and TLR-2/-6 can even discriminate between tiny differences, such as the difference between triacyl-lipopeptide and diacyl-lipopeptide. What's more, various non-TLR molecules, such as adaptors, may influence the TLRs' specific repertoire (Akira and Takeda, 2004). For example, LPS first needs to bind LPS-binding protein (LBP) in serum. This complex would serve to facilitate the binding of LPS to CD14 and TLR-4 on the cellular surface, which enhances both binding affinity and specificity.

Binding between ligands and TLRs can induce signal transduction pathways and activate transcription factors within the host cells. The two common pathways are nuclear factor kappa-B (NFkB) pathway and the interferon regulatory factor (IRF) pathway. The first step in signal transduction requires the interaction between adaptor proteins and TLRs. The common adaptor proteins are MyD88 (myeloid differentiation primary-response protein 88), Mal (MyD88-adaptor-like protein, also known as TIR-domain-containing adaptor protein or TIRAP), TRIF (TIR-domain-containing adaptor protein inducing IFN-beta), TRAM (TRIF-related adaptor molecule) and SARM (sterile-alpha and HEAT-Armadillo motifs) (O'Neill et al., 2003). Different TLRs may recruit

different adaptor proteins to induce different signaling cascades. During the transduction pathways, a series of cytoplasmic intermediates, such as IRAK (IL-1 receptor-associated kinase) and TRAF (TNF receptor-associated factor), are also recruited and phosphorated, in turns. Finally, the transcription factors, such as NFkB and IRF-3, are activated in host cells (Akira and Takeda, 2004). After being translocated into the nucleus, these activated transcription factors can bind to the transcription factor binding sites (TFBSs) on target genes and induce the expression of pro-inflammatory cytokines, chemokines and other immune-related factors, such as interleukin-6 (IL-6), IL-1, tumor necrosis factor-alpha (TNF-alpha) and interferon (IFN).

Most of the time, only a moderate level of inflammatory cytokines or chemokines are produced during the TLR signaling pathways; however, sometimes excessively inductive expressions of these immune-related molecules result in serious systemic disorders within the host, such as endotoxic shock or chronic rheumatoid arthritis. Fortunately, multi-cellular organisms also develop mechanisms to regulate/modulate the TLR signal response and to maintain an inner homeostasis (Akira and Takeda, 2004; Dunne and O'Neill, 2005). IRAK-M provides a good example to illustrate this negative feedback regulation. IRAK-M is one member of the IRAK family (Janssens and Beyaert, 2003) and is only expressed in limited cell types such as macrophages and monocytes (Wesche et al., 1999). As compared to the wild type, the IRAK-M deficient mice exhibit an increased inflammatory response and cytokine production, and significantly reduced endotoxin tolerance with the challenge of TLR ligands. Close investigation has revealed that IRAK-M functioned to prevent the

dissociation of IRAK-1 and IRAK-4 complexes from adaptor MyD88 and further inhibit the following phosphorylation cascades. IRAK-M increases its expression with the activation of TLRs, which counteracts the inductive expression of pro-inflammatory cytokines via a negative feedback control (Kobayashi et al., 2002). Suppressor of cytokine signaling (SOCS)-1 is another regulatory molecule and is induced to express by the pro-inflammatory cytokines. The activated SOCS-1 can also repress the TLR signaling cascades indirectly (Baetz et al., 2004). SOCS-1 deficient mice are hypersensitive to LPS-induced shock, and increase the expression of inflammatory cytokines with LPS challenge (Kinjyo et al., 2002). Recent studies also reveal other molecules functioning to down-regulation TLR signaling pathways such as single immunoglobulin IL-1-related (SIGGIR), MyD88 short (MyD88s) and TIR-containing proteins ST2 (Mansell et al., 2006).

TLRs also play a critical role in adaptive immunity. It is well documented that the maturation of dendritic cells (DCs) involves the increasing expression of specific co-stimulatory molecules, which is dependent upon the activation of TLR signaling pathways (Banchereau and Steinman, 1998; Medzhitov, 2001). During pathogen infection, the interaction of various ligands and TLRs on DCs induces the up-regulation of both co-stimulatory molecules and major histocompatibility complex (MHC) molecules, both of which facilitate the maturation of naïve T cells in adaptive immunity. As expected, the MyD88-deficient mice failed to produce IRF-gamma or active T helper type I cells with stimulation in Schnare's experiment because of the interruption of the TLRs signaling cascades (Schnare et al., 2001). Also in this experiment, the immature

DCs failed to be activated, which strongly illustrated the critical role of TLRs in the activation and induction of co-stimulation molecules in adaptive immunity. Also, the increased expression of various cytokines or chemokines induced by TLRs can significantly contribute to adaptive immunity (Drakesmith et al., 2000; Pasare and Medzhitov, 2004).

Chicken TLRs (chTLRs) were the first identified non-mammalian vertebrate TLRs. Based on the consensus sequences of *Drosophila* and mouse Toll families, Fukui and his colleagues first isolated chicken TLRs (type 1 and 2) with degenerate primers from chicken bursa cDNA library (Fukui et al., 2001). Because of their high homologies to human TLR-2, these two chicken TLRs were named TLR-2 type 1 and type 2. Further experiments showed that these TLRs were expressed in a wide range of tissues and organs especially in the connective tissues. The close location of these two genes on the same chromosome and their significant similarity suggested a duplication origin during evolution. However, it is interesting that only type 2 could recognize and signal both lipoproteins and LPS, but no tested microbial ligands were signaled by type 1 receptor. Chicken TLR-4 was identified two years later, and it was also expressed in almost all tissues tested (Leveque et al., 2003). Other chicken TLRs (TLR-1 type 1 and 2, TLR-3, -5, -7, -15 and -21) were found in 2005 and 2006 respectively (Yilmaz et al., 2005; Roach et al., 2005; Higgs et al., 2006). In total, there are ten chicken TLRs identified so far including two different types (TLR-1 and -2 both have two types). Further investigation has revealed that the chromosomal locations of TLRs showed a high similarity between chicken and human (Yilmaz et al., 2005). TLR-1, -2 and -3 are all located on

chromosome 4 in both species except chTLR-1 is positioned on an un-determined micro-chromosome. In chicken, TLR-4, -5 and -7 are unlinked on different chromosomes 17, 3 and 1 respectively, which is very similar in human (chromosomes 9, 1 and X, respectively). The latest found chicken TLR-15 and -21 are located on chromosomes 3 and 11 respectively, but there was no homology in human or other mammals. The results from Yilmaz's research also revealed that similar TLRs gene structures existed in both chicken and human. For example, most TLRs contain the same number of exons in both species, which shows a conservative evolution for the TLRs family (Yilmaz et al., 2005). With the improvement of powerful bioinformatics tools and chicken genome quality, more chTLRs may be identified and annotated. CpG oligodeoxynucleotide (CpG-ODN) is a strong stimulus to chicken monocytes (Kogut et al., 2005; He et al., 2006). In mammals, CpG-ODN is a specific ligand of TLR-9. However, no chicken TLR-9 ortholog is found. There may have other TLRs for CpG-ODN in chicken, but further experiments are needed to validate this hypothesis.

The chicken also shares similar evolutionary conservative signaling transduction pathway with mammals. A series of signaling pathway genes were identified by O'Farrelly's lab with bioinformatic approaches (Lynn et al., 2003). These genes included MyD88, Mal, IRAK-4, TRAF-6, TGF beta-activated kinase 1 (TAK1), TGF beta-activated kinase 1 binding protein 1 (TAB1), TAB2, inhibitor of NF-kB kinase alpha (IKK-alpha), IKK-beta, and Toll-interacting protein (Tollip).

However, the function of chicken TLRs was only investigated in limited labs, and not much information and results have been obtained so far. In human, only TLR-4

can specifically recognize LPS from gram-negative bacteria; however, in chicken, both TLR-2 and -4 can respond to LPS. In Kogut and his colleagues' experiments, after pre-incubation with mammalian antibody (anti-TLR-2 or anti-TLR-4), the chicken heterophils could significantly decrease oxidative burst with the challenge of LPS from *Salmonella enteritidis* (SE) (Farnell et al., 2003). Also, these researchers found that CD14 and chTLR-2 are essential for chicken heterophils to respond to lipoteichoic acid (LTA) from gram-positive *Staphylococcus aureus* (SA). Understanding the chTLRs functions and regulation mechanisms will surely promote poultry health and benefit both the research and the industry.

**Antimicrobial peptides and defensins**

Antimicrobial peptides are very important innate immune effectors, and generally, they are either already stored in cell granules or easily induced to provide a prompt response during the early stage of pathogen's invasion. At physiologic conditions, the antimicrobial peptides exert multiple functions including a broad spectrum of antimicrobial activities (anti-bacteria, -viruses, -yeasts, -protozoa and -fungi), anti-steroidogenic activity, anti-cancer, chemotaxis and even inducing or regulating the adaptive immune system (Boman, 2003; Zhang and Falla, 2004; Ganz, 2004 and 2005; Brown and Hancock, 2006). Nowadays, hundreds of different antimicrobial peptides have been found in various species and these peptides are all small (less than 100 amino acids), cationic (rich in histidine, lysine, and arginine), amphipathic and evolutionally conserved (Hancock and Lehrer, 1998). Also, the antimicrobial peptides all result from larger precursors with a signal leading sequence

after transcription (Zasloff, 2002). The exact antimicrobial mechanism of each peptide is variable and still not fully understood now, but the cationic characteristics are generally considered to have a close relationship with its function. According to the composition and structure, these antimicrobial peptides can be divided into three main subfamilies: the cecropins, the cathelicidins and the defensins (Lehrer and Ganz, 2002; Boman, 2003; Zanetti, 2004). Cecropins are a family of linear alpha-helical peptides without cysteine residue and they were first found in insects in the early 1980s (Steiner et al., 1981; Boman, 2003). Later, cecropins were also found in other organisms including mammals (Brogden et al., 2003). These cecropins can lyse and kill foreign bacteria after integrating into the pathogen's membrane (Durell et al., 1992). Cathelicidins were first identified in bovine myeloid cells in the 1990s and named because of their pro-region highly homologous to that of cathelin protein (Zanetti et al., 1995). Also, cathelicidins were found in many other mammals including porcine, rabbit, mice, rats, and human (Tomasinsig and Zanetti, 2005). The members of cathelicidins are conserved in their N-terminal domain but substantially heterogeneous in their C-terminal (Hancock and Diamond, 2000).

Defensins are cysteine-rich antimicrobial peptides, and typically contain six to eight cysteines motif, which can form three disulphide bonds (Schutte et al., 2002; Ganz, 2003). Generally, defensins share a structure of triple-stranded beta-sheet with a beta-hairpin turn loop. Currently, various different defensins are found in a wide range of organisms including animals, insects and plants, and these defensins are widely distributed in host tissues/cells. The presence of defensins from lower to higher

organisms significantly indicates their ancient origins, and that all these defensins may evolve according to a primordial immune mechanism (Raj and Dentino, 2002). The first defensin was identified and purified from rabbit granulocytes (Selsted et al., 1984). Later, from normal human neutrophils, Ganz and his colleagues found three human defensins: human neutrophil peptide (HNP)-1, -2, and -3 and all of these peptides can effectively kill *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* under experimental conditions (Ganz et al., 1985). The insect defensins were first purified from the cell culture medium of a flesh fly, *Sarcophaga peregrine* (Matsuyama and Natori, 1988), and currently defensins are found in almost all insect species investigated. In 1990, Mendez et al. found a new basic and sulfur-rich polypeptide from barley endosperm and named it gamma-hordothionin (Mendez et al., 1990). Also in the same year, Colilla et al. found another two gamma-purothionins from wheat endosperm (Colilla et al., 1990). However, the structures of these new plant peptides were more homologous to defensins instead of plant thionins, so Terras and his colleagues coined a new name, “plant defensins”, for this new group of plant antimicrobial peptides (Terras et al., 1995).

Interestingly, these defensins show considerable variations in their sequence and structure perhaps because of selective pressure within different species or different living conditions during long time evolution (Oppenheim et al., 2003). Based on species specificity, disulfide bonds connectivity and cysteine spacing, these defensins can be grouped into five families: alpha, beta, theta, invertebrate and plant defensins (Raj and

Dentino, 2002). The first three mainly exist in vertebrates and are currently under intensive study.

Alpha-defensins only exist in mammals and they were first identified from human neutrophil granules (Ganz et al., 1985). Generally, alpha-defensins contain 29-35 amino acids and their expression patterns are both species- and tissue-specific. An inbred laboratory mouse could express nearly 20 alpha-defensins in paneth cells, whereas it didn't express any in polymorphonuclear (PMN) leukocytes (Eisenhauer and Lehrer, 1992). However, rats are more similar to human and express several alpha-defensins in both paneth cells and PMN leukocytes (Eisenhauer et al., 1998 and 1990). Currently, there are totally six alpha-defensins isolated from human and all these defensins are closely located on chromosome 8 (Linzmeier et al., 1999). HNP-1 to -4 are produced in neutrophils, with HNP-4 expressed lower as compared to other three (Ganz et al., 1990). Human alpha-defensin-5 and -6 (HD-5 and -6) are identified as enteric defensins in paneth cells of the small intestine (Jones and Bevins, 1992). The expressions of these alpha-defensins are regarded as constitutive in human (Cowland and Borregaard, 1999).

Theta-defensins are only found in limited species such as rhesus monkey (*Macaca mulatta*), and the first theta-defensin was identified in 1999 from the granules of neutrophils and monocytes (Tang et al., 1999). Linked with two alpha-defensin-like sequences, the theta-defensin shows a distinctively cyclic structure of 18 amino acid residues, which confers it higher anti-viral activity than alpha-defensins (Lehrer, 2004). However, human only contain a mutated pseudogene of theta-defensin and this gene silences its expression (Levy, 2004).

The beta-defensins are the largest group of defensins and exist in a wide range of vertebrates including mammals, reptiles, fish and birds (Sugiarto and Yu, 2004; Lehrer, 2004). In 1991, the beta-defensin was first identified from bovine tracheal epithelia, but was named as tracheal antimicrobial peptide (TAP) at that time (Diamond et al., 1991). With more similar peptides found, this group of defensins was discovered to differ from other peptides in both consensus sequences and tri-disulfide motifs. So, they were renamed beta-defensins (Selsted et al., 1993). These beta-defensins contain a similar 3-dimensional structure (three intra-molecular disulfide bonds forming a beta-sheet) to that of alpha-defensins. But beta-defensins are generally larger and the spacing and connection of cysteine residues are different from those of alpha group. The structure of beta-defensins is characteristic with a short alpha-helix (or turn) juxtaposed with two or three anti-parallel beta-strands. However, there are still limited variations in the secondary structure of beta-defensins suitable for specific functions. For example, bovine beta-defensin-12 contains a turn-like configuration and is absent the short helix (Torres and Kuchel, 2004). From phylogenetic analysis, the beta-defensins are regarded as arising much earlier than alpha- and theta-defensins. Now it has been conceived that both alpha- and beta-defensin genes originated from a beta-defensin-like gene by a series of duplications (or mutations) and selection pressure, and theta-defensins arose from a pre-existed alpha-defensin gene (Nguyen et al., 2003; Xiao et al., 2004; Radhakrishnan et al., 2005).

The expressions of beta-defensins are generally induced by foreign stimulations or signals at transcriptional level in various tissues. IL-1 and TNF-alpha are important

pro-inflammatory cytokines regulating defensins' expression (Harder et al., 2000; Abbas and Lichtman, 2003). In human, the expressions of most HBDs (human bete-defensins-2, -3 and -4) are highly inducible (Harder et al., 2001; Garcia et al., 2001; Schutte and McCray, 2002). In fact, HBD-2 has recently been intensively investigated and much information about its regulation mechanism has already been elucidated. HBD-2 was first identified from the surface of lung epithelia in 1998 (Bals et al., 1998). But it was only detected in lung disease patients instead of the normal ones (Singh et al., 1998). Numerous experiments had already proved that HBD-2 was induced to express by various pro-inflammatory signals including cytokines and bacterial chemicals. As early as 1996, Bevins' lab found that bovine beta-defensins (TAP) were significantly induced to express with the challenge of LPS in cultured tracheal epithelial cells (Diamond et al., 1996; Russell et al., 1996). In human, HBD-2 showed the same characteristics and was induced to express with the treatment of LPS or pro-inflammatory cytokines such as IL-1-beta or TNF-alpha (Singh et al., 1998; Mathews et al., 1999; Hao et al., 2001). HBD-3 and -4 are also under similar regulation mechanisms. HBD-3 increases its expression with the stimulation of bacteria, TNF-alpha and IL-1-beta, and HBD-4 is induced when responding to PMA (phorbol myristate acetate), LPS and TNF-alpha (Garcia et al., 2001; Yanagi et al., 2005; Vankeerberghen et al., 2005).

The signaling transduction pathways involving the inductive expression of HBD-2 are very complex, and it is generally regarded nuclear factor (NF)kB playing the most important role in this regulation. Blocking the activation of NF-kB completely inhibits the inducible expression of HBD-2 by IL-1-alpha in intestinal epithelia (O'Neil et al.,

1999). Sequence analysis revealed that several putative transcription factor binding sites (TFBSs) for NFkB were located at both 5' proximal promoter region and introns of HBD-2 gene. Mutation or deletion of -208nt of HBD-2 gene (the NFkB binding site) would decrease or even silence its transcription (Wada et al., 2001). Also, from the experiments of luciferase reporter gene, the activator protein (AP)-1 binding site (positions -127 to -121) was found in the promoter region, which meant that AP-1 also played a critical role in inducing HBD-2's expression with the stimulation of IL-1-beta or PA (pseudomonas aeruginosa) (Wehkamp et al., 2006).

Beta-defenins exert multiple microbicidal functions in innate immune response to a wide range of pathogens and infections, and the deficiency of beta-defensins is associated with various dysfunctions or diseases such as inflammatory bowel diseases in human (Cobrin and Abreu, 2005). However, the killing abilities depend upon the salt concentration as well as other physiological conditions. For example, a high salt environment would inhibit the microbicidal functions of beta-defensins (Bals et al., 1998). The positive-charged beta-defensins would effectively interact with the negative-charged microbial membrane components. This electrostatic interaction permeabilizes the microbial cytoplasmic membrane and kills the invasion microbes in a multimer manner (Raj and Dentino, 2002; Chen et al., 2006). Besides direct killing mechanisms, beta-defensins also exert a serious of other functions such as chemotaxis and immune regulation. For example, HBD-1 and -2 are selectively chemotactic for memory T cells and immature dendritic cells (Yang et al., 1999).

Currently, antimicrobial peptides have become very popular in medical field because of their unique microbicidal mechanisms differing from those of classic antibiotics. Extensive clinical use of conventional antibiotics results in potential dangers of drug resistance or side effects. However, beta-defensins are produced by vertebrates themselves. These natural antimicrobial peptides do not involve new selection pressure and will surely be beneficial to the host.

Since the first chicken beta-defensin (cBDs, also named gallinacin) was found in 1994 from leukocytes (Harwig et al., 1994), there have been thirteen cBDs (Gallinacins-1 to -13) identified to date (Lynn et al., 2004; Xiao et al., 2004). All of the cBD genes are densely located in an 86-Kb nucleotide fragment on chromosome 3q3.5-q3.7, which contrasts with several clusters of beta-defensin genes on different chromosomes in other vertebrates such as human and mouse (Schutte et al., 2002; http://genome.ucsc.edu/cgi-bin/hgGateway). The cBDs are predominantly expressed in immune-related tissues such as bone marrow and liver. Gallinacin-13 (named gallinacin-11 in Higgs's paper) was demonstrated to effectively kill a wide range of bacteria, especially the intestinal pathogens such as *Listeria monocytogenes* and *Salmonella typhimurium*. Generally, beta-defensins are more important in innate immune response for avian than mammal because of the lack of oxidative mechanisms in avian heterophils (Sugiarto and Yu, 2004).

**Acute phase response and acute phase proteins**

The acute phase response (APR) is an early and very complex innate immune response induced by infection, stress or trauma (Ceron et al., 2005). This response is a

primary reaction that defends against bacterial endotoxin to ensure host survival (Ebersole and Cappelli, 2000). During the acute phase response, there are systemic changes of behavior, physiology and metabolism in host including fever, hyperglycemia, hypoferremia, leukocytosis and hormonal turbulence. In this fast immune response, the serum concentrations of some proteins, such as C-reactive protein (CRP) and serum amyloid A (SAA), can be detected to significantly change within several hours (Kushner and Mackiewicz, 1987; Gabay and Kushner, 1999). Generally, APR begins within several minutes after stimulation and lasts for 1 to 2 days, but it can be prolonged if a chronic inflammation developed (Baumann and Gauldie, 1994). During a typical APR, the host mobilizes various defense mechanisms to neutralize the invasion agents and repair the damaged tissues. The main purposes of APR include: restriction of the damage area, isolation or elimination of the damaging agents, and finally restoration of the homeostasis and maintenance vital physiological functions.

One of the most important characteristics of APR is the changing of the concentrations of some plasma proteins, most of which are synthesized in liver. These proteins will increase or decrease (at least 25 percent) to regulate host homeostasis (Baumann and Gauldie, 1994). These plasma proteins are called acute phase proteins (APPs) or acute phase reactants. Numerous experiments have proved that APPs exert a very wide range of functions including metabolic, hematopoietic, hepatic and neuroendocrine abilities during the APR (Gabay and Kushner, 1999; Ebersole and Cappelli, 2000). Some APPs are complement components and kill foreign bacteria through complement pathways; some APPs, such as ceruloplasmin, compete with the

ion to starve bacteria; some APPs play an essential role in wound healing by fibrinolysis or inhibition of proteases. In a word, the induced APPs eliminate the infectious microbes and restore the healthy state in a systemic and complex mechanism.

Since the first APP was discovered in 1930, there have been approximately forty different serum proteins generally considered to be APPs and these APPs were found in a wide range of vertebrates. In 1930, the first APP was isolated from *pneumococcal* infectious pneumonia patients and named C-reactive protein (CRP) because of its high affinity to the C-polysaccharide of *pneumococcus* (Tillett and Fransic, 1930). Later studies revealed that CRP could also facilitate phagocytosis via complement pathways after binding to foreign bacteria. Serum amyloid A (SAA) was originally found to be related to amyloid A fibrils and later it was proved to be an apolipoprotein of high density lipoprotein (Gruys and Snel, 1994). Another important APP, alpha-1-acid glycoprotein (AGP) moderately increases its expression by 2- to 4-fold in most vertebrates and plays important roles in both drug binding and immuno-modulation (Fournier et al., 2000).

Even though APPs are generally regarded to be hepatocyte-derived, some other tissues or cells can produce certain amounts of APPs as well, which have been documented in several experiments. AGP is a typical APP significantly increasing its expression in liver cells during an APR. However, AGP is also produced in other tissues such as intestine, heart, or white blood cells (Fournier et al., 2000). Other experiments have also revealed that CRP, SAA, and haptoglobin (Hp) all could be expressed in kidney (Dobryszycka, 1997; Eckersall et al., 2001; Jabs et al., 2003; Upragarin et al.,

2005). Therefore, the production of APPs from both the liver and other tissues may function together to maintain the inner homeostasis during an infection, even though the liver APPs provide the main part of the process.

According to the changes in plasma concentrations, these APPs can be divided into positive and negative groups. The positive APPs can be further sub-divided into major, moderate and minor APPs. The positive group mainly contains collectins, pentraxin, protease inhibitors, coagulation factors, complement proteins, and scavengers (Johnson et al., 1999), which all increase expression during APR. For major APPs, the concentration in plasma can increase more than 10 times, sometimes up to 1000 times during a typical APR (Ceciliani et al., 2002). The moderate APPs will increase expressions from 3- to 10-fold and the minor less than 3-fold during APR (Bayne and Gerwick, 2001). The negative APPs (mainly include albumin and transferrin) significantly decrease expression during APR. Albumin is the most abundant (about 35-50%) protein in plasma, and transferrin is a glycoprotein that functions in transporting iron. Even though there is no exact explanation for the down regulation of the negative APPs, it is generally regarded that the decreasing expression of negative APPs mainly functions to balance the induction expression of positive APPs because more amino acids and nucleotides are needed for synthesizing the positive APPs in liver (Mizock, 1995). Also, the decreasing expression of some enzymes has been detected accomplishing the increased expression of positive APPs in APR. This balance regulation plays a critical role in maintaining normal plasma osmosis and host homeostasis.

However, different individuals may have different qualities and quantities of acute phase reactions depending upon species-specific or agent-specific patterns. Generally, CRP and SAA significantly increase expression during a typical APR and both are regarded as major APPs in most vertebrates (Koj et al., 1988; Baumann and Gauldie, 1994; Moshage, 1997; Ebersole and Cappelli, 2000). But significant up-regulation expression of fibrinogen instead of CRP or SAA can be found in the serum of heart infarction patients (Cavusoglu et al., 2001). During the APR, a significant induction (more than 100-fold) of serum amyloid A was found in mouse, whereas it was the alpha-2-macroglobulin increased expression in rat in Ramadori's research (Ramadori and Christ, 1999). In another example, there were significant changes in serum concentration of serum amyloid P-component (SAP) and SAA in mouse, whereas there was no change in rat at all (Petersen et al., 2004). Because the complex regulatory mechanisms exist in different patho-physiologic states, the changes of each APP aren't uniform in different individuals even with the same challenge.

APPs can also be divided into type I and type II groups depending upon regulation patterns. Type I APPs are induced by IL-1-like cytokines such as IL-1 and TNF-alpha, whereas type II APPs are mainly induced by IL-6-like cytokines such as IL-6. Because IL-6 synergizes IL-1-like cytokines in APR, IL-6 is typically regarded as the principal inducer for APPs (Ceciliani et al., 2002). Type I APPs are comprised of CRP, SAA, AGP, etc., whereas type II mainly contains fibrinogen, alpha-2-macroglobulin, and hemopexin.

Even though the stabilization of APPs mRNA is also improved during APR, the expression of APPs is mainly regulated at the transcriptional level (Sevaljevic et al., 1989; Baumann and Gauldie, 1994). The stimulation of foreign pathogens induces and increases the transcription of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF-alpha, mainly in local cells (macrophages or endothelia). These immune related cytokines circulate to the liver and regulate the hepatic APPs' synthesis (Alsemgeest et al., 1996; Yoshioka et al., 2002). In the liver, the pro-inflammatory cytokines bind to membrane receptors on hepatocytes and activate signaling transduction pathways. Generally, the IL-1-like cytokines will activate and translocate the transcription factors NFkB and activating protein-1 (AP-1); whereas the IL-6-like cytokines function through JAK/STAT (Janus kinases/signal transducers and activators of transcription) pathway. Also, a common pathway shared by both IL-1- and IL-6-like cytokines is through mitogen activated protein kinases (MAPKs) and transcription factor NF-IL-6 (Wu et al., 2003). All of these signaling pathways result in an increased expression of the positive APPs. However, the host also develops multiple mechanisms to down-regulate the increased pro-inflammatory cytokines and APPs, and finally to restore the inner homeostasis. These regulatory molecules include glucocorticoids and other anti-inflammatory cytokines such as IL-4 and IL-10 (Ceciliani, et al., 2002).

APPs have significant value in both clinical and diagnostic areas. CRP and SAA are often used as markers in assessing the infection status in human or animals (Sipe, 1995; Ferard et al., 2002). The serum concentration of CRP changes rapidly during the worsening or improving conditions in infectious patients. In a normal physiological

situation, the plasma CRP concentration is about 2 mg/L or less in human. However, more than eighty percent of infectious patients are detected to have a CRP concentration as high as 100 mg/L in serum (Morley and Kushner, 1982). Also, a significant increase of serum CRP is found in other human diseases such as osteoarthritis and Crohn's disease (Spector et al., 1997; Beaven and Abreu, 2004). Alpha-1-acid glycoprotein (AGP) is another diagnostic marker and also widely used to monitor animal inflammatory process. After infection by meningitis or pneumonia, the plasma AGP significantly increases its expression in pig (Itoh et al., 1993). However, the marker function should be used with caution in clinic because the expression levels of APPs are variable among different species, sexes, ages, or even different measuring times on the same animal. So a combination of several APPs or other methods should be used. This combination method is more meaningful and accurate, and increases the diagnostic power than a single APP. Further studies on APPs will surely help monitor both human and animals' health, and provide much cheaper and accurate methods for routine practice.

In chicken, there is limited research and only a couple of chicken APPs have been found, such as transferrin, fibronectin and AGP. It is already widely recognized that the typical APR also exist in chicken (Xie et al., 2000). A positive APP, ovotransferrin (or transferrin), was identified in chicken as well as two other negative APPs (serum albumin and a 56-KDa protein) from SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel (Xie et al., 2002). Interestingly, the transferrin decreases expression in other vertebrates, but it is detected to increase expression in

chicken during infection. Also, the serum concentration of AGP significantly increased in hen after infection with *Salmonella enteritidis* and therefore AGP was regarded as a positive APP in chicken (Holt and Gast, 2002).

**Protective effects of induced innate immunity**

Because of the important roles of innate immunity, pathogens must overcome these defense mechanisms in order to establish their infections. Many pathogens evolve to possess virulence factors to evade innate immunity (Hackett, 2003). These evasion mechanisms indicate that innate immunity is important for host to resist infections. Evidence shows that stimulating the innate immune system provides both prophylactic and post-exposure protection. A sterile inflammation induced by a subcutaneous injection of casein 24 hours before disease challenges protected mice from lethal infection of gram-positive or negative bacteria (Noursadeghi et al., 2002). CpG-ODN injection protected rodents and non-human primates against bacterial, viral, fungal, and parasitic infections (Klinman, 2004). In poultry, a Newcastle disease virus vaccine induced nonspecific immunity against subsequent infection with pathogenic *Escherichia coli* (Huang and Matsumoto, 2000). The protection effects were suppressed with corticosterone and could not be induced by secondary vaccination. Subcutaneous and intramuscular injection of CpG-ODN in broilers 3 days before challenge with a virulent strain of *Escherichia coli* could reduce the mortality significantly ($P < 0.0001$) (Gomis et al., 2003). These results indicate that innate immune stimulation protects hosts from infectious diseases.

## Tibial Dyschondroplasia (TD)

TD is a genetic leg disease in broilers. A lesion of avascular and non-calcified cartilage is found below the growth plate of the proximal tibiatarsus in TD birds (Farquharson and Jeffereies, 2000). TD is mostly restricted to rapid growing broilers, and it is strongly associated with genetic components (Leach and Nesheim, 1965; Riddel, 1976). Also, a major sex-linked gene and recessive genetic effects are detected to be associated with an increased TD incidence (Sheridan et al., 1978). Generally, TD is regarded as resulting from the inability of proliferating chondrocytes to undergo terminal differentiation to hypertrophic chondrocytes based on the typical accumulation of pre-hypertrophic chondrocytes in the growth plates (Farquharson and Jeffereies, 2000). Chondrocyte differentiation is mainly regulated by a negative feedback control loop consisting of parathyroid hormone-related protein (PTHrP) and Indian hedgehog (IHH) (Shum and Nuckolls, 2002). Thyroid hormones play profound roles on the bone growth because these hormones can promote chondrocyte differentiation and maturation (Wakita et al., 1998; Stevens et al., 2000; Siebler et al., 2002). In chicks, the 3,3',5-Triiodo-$_L$-thyronine ($T_3$) plays more important bioactive roles than $_L$-thyroxine ($T_4$). Three iodothyronine deiodinases, types 1, 2 and 3 (DIO1, DIO2, and DIO3, respectively) are regarded to regulate the local availability of $T_3$ (Kohrle, 2000; Bianco et al., 2002). Understanding the regulation mechanisms of thyroid hormones at the molecular level will surely benefit the TD birds in poultry industry.

## Association Between Disease Susceptibility and Rapid Growth

For over 70 years, commercial broilers have undergone intensive genetic selection for rapid growth. As a result, tremendous improvements have been achieved, especially recently. Modern broilers have shown more than 340% increase in live weight and have reached market 42 days earlier compared to a 1957 strain (Havenstein et al., 1994). The growth rate has continued to improve a great deal since this report, and is expected to increase at a 2-5% annual rate. Unfortunately, genetic selection has also resulted in health complications including lower reproductive performance and ascites (Emmerson, 1997). It is well known that rapidly growing chickens are more susceptible to infectious diseases. A chicken line selected for high body weight showed higher mortality than a line divergently selected for low body weight after *Streptococcus faecalis* challenge (Siegel et al., 1987). Chicken lines with higher body weights had significantly more severe lesions after *Escherichia coli* challenge and a lower antibody response than those with lower body weights (Rao et al., 1999). Broilers had a higher incidence of viraemia and tumors than layers after avian leukosis virus inoculation (Payne et al., 1992). Avian adenovirus and reovirus infections caused more lesions in broilers than in White Leghorns (Okuda et al., 2001; Songserm et al., 2003). Differences between foreign native chickens and broilers that were bred in the US but raised in foreign countries were also well recognized. The association of rapid growth with disease susceptibility was also observed in turkeys (Nestor et al., 1999). In contrast, layers have undergone genetic selection for egg production comparable to broilers in

terms of selection intensity and history, but layers do not display significant changes in disease resistance.

The results of immunological assays further indicate that immune competence may be compromised in broilers. White Leghorns produced higher antibody titers than broilers (Toro et al., 1996). Broilers had weaker febrile response and lower inflammatory cytokines production than layers after LPS stimulation (Leshchinsky and Klasing, 2001). Broilers produced lower IgG titers and shorter antibody responses than layers and the antigen-specific cellular immune response was detectable only in layers (Koenen et al., 2002). Innate immunity has been found to play important roles in disease resistance in chickens (Kogut et al., 1994; Dil and Qureshi, 2002a, 2002b; Lamont et al., 2002; Wigley et al., 2002; Juul-Madsen et al., 2003; Leveque et al., 2003). However, the mechanisms of disease susceptibility in broilers are still unknown.

# CHAPTER III

# DIFFERENTIAL EXPRESSION OF ACUTE PHASE PROTEINS BETWEEN COMMERCIAL BROILERS AND LAYERS

## Overview

Acute phase response (APR) is an acute innate immune response to infections with a characteristic of significant change in the concentration of many plasma proteins. These proteins are called acute phase proteins (APPs) and most of them are important innate immune effectors. The APPs are primarily synthesized in the liver and induced by pro-inflammatory cytokines, such as IL-6 and IL-1. The expression levels of thirty-one (including nineteen newly annotated) chicken APPs in broilers and layers were investigated with relative quantitative real-time PCR (Q-PCR) after the injection of lipopolysaccharide (LPS). The expression levels of serum amyloid A (SAA) and lipopolysaccharide binding protein-like (LBP-like) increased most at transcriptional level after LPS stimulation, and these two APPs are regarded as major APPs in birds. In this experiment, the expression levels of most APPs (24 from 31) were higher in layers than in broilers either before or after LPS stimulation. Furthermore, the critical regulative inflammatory cytokine IL-6 was detected to be differentially expressed between broilers and layers at all three time points (0-, 3-, and 8-hour), with lower expression levels in broilers than in layers. These results all indicate that the differential expression of APPs in liver between these two strains of chickens may partially explain why broilers are more susceptible to infectious diseases than layers.

## Introduction

Tremendous improvements in economic traits have been achieved in commercial poultry through intensive genetic selection. Unfortunately, the selection for rapid growth has also resulted in higher susceptibility to infectious diseases, as well as ascites and skeletal problems (Julian, 1998). Chickens selected for rapid growth showed significantly higher morbidity and mortality to various bacterial and viral disease challenges than those with slow growth (Siegel et al., 1987; Payne et al., 1992; Rao et al., 1999; Okuda et al., 2001; Songserm et al., 2003). Experimental data also indicate that broilers showed poorer innate (Leshchinsky and Klasing, 2001) and adaptive immune responses than layers (Toro et al., 1996; Koenen et al., 2002). The association of rapid growth with disease susceptibility was also observed in turkeys (Nestor et al., 1999). However, the causative factors and immune mechanisms affected are still mostly unknown.

The acute phase response (APR) is an important innate immune response induced by tissue injury or infection. There are systemic changes in physiology and metabolism at the earlier stage of acute phase response such as fever and leukocytosis. The most important characteristic of the APR is that the concentration of some plasma proteins will increase or decrease to restore the animals' homeostasis. These plasma proteins are called the acute phase proteins (APPs) or acute phase reactants (APRs), and most of them are synthesized in the liver. APPs exert a very wide rang of functions including the metabolic, hematopoietic and neuroendocrine activities in infected animals. Some APPs can even be used as clinical markers for infectious inflammations (Liuzzo, 1994;

Ebersole and Cappelli, 2000). Currently, there are nearly forty APPs, and the number may slightly differ between different species. The APPs can be divided into two major groups: positive and negative APPs depending upon the changes of serum concentration. Generally, the serum concentration of positive APPs will increase expression by at least 25%, whereas negative APPs will decrease by at least 25% in a typical APR (Baumann and Gauldie, 1994). The positive APPs can be further divided into major, moderate (or intermediate) and minor APPs. For positive APPs, such as the C-reactive protein (CRP), the expression level in serum can increase more than 10-fold and sometimes even up to 1000-fold during the APR. These APPs are called major APPs. The moderate APPs will increase expression from 3- to 10-fold and the minor are less than 3-fold. Because of complex regulation mechanisms in different patho-physiologic states, each APP doesn't uniformly change in all animals (Gabay and Kushner, 1999). Generally, the expression of APPs in liver is mainly regulated by inflammatory or neuro-endocrine mediators, such as IL-6 type cytokines and IL-1 type cytokines. IL-6 is considered to be the most important inducer of the liver APPs (Ceciliani et al., 2002). LPS stimulation activates and increases the transcription of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF-alpha. These immune related cytokines circulate to the liver and induce or regulate hepatic APPs' synthesis (Alsemgeest et al., 1996). However, these cytokines regulate the expression of APPs in a very complex signaling network. Numerous experiments have been done, especially in rat or mouse, to illustrate the inductive mechanisms of the APP by IL-6 with the stimulation of endotoxins (Haziot et al., 1998; Lyoumi et al., 1998; Ostberg et al., 2000).

Up to 2005, there were only twelve chicken APPs identified, and current research mainly focuses on limited APPs, such as transferrin, fibronectin and alpha-1-acid glycoprotein (AGP). In 2000, Xie et al. did some experiments on LPS-challenged broilers and found that the cloacal temperature elevated and body weight decreased after stimulation (Xie et al., 2000). Also, a significantly increased concentration of some plasma proteins validated a typical APR existing in chicken. From SDS-PAGE gel, a 65-kDa protein was found to increase expression in both broilers and layers. Later, this protein was identified as chicken ovotransferrin (Xie et al., 2002). At the same time, these researchers found two other negative APPs (serum albumin and a 56-KDa protein) from the SDS-PAGE gel as well. Also from chicken, Holt and Gast found another APP, alpha-1-acid glycoprotein (AGP). Its serum concentration showed a significant increase in hen after infection with *Salmonella enteritidis* (Holt and Gast, 2002). The regulative expression of chicken APPs was also investigated in some experiments. Amrani and his colleagues found that the production of fibrinogen was increased in primary chicken hepatocytes in a dose-dependent manner by IL-6 stimulation. They also proved that IL-6 was a major mediator in regulating the production of APPs in chicken (Amrani, 1990; Samad et al., 1993). Xie and Leshchinsky's groups all found an increased concentration of plasma IL-6 and blood heterophil in LPS-injected birds (Xie et al., 2000; Leshchinsky and Klasing, 2001). However, the regulation mechanisms for chicken APPs are still not fully understood and lag behind those in mammals.

The aim of this research was to identify and annotate more chicken APP genes and to compare the acute phase response between broilers and layers. Relative

quantitative RT-PCR was utilized to analyze the expression of thirty-one chicken acute phase proteins in liver from both types of birds at three time points (0, 3, and 8 hours post-injection). Also, the critical regulative pro-inflammatory cytokine IL-6 was investigated at the transcriptional level between these broilers and layers as well.

## Materials and Methods

### Identification and annotation of new chicken acute phase protein genes

Combined the human and chicken genomes from the UCSC Genome Browser (http://genome.ucsc.edu/), the genes of nineteen chicken APPs or APPs-like were identified from the chicken genome with the synteny method. The CAP3 (http://deepc2.psi.iastate.edu/aat/cap/cap.html) program was utilized to assemble these chickens' EST or mRNA sequences available in UCSC database. A BLASTX program (Altschul et al., 1997) was used to analyze the assembled sequences against the NCBI database (http://www.ncbi.nlm.nih.gov/), using the default settings. After extraction the chicken APP genes sequences (putative, predicted or assembled sequences) from the UCSC database, a SMART program (http://smart.embl-heidelberg.de/) was used to analyze both chicken and human sequence to compare the functional domains among these proteins.

### Experimental animals

Eighteen broilers and eighteen leghorns from a local company were used in this study. After wing-banded, these birds were reared in two cages according the species at Texas A&M University Poultry Science Department. During all the experiments, these

birds were fed and watered ad libitum formulated according to NRC recommendations. All experimental procedures were approved by the TAMU Animal Use and Care Committee.

At the age of 5 weeks, both the broilers and the leghorns were randomly divided into three groups (broilers into B0, B3, and B8, and layers into L0, L3, and L8 respectively), each including six birds. Four groups (B3, B8, L3 and L8) were injected intravenously with LPS (Sigma, St Louis, MO) solution (5 mg/ml, dissolved in PBS) at an approximate dose of 2.0 mg per kg body weight. At the same time, the first group of each type of birds (B0 and L0) was killed with $CO_2$ to collect liver tissues labeled as 0-hour. After 3 and 8 hours, the 3rd (B3 and L3) and 8th (B8 and L8) groups were also killed with $CO_2$ to collect the liver tissues labeled as 3-hour and 8-hour, respectively. All of the liver samples collected from these six groups were immediately frozen in dry ice and stored at -80°C for further experiments.

**Quantitative real-time PCR**

TriZol reagents (Invitrogen, Carlsbad, CA) were used to isolate the total RNA according to standard procedure from every liver sample within a week after the samples' collection. After measuring the concentration, equal amounts of total RNA from each of the six samples in one group were mixed together to make a pooling total RNA. After quantification of the pooling samples, all the RNA samples were treated with DNase (Invitrogen, Carlsbad, CA) as the protocol attached. Then, approximately 100 ng of each pooling RNA sample were directly used as template for PCR amplification with the intron primers of beta-actin (GenBank accession number X00182,

**Table 3.1**) to confirm the absence of genomic DNA contamination in the RNA samples. cDNA was synthesized from equal amounts (1 mg) of both the individual and the pooling total RNA samples with the random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according the instruction.

Primers were designed according to the thirty-five sequences (thirty one APPs, two internal controls, one intron control and one cytokine gene) from the assembled or putative chicken mRNA sequences with the Primer Express 2.0 software (Applied Biosystems, Foster City, CA) for PCR amplification. All the designed amplification fragments were approximately 50 to 150 base pairs (except intron control primers) in length, and each pair of primers were designed on different exons to further distinguish PCR products amplified from the cDNA to the genomic DNA. The specificity of every pair primer sequences was confirmed at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. Each amplified PCR fragment was checked for its size and specificity in 2.0% to 2.5% agarose gel electrophoresis. These primers and Genbank access numbers are listed in **Table 3.1**.

SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the real-time PCR analysis with the total reaction volume of 20 ul, containing 50 ng of synthesized cDNA, 10 ul of 2X SYBR Green PCR Master Mix, and 0.3 uM of each oligonucleotide primer. A two-step RT-PCR protocol was used with the cycle of 40 times, 15s at 95°C and 30s at 59°C in an ABI 7900HT DNA Sequence Analysis System (Applied Biosystems, Foster City, CA). At the end, samples were heated at 95 °C for 20 minutes to construct dissociation curves to further identify single PCR product. Every

reaction in the real-time PCR was done in triplicate and the average threshold cycles (Ct) were used for data analysis. The chicken beta-actin and 18S-rRNA genes were used as references and the others as target genes.

The Ct was defined as the PCR cycles at which the fluorescent signal reached a fixed threshold. A higher Ct value means a lower expression level of the gene. The comparative expression (ΔΔCt) between different time points or different species with LPS injection was calculated with the following formula:

$$\Delta\Delta Ct = \Delta Ct_{\text{treatment 1}} - \Delta Ct_{\text{treatment 2}}$$

$$= (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 1}} - (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 2}}$$

A positive value of ΔΔCt means treatment 1 expressed a lower level of target gene than treatment 2.

**Statistical analysis**

Statistical analyses were performed with the correlation and general linear model procedures of SAS statistical software, Release 8.01 (SAS Institute, Inc., Cary, NC). The different expression levels for a given gene were compared between different time points within one type of birds or between broilers and layers at a specific time point, with the significance as $P < 0.05$ by Student's t-test.

**Table 3.1** The primer sequences (APPs) used in relative quantitative real-time PCR (Q-PCR) analysis

| Gene Name | Access number | Primer Sequence [a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| alpha-1-acid glycoprotein | AY584568 | F[b] GGAGCTGAAGTACGCGACCTT | 91 |
| | | R[c] GCATGTTTCATTCAGCCTCATG | |
| alpha-1-antitrypsin | BX935008 | F AACAATCCCACAGAGGCTGAGA | 121 |
| | | R CAAAGCTAGCCAGGAGCATTACA | |
| alpha-2-antiplasmin | BX935009 | F AGTCAGCGCCATGTTCTTCCT | 132 |
| | | R GGCAAGCTTAGTACAGCATGCA | |
| alpha-2-macroglobulin | XM_425448 | F ACACACGAGCCCAGAGACATG | 122 |
| | | R GCATTTTCACATCGATAATCACCAT | |
| antithrombin | S79838 | F CGTCGGACAAGCTGCAAGA | 121 |
| | | R AGCCCCATTTTTCTCAGCTTCT | |
| bactericidapermeability-increasing protein | BI067247 | F AGCCCACCGTGAGCACTTC | 134 |
| | | R GCCCTCCAAGATTTTCCTGAA | |
| complement C1 inhibitor | BU144418 | F TCTCTCCGTGCTTCCCCATAT | 114 |
| | | R GGCTGGAGGGTTGTTGCA | |
| complement C1s | AJ445808 | F TCACCCAATTATCCTCAGGCATA | 122 |
| | | R CAGTTCTGCGAAGGTTCAAGATC | |
| complement C3 | U16848 | F ATAGTGGCCGAGGCGTATCA | 143 |
| | | R ACGGATTTGCGGACGTCAT | |

**Table 3.1 continued**

| Gene Name | Access Number | Primer Sequence [a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| complement C4a-like | CR387078 | F[b] ACTGGATCAGTCACTATGAGTTGGAA | 134 |
| | | R[c] ATTGCCGGCTGCATGTG | |
| complement C5 | BU463842 | F TTGGTTAGTGGATTGGAAGCAAAT | 131 |
| | | R CGATCAGCTGGCACAGAATCTAT | |
| complement C6 | XM_429140 | F GCAGGAATCTCGTGCGTGTAA | 130 |
| | | R GGATGGCCGCAGGAGTTT | |
| complement C7 | BX934541 | F TGGTGATGCCTTTGAAACAAGA | 122 |
| | | R CATTGCACACGAGAGATCTGCTA | |
| complement C8 alpha | BU125345 | F GCACGGCCCATAAGCAGTT | 133 |
| | | R TGTTGCCAGCATCGCTTCT | |
| complement C8 beta | BX934795 | F GAGCATGGGACAACTGCAAAG | 122 |
| | | R GGTGTTACCTGACTGGCTTGCTA | |
| coagulation factor VIII | AF465272 | F CACCTCAAGATCATTCATGGCATA | 131 |
| | | R GTGGTGTTGCCCTTGTATTTCTTC | |
| ferritin | Y14698 | F TGAACATGCTGAGAAGCTGATGA | 121 |
| | | R GGCACACTCCATTGCAGTCA | |
| fibrinogen alpha | M34096 | F CAATTCTTTTTAATGTTGCCTGTCACT | 136 |
| | | R CTGGTTTGACAGAAGTTATCAGTAAAGCT | |
| fibronectin | U21327 | F TATGGTCAGCGTCTATGCTCAGAA | 128 |
| | | R CAATTTTGATGGAATCAACATCCA | |

**Table 3.1 continued**

| Gene Name | Access number | Primer Sequence [a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| hemopexin | M37319 | F[b] GGGCACAGACATGGGAACAC | 148 |
| | | R[c] ACCCATCCCGCCATGAGT | |
| inter-alpha-trypsin inhibitor | BU125793 | F TACAGGCATCACAGTAAATGGAGAAC | 121 |
| | | R CACTTTTATATCCAGGTGCTTGTTTG | |
| interleukin 6 | NM_204628 | F AGGACGAGATGTGCAAGAAGTTC | 78 |
| | | R TTGGGCAGGTTGAGGTTGTT | |
| lipopolysaccharide binding protein-like | CR386046 | F GCTCTGCTGAGGCCTTCGT | 120 |
| | | R CTGTGCCTCCCAGCCTGTT | |
| mannose-binding lectin | AF231714 | F TCAGCCTTTCAGTGCCTTACTACTTT | 131 |
| | | R ATCCATTGACTGCAGGAGCACTA | |
| pentaxin 3 | AY672618 | F CTCTCCTCCCTCACCGTTTGT | 102 |
| | | R CTGGATCTCATAGGGATTGAGCTTT | |
| plasminogen | XM_419618 | F GTGCTCTCCTGCTCAGTATGAGTGT | 131 |
| | | R CATAACTTGTCCGGAGACTGATTTG | |
| prothrombin | M81391 | F ACCGGTCATCTTCAGCGACTAC | 131 |
| | | R TAGTGGCCCACGTTTCTTTCA | |
| Serum amyloid A | BX929815 | F GGCTCTGTATCTGCTTCGTGTTG | 131 |
| | | R GTCCCGGTATGCTCTCCACATA | |
| serum amyloid P-component-like | Contig[d] | F TGGCCACCTACAACCTTTGG | 131 |
| | | R CAGGCACACGGTGAAGTTGA | |

**Table 3.1 continued**

| Gene Name | Access number | Primer Sequence [a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| tissue plasminogen activator | BX933490 | F[b] CAGCTCACATGGGAGTACTGTGAT | 123 |
| | | R[c] TTGCCATGGGTGAGCTTCA | |
| ovotransferrin | X02009 | F CGCTCCCCCCAAGTCAGT | 145 |
| | | R GGCTTTAATGCAGTCAAGGTACGT | |
| von Willebrand factor | BQ037193 | F CACTCAATTCAACAGAGGGAACAA | 129 |
| | | R CACAATGACAATTACCACCTTCGA | |
| beta-actin | L08165 | F CTGATGGTCAGGTCATCACCATT | 78 |
| | | R TACCCAAGAAAGATGGCTGGAA | |
| beta-actin (intron) | X00182 | F AGTGACTTAGAGACTGGCCACA | 205 |
| | | R GGACCTACTGCTATGGGAAAAC | |
| 18S rRNA | AF173612 | F ATTGTGCCGCTAGAGGTGAAAT | 71 |
| | | R CATTCTTGGCAAATGCTTTCG | |

[a]: Annealing temperature for all primers was 59$^{o}$C.
[b]: Forward primer.
[c]: Reverse primer.
[d]: Contig sequence from XM_427341and BG711874 with CAP3 program.

**Table 3.2** Synteny of APP genes between chicken and human

| | | | | | | |
|---|---|---|---|---|---|---|
| alpha-2-antiplasmin | H[a] | PRPF8 | FLJ33817 | SERPINF2 | SERPINF1 | |
| | C[b] | PRPF8 | FLJ33817 | SERPINF2 | SERPINF1 | |
| alpha-2-macroglobulin | H | A2M | PZP | | | |
| | C | A2M | PZP | | | |
| alpha-1-antitrypsin | H | KIAA1622 | SERPINA 10 | SERPINA6 | SERPINA1 | GCS |
| | C | | KIAA1622 | antitrypsin | GCS | |
| bactericidalpermeability-increasing protein | H | TGM2 | BPI | | | |
| | C | BPI | TGM2 | | | |
| complement component 1 inhibitor | H | SERPING1 | YPEL4 | HEAB | ZDHHC5 | |
| | C | ZDHHC8 | HEAB | YPEL3 | C1i (SERPING1) | |
| complement component 1, s subcomponent | H | C2F | C3F | C1s | C1r | |
| | C | C2F | C3F | C1s | C1r | |
| complement component 5 | H | TRAF1 | C5 | CEP1 | | |
| | C | CEP1 | C5 | TRAF1 | | |
| complement component 6 | H | C7 | C6 | OXCT1 | | |
| | C | C7 | C6 | OXCT1 | | |
| complement component 7 | H | PRKAA1 | RPL37 | C7 | C6 | |
| | C | PRKAA1 | RPL37 | C7 | C6 | |
| complement component 8, alpha polypeptide | H | PRKAA2 | C8a | C8b | DAB1 | |
| | C | PRKAA2 | C8a | C8b | DAB1 | |

**Table 3.2 continued**

| | | | | | | |
|---|---|---|---|---|---|---|
| complement component 8, beta polypeptide | H[a] | PRKAA2 | C8a | C8b | DAB1 | |
| | C[b] | PRKAA2 | C8a | C8b | DAB1 | |
| inter-alpha-trypsin inhibitor | H | NEK4 | ITIH3 | MUSTN1 | | |
| | C | NEK4 | ITIH3 | MUSTN1 | | |
| plasminogen | H | SLC22A1 | SLC22A2 | SLC22A3 | PLG | MAP3K4 |
| | C | MAP3K4 | PLG | SLC22A3 | SLC22A2 | |
| serum amyloid A | H | TPH1 | LOC113174 | SAA | HPS5 | GTF2H1 |
| | C | TPH1 | LOC113174 | SAA | HPS5 | GTF2H1 |
| tissue plasminogen activator | H | AP3M2 | PLAT | IKBKB | | |
| | C | AP3M2 | PLAT | IKBKB | | |
| von Willebrand factor | H | TMEM16B | VWF | CD9 | | |
| | C | TMEM16B | VWF | CD9 | | |

[a]: human.
[b]: chicken.

# Results

## Annotation of new chicken APP genes

From the UCSC Genome Browser, nineteen new chicken APP or APP-like genes were found with accurate locations on chicken genome. Sixteen APP genes syntenies in human and chicken were shown in **Table 3.2**. These APP genes were arranged in the same order (either the same direction or reverse direction) in both human and chicken genomes. Twelve chicken APP genes were arranged in the same direction. Alpha-2-antiplasmin belongs to serine proteases inhibitors (serpin) superfamily (serpin F family) and serpin F2 is the precursor of alpha-2-antiplasmin. The synteny of serpin F2 genes arrange identically in both human and chicken. Alpha-1-antitrypsin is a member of serpin A superfamily (Salzet et al., 1999). In human, there are three genes tandem-linked together, serpin A10, serpin A6 and serpin A1, whereas there is only one in chicken, alpha-1-antitrypsin. During evolution, serpin A could have duplicated several times in human (Barbour et al., 2002) but not in chicken. Also, there are four other chicken APP genes arranged in a reverse order on the chromosomes between these two species, which may result from chromosome reversion during evolution. These four APPs are bactericidalpermeability-increasing protein (BPI), complement component 5, complement component 1 inhibitor and plasminogen. Complement component 1 inhibitor, one member of serpin G family, is reversely arranged in these two species. Yippee-like (YPEL) and zinc-finger-DHHC-domain-containing (ZDHHC) are two different gene families. In human, there are YPEL type-4 and ZDHHC type-5 genes linked to serpin G1, whereas there are YPEL type-3 and ZDHHC type-8 in chicken,

which makes the gene synteny not display exact similarity between human and chicken. But generally, these syntenies reveal a conservative evolution of these APP genes in these two species.

The syntenies of complement component 4, lipopolysaccharide binding protein (LBP) and serum amyloid P-component (SAP) are not identical between chicken and human because of sequence gaps within the chicken genome. However, the results from the translated blast (blastx) (http://www.ncbi.nlm.nih.gov/BLAST/) for these APP-like genes all show very high scores to corresponding APP sequences of chicken or other mammals (data not shown). The amino acid sequences of chicken C4 (AAY45788 and T28153) are available in NCBI database, and the blastx results from chicken C4-like mRNA appear to be nearly identical to chicken C4's amino acid sequence. Also, both the amino acid and nucleotide sequences for chicken LBP-like are not available now. However, the amino acid sequence is only predicted to be similar to bactericidal permeability-increasing protein/lipopolysaccharide-binding protein (XP_417449). The translated chicken EST sequence CR386046 matches the predicted amino acid sequence (XP_417449) very well. For chicken SAP-like protein, a predicted nucleotide sequence of XM_427341 is named "similar to chicken SAP", and chicken EST sequence BG711874 is "chicken liver mRNA similar to mouse serum amyloid P-component". After the CAP3 program was utilized to assemble these two nucleotide sequences, the contiged sequence showed a very high similarity (data not shown) to that of the predicted chicken serum amyloid P-component amino acids with the blastx program.

The protein function domains of these chicken APPs are also very similar to those of human's (data not shown). Three APPs, alpha-1-antitrypsin, alpha-2-antiplasmin and complement 1 inhibitor all contain the serpin functional domain. In fact, these three APPs belonged to different serpin subfamilies. Alpha-1-antitrypsin is a type A serpin, whereas alpha-2-antiplasmin and C1 inhibitor are type F and G, respectively. Even though these three APPs exert similar inhibition mechanisms to the target molecules in the cell-signaling pathway, diversity functions may exist for different serpin subfamilies (Silverman et al., 2001). Other three APPs, C1 s subcomponent, plasminogen and tissue plasminogen activator all contain the Tryp_SPc domain, which functions as a trypsin-like serine protease in the cells. The complement components 6, 7, 8 (alpha subunit and beta subunit) all contain the MACPF domain (membrane-attack complex/perforin), which is directly related to the killing activity of the complement components.

**Quantitative PCR analysis**

In this experiment, the relative expression levels of thirty-one chicken APP genes and two internal controls were investigated in quantitative real-time PCR. First, the stability of two internal control genes (18S rRNA and beta-actin) was compared and the results from three independent experiments were shown in **Table 3.3**. The cycle ranges for beta-actin were 0.74(experiment 1), 0.9 (experiment 2) and 0.75 (experiment 3), respectively; whereas the ranges for 18S rRNA were 0.96 (experiment 1), 1.06 (experiment 2) and 0.91 (experiment 3), respectively. Obviously, the expression of beta-actin is more stable than that of 18S rRNA. Also, the expression level of beta-actin was

much lower than that of 18S rRNA, and was more similar to those of the APPs (data now shown). Therefore, beta-actin was regarded to be more accurate than 18S rRNA in chick liver tissue and was used as the internal control in the following analysis. Using beta-actin as internal control, the relative expression levels of all the APPs were obtained by subtracting the Ct of the internal control gene from that of the target genes.

**Table 3.3** Normalized threshold cycle (ΔCt) of triplicate reactions for internal control genes in three independent experiments

| | Gene | B0[a] | L0[b] | B3 | L3 | B8 | L8 |
|---|---|---|---|---|---|---|---|
| **Experiment I** | beta-actin | 19.92 | 19.44 | 19.87 | 19.77 | 19.18 | 19.70 |
| | 18S rRNA | 11.33 | 10.37 | 10.92 | 10.70 | 11.13 | 10.82 |
| **Experiment II** | beta-actin | 20.24 | 20.19 | 20.29 | 20.52 | 19.62 | 19.64 |
| | 18S rRNA | 10.61 | 11.00 | 11.08 | 11.06 | 11.27 | 10.67 |
| **Experiment III** | beta-actin | 19.19 | 19.20 | 19.42 | 19.49 | 18.74 | 19.07 |
| | 18S rRNA | 12.82 | 13.25 | 13.52 | 13.66 | 13.10 | 13.73 |

[a]: B0, B3, and B8 mean broilers at 0-, 3-, and 8-hour time points respectively.
[b]: L0, L3, and L8 mean layers at 0-, 3-, and 8-hour time points respectively.

**Major acute phase proteins**

For major APPs, the serum concentrations will increase from 10 times to nearly 1000 times. Both the CRP and Serum amyloid P-component (SAP) belong to the pentraxin family and are major APPs in human, mouse and fish (Bayne and Gerwick, 2001). In chicken, no C-reactive protein (CRP) was found or reported at this time, so only SAP-like protein was used for quantitative PCR analysis in this study. However, the expression of SAP-like protein didn't significantly increase at the transcriptional level among different time points, and it was not considered to be a major APP in chicken at least at the transcriptional level. AGP is another major acute phase protein in some mammals such as rats or mice (Hochepied et al., 2003). In fact, AGP is currently regarded as a positive APP in all mammals investigated. But as a major APP, it seems to only in broilers (3.4 PCR cycles, or 10.56-fold). In layers, AGP increases only 1.2 PCR cycle (2.3-fold) and there is not enough evidence to consider it to be a major APP at transcriptional level (**Fig. 3.1**). However, in this study, the expressions of LBP-like protein and SAA changed mostly with the stimulation of LPS (more than 4 and 6 PCR cycles respectively), and these proteins were considered to be major APPs in chicken. So, in broilers, there are three major APPs: LBP-like protein, SAA and AGP, whereas in layers there are only two major APPs, LBP-like protein and SAA.

**Differential expressions of APPs between broilers and layers**

After normalization, these thirty-one genes were divided into four groups according to the expression patterns between different time points and different species.

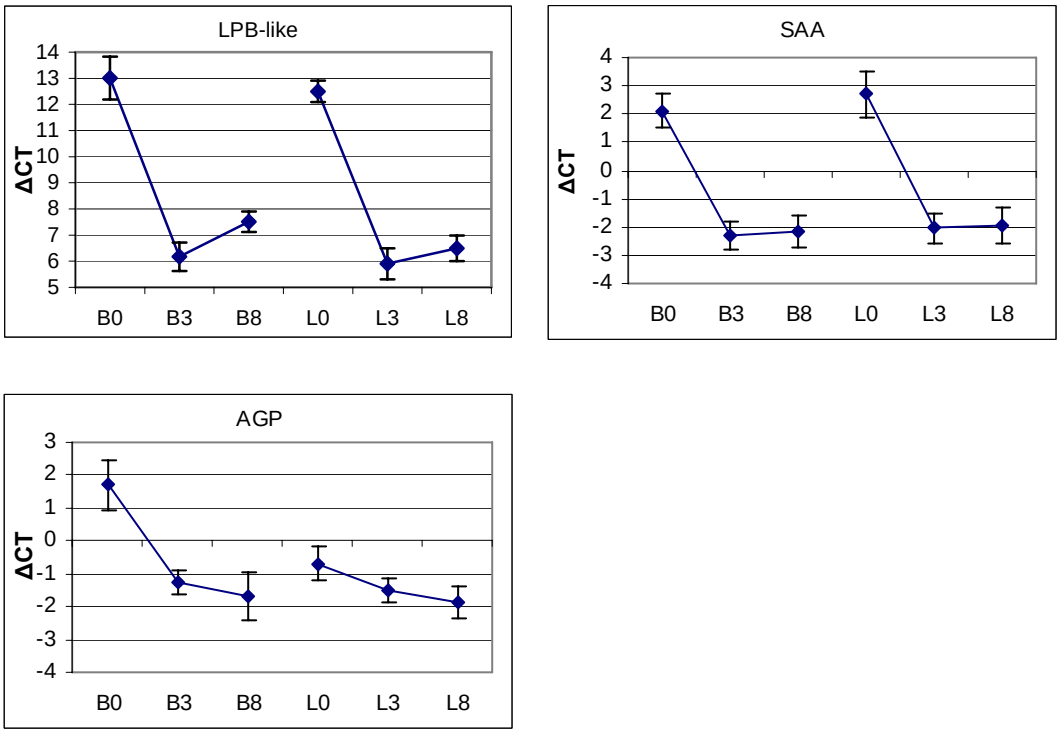



**Fig. 3.1** Normalized threshold cycles (ΔCt) with the stimulation of LPS between broilers (B) and layers (L). LBP-like, lipopolysaccharide binding protein like; SAA, serum amyloid A; AGP, alpha-1-acid glycoprotein. Beta-act gene was used as reference to normalize the target genes to obtain the ΔCt value. A high ΔCt value means a low expression level.

**Table 3.4** Relative expression levels (ΔΔCt) of chicken APPs significantly higher in layers' pooling liver samples with the stimulation of LPS

| Acute Phase Protein | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| alpha-1-acid glycoprotein | **2.39**[b,c] | 0.24 | 0.17 |
| alpha-2-antiplasmin | **1.83** | **1.21** | **1.65** |
| complement C8 beta | **1.66** | 0.43 | 0.77 |
| antithrombin | **1.63** | **1.27** | **1.54** |
| fibrinogen alpha | **1.57** | **1.13** | **1.48** |
| mannose binding lectin | **1.52** | 0.63 | **1.15** |
| complement C8 alpha | **1.42** | **1.13** | **1.11** |
| hemopexin | **1.39** | 0.33 | 0.37 |
| alpha-2-macroglobulin | **1.35** | **1.09** | -0.38 |
| ferritin | **1.3** | 0.59 | **0.95** |
| BPI[d] | **1.03** | -0.07 | 0.26 |
| inter-alpha-trypsin inhibitor | **0.97** | 0.46 | 0.58 |

**Table 3.4 continued**

| Acute Phase Protein | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| complement C5 | **0.93** [b,c] | 0.13 | 0.06 |
| complement C6 | **0.89** | 0.47 | -0.06 |
| c1 inhibitor | 0.68 | 0.39 | **0.98** |
| LPS binding protein like-protein | 0.51 | 0.27 | **1.02** |
| coagulation factor VIII | 0.5 | 0.66 | **1.05** |
| serum amyloid P-like protein | 0.46 | 0.46 | **1.13** |
| pentaxin 3 | -0.3 | **0.93** | **1.82** |

[a]: Six individuals from each group were pooled together.
[b]: Significant differences (P<0.05) were highlighted in bold.
[c]: A positive value means that broilers expressed at a lower level.
[d]: Bactericidalpermeability-increasing protein.

**Table 3.5** Relative expression levels (ΔΔCt) of chicken APPs higher in layers' pooling liver samples with the stimulation of LPS

| | Pooling Samples[a] | | |
|---|---|---|---|
| Acute phase protein | 0 hr | 3 hr | 8 hr |
| prothrombin | 0[b] | -0.08 | 0.62 |
| plasminogen | 0.56 | 0.74 | 0.29 |
| Von Willebrand factor | 0.63 | 0.02 | 0.52 |
| C1 s subcomponent | -0.12 | 0.59 | 0.43 |
| Complement C4 like protein | 0.09 | 0.39 | 0.66 |

[a]: Six individuals from each group were pooled together.
[b]: A positive value means that broilers expressed at a lower level.

**Significantly lower expressions of APPs in broilers.** There are nineteen APPs showing significantly different expressions between broilers and layers either with or without the stimulation of LPS, and most of the differences exist at 0-hour (14 from 19), as shown in **Table 3.4**. Alpha-1-acid glycoprotein (AGP) showed the biggest difference between broilers and layers at 0-hour (about 2.4 PCR cycles or 5.28-fold lower expression in broilers than layers). Most APPs differ approximately 1 to 2 PCR cycles (2- to 4-fold). By closing investigation, the significantly lower expression APPs generally belonged to the positive APPs except the inter-alpha-trypsin-inhibitor. These positive APPs include four types: one major APP (the serum amyloid P); two coagulation factors (fibrinogen alpha and coagulation factor VIII); three proteinase inhibitors (alpha-2-antiplasmin, antithrombin and alpha-2-macroglobulin) and six complement components (C8-alpha, C8-beta, mannose binding lectin, C5, C6 and C1inhibitor).

**Lower expression of APPs in broilers.** There are five APPs lower expressed in broilers, but the difference between broilers and layers are not regarded as significant ($p<0.05$) at transcriptional level, which is shown in **Table 3.5**. The prothrombin, plasminogen and von Willebrand factor all function as coagulation factors, whereas C1s and C4 belong to complement components. Plasminogen and von Willebrand factor are lower expressed in broilers in un-stimulated status as compared to the layers (0.56 and 0.63 PCR cycles, respectively). After LPS stimulation, all five APPs are lower expressed in broilers than layers at 8-hour time point, and the differences of prothrombin, von Willebrand factor and C4 are all bigger than 0.5 PCR cycles (0.62, 0.52 and 0.66 PCR

cycles, respectively). Plasminogen and C1s are mainly lower expressed at 3-hour time point in broilers (0.74 and 0.59 PCR cycles, respectively), but there was no difference at 8-hour time point (0.29 and 0.43 PCR cycles, respectively).

**Similar expression of APPs between broilers and layers.** There are five APPs that show similar expression patterns between broilers and layers both before and after LPS stimulation, which are shown in **Table 3.6**. All the differences were smaller than 0.5 PCR cycles and regarded as no difference at transcriptional level in this study. These APPs contained two complement components (C3 and C7), one major APP (SAA), one coagulation factor (tissue plasminogen activator) and transferrin, a negative APP in most mammals.

**Significantly higher expression of APPs in broilers.** There are two APPs significantly higher expressed in broilers than layers especially before the stimulation, as shown in **Table 3.7**. Fibronectin shows significantly higher expression (1.79 PCR cycles or 3.46-fold) in broilers than in layers at the transcriptional level in un-stimulated status. After LPS stimulation, both alpha-1-antitrypsin and fibronectin are significantly higher expressed in broilers compared to layers (1.57 PCR cycles at 8-hour for alpha-1-antitrypsin and 1.35 PCR cycles at 3-hour for fibronectin, respectively).

**Expression of individual APPs**

In order to validate the results from the pooling samples, six APP genes with strong antimicrobial activities were used to analyze the different expressions between the individual samples at 0-hour, with six samples contained in each group. The normalized Ct ($\Delta$Ct) of these six APP genes and the different expression ($\Delta\Delta$Ct) between broilers

and layers at 0-hour are all shown in **Table 3.8**. Both the pooling and individual samples show significantly different expression for these six genes between broilers and layers, and the individual results validate the pooling results. Therefore the results from the polling samples are reliable and can be used in the analysis of this study.

**Table 3.6** Relative expression levels (ΔΔCt) of chicken APPs similar between the broilers' and layers' pooling liver samples with the stimulation of LPS

| | Pooling Samples[a] | | |
|---|---|---|---|
| Acute phase protein | 0 hr | 3 hr | 8 hr |
| Complement C3 | 0.28[b] | -0.07 | 0.04 |
| Complement C7 | 0.08 | -0.31 | 0.15 |
| serum amyloid A | -0.41 | -0.23 | -0.21 |
| tissue plasminogen activator | 0.28 | -0.08 | -0.1 |
| transferrin | 0.08 | -0.09 | -0.31 |

[a]: Six individuals from each group were pooled together.
[b]: A positive value means that broilers expressed at a lower level.

**Table 3.7** Relative expression levels (ΔΔCt) of chicken APPs higher in broilers' pooling liver samples with the stimulation of LPS

| Acute phase protein | Pooling Samples[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| alpha-1-antitrypsin | **-0.88**[b,c] | -0.06 | **-1.57** |
| fibronectin | **-1.79** | **-1.35** | -0.5 |

[a] : Six individuals from each group were pooled together.
[b] : Significant differences (P<0.05) were highlighted in bold.
[c] : A positive value means that broilers expressed at a lower level.

**Table 3.8** Comparing the relative expression levels (ΔΔCt) of selective chicken APP genes between the individual and the pooling liver samples before the stimulation of LPS

| | Individual sample | | | Pooling sample |
|---|---|---|---|---|
| Acute phase protein | Broilers | Layers | Δ Δ Ct | Δ Δ Ct |
| hemopexin | 0.51±0.67[a] | -0.76 ±0.37 | **1.27**[b,c] | **1.39** |
| mannose binding lectin | 1.59 ±0.24 | 0.16 ±0.40 | **1.43** | **1.52** |
| Complement C8 alpha | 3.11 ±0.29 | 2.14 ±0.32 | **0.97** | **1.42** |
| Complement C8 beta | 2.27 ±0.46 | 0.93 ±0.32 | **1.34** | **1.66** |
| BPI[d] | 4.15 ±0.66 | 3.10 ±0.25 | **1.05** | **1.03** |
| alpha-1-acid glycoprotein | 1.67 ±1.01 | -0.62 ±0.18 | **2.29** | **2.39** |

[a]: Normalized expression levels are shown as mean ± standard deviation (N=6).
[b]: Significant differences were highlighted in bold ($p<0.05$).
[c]: A positive value means broilers expressed at a lower level.
[d]: bactericidalpermeability-increasing protein.

**Differential regulation mechanisms**

With the stimulation of LPS, the expression level of interleukin 6 (IL-6) is significantly increased at 3-hour, then decreased at 8-hour time point, but it was still higher expressed at 8-hour than that of un-stimulated birds (0-hour time point) in this study (**Fig. 3.2**). The expression of IL-6 increases more than 2 PCR cycles (4-fold) at 3-hour after LPS stimulation in both types of birds. Also, the expression of IL-6 shows a significant difference between broilers and layers after LPS stimulation. Broilers significantly lower express IL-6 at both 3- and 8-hour time points, with the biggest difference existing at 3-hour, in which IL-6 expresses 2.4 PCR cycles (5.28-fold) higher in layers than in broilers.

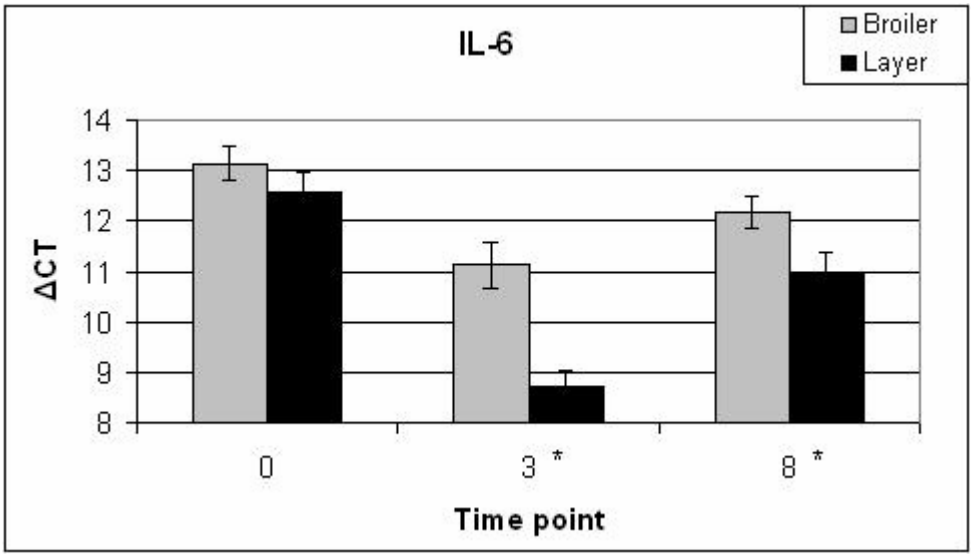



**Fig. 3.2** Normalized threshold cycles (ΔCt) of interleukin 6 with the stimulation of LPS. A positive value means broilers expressed at a lower level. Star means significantly different expressions between broilers and layers within the specific time point ($p<0.05$).

## Discussion

In this study, nineteen chicken acute phase protein genes are identified and annotated from chicken genome via bioinformatics methods. However, because of inadequate sequence information such as genome gaps and low quality sequences, C4, lipopolysaccharide binding protein and serum amyloid P-component (SAP) were only partially annotated and named as "-like protein" genes. C-reactive protein (CRP) is a very important APP, but there is no convincing evidence to assert that CRP also exists in chicken. Both the serum amyloid P-component (SAP) and CRP belong to the pentraxin superfamily. Jensen and his colleagues thought that CRP emerged from the duplication of ancestral classical pentraxin gene, which happened after the mammalian lineage divergence (Jensen et al., 1997). But Bayne and Gerwick found that there were both CRP and SAP in some kinds of fishes. And they proposed that the ancestral pentraxin duplicated much earlier, maybe at the stage of gnathostomes appearance (Bayne and Gerwick, 2001). Even though the chicken genome draft came out in April 2004, some gaps and low-quality sequences still remained in some areas such as the CRP and SAP gene location sites. Based on the comparisons of the human and mouse genome, there should be both CRP and SAP in chicken, but further sequencing is needed to validate this hypothesis.

Current, TaqMan probe and SYBRgreen fluorescence reagents are most widely used in real-time PCR reaction. Compared to TaqMan probe, SYBRgreen is less expensive and easily manipulated. But the main disadvantage is that additional procedures are needed to check the specificity of PCR amplification (Freeman et al.,

1999; Bustin 2002; Ponchel et al., 2003). In this study, the primer's specificity was checked at three points: bioinformatics analysis, agar gel electrophoresis in regular PCR and dissociation curve analysis in real-time PCR. These measures guaranteed the specificity of PCR amplification in this research.

In relative quantitative RT-PCR analysis, the internal control (or endogenous control, reference or housekeeping gene) plays a critical role for the sensitivity, accuracy and reliability of this method. Generally, there are some basic requirements for the internal control. First of all, a good internal control should be stable expression independent of the impacts from different treatments or different tissue/cell types. Abundant expression should be also evaluated in the experimental design (Overbergh et al., 2003). Ribosomal RNAs (18S or 28S rRNA in eukaryote and 16S rRNA in prokaryote, respectively) are widely used in real-time PCR experiments. Other stable expression genes include beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the ribosomal RNAs sometimes don't truly reflect the overall RNA. Also, the beta-actin and GAPDH may be up-regulated expression in specific tissue or cell types such as the proliferating cells (Suzuki et al., 2000). So, it is recommended that combined internal controls or tissue- or cell-specific controls should be used in real-time PCR procedures (Vandesompele et al., 2002; Schmid et al., 2003). This study compared the expression patterns of both 18S rRNA and beta-actin, and concluded that beta-actin was more suitable to be internal control in liver tissues (tissue-specific control).

It is well known that rapidly growing chickens are more susceptible to infectious diseases. Results of the present study, in combination with other studies, support this opinion. In 2001, Leshchinsky and Klasing compared inflammatory responses between broilers and layers after LPS stimulation. They found that the fast growth broilers decreased expression of pro-inflammatory cytokines and increased the immunosuppressive cytokines such as TGF-beta, which resulted in a blunted febrile response in fast growth broilers (Leshchinsky and Klasing, 2001). Our study also showed that the fast growth broilers lower expressed most APP genes as compared to the controlled layers. Further, this experiment provided more detailed information about the expression profile of APP in both chicken species. From all of the thirty-one chicken APP genes investigated, nineteen were significantly lower expressed and five were lower expressed in broiler birds. Lower expression of immune effectors means lower immune competence. Broilers have undergone intensive growth selection in the last century and have shown a compromised immune competence (Praharaj et al., 1996; Yunis et al., 2000). Also from this research, the most important regulation cytokine, IL-6, was found to be lower expressed in broilers at all three time points (significant differences at both 3- and 8-hour time points). The differences in signaling transduction pathways should partially explain the different immune response between broilers and layers, but further research is needed to elucidate the full aetiology of this difference.

# CHAPTER IV

# EXPRESSION PROFILE OF BETA-DEFENSINS BETWEEN COMMERCIAL BROILERS AND LAYERS

## Overview

Defensins are widely expressed in plants and animals and comprise a large family of antimicrobial peptides including alpha, beta, theta, plant and invertebrate defensins. During an innate immune response, these antimicrobial peptides would play an important role in first-line host defense including a broad spectrum of antimicrobial activities, anti-steroidogenic activity, chemotaxis, and even the induction or regulation of the adaptive immune system. In chicken, there are thirteen defensins all belonging to the beta-defensins subfamily and densely located in an 86-Kb nucleotide fragment on chromosome 3. Using quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), the expression profiles of these chicken beta-defensins (cBDs) were investigated at transcriptional level in this experiment. From these results, most cBDs significantly increase their expressions at 3-hour time point in both liver and spleen tissues, and then decrease their expression at 8-hour with the challenge of endotoxin (lipopolysaccharide, LPS). This study also reveals that nearly all of these 13 cBDs show significantly different expression between broilers and layers in both types of tissues, which further validates that a compromised innate immune response is associated with fast growth broilers resulting from long term divergent selection.

## Introduction

Antimicrobial peptides have gained extraordinary attention recently because of their critical functions in innate immune response (Ganz, 2005). Defensins are a large family of cationic, cysteine-rich antimicrobial peptides containing fewer than 100 amino acid residues (Ganz, 2002). The most significant structure characteristic is their six-cysteine motif that forms three disulphide bonds. Defensins provide multiple functions including antimicrobial and chemotactic activity against the invading foreign microorganisms. The positive-charged defensin peptides can effectively interact with the negative-charged microbial membrane components that specifically exist in prokaryotic organisms. With the accumulation of defensin peptides on pathogen's membrane, foreign bacteria become distorted and the membrane integrity is disrupted (Raj and Dentino, 2002). According to the sources and structures, defensins could be divided into plant, invertebrate, alpha, beta and theta subfamilies, and the last three mainly exist in vertebrates (Ganz, 2002; Raj and Dentine, 2002; Yang et al., 2002; Xiao et al., 2004).

The beta-defensins are the largest group and exist in a wide range of vertebrates including human, rats, mice, cattle, goats, pigs, turkey and chicken (Sugiarto and Yu, 2004; Davis et al., 2004; Ganz, 2004). From phylogenetic analysis, the beta-defensins arise much earlier than alpha- and theta-defensins. Generally, the alpha- and beta-defensin genes are regarded as to origin from a beta-defensin-like gene by a series of duplications or mutations, whereas theta-defensins arise from a pre-existing alpha-defensin gene (Nguyen et al., 2003; Xiao et al., 2004; Radhakrishnan et al., 2005). The

structure of beta-defensins is characteristic with a short alpha-helix (or turn) juxtaposed with several anti-parallel beta-strands (Torres and Kuchel, 2004).

The expression patterns of beta-defensins significantly differ among different species. For example, defensins (both alpha and beta) are expressed in mice leukocytes but not in rat leukocytes (Eisenhauer et al., 1989; Eisenhauer and Lehrer, 1992). The expression of most beta-defensins in vertebrates are mainly regulated by pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) at transcriptional level during the immune response (Harder et al., 2000; Abbas and Lichtman, 2003). In human, the beta-defensin-2 (hBD-2) is strongly induced to express in keratinocytes by IL-1-beta (Wehkamp et al., 2006). Also, Hao and Liu both found that IL-1 could promote the expression of hBD-2 in cell culture stimulated by LPS (Hao et al., 2001; Liu et al., 2003). Even though beta-defensins are widely distributed in various tissues or cells, different signal transduction pathways may be activated in different tissue or cell types to produce tissue-specific beta-defensins (Chen et al., 2006). For instance, bovine neutrophil beta-defensin-4 (BNBD-4) was found abundantly in bovine granulocytes, but other defensins are expressed in the tongue (such as lingual antimicrobial peptide, or LAP) and the intestine (such as enteric beta-defensin, or EBD) (Schonwetter et al., 1995; Yount et al., 1999).

Nowadays, only beta-defensins are found to be expressed in chicken and all thirteen cBDs (Gallinacin 1–13) are densely located in an 86-Kb nucleotide fragment on chromosome 3q3.5-q3.7 (Evans et al., 1995; Xiao et al., 2004), which is in contrast to several clusters of beta-defensin genes on different chromosomes in other vertebrates

such as human and mouse (Schutte et al., 2002; http://genome.ucsc.edu/cgi-bin/hgGateway). The chicken beta-defensins (cBDs) are predominantly expressed in immune related tissues such as bone marrow, respiratory tract and liver. In this experiment, the expression patterns of all thirteen chicken beta-defensins are investigated in liver and spleen tissues. And, the different expression patterns between divergently selected commercial chicken lines, broilers and layers, are studied with the stimulation of LPS as well.

## Materials and Methods

### Experimental animals

Eighteen broiler and eighteen leghorns were obtained from a local company. After wing-banded, these birds were placed in two cages according the species at Texas A&M University Poultry Science Department. During all the experiment, these chickens were fed and watered ad libitum formulated according to NRC recommendations (National Research Council, 1994). All experimental procedures were approved by the TAMU Animal Use and Care Committee.

At the age of 5 weeks, both the broilers and the leghorns were randomly divided into three groups (broilers into B0, B3, and B8, and layers into L0, L3, and L8 respectively), each including six birds. Four groups (B3, B8, L3 and L8) were injected intravenously with LPS from *Salmonella typhimurium* (Sigma, St Louis, MO) solution (5 mg/ml, dissolved in PBS) at an approximate dose of 2.0 mg per kg body weight. At the same time, the first group of each type of birds (B0 and L0) was killed with $CO_2$ to

collect liver and spleen tissues labeled as 0-hour. After 3 and 8 hours, the 3rd (B3 and L3) and 8th (B8 and L8) groups were also killed with $CO_2$ to collect the liver and spleen tissues labeled as 3-hour and 8-hour, respectively. All of the liver and spleen samples collected from these six groups were immediately frozen in dry ice and store at -80°C for further experiments.

**Quantitative real-time PCR**

Total RNA was isolated within a week after the samples' collection from all the tissues with TriZol reagents (Invitrogen, Carlsbad, CA) according to standard procedure. After measuring the concentration and checking the quality of all RNA samples with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA), equal amounts of total RNA from each of the six samples in one group were mixed together to make a pooling total RNA. After quantification again for the pooling samples, both the individual and pooling RNA samples were treated with DNase (Invitrogen, Carlsbad, CA) as the protocol attached. cDNA was synthesized from equal amounts (1 mg) of both the individual and pooling total RNA samples with the random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the instruction. After incubating for 30 min at 42°C followed by 85°C for 5 min and 5°C for 5 min, the prepared cDNA templates were stored at -20°C prior to use in the PCR assay.

Primers were designed according to fourteen (thirteen chicken beta-defensins and one internal control) chicken mRNA sequences with the Primer Express 2.0 software (Applied Biosystems, Foster City, CA) for PCR amplification. All the designed amplification fragments were approximately 50 to 150 base pairs in length, and each pair

of primers were designed on different exons or intron-exon boundaries to further distinguish PCR products amplified from the cDNA to the genomic DNA. The specificity of every pair primer sequences was also confirmed at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

In regular PCR reaction, ten microliter total volume contains approximately 50 ng of first-strand cDNA, 0.2 uM of each pair primers, 1 ul of 10X Taq reaction buffer [15 mM $Mg(OAC)_2$, 100 mM Tris-HCl, and 500 mM KCl, pH 8.3], 0.2 uM of dNTP mix and 0.2 U of Taq DNA polymerase (New England Biolabs, Beverly, MA). The reaction was performed on a thermocycle (MJ Research, Watertown, MA) with the program of an initial denaturation at 94$^{o}$C for 2 min followed by 35 cycles of 94$^{o}$C for 30s, 59$^{o}$C for 30s, and 72$^{o}$C for 30s, and ended with an extension at 72$^{o}$C for 10 min. Each amplified PCR fragment was checked for its size and specificity in 2.0% to 2.5% agarose gel electrophoresis. These primers and Genbank access numbers are listed in **Table 4.1**.

**Table 4.1** The primer sequences (cBDs) used in relative quantitative real time PCR (Q-PCR) analysis

| Gene Name | Access number | Primer Sequence[a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| cBD-1 | AF033335 | F[b] CCTTGCTGTACCCTGAGAAACC | 77 |
| | | R[c] AGGTACACGATCCGCATGGT | |
| cBD-2 | AF033336 | F CCAGGTTTCTCCAGGGTTGTC | 65 |
| | | R GGCAGGACCCTCCTTTACAGA | |
| cBD-3 | AF181952 | F CTGTGGAAGAGCATATGAGGTTGAT | 127 |
| | | R CACGGTCATACCATGGGAGACT | |
| cBD-4 | AY621306 | F TTCTCTGCAGTGACAGGATTTCC | 101 |
| | | R AAGCCCACAGCTCCATGAACT | |
| cBD-5 | AY621307 | F CATGCAGATCCTGACTCTCCTCTT | 131 |
| | | R GACATGACTTGTGGGAGCAGAA | |
| cBD-6 | AY621308 | F CCAGCCCTATTCATGCTTGTAGA | 121 |
| | | R CTGTTCCTCACACAGCAAGATTTTAG | |
| cBD-7 | AY621309 | F TGCAGGTCAGCCCTTCATTC | 121 |
| | | R GCCTATTCCATTGTTACATGTTCCA | |
| cBD-8 | AY621310 | F TTGGCCGTTCTCCTCACTGT | 137 |
| | | R TGCCCAAAGGCTCTGGTATG | |

**Table 4.1 continued**

| Gene Name | Access number | Primer Sequence[a] ( 5' → 3' ) | Size(bp) |
|---|---|---|---|
| cBD-9 | AY621311 | F[b] GCCGTGCTCCTTCAGTTGA | 67 |
| | | R[c] GGTGCCCATTTGCAGCAT | |
| cBD-10 | AY621312 | F CAAGATTCCGGCGCAGTAAG | 74 |
| | | R CAAGGCAGTGGAAATGTTGCT | |
| cBD-11 | AY621313 | F CTCTTCCTCCTCCAGGCTGTT | 131 |
| | | R CAAGAGCATGTTCCAAATGCA | |
| cBD-12 | AY621314 | F CCTTTGTTTCGTGTTCATCTTCATC | 137 |
| | | R CAAAGCAGTACTTAGCCAGGTATTCC | |
| cBD-13 | AY621315 | F GGAGGCTCTGCTTCCACATG | 134 |
| | | R AAGGGTCCTGCTCTGCTGTGT | |
| beta-actin | L08165 | F CTGATGGTCAGGTCATCACCATT | 78 |
| | | R TACCCAAGAAAGATGGCTGGAA | |

[a]: Annealing temperature for all primers was 59°C.
[b]: Forward primer.
[c]: Reverse primer.

SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the real-time PCR analysis with the total reaction volume of 20 ul, containing 50 ng of synthesized cDNA, 10 ul of 2X SYBR Green PCR Master Mix, and 0.3 uM of each oligonucleotide primers. These reagents were loaded on a 384 wells Thermo-Fast PCR plate (Abgene, Surrey, KT, UK) with an Eppendorf epMotion™ 5070 Workstation (Eppendorf, Westbury, NY) to decrease the manual variation for each well. A two-step real-time RT-PCR protocol was used with the cycle of 40 times, 15s at 95°C and 30s at 59°C in an ABI 7900HT DNA Sequence Analysis System (Applied Biosystems, Foster City, CA). At the end, the samples were heated at 95°C for 20 minutes to construct dissociation curves to further verify single PCR product. Every reaction in the real-time PCR was done in triplicate and the average threshold cycles (Ct) were used for data analysis. The chicken beta-actin gene was used as endogenous reference and the others as target genes.

The Ct was defined as the PCR cycles at which the fluorescent signal reached a fixed threshold. A higher Ct value means a lower expression level of that gene. The comparative expression ($\Delta\Delta$Ct) between different time points or different species with LPS injection was calculated with the following formula:

$\Delta\Delta Ct = \Delta Ct_{\text{treatment 1}} - \Delta Ct_{\text{treatment 2}}$

$= (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 1}} - (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 2}}$

A positive value of $\Delta\Delta Ct$ means treatment 1 expresses a lower level of target gene than treatment 2.

**Statistical analysis**

Statistical analyses were performed with the correlation and general linear model procedures of SAS statistical software, Release 8.01 (SAS Institute, Inc., Cary, NC). The different expression levels for a given gene were compared between different time points within one type of birds or between broilers and layers at a specific time point, with the significance as $P < 0.05$ by Student's t-test. The statistical model is $Y_t = u + l + m_l + m_b + \varepsilon$ where, $Y_t$ is the observed expression level at "t" time point, u is the population mean in layers at un-stimulated, l is the line effects (0 in layers), $m_l$ is the induction effects in layers at "t" time point, $m_b$ is the induction effects in broilers at "t" time point, and $\varepsilon$ is the random residual.

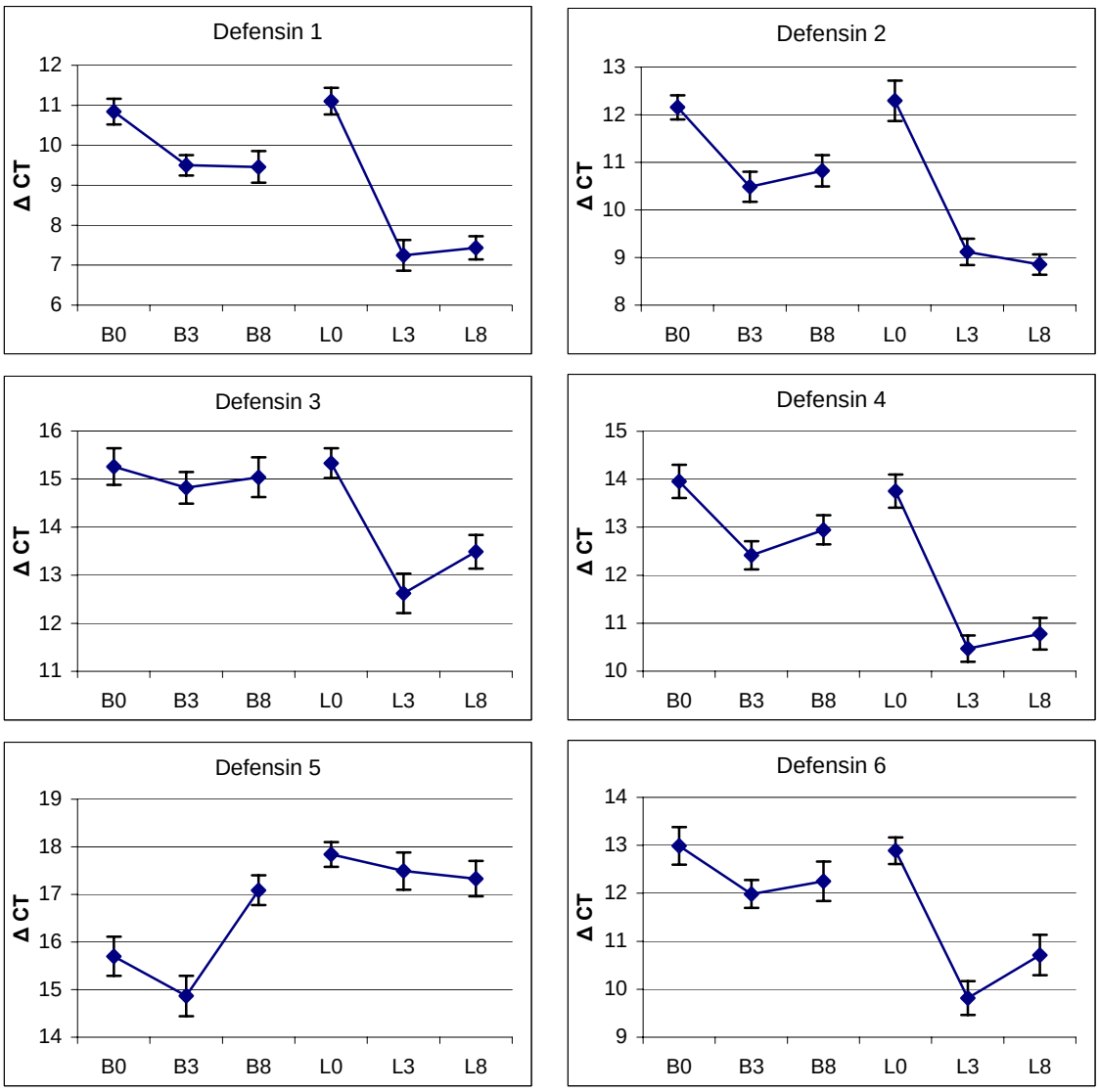



**Fig. 4.1** Normalized threshold cycles (ΔCt) of chicken beta-defensins (cBDs) expressions in liver pooling samples before and after LPS stimulation. B0, B3, and B8, broilers at 0-, 3-, and 8-hour time point respectively; L0, L3, and L8, layers at 0-, 3-, and 8-hour time point respectively. Beta-act gene was used as reference to normalize the target gene to obtain the ΔCt value. A high ΔCt value means a low expression level.

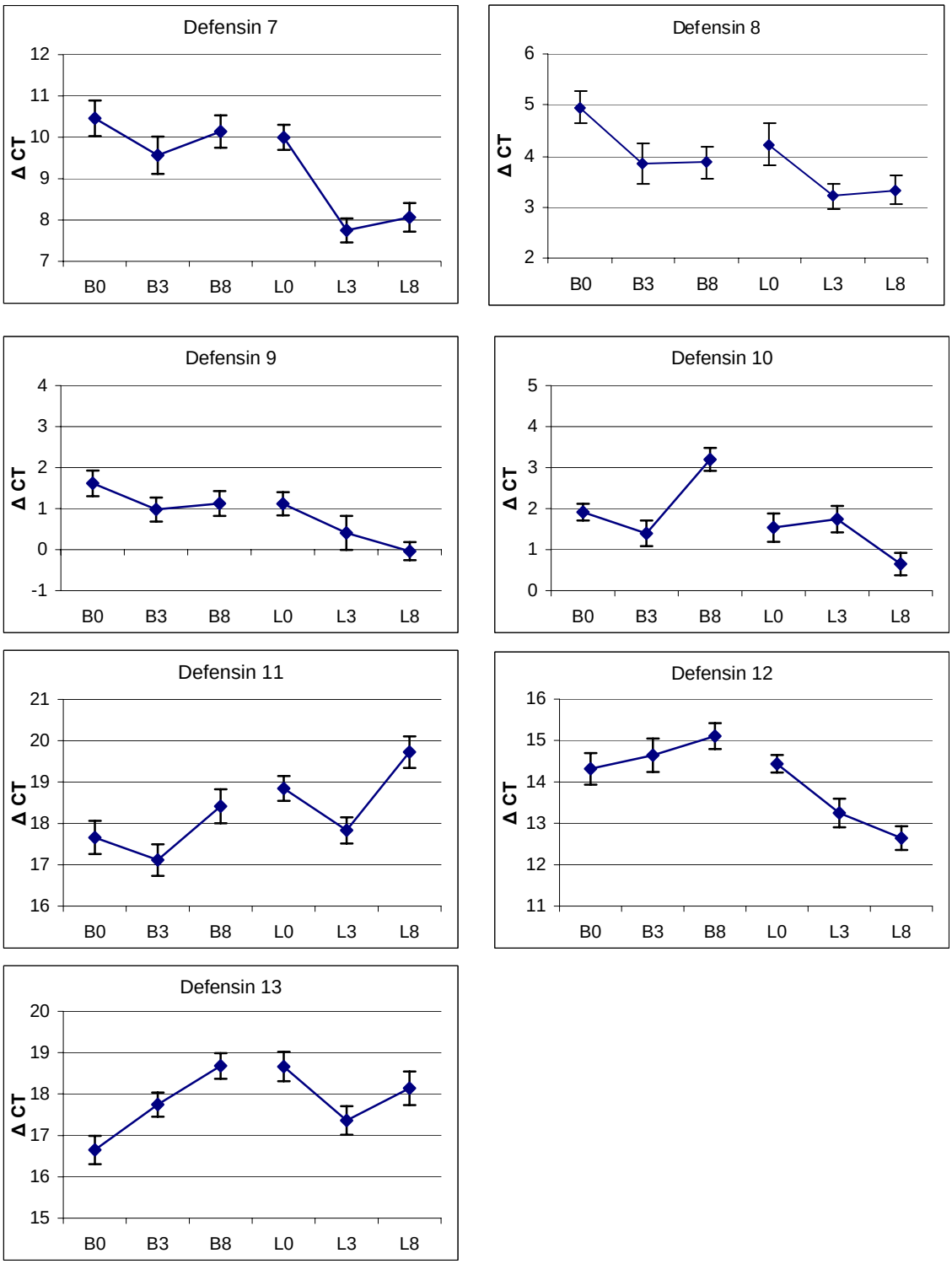



**Fig. 4.1** continued

## Results

With the stimulation of LPS, the expression of thirteen chicken beta-defensins (cBD) were investigated at transcriptional level in liver and spleen tissues at 0-, 3-, and 8-hour time points in both broilers and layers. The expression profiles of these cBDs along the plotted time course were obtained to illustrate the up- or down-regulation expressions with the stimulation.

### Expression profile of cBD in liver

**Induction expression of cBDs in liver.** Almost all cBDs are induced to express at early phase (from 0- to 3-hour) in both broilers and layers' pooling liver samples after the challenge of LPS, and the normalized threshold cycles were shown in **Fig. 4.1**. Ten from thirteen cBDs (cBD-1, -2, -3, -4, -6, -7, -8, -11, -12, and -13) significantly increased their expression in layer at early phase, whereas in broiler birds, only five cBDs (cBD-1, -2, -4, -6, and -8) showed significant up-regulations. At 3-hour time point after LPS stimulation, the cBD-1 increased most (3.86 PCR cycles or 14.5-fold) in layers. Also, the cBD-2, -4 and -6 were all up-expressed more than 3 PCR cycles (or 8-fold) in layers. However, in broilers, the cBD-2 increased only 1.67 PCR cycles (merely more than 3-fold) at transcriptional level. Also, there were several cBDs decreasing their expression in pooling liver samples with LPS stimulation at early phase. From **Fig. 4.1**, cBD-12 and -13 in broilers, as well as cBD-10 in layers, all decreased their expressions with the challenge of LPS at early phase, but only cBD-13 showed a statistical difference ($p<0.05$). At the later phase (from 3- to 8-hour), most cBDs decreased expression to counteract the effects of increasing transcription at the early phase, but the overall effect

(from 0- to 8-hour) of LPS stimulation was still regarded as induction for most cBDs especially in layers' pooling liver samples. In layer birds, except cBD-11, all other cBDs were generally induced to express during the whole course (0- to 8-hour), and the expression levels of most defensins (cBD-1, -2, -3, -4, -6, -7, -8, -9, -10, and -12) were significantly higher ($p<0.05$) at 8-hour time point than the un-stimulated status. However, in the broilers' pooling liver samples, the overall effect was suppressed for several beta-defensins (cBD-5, -10, -11, -12, and -13). These cBDs significantly decreased expression from 0- to 8-hour with the LPS stimulation.

**Differential expression of cBDs between broilers and layers in liver.** The different expressions of cBDs between broilers and layers' pooling liver samples at specific time points were also compared in this study, as shown in **Table 4.2**. Most cBDs didn't show significantly different expressions between these two types of birds at un-stimulated status except cBD-5, -11 and -13. But after LPS stimulation, most cBDs (8 from 13 at 3-hour and 10 from 13 at 8-hour) showed significantly different expressions between these two types of birds. All of these differences reflected higher expressions of cBDs in layers' liver samples except cBD-5 and -11. Close investigation revealed that most cBDs (cBD-1, -2, -3, -4, -6, -7, and -12) showed significantly high expression at both time points (3- and 8-hour) after LPS stimulation. Also, from **Fig. 4.1**, there were more cBDs increasing their expressions in layers than in broilers with the stimulation of LPS, and the degrees of increase in layers were much higher than those of broilers, which further showed a differential immune response between broilers and layers.

**Table 4.2** Relative expression levels (ΔΔCt) of chicken beta-defensins (cBDs) between broiler' and layer' pooling liver samples with the stimulation of LPS

| Chicken beta-defensins | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| beta-defensin-1 | -0.26[b] | **2.25**[c] | **2.02** |
| beta-defensin-2 | -0.14 | **1.37** | **1.97** |
| beta-defensin-3 | -0.07 | **2.19** | **1.55** |
| beta-defensin-4 | 0.21 | **1.94** | **2.17** |
| beta-defensin-5 | **-2.14** | **-2.63** | -0.24 |
| beta-defensin-6 | 0.10 | **2.17** | **1.54** |
| beta-defensin-7 | 0.46 | **1.82** | **2.07** |
| beta-defensin-8 | 0.71 | 0.65 | 0.54 |
| beta-defensin-9 | 0.50 | 0.57 | **1.16** |
| beta-defensin-10 | 0.38 | -0.35 | **2.55** |
| beta-defensin-11 | **-1.19** | -0.72 | **-1.31** |
| beta-defensin-12 | -0.12 | **1.39** | **2.46** |
| beta-defensin-13 | **-2.02** | 0.38 | 0.54 |

[a]: Six individuals from each group are pooled together.
[b]: A negative value means broiler expression at a higher level.
[c]: Significant differences are highlighted in bold.

**Expression profile of cBDs in spleen**

**Induction expression of cBDs in spleen.** Under the experimental conditions, the expressions of cBD-11 and -12 were not detected from pooling spleen samples neither in broilers nor layers. However, the expression of other cBDs was generally induced in both type birds with the stimulation of LPS, and the normalized threshold cycles were shown in **Fig. 4.2**. In broiler birds, even though almost all cBDs seemed to be induced to express at early phase with the stimulation of LPS, only three (cBD-3, -5, and -13) show significant induction ($p<0.05$). The cBD-13 was the most inductive expression one and it increased its expression about 5-fold (or 2.17 PCR cycles). On the other hand, in layers, more cBDs (seven: cBD-1, -2, -4, -5, -6, -7, and -9) were detected to significantly induce expression at early phase. In fact, cBD-5 increased expression for nearly 5 PCR cycles (30-fold) in layers at early phase with LPS stimulation. At later phase (3- to 8-hour), most cBDs decreased their expressions at transcriptional level in broiler birds, which made almost no significant difference in broilers spleen samples during the whole phase (0- to 8-hour). But in layer spleens, the net effects were still induction for most cBDs from 0- to 8-hour, and five cBDs (cBD-1, -2, -4, -6, and -7) were detected to be significantly inductive expression.

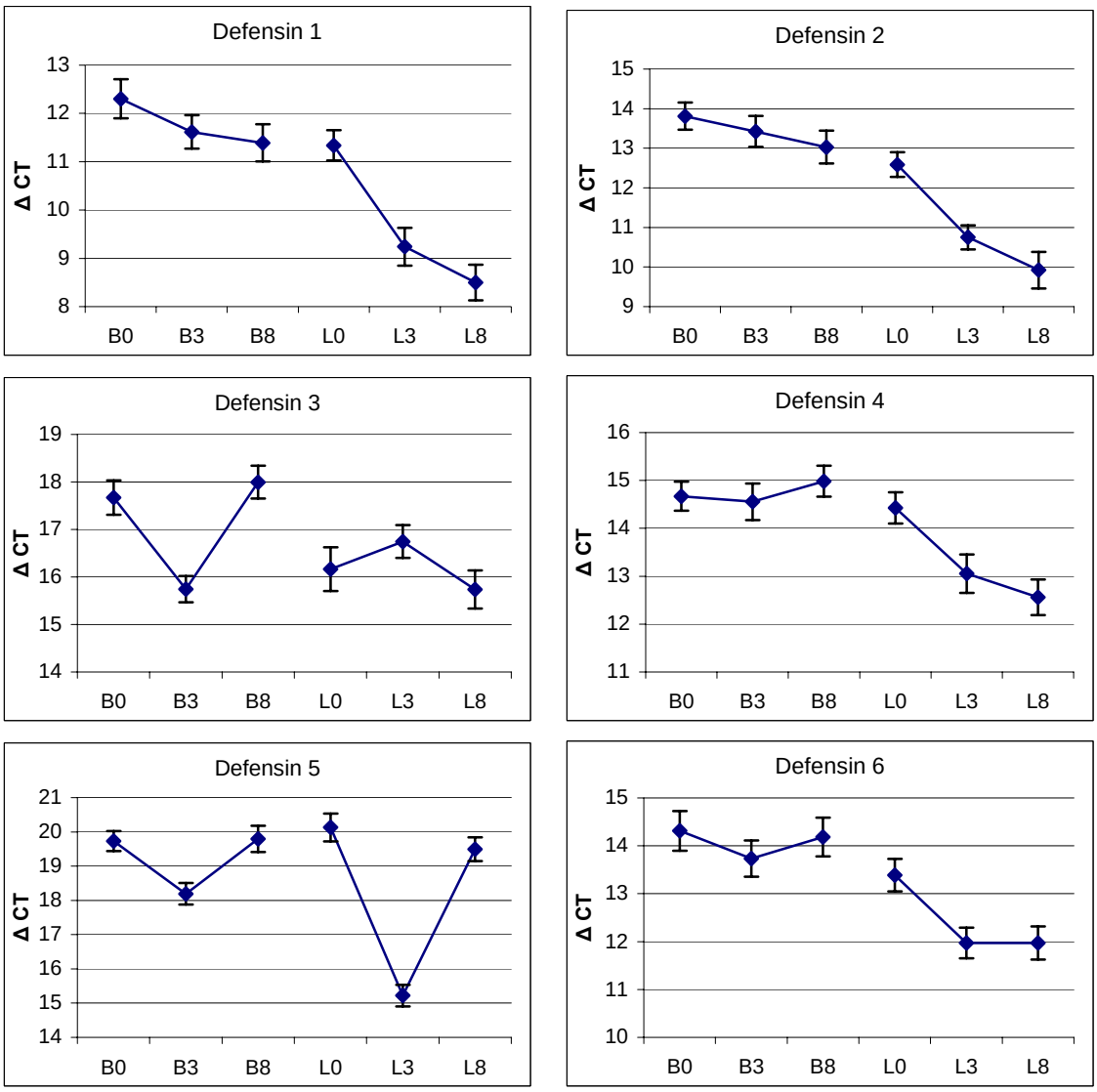



**Fig. 4.2** Normalized threshold cycles (ΔCt) of chicken beta-defensins (cBDs) expressions in spleen pooling samples before and after LPS stimulation. The cBD-11 and -12 were not detected with 40 cycles. B0, B3, and B8, broiler birds at 0-, 3-, and 8-hour time point respectively; L0, L3, and L8, layer birds at 0-, 3-, and 8-hour time point, respectively. Beta-act gene was used as reference to normalize the target gene to obtain the ΔCt value. A high ΔCt value means a low expression level.

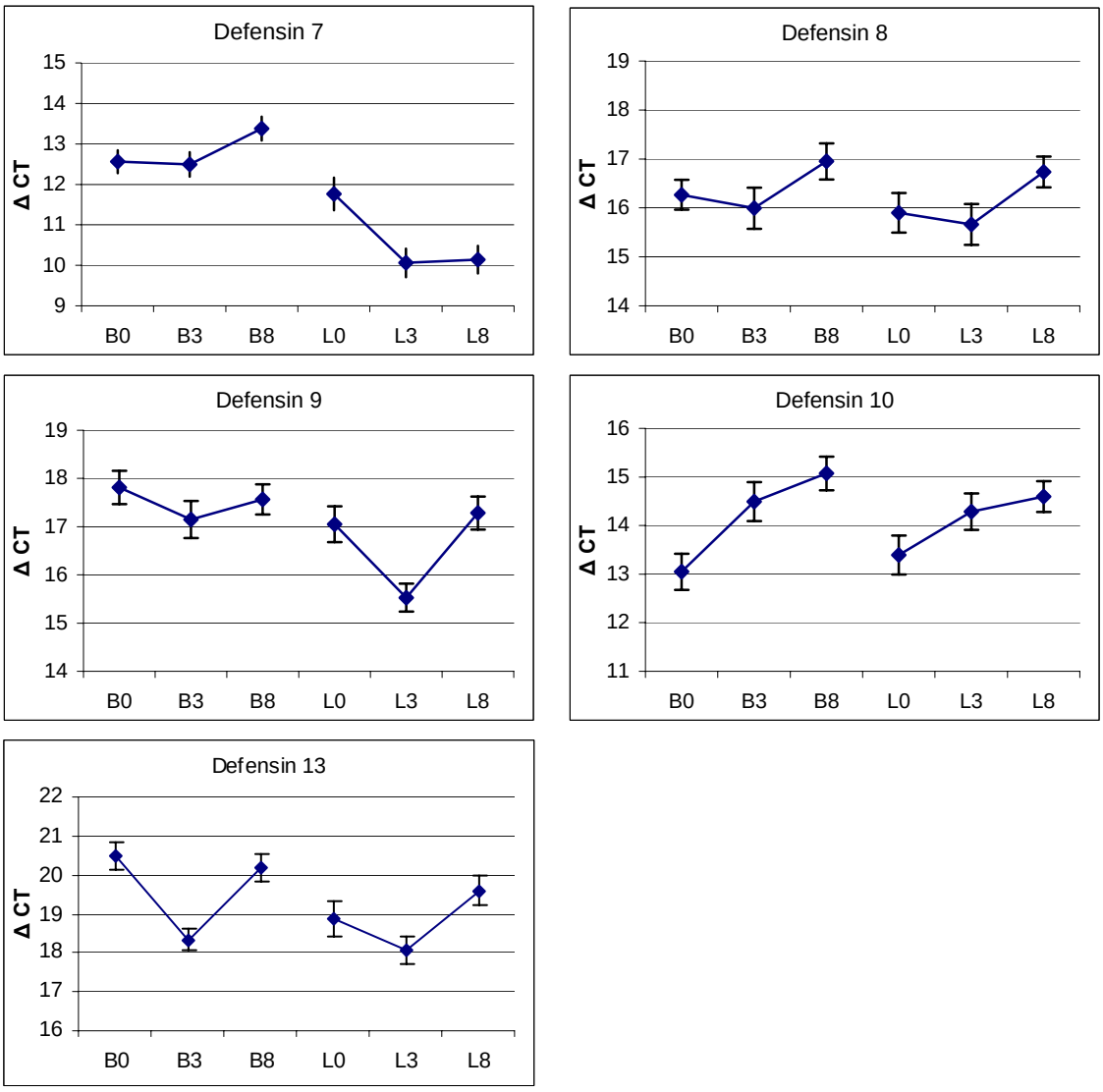



**Fig. 4.2 continued**

**Table 4.3** Relative expression levels (ΔΔCt) of chicken beta-defensins (cBDs) between broiler' and layer' pooling spleen samples with the stimulation of LPS

| Chicken beta-defensins | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| beta-defensin-1 | 0.96[b] | **2.37**[c] | **2.89** |
| beta-defensin-2 | **1.23** | **2.68** | **3.11** |
| beta-defensin-3 | **1.51** | **-1.00** | **2.26** |
| beta-defensin-4 | 0.25 | **1.50** | **2.43** |
| beta-defensin-5 | -0.40 | **2.97** | 0.31 |
| beta-defensin-6 | 0.92 | **1.76** | **2.21** |
| beta-defensin-7 | 0.80 | **2.43** | **3.24** |
| beta-defensin-8 | 0.37 | 0.33 | 0.21 |
| beta-defensin-9 | 0.76 | **1.62** | 0.28 |
| beta-defensin-10 | -0.35 | 0.21 | 0.47 |
| beta-defensin-11 | ND | ND | ND |
| beta-defensin-12 | ND | ND | ND |
| beta-defensin-13 | **1.63** | 0.26 | 0.58 |

[a]: Six individuals from each group are pooled together.
[b]: A positive value means broiler expression at a lower level.
[c]: Significant differences are highlighted in dark ($p<0.05$).
ND: not detectable with 40 cycles.

**Table 4.4** Comparing the relative expression levels (ΔΔCt) of selective chicken beta-defensin (cBD) genes between the individual and the pooling samples at 0- and 8-hour time points

In liver

| | | Individual sample | | | Pooling sample |
|---|---|---|---|---|---|
| Chicken beta-defensins | | Broiler | Layer | Δ Δ Ct | Δ Δ Ct |
| cBD-2 | 0h | 11.99 ±0.24[a] | 12.23 ±0.23 | -0.24[b] | -0.13 |
| | 8h | 11.1 ±0.30 | 8.97 ±0.25 | **2.13**[c] | **1.97** |
| cBD-10 | 0h | 1.70 ±0.33 | 1.39 ±0.31 | 0.31 | 0.37 |
| | 8h | 3.20 ±0.34 | 0.73 ±0.25 | **2.47** | **2.55** |

In spleen

| | | Individual sample | | | Pooling sample |
|---|---|---|---|---|---|
| Chicken beta-defensins | | Broiler | Layer | Δ Δ Ct | Δ Δ Ct |
| cBD-2 | 0h | 13.97 ±0.33 | 12.73 ±0.31 | **1.24** | **1.23** |
| | 8h | 13.29 ±0.23 | 10.01 ±0.30 | **3.28** | **3.11** |
| cBD-10 | 0h | 13.03 ±0.34 | 13.33 ±0.24 | -0.30 | -0.34 |
| | 8h | 15.16 ±0.32 | 14.74 ±0.33 | 0.42 | 0.47 |

[a]: Normalized expression levels are shown as mean ± standard deviation (N=6).
[b]: A negative value means broilers expressed at a higher level.
[c]: Significant differences were highlighted in bold ($p<0.05$).

**Different expression of cBDs between broilers and layers in spleen.** After subtracting the normalized threshold cycles (ΔCt) value of layers from those of broilers, the relative expression levels (ΔΔCt) of all these cBDs between these two types of birds were shown in **Table 4.3**. Generally, broilers lower expressed most cBDs both before and after LPS stimulation. At un-stimulated status, there were three cBDs (cBD-2, -3, and -13) significantly higher expressed in layers. But after LPS challenge, nearly all eleven cBDs are detected to be higher expressed in layer pooling spleen samples. cBD-1, -2, -4, -6 and -7 were all significantly higher expressed in layers than broilers at both 3- and 8-hour and interestingly, much bigger differences were detected at 8-hour instead of 3-hour. There were also several cBDs showing significant differences between broilers and layers only at either 3- or 8-hour time point. Layer birds expressed significantly higher cBD-5 and -9 only at 3-hour, and cBD-3 only at 8-hour after LPS stimulation. Combining both **Fig. 4.2** and **Table 4.3**, the layer birds generally expressed higher levels of cBDs and bigger degrees of up-regulation of these antimicrobial peptides than broiler birds.

**Comparing results from pooling samples to individual samples**

At the end of the study, the standard deviations within groups were also analyzed to compare the pooling results with the individual ones. The individual samples from 0- and 8-hour (B0, L0, B8, and L8) were used to analyze the relative expression levels (ΔΔCt) of cBD-2 and -10 in both liver and spleen tissues (**Table 4.4**). These two cBDs were selected because of the expression levels and the induction effects. In individual liver samples, both cBD-2 and -10 were significantly lower expression in broilers

comparing to layers at 8-hour after LPS stimulation (2.13 and 2.47 PCR cycles, respectively), which was very consistent ($p<0.01$) with the pooling samples (1.97 and 2.55 PCR cycles, respectively). Similar for spleen individual samples, cBD-2 was significantly higher expressed in layers at both 0- and 8-hour (1.24 and 3.28 PCR cycles respectively); whereas they were 1.23 and 3.11 PCR cycles respectively in pooling samples. This experiment demonstrated that the pooling method was reliable in this study.

## Discussion

As an important immune effector family, beta-defensins mainly provide immediate and first line defense to directly kill the foreign pathogen and protect the host (Boman, 2003). They also provide chemotactic function for adaptive immunity (Yang et al., 1999; Yang et al., 2002; Biragyn et al., 2001). The wide and intensive study on antimicrobial peptides especially the beta-defensins is very important and meaningful in both pure and applied science. Understanding the expression mechanisms of cBDs may provide efficient methods in animal breeding to produce high immune competent animals.

Evolutionary analysis of defensins families revealed that a relatively conservative characteristic existed among them and all vertebrate defensins were originated from a single beta-defensin like ancestor (Xiao et al., 2004). Powerful bioinformatics tools made it feasible and easier to identify and annotate new chicken beta-defensins from the genome drafts in recent years (Xiao et al., 2004; Lynn et al., 2004; Higgs et al., 2005).

The expression of beta-defensins is both species- and tissue- specific. In human, there are at least thirty different beta-defensins widely expressed at different levels in different tissues (Bensch et al., 1995; Harder et al., 1997; Harder et al., 2001; Yamaguchi et al., 2002; Schutte et al., 2002). In chicken, there are only thirteen cBDs identified so far and all these defensins are expressed in a wide range of tissues including bone marrow, liver and spleen (Xiao et al., 2004; Lynn et al., 2004; Higgs et al., 2005). It is interesting that hBD-1 is constitutively expressed in human, whereas hBD-2 and -3 are all induced to express in epithelia responding to stimuli such as the pro-inflammatory cytokines or bacterial infection. However, in chicken, both cBD-1 and -2 are significantly induced to express in liver and spleen tissues in this study. In liver, these two cBDs are induced to express with a much bigger extend at early phase in layers than those in broilers (more than 3 PCR cycles in layers, whereas only about 1.5 PCR cycles in broilers). Noticeable, in spleen samples, the induction effects are not significant for cBD-1 and -2 in broilers in this study ($p>0.05$). But in layers spleen, the expressions of these two defensins are all significantly induced (nearly 2 PCR cycles) at 3-hour compared to the un-stimulated status. In fact, the expression patterns of cBD-1 and -2 show a very high similarity, as in **Fig. 4.1 and 4.2**. With the stimulation of LPS, cBD-1 and -2 are all induced to express in both tissues and both bird types at early phase even though it is not significant for broilers spleen.

Generally, real-time quantitative PCR provided a more sensitive and accurate method in detecting gene expression at transcriptional level than others. In liver, the expression levels of cBD-8, -9, and -10 are much higher than those of other defensins

(data not shown) and the results are consistent with Xiao and Lynn's (Xiao et al., 2004; Lynn et al., 2004). In Xiao and his colleagues' research, different PCR cycles were used to amplify different cBD genes and optimize the detection of PCR products from agar gel electrophoresis. This research provided much detail information about the expression profiles of all thirteen cBDs in these two tissues of both broilers and layers. The different expression levels between cBD-8, -9, and -10 to other cBDs are easily obtained from the normalized expression levels in real-time PCR analysis. This study also utilized pooling samples method in real-time PCR reactions. The individual samples from four groups (B0, L0, B8, and L8) were tested for selected cBDs in this experiment as well. All of the individual results are consistent with the pooling results. It means that the pooling method provide a time-, labor- and money-saving method in experimental designing, which is especially suitable for analysis large amount samples when funding is concerned.

It is well known that most beta-defensins are induced to express with the stimulation of bacterial LPS or other inflammatory mediators in mammals (Selsted and Ouellette, 2005). As a common pathogen associated molecule patterns (PAMPs), lipopolysaccharide (LPS) is recognized by host pattern recognition receptors (PRRs), mainly Toll-like receptors (TLRs). After recognition, the antimicrobial machinery is recruited to activate complex signaling transduction pathways within the host. The immune related transcription factors, such as nuclear factor kappa B (NFkB) and NF-IL-6, are induced to expression during the signaling pathway (Mineshiba et al., 2005; Tsutsumi-Ishii and Nagaoka, 2002). These activated transcription factors translocate into

the nucleus and bind to transcription factor binding sites (TFBSs) on target genes to induce the transcription of target genes. Finally, the innate immune effectors increase their expression to eliminate the foreign bacteria. Our results also show that NFkB is significantly induced at 3-hour time point after LPS stimulation in both liver and spleen samples (manuscript in preparation), which indicates that similar signaling transduction pathways may exist in both mammals and birds because of selection pressure during the evolution.

This is the first experiment in chicken focusing on the expression of beta-defensins during the acute inflammation (0- to 8-hours) after LPS stimulation. It clearly demonstrates the inductive expression of most cBDs in both liver and spleen tissues (**Fig. 4.1 and 4.2**). Most important, our research also focused on the different innate immune response between divergently selected commercial broiler and layer birds. Due to the different genetic background, it is well known that the immune competence is different between commercial broilers and layers (Siegel et al., 1987). Our data show a generally compromised immune competence associated with fast growth broilers. In this study, most cBDs lower expressed or are induced at lower extend in broilers compared to layers with the stimulation of LPS. Selection for fast growth in chicken could result in undesirable disease resistant traits and decrease the broiler immunological competence (Julian, 1998; Yunis et al., 2000). Similar results are also obtained from the immunological assays. For example, researchers have reported that the damaging immune response existed in fast growth broilers (Leshchinsky and Klasing, 2001; Koenen et al., 2002), but layers show much stronger immune competence (Toro et al.,

1996). Our study obtains similar conclusions and provides further detailed information about broilers' weaker immune competence at molecular level.

# CHAPTER V

# DIFFERENTIAL EXPRESSION OF TLRS BETWEEN COMMERCIAL BROILERS AND LAYERS WITH LPS STIMULATION

## Overview

As a main family of cellular receptors, Toll like receptors (TLRs) play an important role in recognition of foreign pathogens and induction of the following signaling cascades in host cells. Currently, there are eight chicken TLRs (chTLRs) found. In this study, the expression patterns of these chTLRs are systematically investigated during early inflammation with the challenge of lipopolysaccharide. chTLR-2, -3, -4, and -5 significantly change their expression at transcriptional level in liver and spleen tissues of both broilers and layers. This study reveals that the expression levels of chTLR-2, -3, -4, and -5 are significantly different between these two type commercial selected birds. Broilers generally lower express these chTLRs comparing to layers. Two important transcription factors (NFkB and IRF-3) associated with TLRs signaling pathways are also investigated in liver and spleen tissues in this study, and the results are consistent with those of chTLRs. All the data validate a different innate immune response between broilers and layers, and the broilers show a much lower immune competence partially because of long-term intensive selection for fast growth.

## Introduction

Toll receptors were first discovered in *Drosophila* as important cell surface receptors for dorsal/ventral development (Stein et al., 1991) and antifungal defense (Lemaitre et al., 1996). Similar receptors found in mammals, birds and fish were named as toll-like receptors (TLRs). Since the first mammalian toll-like receptor was identified in human in the 1990s (Medzhitov et al., 1997), eleven TLRs have been discovered so far in mammals (TLR-1 to -11), and more have been identified in non-mammalian species such as fish and birds (Fukui et al., 2001; Akira et al., 2001; Takeda et al., 2003; Iqbal et al., 2005; Owens et al., 2005). Toll-like receptor family shares a similar structure including an extra-cellular domain with several leucine-rich repeats (LRR), a transmembrane domain, and an intra-cytoplasmic Toll/Interleukin-1 receptor (TIR) homology domain.

As an important family of host pathogen recognition receptors (PRRs), TLRs play a critical role in innate immune response. Different TLRs recognize different spectrums of pathogen-associated molecular patterns (PAMPs). Briefly, TLR-1, TLR-2 and TLR-6 specifically respond to lipoprotein or peptidoglycan (PDG) from gram-positive bacteria, TLR-3 interacts with double-stranded viral RNA (dsRNA), TLR-4 recognizes LPS from gram-negative bacteria, TLR-5 responds to bacterial flagellin, TLR-7 and TLR-8 mainly recognize single-stranded RNA (ssRNA) as well as imidazoquinolines, and TLR-9 responds to un-methylated CpG DNA motif from bacterium or virus as well as hemozoin from malaria (Iqbal et al., 2005; Kawai and Akira, 2006b). Currently, the exact ligands for TLR-10 and TLR-11 are still unknown.

However, TLR-10 shows similar structure to TLR-1 and TLR-6, and it can heterodimerize with them. Therefore, TLR-10 may exert similar functions as TLR-1 or TLR-6 (Hasan et al., 2005). The latest one, TLR-11, is thought to recognize profiling-like ligand (Yarovinsky et al., 2005). The specific repertoire of TLRs can be extended by the heterodimerized or homodimerized capability among these receptors. The heterodimer of TLR-2 and TLR-1 can specifically recognize bacterial lipopeptide (Wyllie et al., 2000), whereas the TLR-2 and TLR-6 heterodimer responds to mycoplasma lipoprotein and peptidoglycan (Wetzler, 2003). Further, various non-TLR molecules may influence the TLRs specific repertoire (Akira and Takeda, 2004). For example, LPS firstly needs to bind LPS-binding protein (LBP) in serum and this complex will facilitate the binding of LPS to CD14 and TLR-4 on the cellular surface, which enhances both the binding affinity and specificity.

The binding between ligands and TLRs activates transcription factors signaling transduction pathways, such as nuclear factor kappa-B (NFkB) and interferon regulatory factor (IRF) pathways, within the host cells. The first step in the signal transduction requires the interaction between the adaptor proteins and TLRs. The common adaptor proteins are MyD88 (myeloid differentiation primary-response protein 88), Mal (MyD88-adaptor-like protein; also known as TIR-domain-containing adaptor protein, TIRAP), TRIF (TIR-domain-containing adaptor protein inducing IFN-beta) and TRAM (TRIF-related adaptor molecule). Different TLRs may recruit different adaptor proteins to induce different signaling cascades (Kawai and Akira, 2006a). The activated TLRs complex will recruit and phosphorylate a series of intermediates, such as IRAK (IL-1

receptor-associated kinase) and TRAF (TNF-R associated factor) to finally activate the transcription factors, such as NFkB and IRF-3 (Akira and Takeda, 2004). After translocation into the nucleus, these transcription factors will bind to the transcription factor binding sites (TFBSs) on the target genes to induce the expression of immune related genes, such as interleukin-6 (IL-6), IL-1 and tumor necrosis factor-alpha (TNF-alpha).

There are eight chicken TLRs (chTLRs) reported so far, and they are chTLR-1 (type 1 & 2), chTLR-2 (type 1 & 2), chTLR-3, -4, -5, -7, -15 and -21 (Fukui et al., 2001; Leveque et al., 2003; Yilmaz et al., 2005; Roach et al., 2005). Some genes involving TLRs pathway, such as IRAK-4 and TRAF-6, are also identified in chicken via bioinformatics approach (Lynn et al., 2003). Most chTLRs are expressed in a wide range of immune related tissues or cells including liver and spleen (Iqbal et al., 2005). In this study, all of these eight chTLRs were investigated at transcriptional level to analyze the expression patterns and the different expression between broilers and layers with the stimulation of LPS.

## Materials and Methods

### Experimental animals

Eighteen broilers and eighteen leghorns were used in this study from a local company. After wing-banded, these birds were reared in two cages according to the species at Texas A&M University Poultry Science Department. During all the experiments, these chickens were fed and watered ad libitum formulated according to

NRC recommendations. All experimental procedures were approved by the TAMU animal use and care committee.

**Experimental design**

At the age of five weeks, both the broilers and leghorns were randomly divided into three groups (broilers into B0, B3, B8 and layers into L0, L3, L8) respectively, each including six birds. The four groups (B3, B8, L3 and L8) were injected intravenously with LPS (Sigma, St Louis, MO) solution (5mg/ml, dissolved in PBS) at an approximate dose of 2.0 mg per kg body weight. At the same time, the first group of each species (B0 and L0) was killed with $CO_2$ to collect liver and spleen tissues labeled as 0-hour. After 3 and 8 hours, the groups of 3 hours (B3 and L3) and 8 hours (B8 and L8) were also killed with $CO_2$ to collect the tissues (liver and spleen) labeled as 3-hour and 8-hour respectively. All these liver and spleen samples collected from these six groups were frozen immediately in dry ice and then store at -80°C.

**Relatively quantitative real-time PCR**

The total RNA was isolated within a week after the samples' collection. TriZol reagents (Invitrogen, Carlsbad, CA) were used to isolate the total RNA according to standard procedure. After measuring the concentration and checking the quality of all these total RNA samples with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA), equal amounts of total RNA from each of the six samples in one group were mixed together to make a pooling total RNA. After quantification again for the pooling samples, both the individual and the pooling RNA samples were treated with DNase (Invitrogen, Carlsbad, CA) as the protocol attached.

**Table 5.1** The primer sequences (TLRs) used in relative quantitative real time PCR (Q-PCR) analysis

| Gene Name | Access number | Primer Sequence[a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| chTLR 1 | AB109401 | F[b] CCCAGAAGACTTGAGCGGAA | 151 |
| | | R[c] CCACGGCACATCCAGGTAG | |
| chTLR 2 | AB046119[d] | F GGCCTGAAAACCTGAAATATCTGA | 79 |
| | | R TCAAGAGTTGAGGGAATGCAAGT | |
| chTLR 3 | AY633575 | F TTGACAGACTACCGGGAGTGTTT | 94 |
| | | R TCCAGAGAGGTGAAGTTTGTCAAC | |
| chTLR 4 | AY064697 | F AGATGCAGAACCGAAGGCAA | 76 |
| | | R TTGTGATGCTTTCCCACGTG | |
| chTLR 5 | AY633576 | F TGACATACGATGACTGCGATGA | 84 |
| | | R TCAGAAGGGTGACAGATAGGAAAAC | |
| chTLR 7 | AY633577 | F TGACAACCTTTCCCAGAGCAT | 91 |
| | | R TGTTGTTTTGAAAGTGCCACTTTTA | |
| chTLR 15 | DQ267901 | F TGCTGCCACATTTGGAAGATC | 131 |
| | | R GATCGGTGCTCCACACAAGTC | |
| chTLR 21 | AJ720600 | F TCACAGGCGGAGGTCTTCAC | 139 |
| | | R GCACCAACCCAGAGAAATCC | |

**Table 5.1 continued**

| Gene Name | Access number | Primer Sequence[a] ( 5' → 3' ) | Size (bp) |
|---|---|---|---|
| NFkB p50 subunit | D13719 | F[b] GAAGGAATCGTACCGGGAACA | 131 |
| | | R[c] CTCAGAGGGCCTTGTGACAGTAA | |
| IRF-3 | U20338 | F CCATCTTCGACTTCAGGGTGTT | 134 |
| | | R CTTGGACTCCTTGGGCTTTGT | |
| beta-actin | L08165 | F CTGATGGTCAGGTCATCACCATT | 78 |
| | | R TACCCAAGAAAGATGGCTGGAA | |

[a] Annealing temperature for all primers was 59°C.
[b] Forward primer.
[c] Reverse primer
[d] this primers also exist in other chicken TLR-2 sequences (AB046533 and AB050005).

First strand cDNA was synthesized from equal amounts (1 mg) of both individual and pooling total RNA samples with the random hexamer primer of a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according the instruction. After incubating at 25°C for 10 min followed by 50°C for 50 min and terminating at

85°C for 5 min, the prepared cDNA templates were stored at -20°C prior to use in the PCR assay.

Primers were designed according to eleven (eight chicken TLRs, two transcription factors and one internal control) chicken mRNA sequences with the Primer Express 2.0 program (Applied Biosystems, Foster City, CA) for PCR amplification. All the designed amplification fragments were approximate 50 to 150 base pairs in length. The specificity of each pair primer sequences was confirmed at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. Each amplified PCR fragment was also checked for their size and specificity in 2.0% to 2.5% agarose gel electrophoresis. These primers and genbank access numbers were listed in **Table 5.1**.

SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the Real-time PCR analysis with the total reaction volume of 20 ul, containing 50 ng of synthesized cDNA, 10 ul of 2X SYBR Green PCR Master Mix, and 0.3 uM of each oligonucleotide primer. These reagents were loaded on a 384 wells Thermo-Fast PCR plate (Abgene, Surrey, KT, UK) with an Eppendorf epMotion™ 5070 Workstation (Eppendorf, Westbury, NY) to decrease the manual variation in each well. A two-step RT-PCR protocol was used with the cycle of 40 times, 15s at 95°C and 30s at 59°C in an ABI 7900HT DNA Sequence Analysis System (Applied Biosystems, Foster City, CA). At the end, samples were heated at 95°C for 20 minutes to construct dissociation curves to identify single PCR products. Every reaction in the real-time PCR was done in triplicate. The average threshold cycles (Ct) were obtained and used for data analysis. The chicken beta-actin was used as endogenous reference and the others as target genes.

The Ct was defined as the PCR cycles at which the fluorescent signal reached a fixed threshold. A higher Ct value means a lower expression level of the gene. The comparative expression (ΔΔCt) between different time points or different species with LPS stimulation was calculated with the following formula:

$$\Delta\Delta Ct = \Delta Ct_{\text{treatment 1}} - \Delta Ct_{\text{treatment 2}}$$

$$= (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 1}} - (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 2}}$$

A positive value of ΔΔCt means that treatment 1 expresses lower level of target gene than treatment 2.

**Statistical analysis**

Statistical analyses were performed with the correlation and general linear model procedures of SAS statistical software, Release 8.01 (SAS Institute, Inc., Cary, NC). The different expression levels for a given gene were compared between different time points within one type of birds or between broilers and layers at a specific time point, with the significance as $P < 0.05$ by Student's t-test.

## Results

In order to obtain the expression profile of chicken TLRs in innate immune related tissues (liver and spleen), relative quantification RT-PCR was utilized to analyze how these eight receptors change their expressions between different time points (0-, 3-, and 8-hour) and different bird types with the stimulation of LPS.

### Expression profile of chicken TLRs in liver

**Inductive expression of chicken TLRs in liver**. The expression levels of all chTLRs between different time points with the stimulation of LPS were investigated in this study and the results of normalized threshold cycles (ΔCt) were shown in **Fig. 5.1**. With the stimulation of LPS, chicken TLR-2, -3, -4, and -5 were significantly induced or suppressed to express in either broilers or layers with LPS challenge. As a most important PRR for LPS, chTLR-4 significantly increased its expression in both bird types at 3-hour time point (1.08 and 1.59 PCR cycles respectively). It was interesting that the expression of chTLR-2 was only detected to significantly change in layer birds. It decreased expression (2.35 PCR cycles) at early phase (0- to 3-hour) and then increased (1.99 PCR cycles) at later phase (3- to 8-hour). In broilers, the expressions of both TLR-3 and -5 were significantly suppressed at early phase (1.78 and 2.49 PCR cycles respectively). At later phase, however, only chTLR-5 significantly increased expression to counteract the suppression effects. So there was no significant change for chTLR-5 in broiler liver during the whole course (0- to 8-hour time point). In layer, chTLR-3 was induced first and suppressed later with the stimulation of LPS, but the total effect was also not significant (0.26 PCR cycle, $p>0.05$) during the whole course.

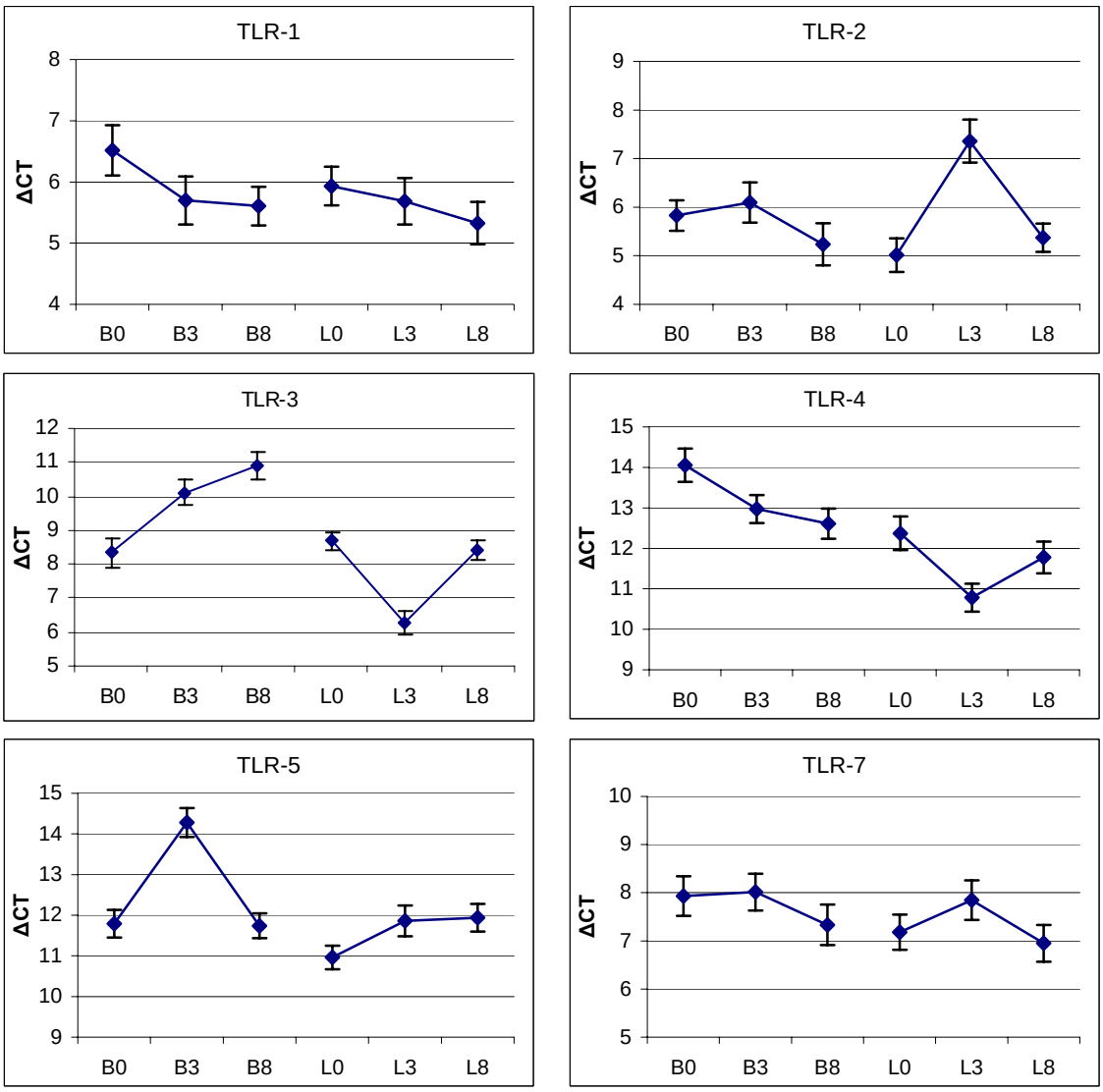



**Fig. 5.1** Normalized threshold cycles (ΔCt) of chicken TLRs expressions in liver pooling samples before and after LPS stimulation. B0, B3, and B8, broiler birds at 0-, 3-, and 8-hour time point respectively; L0, L3, and L8, layer birds at 0-, 3-, and 8-hour time point respectively. Beta-act gene was used as reference to normalize the target gene to obtain the ΔCt value. A high ΔCt value means a low expression level.

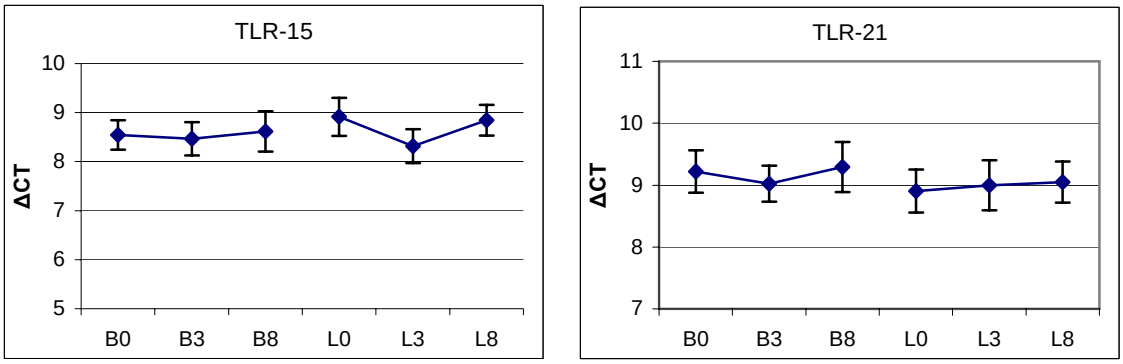



**Fig. 5.1 continued**

**Different expression of chTLRs between broilers and layers in liver.** The relative expression levels of chTLRs between broilers and layers pooling liver samples at all three time points were shown in **Table 5.2**. The chTLR-2, -3, -4, and -5 were all significantly different expression between these two type birds at certain time points. Except chTLR-2, all other three chTLRs were significantly lower expressed in broilers especially at 3-hour (3.81, 2.19, and 2.42 PCR cycles for chTLR-3, -4, and -5, respectively). chTLR-2 was lower expressed (0.81 PCR cycle) in broilers than layers at un-stimulated status. But it was significantly higher expressed (1.26 PCR cycles) in broilers at 3-hour after LPS stimulation. The **Table 5.2** revealed that the layers generally higher expressed chTLRs in liver than broilers both before and after LPS stimulation, which was consistent with the hypothesis that a weak and not effective innate immune response was associated with fast growth broilers.

**Table 5.2** Relative expression levels (ΔΔCt) of chicken TLRs between broiler' and layer' pooling liver samples with the stimulation of LPS

| Chicken Toll-like receptors | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| Chicken TLR 1 | 0.58[b] | 0.01 | 0.28 |
| Chicken TLR 2 | 0.81 | **-1.26**[c] | -0.13 |
| Chicken TLR 3 | 0.35 | **3.81** | **2.47** |
| Chicken TLR 4 | **1.68** | **2.19** | 0.83 |
| Chicken TLR 5 | 0.83 | **2.42** | -0.20 |
| Chicken TLR 7 | 0.75 | 0.17 | 0.38 |
| Chicken TLR 15 | -0.37 | 0.15 | -0.23 |
| Chicken TLR 21 | 0.32 | 0.03 | 0.24 |

[a]: Six individuals from each group are pooled together.
[b]: A positive value means broiler expression at a lower level.
[c]: Significant differences are highlighted in bold ($p<0.05$).

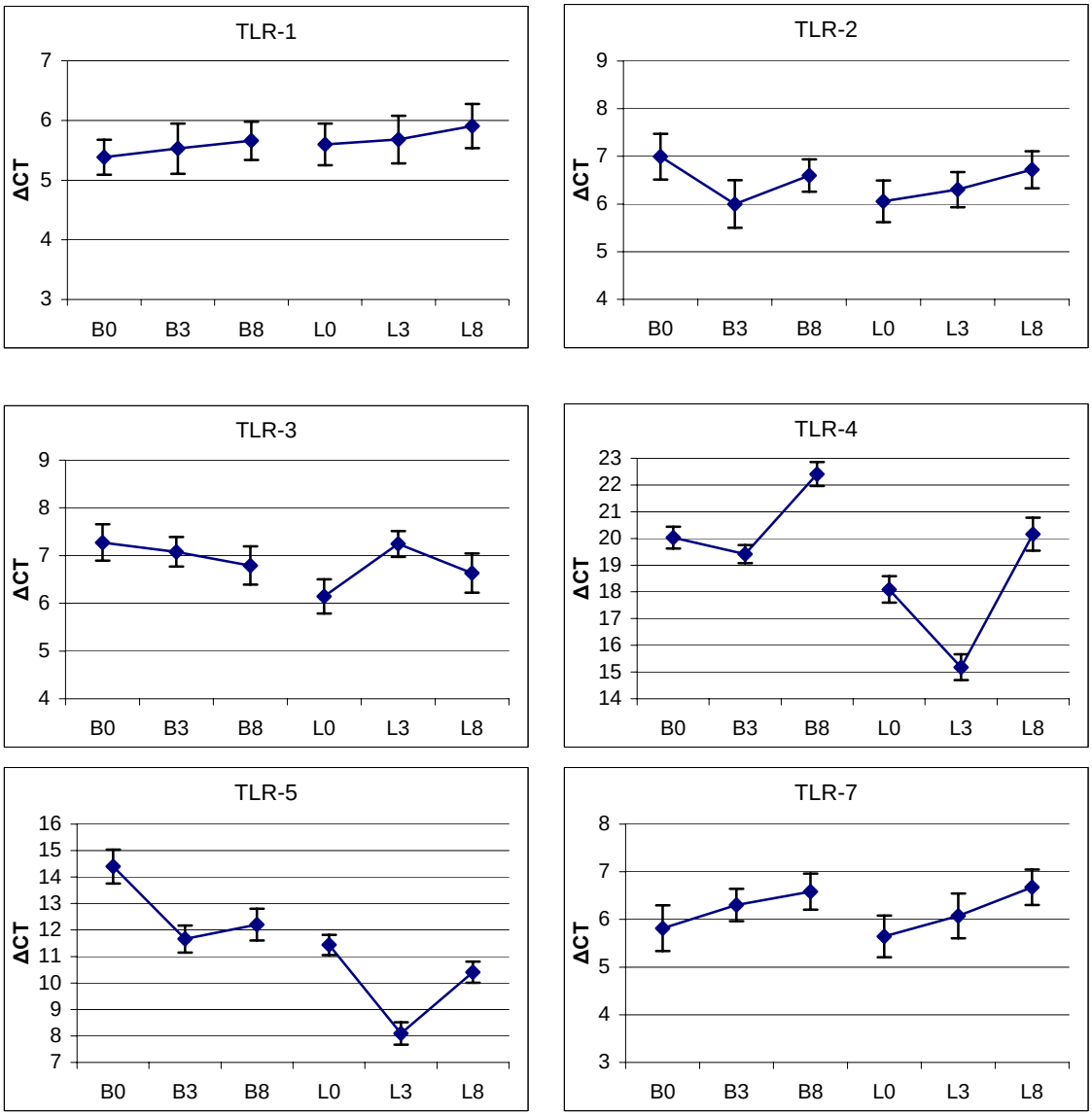



**Fig. 5.2** Normalized threshold cycles (ΔCt) of chicken TLRs expressions in spleen pooling samples before and after LPS stimulation. B0, B3, and B8, broiler birds at 0-, 3-, and 8-hour time point respectively; L0, L3, and L8, layer birds at 0-, 3-, and 8-hour time point respectively. Beta-act gene was used as reference to normalize the target gene to obtain the ΔCt value. A high ΔCt value means a low expression level.

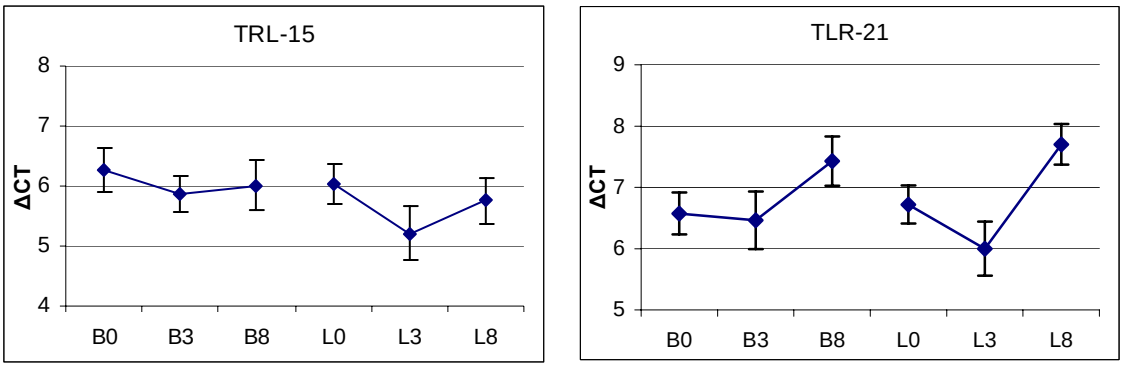



**Fig. 5.2 continued**

**Expression profile of chicken TLRs in spleen**

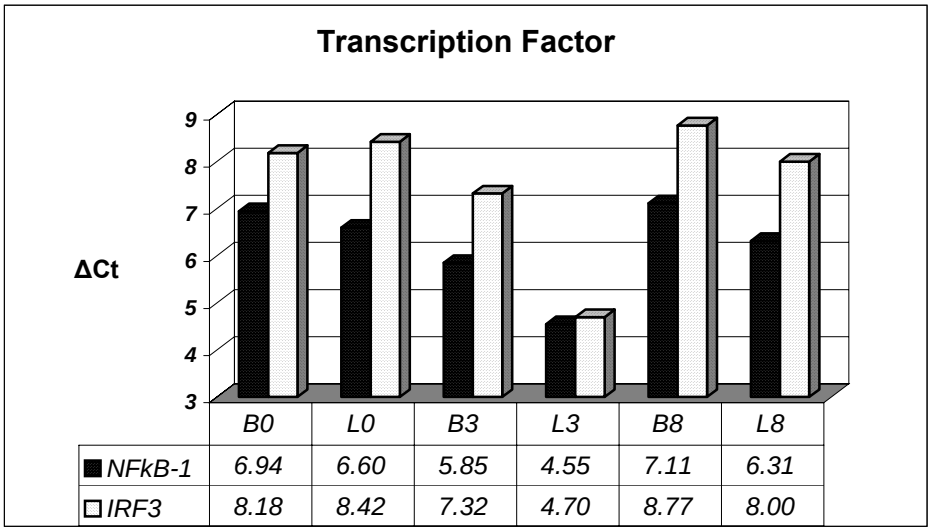
**Inductive expression of chicken TLRs in spleen.** In spleen, the chicken TLR-4 and -5 significantly changed their expressions with the challenge of LPS, as shown in **Fig. 5.2**. chTLR-5 was significantly induced to expression in both type birds at early phase (2.74 and 3.35 PCR cycles in broilers and layers respectively). However, chTLR-4 was only significantly induced in layers (2.91 PCR cycles) at early phase. At later phase, the expressions of both chTLR-4 and -5 were all suppressed in both type birds except chTLR-5 was not significant in broilers. However, from 0-hour to 8-hour, the total effects of LPS stimulation were suppression for chTLR-4 in both broilers and layers; whereas they were induction for chTLR-5. In layer, chTLR-3, -7, and -21 significantly decreased expression at certain time points with the stimulation of LPS. chTLR-3 was suppressed (1.10 PCR cycles) at early phase, whereas chTLR-21 (1.70 PCR cycles) was at later phase. chTLR-7 only significantly decreased its expression from 0-hour to 8-hour time point (1.03 PCR cycles).

**Table 5.3** Relative expression levels (ΔΔCt) of chicken TLRs between broiler' and layer' pooling spleen samples with the stimulation of LPS

| Chicken Toll-like receptors | Pooling Sample[a] | | |
|---|---|---|---|
| | 0 hr | 3 hr | 8 hr |
| Chicken TLR 1 | -0.21[b] | -0.15 | -0.25 |
| Chicken TLR 2 | **0.94**[c] | -0.30 | -0.12 |
| Chicken TLR 3 | **1.13** | -0.17 | 0.16 |
| Chicken TLR 4 | **1.94** | **4.23** | **2.25** |
| Chicken TLR 5 | **2.96** | **3.57** | **1.79** |
| Chicken TLR 7 | 0.17 | 0.22 | -0.09 |
| Chicken TLR 15 | 0.23 | 0.65 | 0.26 |
| Chicken TLR 21 | -0.15 | 0.46 | -0.27 |

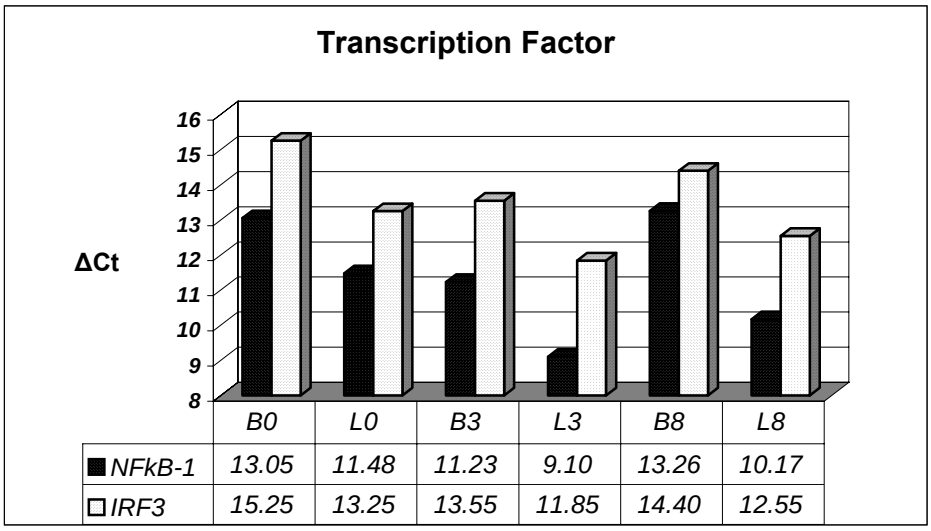
[a]: Six individuals from each group are pooled together.
[b]: A negative value means broiler expression at a higher level.
[c]: Significant differences are highlighted in dark ($p<0.05$).

**Liver**




| | B0 | L0 | B3 | L3 | B8 | L8 |
|---|---|---|---|---|---|---|
| NFkB-1 | 6.94 | 6.60 | 5.85 | 4.55 | 7.11 | 6.31 |
| IRF3 | 8.18 | 8.42 | 7.32 | 4.70 | 8.77 | 8.00 |

**Spleen**




| | B0 | L0 | B3 | L3 | B8 | L8 |
|---|---|---|---|---|---|---|
| NFkB-1 | 13.05 | 11.48 | 11.23 | 9.10 | 13.26 | 10.17 |
| IRF3 | 15.25 | 13.25 | 13.55 | 11.85 | 14.40 | 12.55 |

**Fig. 5.3** Normalized threshold cycles (ΔCt) of transcription factors (NFkB-1 and IRF3) in liver and spleen tissues with the stimulation of LPS in both broiler and layer birds. B0, B3, and B8: broiler birds at 0-, 3-, and 8-hour time point respectively; L0, L3, and L8: layer birds at 0-, 3-, and 8-hour time point respectively. Beta-act gene was used as reference to normalize the target gene to obtain the ΔCt value. A high ΔCt value means a low expression level.

**Different expression of chTLRs between broilers and layers in spleen.** The different expression of chicken TLRs in spleen was also investigated in this study and the results were shown in **Table 5.3**. At un-stimulated status, chTLR-2, -3, -4, and -5 were all significantly different expression between broilers and layers. Broilers lower expressed all these four chTLR than layers, and the expression level of chTLR-5 in broilers was only 1/8 of that in layers at un-stimulated status. After LPS stimulation, chTLR-4 and -5 all showed significantly different expression between broilers and layers. Layers higher expressed both chTLR-4 and -5 than broilers especially at 3-hour time point. The **Table 5.3** revealed that broilers generally lower expressed chTLRs both before and after LPS stimulation, which reflected a weaker innate immune competence within broiler birds.

**Inductive and different expression of transcription factors**

With the activation of TLRs signaling pathways, the expression levels of transcription factors were also investigated in liver and spleen tissues of both type birds, as shown in **Fig. 5.3**. Generally, NFkB p50 subunit (also NFkB-1) and IRF-3 significantly increased expression at early phase and decreased at later phase in both tissue types of both birds. Close investigation revealed that these factors were induced to express with bigger degree in layers than in broilers except IRF-3 in spleen. Also from the results, significantly different expressions of these two major transcription factors were detected between broilers and layers at certain time points. In liver, there was no significant difference in the expression of NFkB-1 and IRF-3 before LPS stimulation. But at 3-hour after challenge, both transcription factors are significantly higher

expressed in layers than broilers (1.30 and 2.58 PCR cycles for NKkB and IRF-3 respectively). In spleen, however, these two transcription factors were consistently significantly higher expressed in layers than in broilers independent of LPS stimulation. These results clearly demonstrated that broilers generally lower expressed transcription factors than layers, and these transcription factors were induced at a smaller degree in broilers than in layers.

**Comparing results from pooling samples to individual samples**

In order to validate the data from pooling samples, the individual liver and spleen samples from both broilers and layers were utilized to run relative Q-PCR. Because LPS was used as stimulus in this study, the chTLR-2 and -4 genes were selected to analyze at 0- and 3-hour time points. The comparisons of relative expression levels between pooling and individual samples are shown in **Table 5.4**. The results from individual samples were highly consistent with those of pooling samples, which meant the pooling methods are applicable and reliable in this experiment.

**Table 5.4** Comparing the relative expression levels (ΔΔCt) of selective chicken TLRs genes between the individual and the pooling samples at 0- and 3-hour time points

In liver

| Chicken TLRs | | Individual sample | | | Pooling sample |
|---|---|---|---|---|---|
| | | Broiler | Layer | Δ Δ Ct | Δ Δ Ct |
| TLR-2 | 0h | 5.70 ±0.54[a] | 4.94 ±0.35 | 0.76[b] | 0.82 |
| | 3h | 6.20 ±0.13 | 7.27 ±0.34 | **-1.07**[c] | **-1.26** |
| TLR-4 | 0h | 13.95 ±0.18 | 12.40 ±0.27 | **1.55** | **1.68** |
| | 3h | 12.79 ±0.21 | 10.90 ±0.32 | **1.89** | **2.19** |

In spleen

| Chicken TLRs | | Individual sample | | | Pooling sample |
|---|---|---|---|---|---|
| | | Broiler | Layer | Δ Δ Ct | Δ Δ Ct |
| TLR-2 | 0h | 7.08 ±0.36 | 6.27 ±0.29 | **0.81** | **0.93** |
| | 3h | 6.19 ±0.27 | 6.65 ±0.38 | -0.46 | -0.30 |
| TLR-4 | 0h | 20.29 ±0.34 | 18.28 ±0.22 | **2.01** | **1.94** |
| | 3h | 19.60 ±0.34 | 15.31 ±0.19 | **4.29** | **4.23** |

[a]: Normalized expression levels are shown as mean ± standard deviation (N=6).
[b]: A positive value means broilers expressed at a lower level.
[c]: Significant differences were highlighted in bold ($p<0.01$).

## Discussion

Innate immune response functions as the first physiological line for host to defend against the infectious pathogens. Multi-cellular organisms develop various mechanisms specifically recognizing different compounds of foreign microbes. TLRs are a group of evolutional conserved host membrane receptors that are widely expressed in both vertebrates and invertebrates (Akira and Takeda, 2004; Roach et al., 2005). As a main family of pathogen recognition receptors (PRRs), TLRs play an essential role in inducing and activating host immune response. In human, there are at least eleven TLRs recognizing different pathogens components (Uematsu and Akira, 2006). With the improvement of bioinformatics tools, more TLRs may be identified from human genome. Also, the recognition ligands and functions for each receptor will be more clear in the foresee future (Roach et al., 2005).

Currently, the studies on TLRs in human and other mammals are well documented. In chicken, however, only limited papers have been published. The first identified chTLR-2 can recognize two major PAMPs: the LPS and lipoproteins (Fukui et al., 2001). Kogut and his colleagues further found that, in heterophils, both chTLR-2 and -4 can respond to LPS, whereas chTLR-2 also recognizes lipoteichoic acid (LTA) from Gram-positive bacteria with the participation of CD14 (Farnell et al., 2003). In fact, LPS was utilized in this study because it was a very potent stimulus in immune response. For example, LPS and CpG-oligodeoxydinucleotide (CpG-ODN) stimulated the highest production levels of nitric oxide (NO) in chicken monocytes among several common PAMPs (He et al., 2006). Also, LPS and flagellin (FLG) induced the most highly

expression of the kinases (p38 and ERK1/2) among several TLRs agonists including LPS, PAM, FLG, PGN, LOX and poly I:C (Kogut et al., 2005).

As main structures in the outer membrane of Gram-negative bacteria, LPS specifically interacts with TLR-4 in mammals. The LPS utilized in this experiment is extracted from *Salmonella typhimurium* with trichloroacetic acid (TCA). Generally, there can be small amount of contaminations such as nucleic acid and/or protein. In fact, mammalian TLR-2 was often mistakenly regarded to be LPS signaling receptor in most experiments several years ago. In 1998, a paper from Nature reported that TLR-2 could mediate cellular signaling stimulated by LPS in human monocytes and macrophages (Yang et al., 1998). Also, Carsten and his colleagues claimed that TLR-2 mediated NFkB activation with LPS stimulation in human embryonic kidney 293 cells (Kirschning et al., 1998). However, the results from knockout mice revealed that TLR-4 deficient mice didn't respond to LPS stimulation, whereas both TLR-2 deficient and wild-type mice all responded to LPS stimulation with the same patterns (Takeuchi et al., 1999). Finally, Tapping's group found that impurities in LPS could be the reason of TLR-2 signaling pathway activation. After re-purification, they confirmed that no TLR-2 pathway was activated in human by commercial LPS (Tapping et al., 2000).

The main purpose of this research is to compare the innate immune response between commercial broilers and layers, and the purity of LPS is not so important. On the other hand, with the protein and nucleic acid contamination in LPS, more chTLRs may be activated, and a much wide and general comparison can be made between these two type birds. However, there could be another explanation for multiple chTLRs'

activation in this research. The activation of chTLR-4 and -2 induces the signaling cascades to increase the expression of pro-inflammatory genes as well as other chTLRs, such as chTLR-3 and -5. Further experiments are needed to find out which one is more predominant in this study.

This study systematically analyzes the expression patterns of eight chicken TLRs at three time points (0-, 3-, and 8-hour) with the stimulation of commercial lipopolysaccharide (LPS). In fact, chTLRs are widely expressed in a wide range of tissues or cell types. The expression patterns of chTLRs in our study are very similar to those of others (Iqbal et al., 2005; Yilmaz et al., 2005). At un-stimulated status, the expression levels of TLR-1, -2, -3, and -7 are generally higher than TLR-4 and -5 in both liver and spleen tissues. Also, in our research, the expressions of chTLR-2, -3, -4, and -5 are significantly changed (induction or suppression) either at 3- or 8-hour time points in liver (**Table 5.2**) and spleen (**Table 5.4**). In fact, the inductive expression of chTLRs and effector molecules is both dose- and time- dependent. For example, the expression level of iNOS (inducible nitric oxide synthase) is highest in chicken peripheral blood monocytes at 4-hour after TLR agonists stimulation, and then decreases at 18- and 24-hour time points (He et al., 2006). However, because of very complex regulation mechanisms in TLRs signaling pathways, the results heavily depend upon specific experimental conditions such as physiological conditions of animals and stimuli types. Different chTLRs may induce different signaling transduction pathways to differential expression of immune related molecules. In chicken, most current studies on chTLRs focuses on cell culture or cell lines (Bliss et al., 2005; He et al., 2006; Kogut et al.,

2005). This research, however, focuses on the in vivo expression of chTLRs, which means more challenge and more complex regulation mechanisms involved. For example, the chTLR-1, -15, and -21 don't show any significant changes with the stimulation of LPS in this experiment. It is noticeable that none of the previously research discovered differential expression patterns of chTLR between commercial broilers and layers. Our data show that chTLR-2, -3, -4 and -5 all significantly differential express between these two type birds. Generally, the layers express higher levels of chTLRs and show more robust inductive expression of these receptors than broilers in both liver and spleen tissues.

The ligand binding on various membrane TLRs can induce a series of signaling cascades within the host. Finally, some transcription factors, such as NFkB and IRF-3, are activated and translocated into the nucleus to regulate the expression of immune related genes. Two important transcription factors (NFkB-1 and IRF-3) are also investigated in liver and spleen at transcriptional level in this study. The results from this study reveal that both NFkB-1 and IRF-3 are induced to express at early phase and decrease at later phase. In Keeler and his colleagues' microarray analysis (Bliss et al., 2005), the expression level of NFkB also significantly increased in macrophages at 2-hour after the stimulation of LPS. Further, their research confirmed that cytokine IL-6 was significantly induced to express in macrophages, which was consistent to our results (manuscript in preparation). Kogut et al. obtained same results in chicken heterophils (Kogut, et al., 2005). They found that the expressions of pro-inflammatory cytokines (IL-1-beta and IL-6) significantly increased with the stimulation of specific TLR

agonists. It is interesting that the inductive expressions of chTLRs are not as significant as those of immune effectors such as defensins. In fact, Keeler's experiment showed similar results. They concluded that the transcription factors and the end products of signaling pathway generally increased their expressions with the stimulation, whereas the signaling molecules and cellular receptors would seldom increase or even decrease the expression level (Bliss et al., 2005).

Combining all these results, a general conclusion is drawn that layer birds show higher expression level and stronger activation of chTLRs and important transcription factors (NFkB-1 and IRF-3) in both liver and spleen tissues with the stimulation of LPS. Broilers are weaker in innate immune competence because of long-term selection for growth rate.

# CHAPTER VI

# DIFFERENTIAL EXPRESSION OF IODOTHYRONINE DEIODINASE TYPE 2 IN GROWTH PLATES OF CHICKENS DIVERGENTLY SELECTED FOR INCIDENCE OF TIBIAL DYSCHONDROPLASIA*

## Overview

Tibial dyschondroplasia (TD) is a genetic leg defect in broilers with a lesion of avascular, non-calcified cartilage below the growth plate of the proximal tibiatarsus. This disease is considered to result from the inability of chondrocytes to undergo terminal differentiation. Thyroid hormones are required for chondrocyte differentiation. The thyroid gland produces and secrets mostly L-thyroxine or $T_4$, and $T_4$ plays most of its biological activities through conversion to triiodothyronine or $T_3$ in local tissues by iodothyronine deiodinases type 1 or type 2, which are tissue specific. In this study, no differences were found in the plasma concentrations of total $T_3$ and $T_4$ between two chicken lines divergently selected for the incidence of TD. Plasma $T_4$ was higher than $T_3$, especially in older chickens. Younger birds had much higher $T_3$ than older birds, but there were no significant age differences in $T_4$. The expression level of deiodinase type 2 in the growth plates of broilers with TD was one-eighth of those birds without the disease. The expression levels of deiodinase type 2 (DIO2) in commercial broilers

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*Reprinted with permission from "Differential expression of iodothyronine deiodinase type 2 in growth plates of chickens divergently selected for incidence of tibial dyschondroplasia" by Shen S, Berry W, Jaques S, Pillai S and Zhu J, 2004, A*nimal Genetics*, 35(2):114-8. 

without the disease were much higher than those with TD and lower than those without the disease in the susceptible and resistant lines, respectively. These results indicate that the inadequate expression of DIO2 in the growth plates contributes to the pathogenesis of TD in broilers and that TD is a tissue-specific hypothyroidism.

## Introduction

Tremendous improvements in growth rates and feed efficiency have been achieved in commercial broiler chickens. Compared with a 1957 broiler strain, modern broilers showed a 340% increase in live weight, 40% increase in feed efficiency, and 42 days earlier to reach market weight (Havenstein et al., 1994). Unfortunately, genetic improvements in production performance have also resulted in high susceptibility to a variety of diseases or syndromes, such as ascites, sudden death, and skeletal abnormality (Julian 1998). Tibial dyschondroplasia is one of the diseases associated with rapid growth in broilers.

Dyschondroplasia in chickens was originally described as a spontaneously occurring cartilage abnormality (Leach and Nesheim, 1965). Because this disease is usually observed in tibiatarsus, it is often called tibial dyschondroplasia or TD. A lesion of avascular, non-mineralized cartilage extending from the epiphyseal growth plate into the metaphysis is the characteristic of this disease (Farquharson and Jefferies, 2000). The symptoms can range from subclinic to severe bone deformities and lameness. TD appears to be restricted to rapid growing broilers (Leach and Nesheim, 1965; Havenstein et al., 1994; Julian, 1998) and is strongly associated with genetic components. Divergent

selection alters the incidence of the disease (Riddel, 1976). There has been consistent evidence indicating the presence of a major sex-linked gene and the recessive genetic effects associated with an increased incidence of TD (Sheridan et al., 1978). The heritability and realized heritability of TD at 7 week of age were estimated as 0.42 (Kuhlers and McDaniel, 1996) and 0.437 (Wong-Valle et al., 1993), respectively.

Although the primary causes remain unknown, TD is considered to result from the inability of proliferating chondrocytes to undergo terminal differentiation to hypertrophic chondrocytes based on the typical accumulation of pre-hypertrophic chondrocytes in the growth plates (Farquharson and Jefferies, 2000). The differentiation of chondrocytes is tightly regulated by autocrine, paracrine, and endocrine factors, and fully terminal differentiation of chondrocytes is necessary for bone formation (Harvey et al., 2002). Parathyroid hormone-related protein (PTHrP) and Indian hedgehog (IHH) form an important negative feedback control loop regulating the pace of chondrocyte differentiation (Shum and Nuckolls, 2002). Vascular endothelial growth factor (VEGF) promotes vascular invasion that provides access for osteoblasts and osteoclasts to participate in bone formation (Gerber and Ferrara 2000). Thyroid hormones have profound impacts on the growth of long bone by promoting chondrocyte differentiation and maturation (Burch and Lebovitz, 1982a,b; Wakita et al., 1998; Robson et al., 2000; Stevens et al., 2000; Ballock et al., 2001; Siebler et al., 2002). 3,3',5-Triiodo-$_L$-thyronine ($T_3$) has much higher bioactivity than $_L$-thyroxine ($T_4$). The local availability of $T_3$ is regulated by three iodothyronine deiodinases, types 1, 2 and 3 (DIO1, DIO2, and DIO3, respectively) (Kohrle, 2000; Bianco et al., 2002).

Commercial broilers have undergone intensive genetic selection for rapid growth and high feed efficiency for more than 50 generations. Thyroid hormones play very important roles in regulating the expression of these selected traits (King and May, 1984). The association of thyroid hormones with TD has not yet been investigated. The objective of the present study is to identify the mechanisms involved in the pathogenesis of TD, and examine the hypothesis that hypothyroidism is associated with the susceptibility to TD in broilers.

## Materials and Methods

### Experimental animals

Broiler chickens used in this study were reproduced from two chicken lines divergently selected for the incidence of TD. These lines were derived from commercial broiler breeders and have been selected for 10 generations (Wong-Valle et al., 1993; Yalcin et al., 2000). The base population consisted of 200 primary broiler breeders obtained from a commercial breeder company. From the base population, 15 sires and 58 dams were divergently selected to develop the first generation of a high-TD incidence (HTDL) and a low-incidence (LTDL) lines. The chicks were examined and scored for TD incidence at 7 weeks of age using low-intensity radiographic imaging. From generation 2–10, 15 sires and 60 dams were selected from each line to produce the next generation. In each generation, sires and dams were selected based on half-sibling family performance. A selected sire was randomly mated to four dams within each line,

avoiding matings of pairs with a common parent or grandparent. By generation 10, the incidence of TD in HTDL and LTDL were 98% and 5%, respectively.

Forty fertilized eggs were collected from each chicken line from which 30 chicks hatched. At the day of hatch, chicks were wing-banded and placed in heated Petersime brooder batteries and provided with water and a ration that was formulated according to NRC recommendations ad libitum. From 3 weeks of age to the end of the experiment, the chickens were reared in wood chip-floored pens without specific treatments. The animal use protocol of this experiment was reviewed and approved by an animal use and care committee.

**Assays of plasma thyroid hormones**

One bird in HTDL and two in LTDL died before 2 weeks of age. Nine females and six males in HTDL and seven females and five males in LTDL were used in this study. At 4 weeks of age, 4 ml of blood were collected from each chicken with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Plasma was isolated from the blood samples and frozen at -20°C until analysis. Blood samples were also collected from their 50-week parents (10 males and 10 females from each line) for thyroid hormone analysis (total $T_3$, free $T_3$ and total $T_4$). Commercially available radioimmunoassay reagents (Diagnostic Products Incorporated, Los Angeles, CA, USA) were used in thyroid hormone assay. All plasma samples were assayed in duplicate, and those with a coefficient of variation (CV%) greater than 7.0% were reassayed. Interassay variations determined by using 10 replicates of each of the controls (low, medium and

high) were <12.0% for free $T_3$, total $T_3$ and total $T_4$, whereas intrassay variations were <7.0% for free $T_3$ assay, <6.8% for total $T_3$ and <6.2% for total $T_4$.

**RNA isolation and cDNA synthesis**

Four birds from each line were killed with $CO_2$ at 7 weeks of age due to the reproduction purpose. Tibial growth plates were collected, frozen immediately in dry ice, and stored at -80°C. Individual total RNA was isolated from the growth plates within a week after tissue collection using TriZol reagents (Invitrogen, Carlsdad, CA, USA). Single-stranded cDNA was synthesized with a Thermoscript RT-PCR System kit (Invitrogen) using random primers according to the protocol provided by the manufacturer. For comparison purposes, the growth plates from two male and two female commercial broilers without TD were also collected for real-time polymerase chain reaction (PCR) analysis.

**Real-time PCR analysis**

Chicken DIO1 (GenBank accession no. Y11273), DIO2 (AF125575), DIO3 (Y11110), VEGF (AB011078), PTHrP (X52131), IHH (U58511), β-actin (L08165) and 18S rRNA (AF173612) sequences were retrieved from GenBank to design PCR primers with Primer Express (Applied Biosystem Corp., Foster City, CA, USA) for real-time PCR analysis (**Table 6.1**). Real-time PCR were carried out in 20 μl-reactions containing 10 μl of 2X SYBR Green PCR Master Mix (Applied Biosystem Corp.), cDNA made from 50 ng of total RNA, and forward and reverse primers as listed in **Table 6.1** with an ABI 7900HT DNA Sequence Analysis System (Applied Biosystem Corp.) using a two-step PCR protocol that cycled for 40 times at 95°C for 15 s and at 59 C for 30 s.

Fluorescence intensity was detected at the end of every cycle. Each individual sample was run in triplicates and threshold cycle ($C_T$) values were averaged from triplicates. Dissociation curve analysis was performed after the cycling protocol to examine for non-specific PCR amplification and primer dimers. The primer concentrations of real-time PCR were optimized according the recommendations provided by Applied Biosystem Corp. The expression levels of chicken 18S rRNA and β-actin were used as endogenous reference genes. Differences in $C_T$ between reference and the target genes of the same samples, $\Delta C_T$, were calculated and used in statistical analysis.

The amplification efficiencies of the reference genes and target genes were examined with two-fold serial dilutions of a cDNA sample synthesized from the total RNA with starting cDNA template synthesized from 150 ng of total RNA per 20 µl PCR and ending at $2^5$ dilutions. $\Delta C_T$ between dilutions was 1.4 for the reference genes instead of 1 as expected as for the target genes. Because the amplification efficiency of the reference genes was lower than the target genes, the $C_T$ of 18S rRNA was normalize to be independent on the concentration of cDNA template. The relative expression level of target genes in each chicken was expressed as $\Delta C_T$ that is a difference between the $C_T$ of the target genes and the normalized $C_T$ of the reference genes.

**Statistical analysis**

Statistical analyses were performed using the correlation and general linear model procedures of SAS statistical software, Release 8.01 (SAS Institute, Inc., Cary, NC, USA). The differences in thyroid hormones and $C_T$ for a given gene between lines and between sexes were tested with a statistical model, $Y_{ijk} = \mu + l_i + s_j + \varepsilon_{ijk}$ where, $Y_{ijk}$

is the observation k in the line i and sex j, μ is the population mean, $l_i$ is the effect of line j, $s_j$ is the effect of sex j, and $\varepsilon_{ijk}$ is the random residual.

## Results

### Plasma concentrations of thyroid hormones

Average total $T_4$ level was 60% and 11 times higher than total $T_3$ in the plasma at 4 and 50 weeks of age, respectively, and free $T_3$ was about 0.06% of total $T_3$ in both age groups. There were no significant differences in plasma concentration of total $T_3$ and total $T_4$ between chickens of HTDL and LTDL at 4 and 50 weeks of age (**Table 6.2**). Free $T_3$ concentration was significantly different in females between the two lines with HTDL higher than LTDL ($P < 0.01$). Significant differences between sexes in both lines were found in total $T_3$ with the males 60% higher than the females at 50 week of age ($P < 0.001$ in HTDL, and $P = 0.01$ in LTDL) but not at 4 weeks. Total and free $T_3$ in 4-week chickens were five and six times of those in adult chickens, respectively, whereas there were no significant differences in total $T_4$ between these age groups. Correlations between the concentrations of free $T_3$ and total $T_3$ were significant with correlation coefficients of 0.55 ($P < 0.001$) and 0.45 ($P < 0.05$) at 4 and 50 weeks of age, respectively. There were no correlations between the concentration of $T_4$ and $T_3$ in both age groups.

**Table 6.1** Primers of reference and target genes for real-time polymerase chain reaction (PCR) analysis

| Genes | Primers | Primer sequences (5'→3') | Amplicon size | Final primer concentration (nM) |
|---|---|---|---|---|
| DIO1 | Forward | GGGAGATGCAGCACCTCTTC | 77 | 900 |
| | Reverse | AAGGTCAGGTGCAGCTTCCA | | 900 |
| DIO2 | Forward | GTGGAACCAAGTGCCATCTTCT | 91 | 300 |
| | Reverse | GCTTGTGAATGGTGGTCAGGTA | | 300 |
| DIO3 | Forward | GGACCGGAGGGCTACAAGA | 67 | 900 |
| | Reverse | CTCTGGAGCCGGGTTTTGTAC | | 900 |
| VEGF | Forward | ATCCTGTGTGCCTCTGATGAGAT | 75 | 300 |
| | Reverse | GTACACATCCACAGGGACACATTC | | 300 |
| PTHrP | Forward | CGGAGGATATGATGTTCACGAA | 76 | 900 |
| | Reverse | GGAGGGCACAGAATAACTCAGAAG | | 900 |
| IHH | Forward | AGACAGGGACCGCAACAAGTAC | 108 | 900 |
| | Reverse | TGACTTGACGGAGCAGTGGAT | | 900 |
| 18S rRNA | Forward | ATTGTGCCGCTAGAGGTGAAAT | 70 | 50 |
| | Reverse | CATTCTTGGCAAATGCTTTCG | | 50 |
| β -actin | Forward | CTGATGGTCAGGTCATCACCATT | 77 | 300 |
| | Reverse | TACCCAAGAAAGATGGCTGGAA | | 300 |

**Table 6.2** The mean values and SD of plasma total $T_3$ and $T_4$ and free $T_3$ levels in 4- and 50-week-old chickens of TD- susceptible and resistant lines

| Hormones | Sex | High-TD line | Low-TD line |
|---|---|---|---|
| 4 weeks of age | | | |
| | Female | 3.73 ± 0.59 | 3.47 ± 0.75 |
| Total $T_3$ (ng/ml) | Male | 3.93 ± 0.96 | 3.67 ± 0.37 |
| | Female[1] | 2.73 ± 0.60 | 1.89 ± 0.32 |
| Free $T_3$ (pg/ml) | Male | 2.57 ± 0.90 | 2.10 ± 0.38 |
| | Female | 0.73 ± 0.39 | 0.53 ± 0.34 |
| Total $T_4$ (μg/dl) | Male | 0.58 ± 0.15 | 0.50 ± 0.29 |
| 50 weeks of age | | | |
| | Female | 0.56 ± 0.17 | 0.56 ± 0.17 |
| Total $T_3$ (ng/ml)[2] | Male | 0.95 ± 0.26 | 0.85 ± 0.28 |
| | Female | 0.28 ± 0.32 | 0.28 ± 0.25 |
| Free $T_3$ (pg/ml) | Male | 0.53 ± 0.30 | 0.41 ± 0.24 |
| | Female | 0.81 ± 0.30 | 0.78 ± 0.27 |
| Total $T_4$ (μg/dl) | Male | 0.86 ± 0.34 | 0.80 ± 0.19 |

[1]The differences between the two lines were statistically significant ($P < 0.05$).
[2]The differences between sexes in both lines were statistically significant ($P < 0.05$).
TD, tibial dyschondroplasia.

**Relative expression levels of target genes**

All growth plates isolated from the chickens of HTDL displayed typical TD lesions although the size of lesions varied among individuals. TD lesions were not observed in the chickens from the resistant LTDL line. The relative expression levels of target genes in the growth plates were not significantly different between males and females ($P > 0.05$). Among the genes tested, DIO2 and VEGF were expressed at relative higher levels than others. DIO1 and DIO3 were expressed at very lowest levels among the genes tested with more than 8 $\Delta C_T$ higher than DIO2. There were no differences in the expression levels of genes tested between the two lines ($P > 0.05$) except DIO2 ($P < 0.05$) (**Table 6.3**). The differences in $\Delta C_T$ of DIO2 expression between these lines were more than three, which mean that the expression levels in chicken growth plate of LTDL were approximately eight-fold higher than HTDL. In contrast to these two chicken lines, DIO2 expression in four healthy commercial broilers ($\Delta C_T = 9.23 \pm 0.70$) was approximately half (1 $\Delta C_T$ higher) of LTDL and four times higher (2 $\Delta C_T$ lower) than HTDL, respectively.

**Table 6.3** The mean values and SD of relative expression levels ($\Delta C_T$) of target genes in the growth plates of chickens from TD -susceptible and -resistant lines[1]

| Genes | Low-TD line | High-TD line |
|---|---|---|
| DIO1 | 18.2 ± 1.3 | 19.2 ± 1.1 |
| DIO2[2] | 8.1 ± 0.7 | 11.4 ± 1.7 |
| DIO3 | 16.8 ± 0.7 | 18.5 ± 3.2 |
| VEGF | 8.5 ± 0.9 | 8.6 ± 0.6 |
| PTHrP | 13.7 ± 2.9 | 16.5 ± 0.8 |
| IHH | 10.3 ± 3.0 | 9.1 ± 0.5 |

[1]Differences between sexes were not significant ($P > 0.05$).
[2]The differences between lines were significant ($P < 0.05$).
TD, tibial dyschondroplasia.

## Discussion

Thyroid hormones have been found to play an essential role in stimulating chondrocyte differentiation, and the terminal differentiation that is required for bone formation (Burch and Lebovitz, 1982a,b; Wakita et al., 1998; Robson et al., 2000; Ballock et al., 2001). Results of the present study in combination with other studies support the hypothesis that thyroid hormones are involved in the pathogenesis of TD in broilers and that the inadequate expression of DIO2 in the growth plate causes TD. Feed restriction in broilers can lower TD incidence (Su et al., 1999). Therefore, it may be concluded that TD is the combination of genetic defects in DIO2 expression in growth plates, genes promoting rapid growth, and unrestricted feeding that provides the necessary nutrients for rapid growth. Imbalance between chondrocyte proliferation and differentiation causes TD.

Three deiodinases, DIO1, DIO2, and DIO3, play an important local control mechanism in regulating the availability of $T_3$ within cells (Bianco et al., 2002). $T_4$ is secreted at a much higher level than $T_3$ and $T_4$ plays most of its biological effects via conversion to T3 in extrathyroidal tissues catalysed by DIO1 or DIO2 depending on tissues. Both $T_3$ and $T_4$ are inactivated by DIO3. Our results showed that plasma $T_4$ was higher than $T_3$, especially in older chickens whereas older birds had a much lower $T_3$ level than younger birds but there were no age differences in $T_4$. DIO2 was the major isoform of the deiodinases expressed in the growth plates. It may be concluded that DIO2 plays an increasingly important role in regulating the availability of $T_3$ in chicken growth plates as they become older. Although thyroid hormones have very important

effects on various types of cells, inadequate expression of DIO2 in the growth plates but not low systematic production of thyroid hormones contributes to TD, which suggests that TD is tissue-specific hypothyroidism. This hypothyroidism may be associated with selected traits based on a report that thyroid hormones displayed negative effects on growth and feed efficiency (King and May, 1984). Free $T_3$ was different in females of the two chicken lines. The effect of these differences on the growth plate probably was very limited because free $T_3$ was a very small fraction of the plasma thyroid hormones. Therefore, it may be concluded that the plasma concentrations of thyroid hormones probably were not the factors affecting susceptibility to TD.

It appears that genetic selection in TD incidence affect the expression of DIO2 in the growth plates. Commercial broilers that were not intensively selected for resistance to TD showed lower expression level of DIO2 than LTDL but much higher than HTDL. Further investigation in why DIO2 is expressed at a much lower level in the growth plate of TD broilers will lead to better insight into the aetiology of TD and eventually to the identification of the genetic factors involved.

# CHAPTER VII

# CONCLUSIONS

This study systemically investigated the differential expression of four type genes between commercial broilers and layers, and the conclusions are:

1. Nineteen new chicken acute phase proteins (APPs) genes are identified and annotated from chicken genome via bioinformatics tools. With the stimulation of lipopolysaccharide (LPS), most chicken APPs (24 from 31) higher express in layers' liver than broilers' and nineteen show statistically significant. Further experiment reveals that interleukin-6 also significantly higher expresses in layers' liver at all three time points (0-, 3-, and 8-hour).
2. Most chicken beta-defensins (cBDs) significantly increase expression at 3-hour time point in liver and spleen tissues, and then decrease expression at 8-hour with the challenge of LPS. This study also reveals that almost all thirteen cBDs show significantly different expression between broilers and layers in both type tissues. Broilers generally lower express cBDs in both liver and spleen tissues than layers especially after LPS challenge (3- and 8-hour time points).
3. The chTLR-2, -3, -4, and -5 significantly change their expressions with the challenge of LPS in liver, and only chTLR-4 and -5 in spleen. Generally, these chTLRs significantly lower express in broilers than layers especially at 3-hour after LPS stmulation. Further investigation reveals that the transcription factors (NFkB and IRF-3) also significantly increase expression at early phase and decreasing at

later phase with stimulation, and layers generally higher express NFkB and IRF-3 both before and after LPS stimulation.

4. This research validates that the pooling samples provides a reliable and practical method for individual samples in real-time PCR analysis.

5. Significant lower expression of deiodinase-type 2 (DIO2) is detected in the growth plates of broilers with tibial dyschondroplasia (TD).

6. The results from all these four experiments validate our hypothesis that a compromised disease resistance or innate immune response is associated with fast growth broilers resulting from long term divergent selection.

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Selected Publications and Conference Abstracts

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