DNA Sequence and Haplotype Variation Analysis of Inflammatory Response Genes NLRX1, IL6, and IL8 in the Turkey (*Meleagris gallopavo*)

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ABSTRACT

Genotype-phenotype analyses continue to be the primary goal for genome analyses in livestock and poultry breeding. Essential to accomplish this goal is the need to identify variation at the genomic level. To test the hypothesis that DNA sequence variations in inflammatory response genes are associated with phenotypic differences in the heritage turkey, the primary objective of this project was to search for single nucleotide polymorphisms (SNPs) in candidate inflammatory response genes. A minor objective was to develop a system for inducing inflammatory response in the turkey using a microbe-based lipopolysaccharide (LPS), an approach previously described for the chicken. A total of 16 SNPs was identified in the three genes screened in this project: Interleukin 6 (IL6) and 8 (IL8), and NLRX1. Mortality data from the LPS challenge were not significantly different among the strains. Further gene expression analyses will be part of future work. The SNP data represent the first extensive analyses of candidate inflammatory response genes in the turkey. Combined with the protocols developed for inflammation assessment in the turkey the SNPs described here will be valuable resources for future inflammation: genotype evaluation in the turkey.

ABSTRACT-GENERAL

Though progress has been made in the genome analyses of the turkey, *Meleagris gallopavo*, our understanding of the genotype: phenotype relationships continue to lag those of other agriculturally important animal species. Among the phenotypes for which genetic understanding can be useful is inflammation, a complex trait that is influenced by many interdependent response mechanisms. These mechanisms, regarding differences across heritage turkeys, has been mildly investigated. Single Nucleotide Polymorphism (SNP) screening is a common method used to comprehend the robust effects these differences have on genotype and phenotype. Here, we report initial investigations in our lab of the genetics of inflammation in the turkey using comparative information from the chicken NOD like receptor X1 (NLRX1), turkey Interleukin 6 (IL6), and Interleukin 8 (IL8). These genes were screened for nucleotide variants that may be informative for future studies that will investigate the turkey's response to *Salmonella* derived lipopolysaccharide that can induce inflammation. The rationale for selecting these three genes is that IL8, IL6, and NLRX1 have pro inflammatory and/or anti-inflammatory functions that respond to maintain homeostasis. Primers were designed and investigated using DNA from Broad Breasted White (BBW), Broad Breasted Bronze (BBB), Blue slate (SL), Spanish Black (SBL), Midget White (MW) and Royal Palm (RP). The birds were also challenged with 1.5 mg/kg Lipopolysaccharide (LPS) intra-abdominally to collect tissue post LPS challenge. Tissues was collected from the thymus, spleen, and bursa of fabricius: organs identified to effect inflammation. A total of 2,239 bp for IL8, 2,439 bp for IL 6, and 572 bp for NLRX1 were screened for SNPs. SNP analysis revealed 16 SNPs in the inflammatory response genes mentioned.

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CHAPTER 1

INTRODUCTION

The turkey, *Meleagris gallopavo*, is a popular food source in the USA, the National Turkey Federation estimates 45 million birds are consumed during Thanksgiving each year and almost 800 million annually (Cunnane, 2014). The wild turkey, ancestor of the domesticated breed, is one of only two turkey species native to North America (Brant, 1998). The wild turkey was domesticated 2000 years ago after being brought from Mexico to Europe (Brant, 1998). In 1874, The American Poultry Association (APA) established standards for the turkey that included the five strains common at that time: Bronze, Narragansett, White Holland, Black and Slate and over the course of the next decade, Bourbon Red, Beltsville Small White and Royal Palm were also accepted into the APA (Reese et al., 2010). As the demand for turkeys increased, research on turkeys focused on increased yield, leading to the identification of cost-effective methods to increase breast size. Selection for larger breast muscles began in Washington and Oregon in the 1900s, giving this bird a new name, Mammoth Bronze (Reese et al., 2010). Consumer preference for white meat led to selection for a larger breast, making the market favorable towards the Broad Breasted White, yielding the most meat at the lowest cost. These changes were later determined to cause declines in fertility as the male could no longer mount the female, and an increase in disease susceptibility among flocks (Farm Sanctuary, 2007). To combat the decline in fertility, artificial insemination became a common practice among commercial breeders in the 1960s (Reese et al., 2010). Selection for higher body weight can led to muscle damage (Velleman et al., 2003). Between 2004 and 2006 heritage turkey

breeds were discovered to have more robust immune systems when compared to industrial strains through a research project conducted at Virginia Tech (Reese et al., 2010). Genetic characteristics that make the heritage strains unique have been of recent scientific interest (Aslam et al., 2012).

Disease susceptibility and infection are common among poultry, impacting viability, performance, and mortality. Turkeys are reported to be more vulnerable to disease compared to other avian species (Tumpey *et al.*, 2004). The avian species relies heavily on biological processes like inflammatory response to impede pathogen progression and disease resistance. Inflammation is generally defined as an acute-phase response to pathogens that cause injury. Merriam-Webster's definition of inflammation describes the acute-phase response to include capillary dilation, leukocytic infiltration, redness, heat, and pain. Environment, genetics, and flock management are all contributing factors that influence inflammation and overall bird health (Rath et al., 2009) (Nestor, 1969). Many avian diseases are associated with inflammation (McDonald et al., 2016) like Marek's disease (Witter et al., 2003). Cell mediated immunity is important in Marek's disease as it is highly contagious amongst flocks. Symptoms of this disease include development of lesions that lead to mortality. The occurrence of Marek's disease relies heavily on contributing factors including strain and genetics. Inflammation is observed in this disease by enlargement of the vagus, brachial, and, sciatic. Tumors may be visualized in various organs including the spleen, liver, and kidney.

Discovery of SNP differences in inflammatory response genes across varying strains will provide a basis to potentially explain the prevalence of these diseases.

In experimental investigations, Immunogenic materials like lipopolysaccharides (LPS), are used to induce the inflammatory response in diverse species including the chicken (Poltz and Klasing, 2016) and mouse (Poroyko et al., 2015). Although the inflammatory response is important in slowing down infection, excess inflammation can be disadvantageous to the host. Chronic Inflammation can be detrimental, allowing disease advancement and increased mortality rates in avian populations, ultimately resulting in substantial economic losses (Klasing, 1991). Since so many diseases involve inflammatory response cells, dysregulation of this nonspecific immune response can lead to advancement of these diseases, demonstrating the significance for regulation of the immune response (Klasing, 1991). In order to better understand inflammation and its regulation in the turkey investigation of the genes that contribute to this defense cascade is needed. Interleukin (IL) 6, IL 8, and Nod-Like Receptor X1/leucine rich repeats X1 (NLRX1) are essential to avian mediated inflammatory response (ref). Pro-inflammatory cytokines like IL 6 and IL 8 recruit immune cells to the site of infection using their chemotactic function to regulate inflammation (Wigley et al., 2003). NLRX1, on the other hand, has functionality that attenuates the innate immune response and inflammatory response serving as a checkpoint for overuse of inflammation (Allen et al., 2011). NLRX1 is also involved in the increased IL 6 production (Allen et al., 2011). Literature regarding the investigation of the function of the NLRX1 gene in inflammatory response in the turkey is limited.

Studies that use Whole Genome SNP discovery to examine genetic differences in Heritage turkey strains are influential in providing insight into heritability (Aslam et al., 2012).

Investigation of the genes that influence differences in the immune system across heritage turkey strains have not been previously investigated compared to commercial strains. The turkey genome comprises 33 chromosomes having a total length of 1.101Mb (Dalloul et al., 2014). Though progress has been made in turkey genome discovery and immunological comprehension, discovery of nucleotide and haplotype variants in inflammatory response genes including IL 6, IL8, and NLRX1 can potentially further explain phenotypic differences amongst heritage strains.

Genetic diversity can be detected through Single Nucleotide Polymorphisms (SNPs). The largest amount of genetic diversity in the genome is associated with SNPs. SNPs are instrumental in heritable trait detection across related individuals (Aslam et al., 2012). SNP detection has facilitated the assembly of genetic and physical mapping which has allowed a platform for the analysis of genetic diversity (Appleby et al. 2009). SNP detection has also established the basis for the development of PCR-RFLP (Polymerase Chain Reaction-Randomly Amplified Polymorphic DNA), a genotyping procedure leading to lineage discoveries of strain relatedness (Smith et al., 2003). Analysis using these procedures established Royal palm as genetically distinct from five other domesticated turkey strains (Smith et al., 2003).

The primary goal of this thesis research is to determine SNP differences amongst varying turkey strains by screening the NLRX1, IL 6 and IL 8 genes for sequence and haplotype variants in the turkey, *Meleagris gallopavo*.

Specific goals of this thesis research include:

1. Screen the NLRX1, IL 6, and IL8 genes for sequence and haplotype variants in the turkey;

2. Collect tissue post LPS challenge for future research in determining LPS-induced NLRX1, IL6, and IL8 sequence and haplotype variations are associated with differences among heritage and commercial turkey strains.

The rationale for the thesis project is that discovery of variations in these and other genes can be associated with differences in inflammatory response in the turkey. Distinct correlation between the genotype and phenotype of inflammation can lead to advancements in inflammation biomarkers, flock health, and genetic selection.

Chapter 2
Literature Review

2.1 Turkeys

The wild turkey, *Meleagris gallopavo* originated in North America (ref). The wild turkey belongs to the order Aves and family Galliformes, similar to the chicken, *gallus gallus*. Sub-species of the North American wild turkey belonging to the species *Meleagris* gallopavo include *M.g. silverstirs, M.g. gallopavo, M.g. osceola, M.g. intermedia, M.g. merriami, M.g. mexicana.* Region of habitat is the determining factor of the subspecies, similarities in plumage color and body size can be observed across sub species (Kemp et al., year). This large bird is considered a game bird in the wild, but once domesticated, raised for its meat, feathers, and egg production. The turkey was domesticated about 1100 C.E, although there is believed to be an initial domestication of the wild turkey during 300 C.E (Hirst, 2018). when turkeys were imported from Mexico. Studies investigating care, diet, and tending of the turkey have suggested the 1100 C.E. as the most important domestication even while before, the interest in the turkey was only for feathers (Lipe et al., 2016).

The wild turkey can be found in regions of northern Mexico and areas of the Eastern and Western United States (US). These birds prefer habitats that are temperate and terrestrial, among woody forests and marsh wetlands. The physical body structure of wild turkeys includes long legs and necks. They have bilateral symmetry with fan shaped tails and rounded wings. Though they are ground-dwelling animals, they can fly and often times roost in trees. Hens have a distinct physical appearance when compared to toms. Hens are smaller with a gray head

and feathered neck. Toms are usually double the weight of hens and have varying head color, depending on the season (red, blue, white). Males can be quickly identified by the red fleshy appendage hanging from the beak called a snood. Wild turkeys are polygynous and capable to mate at 10 months of age. Wild turkeys breed on a seasonal basis occurring once per year. Courtship begins in early spring and they can lay 4-17 eggs per season. Female wild turkeys provide all parental care for the poults. Life expectancy for wild turkeys range from 1.3-1.6 years though the oldest documented wild turkey was 13 years old (Eaton, 1992). Wild turkeys are primarily omnivorous feeding on vegetation, seeds, and nuts. Wild turkeys primarily forage on amphibians and insects on the ground and occasionally mount trees for fruit. Though commercialized turkey production has developed for meat, wild turkeys are economically important to humans in other forms. Wild turkeys are among the most popular game birds in the US. Millions of dollars in revenue is collected by the Departments of Natural Resources and the State's through turkey hunting efforts each year.

Though the wild turkey and the domestic turkey are genetically identical, with the domesticated turkey originating from the wild turkey, genetic selection and breeding techniques have altered the lifestyle of the domesticated breeds. Modern practices in the turkey industry use genetic selection in order to select for favorable traits including larger breast yields (Berri et al., 2001). Genetic practices like mitochondrial DNA analysis have been used to trace the lineage and ancestry of archaeological findings (Speller et al., 2010). As the demand for turkey meat increased, pressure to produce a faster growing more efficient, higher yielding turkey arose. The 20th century marks a revolutionary period for the turkey industry, the commercial hybrid white turkey was developed. The hybrid, a cross between two or more

strains, is larger and grows at a faster rate than the wild or purebred strains. The white turkey hybrid has dominated the commercial industry since de-feathering does not leave unsightly pigmentation on the skin like those of darker plumage (Reese et al., 2010).

2.2 Heritage Turkeys

Heritage turkeys occupy the space between the wild variety and the broad breasted commercial breeds. Black, Bronze, Narragansett, White Holland, Slate, Bourbon Red, Beltsville Small White, and Royal Palm are all heritage turkey varieties, by definition of the APA standard (Reese et al., 2010). Though not recognized by APA, Midget White, Chocolate, Jersey Buff, and Lavender are all considered distinct varieties. In order to be deemed a heritage turkey the following criteria must be met;

1. Natural mating: Both grandparent and parent lines must be the result of natural mating pairs. Toms practice breeding for 5-7 years while Hens breed for 3-5 years. Unlike commercial bred turkeys, heritage turkeys can reproduce without the need of artificial insemination.
2. Long productive outdoor lifespan: Heritage Turkeys should be raised in living conditions more resembling that of the wild turkey versus the commercial raising conditions. Heritage turkeys should be raised in an open environment with roaming ability often times in large pins or free-range pastures. Commercial bred turkeys are usually housed in grow out houses until they reach desired market weight.

3. In comparison to commercial- breed turkeys, on average, the heritage breeds have longer lifespans. Commercial turkey's average lifespan is 2-6 years while the lifespan of heritage strains is 10-12 years.
4. Slow growth rate: Skin and meat yields in turkeys are influenced by strain (MacNeil et al., 1968). Selective pressures for larger breast size at the lowest possible cost has altered the natural growth rate of commercial turkeys (Zuidhof et al., 2014). Broad Breasted White reaches market weight between 14-18 weeks at 22.7kg for toms and 16.3kg for hens. Heritage breeds reach market weight at about 28 weeks. This slow growth rate allows full development of skeletal structure and organs before muscle mass development. Long term genetic selection has granted improvements in poultry production but has affected muscle and adipose tissue development (Buzala et al., 2016).

2.2.1 Phenotypes of Heritage Turkeys

Plumage is the most obvious phenotypic difference amongst the heritage turkey variety. Extensively popular, Bronze is the result of the cross between domestic European and eastern American wild turkeys (Reese et al., 2010). The Broad Breasted Bronze (BBB) turkeys have feathers that are covered in a bronze sheen. The American Livestock Breeds Conservancy (ALBC) This variety is most popular for its large size and meatiness, and parallels growth and size of Broad Breasted White. Smaller in size and native to Lake Worth, FL during 1920s, Royal Palm (RP) is an ornamental bird fancied for its contrasting black and white plumage (ALBC). This variety is usually free from selection for growth and muscularity and is smaller than other varieties. Similar to RP in color combination, the Narragansett (N) variety was developed in

Narragansett Bay, RI. This variety is popular for its early maturation, egg production, good meat quality, and good maternal instincts. The Black turkey originated in Europe, the variety adopted names where they became popular like “Black Spanish” from Spain and “Norfolk Black” from Norfolk England. Interest in this variety came from its calmness, rapid growth rates, and early maturation. There is a common misconception that the Slate originated from the Black turkey, instead the ashy blue plumage is a result of a mutation (ALBC). This strain is most popular for its survivability and flavor. As a result of its variability in color, there is little documentation of the Slate turkey. In the late 1800’s a cross between Buff, Bronze, and White Holland gave rise to the Bourbon Red (BR) turkey (ALBC). The BR turkey has a rich chestnut plumage with white wing and tail feathers. The APA recognized the BR variety in 1909 for its heavy breasts and rich flavored meat. Though smaller in size compared to the Bronze, the lack of pinfeather markings gave White Holland market advantages of cleaner carcasses. WH also matured earlier than the darker plumage varieties. To maximize the advantage of clean carcass and larger size, researchers crossed WH and BBB producing the Broad Breasted White (BBW). The BBW is the leader of today’s turkey industry. Also affected by the absence in color mutation, Beltsville Small White was bred in Beltsville, MD between 1934 and 1941 (ALBC). This breed was designed to fulfill consumer demands for a smaller bird. In contrast to the BBW, the BSW has the ability to naturally mate as a result of its smaller stature. Though advantageous, the BBW overshadowed the BSW in market demand, nearly making it extinct.

Variation in DNA as it is correlated to phenotypic differences among heritage turkeys and other Galliformes is of scientific interest. Variations across breeds of the avian species are

associated to disease susceptibility and resistance. Discovery of 5,719 polymorphisms in the major histocompatibility complex (MHC) using a variety of avian species, including the heritage breeds, adds to available resources to study the genotype-phenotype relationship of the agriculturally important avian species (Reed et al., 2016). Similar to variations in the MHC, the examination of variations using mitochondrial sequences offers the opportunity to examine relatedness among varieties of both wild and domesticated turkeys (Guan et al., 2015). The use of a mitochondrial genome sequence to discover SNPs and establish haplotype maps offers insight into the relatedness of 15 turkey varieties. Findings validated 24 SNPs, 18 of which were located in the D-loop, while 6 were found in the 16S rRNA. Construction of haplotype groups and parsimony networks that examined variability, documents that wild and commercial turkeys highly variable while heritage and Broad Breasted varieties have less variability (Guan et al., 2015). Their findings that the Royal Palm (RP) variety is closely related to Narragansett (N) and less related to Blue Slate (BS) and Spanish Black (SB) are consistent with those described by Smith and co-workers (2005).

Studies of genetic relatedness are useful to describe evolutionary change among the heritage strains as well as relatedness across varieties of the domesticated turkey. Rigorous genetic selection among commercial breeds has resulted in an increase in sweeps that span the turkey genome causing haplotype fixations that limit diversity (Aslam et al., 2014). The genetic relationship between commercial (CO) and heritage turkeys was characterized by Kamara and others (2007). Consistent with methodology used by Smith and co-workers (2005), microsatellite markers are used to analyze relatedness between CO and heritage strains N, BS,

SB, RP, and BR. Findings concluded that BS, BR, and N are most genetically related to the CO strains compared to the other strains studied (Kamara et al., 2007). The study of biomarkers, like plasma uric acid levels (Hartman et al., 2006) demonstrates phenotypic differences that are strain specific. As genomic maps and heritage turkey hatcheries are becoming more prevalent there is a need to discover phenotypic differences as it pertains to inflammation. Discovery of these differences provides an opportunity to understand and tailor practices to decrease disease advancement and susceptibility.

2.3 Inflammation and diseases in turkeys

The innate immune system acts as the first line of defense, recognizing harmful stimuli and initiating defense mechanisms in order to eliminate pathogens and hopefully mitigate tissue damage. Inflammation is the most predominant indication of host resistance in response to irregularities in tissue homeostasis and is regulated by innate immune responses which are responsible for perceiving and distinguishing potential disruptions in homeostasis (Klasing, 1991). Innate immune receptors govern the consequences of the elicited inflammatory response. The inflammatory response is contingent upon the type of trigger that activates its cellular response cascade. Moreover, the trigger is directly correlated to the inflammatory phenotype yielded. The specific phenotype is correlated to the location, longevity, and level of destruction to surrounding tissues. Inflammation phenotypes: Pathogenic, Physiological, Metabolic, and Sterile exist in the intestines of poultry (Kogut, 2018).

Inflammation research is devoted to determining if the effects of inflammation are beneficial or detrimental in farm animals (Brown et al., 2018). Selection of broilers with high expression levels of pro-inflammatory mediators, cytokines like IL 6, promote resistance to *Salmonella enterica serovar enteritidis in progeny* (Swaggerty et al., 2014). Increase in pro-inflammatory cytokines allow birds to overcome challenges from *Salmonella* at much faster rate when compared to other birds (Wigley, 2017). Up-regulation of pro inflammatory cytokines and down-regulation of anti-inflammatory cytokines (IL-10, TGFβ) have demonstrated effectiveness in clearing a viral pathogen that is common in poultry, infectious bursal disease virus (IBDV) (Jain et al., 2013).

2.4 Lipopolysaccharide-induced inflammation

LPS is documented to induce a systemic inflammatory response by elevating pro-inflammatory cytokines and chemokine expression in chickens (Kalaiyarasu et al., 2016). LPS is a pathogen-associated molecular pattern (PAMP) that binds to the pattern-recognition receptor (PRR) toll-like receptor 4 (TLR4) that is bound to a serum protein, lipopolysaccharide-binding protein (LBP) and CD 14 co-receptor (Kogut et al., 2005). Binding of these complementary components initiates the innate immune response that induces cytokine production of pro-inflammatory cytokines interleukin 6 (IL 6) and interleukin 8 (IL 8) (Kogut et al., 2005). Chicken heterophils express TLR4 constitutively (Kogut et al., 2005). so that *Salmonella*-derived LPS can induce heterophil activation in the absence of LBP, the vitality of LBP is in question. The role of LBP and CD14 and their ability to recognize LPS and induce the innate immune response as well as cytokine and chemokine gene activation has been investigated. Findings confirmed LBP and

CD14/TLR4 are essential to LPS mediated activation of innate immune gene expression in chicken heterophils (Kogut et al. 2005).

LPS is also used in combination with diet adaptation to examine the effects of immunomodulatory nutrients on potential growth and immune-related gene expression (Wils Poltz et al. 2017). Findings indicated each nutrient (corn oil, fish oil, conjugated linoleic acid, leutin, and vitamin E) addition incorporated in the diet of layer chickens resulted in modified immune response to LPS challenge. This study introduced LPS in the dosage of 1.5mg/kg body weight (BW) by way of intra-abdominal injection on day 14 of the study. Similar dosage methodology was used by Wang and others (2016) to examine the effects of live yeast on inflammatory response in broilers challenged with LPS. This experimental design sampled three live yeast dosages at 0%, 0.05%, and 0.50% using 480 one-day-old male broilers. In this study researchers concluded that yeast supplemented at 0.05% improved antibody response, additionally, supplementation at 0.50% mitigated LPS-induced intestinal damage.

2.5 Candidate inflammatory response genes in the turkey

Pro-inflammatory cytokines and chemokines are key contributors to recognizing stimulus and initiating the innate and adaptive inflammatory response (Staeheli et al., 2001). Cytokines are signaling proteins that function as regulators, affecting cellular environment to regulate homeostasis. Chemokines, like Interleukin 8 (IL 8), are networks of cytokines that are defined by their specificity in structure and chemotactic functionality.

2.5.1 Interleukin 6 (IL 6)

The pro-inflammatory cytokine, IL6, mediates inflammation in a response to rid the body of harmful stimuli. The functionality of IL6 includes immune response, inflammation, and hematopoiesis, B cell maturation, cell proliferation, differentiation, apoptosis, and survival. IL6 affects many organ systems including nervous system, endocrine system, and immune systems (Tanaka et al., 2014). Typical to cytokines, IL 6 is pleiotropic and has functional redundancy. The components of the IL6 receptor includes an IL 6 binding alpha chain and a gp130 signal transducer, a shared component of IL6 subfamily cytokines. This shared signal transducer is common among cytokines, permitting functional redundancy (Hirano, 1991). IL6 receptor alpha is activated when the IL6 protein is secreted into the serum and the transcriptional inflammatory response cascade is induced. Accumulation of IL6 proteins occurs in sites of acute and chronic inflammation. IL6 functionality is associated with susceptibility to several inflammation-associated diseases, like *Eimeria* infection (Lynagh et al., 2000) and rheumatoid arthritis (Wielinska et al., 2018). Human IL-6 contains 212 amino acids and located on chromosome 7p21 (Hirano et al. 1991). IL6 homologs are reported for over a dozen vertebrate species, including that of the turkey (NC_015016.2). *Meleagris gallopavo* IL6 is located on chromosome 6 and contains 3 exons.

The avian pro-inflammatory cytokine IL-6 structure is not conserved with that of the mammalian structure, but functionality in terms of immune response is similar (Staeheli et al., 2001). IL6 expression levels can be measured and compared before and after challenge to assess the effectiveness of the inflammation response. LPS-induced inflammatory response in chickens where natural vitamin E is supplemented expresses lower levels of IL6 RNA in the spleen and significantly lower inflammation (Kaiser et al., 2012).

2.5.2 Interleukin 8 (IL 8)

Also known as CXCL8, Interleukin-8 (IL 8) is a chemokine of the CXC family (an amino acid separated by two cysteines). These chemokines are produced by macrophages and other cells including epithelial cells. CXC family member chemokines have two categories: ELR positive or ELR negative. IL 8 has a glutamic acid-leucine-arginine (ELR) motif before the first cysteine of the CXC motif, indicating its ELR positivity. This amino acid sequence is conserved in all CXC chemokine family members that exhibit neutrophil activation (Clark-Lewis 1993). ELR positive chemokines initiate neutrophil migration by interacting with CXCR1 and CXCR2 chemokine receptors.

IL 8 is an inflammatory cytokine that has chemotactic functionality (Baggiolini et al., 1992) responsible for heterophil (Redmond et al., 2011), monocyte (Barker et al., 1993) and T cell (Min et al., 2001) recruitment to the site of infection. Chemotaxis of target cells takes place predominantly in neutrophils bearing CXCR1 and CXCR2, triggering an immense accumulation of these cells at the sight of infection.

2.5.3 Nod Like Receptor X 1 (NLRX1)

Toll- like receptors (TLRs) are pattern recognition receptors (PRRs) that have been identified and evolutionarily conserved in mammals and avian species (St. Paul, et al., 2013). The interaction of microbe associated molecular patterns (MAMP's) and Toll-like receptors (TLR's) activates the innate immune response. Selective pressures have affected affinity and binding recognition of MAMP's to their pattern recognition receptor (PRR). TLRs are identified as the primary PRR of MAMP's in many animal species (Roach et al., 2005). With six major

vertebrate TLR's, nucleotide-binding oligomerization (NOD)-like receptors (NLRs) fall into the category of cytosolic PRRs identified in mammals. NLRs are activated by detection of intracellular PAMPs. Findings pertaining to NLRs in chickens are limited (Lian et al, 2012).

Previously known as CLR11.3 and NOD 9, Nod like receptor X1 (NLRX1) influences the inflammation phenotype by functioning as an innate immune sensor. Human NLRX1 is believed to function as a modulator of pathogen- associated molecular pattern receptors as well as a potential therapeutic target to enhance antiviral responses (Moore et al., 2008). This NLR protein can be found in the mitochondrial outer membrane which interacts with mitochondrial antiviral signaling adaptor (MAVS). Studies to confirm the mitochondrial antiviral signaling potential of NLR proteins, where bioinformatics were used to identify mitochondria localized NLRs. Through this experiment, scientists were able to confirm the conserved NLR family structure, NLRX1 containing a central putative nucleotide binding domain (NBD) and carboxy-terminal leucine rich repeat (LRR) (Moore et al., 2008).

In contrast, NLRX1 is said not to be a negative regulator of MAVs-dependent antiviral responses through a derived NLRX1 knockout mouse (Soares et al., 2012). Both the wild type and NLRX1 knockout mice revealed unaffected antiviral and inflammatory gene expression post influenza-A virus challenge. These results refute the idea that NLRX1 participates in negative regulation of MAVs-dependent antiviral responses (Soares et al., 2012). NLRX1 has proven to protect macrophages from induced apoptosis allowing the preservation of the antiviral function (Jaworska et al., 2014).

Though NLRX1 findings are limited, homologues have been identified in several vertebrates including human, monkey (Guo et al., 2016), dog, cat, mouse (Soares et al., 2012),

rat, chicken, and zebrafish (NCBI, HomoloGene). Alverez et al (2017) conducted a study investigating NOD-like receptors identified in Oncorhynchus mykiss; OmNLRC3, omNLRC5, and omNLRX1 in rainbow trout. Findings concluded LPS challenge induced an increased accumulation of omNLRs in the kidney and gills of these trout. Similar studies conducted in the turkey, *Meleagris gallopavo,* will provide insight into the genes that are important to inflammatory response and development of a system that could potentially reduce negative inflammatory consequences.

2.6 Single Nucleotide Polymorphisms (SNPs)

Prior to the discovery of SNPs, population studies associating candidate genes to a disease were limited. In the 1980s SNPs became a popular method to examine variation based on fragment length using restriction enzymes and cutting sites. In the early 1990s interest in SNPs declined as simple tandem repeat (STR) became the more popular option to study linkage. SNPs eventually regained popularity in genetic studies. The shift in the diversity study method from STRs to SNPS was encouraged by the type of genetic studies being performed during this era. STRs were more useful when examining monogenic diseases. As the prevalence in multifactorial diseases increased, SNPS became the more accepted method to study the genetics of these diseases. Breeds and researchers alike are interested in how SNPs are related to specific traits that may allow further advancements in poultry health.

Following completion of the most recent turkey genome build 95% coverage (Dalloul 2014), single nucleotide polymorphisms (SNPs) have become a useful tool to detect variability, disease susceptibility, treatment efficacy, and drug response (Duan et al., 2018). Low mutation

frequency and abundance make SNPs more stable and useful markers to examine of complex multifactorial diseases when compared to STRs. SNPs are known to be consequential if occurring in the coding or regulatory regions of a gene (Gray et al., 2000). Examination of SNP markers can be used to gain insight in the association of genotype to phenotype and the effects of alterations. SNPs have aided in characterizing phenotypic variations of turkeys (Guan et al., 2015). The discovery of SNPs across the heritage breeds has added to the network of data that allow for trait selection, productivity, disease susceptibility and resistance, and immune response.

Discovery of SNPs in the inflammatory response genes IL6, IL8, and NLRX1 will contribute to applications of genomic selection and association studies. Contributions of information regarding genetic diversity will allow the incorporation of practices that maximize selection to manage inflammation and ultimately enhance fitness.

Chapter 3

DNA Variation Analysis in Candidate Response Genes in Heritage and Commercial Turkeys

3.1 Abstract

Though progress has been made in the genome analyses of the turkey, *Meleagris gallopavo*, our understanding of the genotype: phenotype relationships continues to lag behind those of other agriculturally important animal species. Among the phenotypes for which genetic understanding can be useful is inflammation, a complex trait that has not been widely investigated in the turkey. The inflammatory response is one of the most essential parts of non-specific immune response in vertebrates including birds. To study inflammation in the turkey, a previously described approach in the chicken was utilized that involved challenging birds with *Salmonella enteritidis*-derived lipopolysaccharides to elicit and induce the inflammatory response. Additionally, three genes were selected with the potential to impact inflammation to screen for DNA sequence variation. The rationale for selecting these three genes is that IL 6, IL 8, and NLRX1 have pro inflammatory and/or anti-inflammatory functions that maintain homeostasis. Primers were designed and tested using three heritage turkey strains given 1.5 mg/kg LPS at seven weeks of age. SNP analysis allows examination of differences among genetically distinct turkey strains in the inflammatory response to LPS. Screening the chicken NLRX1, turkey IL8, and IL6 for sequence and haplotype variation, using a standard protocol in our lab (Adikari et al., 2017), revealed differences in SNP occurrence across strain. In the IL8 gene, a total of 5 SNPs were detected, 1 of the 5 SNPs discovered was specific

to the commercial strain. Six of the 9 discovered SNP in the NLRX1 gene were validated in both the heritage and commercial sequence alignments of the experimental birds. The remining 3 were specific to the commercial strain. Identified SNPs of the IL6 and IL8 genes were located in the non-coding regions of the genes, not having an effect on the amino acid sequence that produce proteins. In the NLRX1 gene, a total of 3 SNPs was identified. This is the first time the NLRX1 gene has been screened to discover variants in the turkey. Conservation of SNPs in both the heritage and commercial breeds could be suggestive of the limitations genetic selection have placed on the poultry industry.

Keywords: Inflammatory Response, SNP analyses, Interleukin 6, Interleukin 8, Nod-Like Receptor X1, turkey

3.2 Introduction

Genetic enhancements of farm animals through selection has led to increased productivity but may have resulted in loss of genetic diversity (Groneveld et al., 2010). Studies of genomic variation at the DNA level allows for assessment of genetic divergences that have resulted from selection as well as add the network of information that allow advances in disease susceptibility and advancement. Cervical dislocation was performed to humanely euthanize the animals post LPS challenge. The Bursa of Fabricius is the primary site for B-cell proliferation in Galliformes like turkeys. It was not until 1956 that Glick and Chang first explained the essential role the bursa of Fabricius played in antibody production (Glick et al. 1956). Glick was influential in demonstrating antibody production against *salmonella* (Glick et al. 1955). Attached to the dorsal part of the cloaca, this fibrous sac has functionality similar to that of the lymph glands in mammals, including similarities in cell type (Calhoun, 1933). Poultry are notorious for lymph

nodes that are undeveloped, instead many organs contain lymphatic tissue including the Bursa of Fabricius, thymus, and spleen.

Serving to assist in immunity and antibody production (FDA) the Bursa of Fabricius regresses in size as the bird matures eventually vanishing. Expressive in its size and diameter the bursa of Fabricius reaches its maximum size at six months old followed by deterioration (Jayachitra et al, 2009). In this experiment, euthanizing was strategically scheduled to visualize differences in bursa size as it is related to age. Also decreasing in size as the birds matures, the thymus is composed of about ten flattened pale pink lobes along the neck just beneath the skin (FDA). Similar in functionality of the bursa of Fabricius, the spleen is essential for antibody and leukocyte production. The spleen is located adjacent to the gizzard. These organs functionality in immune response made them organs of interest to collect tissues post challenge that will be used in a later experiment.

In order to investigate Cell Mediated Immunity (CMI) and the chicken's ability to overcome challenges associated with the detriments of this response, Genome Wide Association (GWA) studies are implemented to associate specific SNPs to pathways that induce inflammation. Conclusions of this study included the discovery of three strongly associated SNPs to chromosome 24 and three suggestively associated SNPs to chromosome 1, 5, and 16 (Raeesi et al., 2017).

3.3 Materials and Methods

3.3.1 Animals

Two hundred thirty-nine one-day-old heritage turkeys were used in this study in two phases:

Pilot: In the initial pilot study, 59 one-day-old poults obtained from Welp Hatchery (Bancroft, IA, USA) including 20 each from Broad Breasted Bronze (BBB), Blue Slate (BS), and 19 Midget White (MW, one died during shipping). The objectives of the pilot trial were to:

1. Understand the ordering and delivery process of birds through the postal system,
2. Implement the rearing and management process of birds at the Virginia Tech Turkey Farm,
3. Identify a low-cost and rapid but tractable method for assessing inflammatory response.

Experimental: Following the "Pilot" study and optimization of the farm and lab protocols, 180 one-day-old turkeys were obtained from Murray McMurray Hatchery (Webster City, IA) including 60 BBB, 60 Black Spanish, and 60 Royal Palm. Additionally, 80 one-day-old Nicholas select commercial poults were obtained from Aviagen (Lewisburg, WV, USA). Following the protocols optimized in the "Pilot" project, all turkeys were raised at the Virginia Tech Turkey Farm using standard protocols (Adikari et al., 2017). Birds were raised in 12ft by 12ft rooms on a sixteen-hour light and eight-hour darkness lighting cycle. The room temperature was set to 85 F and heating lamps were used in order to maintain a viable body temperature for the first fifteen to twenty days of life. At 4 weeks old, on the assumption that the poults have developed substantial immune system and feathers, the room temperature was adjusted to 78 F. All the birds were raised on bedding of pine shavings and fed turkey starter feed and water *ad libitum*.

3.3.2 Tissue and Blood Collection

In both the “pilot” and “experimental” projects, blood was collected by brachial venipuncture on day 28 after weighing. Specifically, from each bird 1mL of whole blood was collected from the brachial vein in the wing using 25 G 5/8 needle, 5mL syringe, and 2.0 mL EDTA (100ul) coated tubes. The whole blood was stored at 4 C until ready for use at which time it was aliquoted according to the DNA isolation protocol used.

Tissue was collected from the bursa of Fabricius, thymus, and spleen of both the treatment and control group. Tissue samples were collected on site in 15mL high clarity polypropylene conical tubes with 8mL of RNAlater solution. Tissue was cut to .25g aliquots and stored in 1.25mL RNAlater in SAF-T- seal screw cap gaskets at -80° centigrade.

3.3.3 Genomic DNA isolation

Genomic DNA from whole blood was isolated using DNeasy Blood and Tissue kit (Qiagen Inc., Hilden, Germany) using a minor modification of the manufacturer’s protocol. The integrity was estimated using 2% agarose gel and the concentration of DNA was checked using Nanodrop spectrophotometer ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was eluted in 200 ul Buffer AE provided in the DNeasy Blood and Tissue kit (Qiagen inc., Hilden, Germany) and stored at -20°C until used.

3.3.4 Primer design and PCR optimization

Three genes including IL6, IL8, and NLRX1, previously reported to influence inflammation, were screened for variation. At the time of the SNP analyses for this project, there was no turkey NLRX1 gene sequence in Genbank. Therefore, a chicken sequence (Accession number NC_006111.4) was used to design primers in the molecular analyses. Primers were designed using NCBI's Primer BLAST software (Table 1).

3.3.5 Quantitative Polymerase Chain Reaction (Q-PCR)

Quantitative Polymerase Chain reaction is a common scientific practice used to amplify target DNA sequences. Amplification of these DNA sequences allows quantitative conclusions to be drawn about gene expression. More conclusive research practices like Sanger Sequencing, permits examination of Single Nucleotide Polymorphisms and their heritability as it is related to inflammatory response. Quantitative PCR uses designed oligonucleotides, primers, to amplify nucleic acids of a targeted DNA region. In this experiment PCR was performed using Geneamp PCR system 9700 (Applied Biosystems Foster City CA, USA). PCR conditions of this experiment are outlined in Table 1. After amplification, PCR products were loaded onto 2% agarose gels for band and integrity inspection (Figurer 1A-1C). Samples free of smear indicate a high likelihood for product amplification and purity, these samples were sequenced. Sequencing was performed by Virginia Tech Biocomplexity Institute using standard protocol for Sanger Sequencing. Sequences were analyzed using DNAStar Seqman Pro alignment to visualize and quantify the prevalence of Single Nucleotide Polymorphisms in genes that are involved with inflammatory response.

QPCR is not only a fundamental step in gene sequencing, but also in Real Time PCR, a technique that uses probes designed using RNA to quantify gene expression levels of target genes. Identification of specific gene expression levels will enable conclusions to be drawn about how a single base alteration, Single Nucleotide Polymorphism, effects inflammation. These experiments are a prelude to future studies of gene quantification of specific inflammatory response genes. To examine LPS effects of lethality, mortality post challenge was documented. 8 CO, 5 BBB, 1 Spanish Black (SB), and 3 MW expired as a result of LPS challenge. BLAST analysis of expressed sequence tags database identified two predicted *Meleagris gallopavo* NLR family member X1 transcript variants. Though quantification of LPS effects were not identified in this experiment, tissue was collected and stored in RNAlater for future experiments.

3.4 Results

BLAST analysis of expressed sequence tags database identified two predicted *Meleagris gallopavo* NLR family member X1 transcript variants (Accession numbers: XM_019623043.1, XM_010723679.2) that showed significant sequence similarity (96%) to the chicken NLRX1 gene (Figure 3). Primer SCP2, of the five designed primers amplified the turkey genomic DNA, verified by way of gel electrophoresis (Figure 1A). NLRX1 conservation and homology across the human, chicken, mouse, dog, cat, and zebrafish indicates a potential NLRX1 homolog for the turkey.

Primers were similarly designed for both IL6 (Accession number: NC_015016.2:29929594-29932033) and IL8 (Accession number: NC_015014.3: 16136160-16138399) (Table 2). Each Primer was designed to be 20-25bp in length and produce amplicons of 550-900 bp in length.

Primers were also designed to include overlapping regions in order to serve as verification of the amplified region (figure 2). All primers used in this research was synthesized by Invitrogen (Thermo scientific).

3.5 Discussion

(Accession numbers: XM_019623043.1, XM_010723679.2) that showed significant sequence similarity (96%) to the chicken NLRX1 gene. Primer SCP2, of the five designed primers amplified the turkey genomic DNA, verified by way of gel electrophoresis (Figure 1A). NLRX1 conservation and homology across the human, chicken, mouse, dog, cat, and zebrafish indicates a potential NLRX1 homolog for the turkey.

A total of 2,239 bp of IL8, 2,439 bp of IL 6, and 572 bp of NLRX1 were screened for SNPs. A total of 16 SNPs was identified in the IL6 gene, 6 of these SNPs are observed in both heritage and commercial strains. In the IL8 gene, a total of 5 SNPs were detected, 1 is specific to the commercial strain. Identified SNPs of the IL6 and IL8 genes were located in the non-coding regions of the genes. In the NLRX1 gene, a total of 3 SNPs were identified. SNP discovery described will aid in comparative genetic analysis studies of heritage and commercial turkeys. The current results represent the first time NLRX1 variation in an avian species has been examined.

Table 1 Primer Sequences, expected amplicon size and qPCR characteristics of IL 8, IL 6, and NLRX 1 genes

Gene	Primer Name[1]	Primer Sequences	Tm (°C)[2]	Amp.[3] (bp)
IL8	IL8P1-F (16137142)	5'-CCTCTCTTTTCCCAACCTAC-3'	56.0	597
	IL8P1-R (16137739)	5'-GTCCTTTAGAGTAGCTCTGC-3'	56.0	
	IL8P4-F(16136664)	5'-TTACAGGGCTCTCTCTTCTT-3'	56.0	434
	IL8P4-R (16137098)	5'-GATTTCAACGTTCTTGCAGT-3'	56.0	
	IL8P5-F (16137646)	5'-ATAGGCACACAAGCTCTAAG-3'	56.0	605
	IL8P5-R (16138251)	5'-TCCAACTCTATCTGCTGTCT-3'	56.0	
	IL8P9-F (16136268)	5'-CACTTGCTGAGATGTTTGTC-3'	56.0	614
	IL8P9-R (16136882)	5'-ACAACATGCTGAAGTCCTAC-3'	56.0	
NLRX1	SCP2-F (4264271)	5'-CGTCCCTTTCCTTCGCTCTTG-3'	50.0	553
	SCP2-R (4264824)	5'-GCTTTGTGCCACGTTTCAGC-3'	50.0	
IL6	IL6P2-F (29930215)	5'-CCATTCCTACCTTCCAGACAAC-3'	60.0	789
	IL6P2-R (29931004)	5-'CCCATCTCAAACAGCCTTACA-3'	60.0	
	IL6P4-F (29930906)	5-'GCTTGTAGGGACTGTTGAGATT-3'	60.0	571
	IL6P4-R (29931477)	5'-CCTCACGGTCTTCTCCATAAAC-3'	60.0	
	IL6P5-F (29929896)	5'-CTGTTCGCCTTTCAGACCTATC-3'	60.0	873
	IL6P5-R (29930768)	5'-GTTGCCTTAAGCCAGGACTATT-3'	60.0	

[1] F, forward primer; R, reverse Primer. Primer binding sites are presented in parenthesis from IL 8 *Meleagris gallopavo* Nicholas breeding stock chromosome 4 (NC_015014.2) IL 6 *Meleagris gallopavo* Nicholas breeding stock chromosome 6 (NC_015016.2) and *Gallus gallus* Red jungle fowl chromosome 24 (NC_006111.4) in Genbank.

[2]Tm (°C) represents the optimized annealing temperature at which a single amplicon of the expected size is obtained

[3]Amp. (bp), Amplicon length in base pairs (bp) of the expected amplicons based on the binding sites of the forward and reverse primers.

Table 2 Validated SNPs in five distinct turkey breeds using *Gallus gallus* Red jungle fowl chromosome 24 (NC_006111.4) as reference

Cons. Pos.[1]	Ref. Pos.[2]	Ref. Base	Called Base	SNP% [3]	Codon
428	4264686	A	G	61.29%	D:GAC -> D:Gat
460	4264718	G	A	21.73%	Non-Coding Region
469	4264727	G	A	52.17%	No-Coding Region

[1] **Consens. Pos**. (Consensus position): Position of SNP using the consensus sequence generated by Seqman Pro during sequence alignment.
[2] **Ref. Pos** (Reference Position): Position of SNP in NLRX 1 gene (NC_006111.4)
[3] **SNP %:** The percentage of sequences the SNP was found in when considering all sequences added to the alignment.

Table 3 Sequence contexts of NLRX1 single nucleotide polymorphisms (SNPs) in five distinct turkey varieties that have variation by in silico analyses of *Gallus gallus* NLRX1 gene.

Sequence context of the SNPs detected in NLRX1	Genotype	Consensus SNP Location	SNP position in NLRX1 gene sequence (Genbank Accession #: NC_006111.4)
GTG-CCCTCTTGCC(A/G)TCCTCACTGATGTCATTGAAATATAA	A/G	428	4264686
TCCTCACTGATGTCATTGAAATATAAACTAT(G/A)GGGTAAAA	G/A	460	4264718
GGGTAAAA(G/A)GAGATGCTGAGGGCTGGGAGGAGGGATGAAG	G/A	469	4264727

Table 4. Characteristics of single nucleotide polymorphisms (SNPs) identified in the IL 6 gene in commercial turkeys**.**

Gene	*Primer ID*	*Consensus Location*	*Nucleotide position*	*Sequence Context*	*Genotype*	*Genotype Frequency (%)*	*Al Freq*
IL 6	IL6P5	69	29929969	GTCGAATCGCT(G/A)TGCTACGG	G/G G/A A/A	0.9 0.01 0	p q
IL 6	IL6P5	142	29930039	GCACTGCCAC(T/G)GCCGCTGGG	T/T T/G G/G	0 0.19 0.81	p q
IL 6	IL6P2/IL6P5	416	29930307	TG-GTCCCAGC(C/A)CCTCGGTGC	C/C C/A A/A	0.37 0 0.63	p q
IL 6	IL6P2/IL6P5	641	29930531	TATAACCCTT(T/C)CTACTTTGT	T/T T/C C/C	0.71 0.24 0.05	p q
IL 6	IL6P2/IL6P5	756	29930642	TTTCAGCAAC(C/T)GAGAAGGAC	C/C C/T T/T	0.25 0.05 0.70	p q
IL 6	IL6P2	951	29930832	GAGACTGGCC(T/G)TCTGAGGAA	T/T T/G G/G	0.73 0.21 0.06	p q
IL 6	IL6P4	1128	29931003	GTTTGA-GATG(G/A)GGATGGCAG	G/G G/A A/A	0.21 0.30 0.49	p q
IL 6	IL6P4	1129	29931004	TTTGA-GATGG(G/A)GATGGCAGG	G/G G/A A/A	0.86 0 0.14	p q
IL 6	IL6P4	1204	29931079	GATACCTAAC(G/A)CACTTGTGA	G/G G/A A/A	0.27 0.26 0.47	p q

SNP specific to the sequence alignments of Nicholas select commercial breed turkeys Aviagen (Lewisburg, WV, USA).

Table 5. Characteristics of single nucleotide polymorphisms (SNPs) identified in the IL 6 gene in five heritage turkey varieties

Gene	*Primer ID*	Cnsensus Location	Nucleotide[1] position	*Sequence Context*[2]	*Genotype*	*Genotype Frequency*	*Al Freq*
IL 6	IL6P5	85	29929969	TCGAATCGCT(G/A)TGCTACGGC	G/A	0.91	p
					G/A	0.07	
					A/A	0.02	q
IL 6	IL6P5	159	29930039	GCACTGCCAC(T/G)GCCGCTGGG	T/T	0.06	p
					T/G	0.31	
					G/G	0.63	q
IL 6	IL6P5	452	29930307	GGTCCCAG-C(C/A)CCTCGGTGCT	C/C	0.51	p
					C/A	012	
					A/A	0.37	q
IL 6	IL6P5/IL6P2	803	29930642	TTTCAGCAAC(C/T)GAG-AAGGAC	C/C	0.10	p
					C/T	0.34	
					T/T	0.56	q
IL 6	IL6P2	1002	29930832	GAGACTGGCC(T/G)T-CTGAGGAA-	T/T	0.68	p
					T/G	0.29	
					G/G	0.03	q
IL 6	IL6P4	1183	29931003	GTTTGA-GATG(G/A)GG-ATG-GCAG	G/G	0.46	p
					G/A	0.20	
					A/A	0.34	q

[1]Posion of SNP identified using DNA Star Seqman Pro on the forward strand of IL 6 *Meleagirs gallopavo* Nicholas breeding stock chromosome 6 (NC_015016.2). [2]Sequence context containing SNP and flanking region, allele at the SNP locus appear in parentheses. Sequence alignments of Broad Breasted Bronze (BBB), Blue slate (SL), Spanish Black (SBL), Midget White (MW) and Royal Palm (RP).

Table 6. Characteristics of single nucleotide polymorphisms (SNPs) identified in the IL 8 gene in five distinct turkey varieties

Gene	*Primer ID*	Cnsensus Location	Nucleotide position[1]	*Sequence Context*[2]	*Genoty*
IL 8	IL8P1	40	16138068	CCTACGTTATGCTGTGGCT(G/A)TG-GGCTTTTCGCAGTCTCTC	G/G G/A A/A
IL 8	IL8P5	448	161367122	AACACCTCATGAAAAATGGG(G/A)CTTTTAATAAATTATCTTC	G/G G/A A/A
IL 8	IL8P9	435	16138068	CTAAAAGACGCAACTGATT(A/G)CATCTTTTGCAAGTAAACAC	A/A A/G G/G
IL 8	IL8P9	463	161367122	GCAAGTAAACACAAAATAC(A/G)TGGTTTTACTTTAAAAAGAC	A/A A/G G/G

[1] Positon of SNP in the *from IL 8 Meleagris gallopavo* Nicholas breeding stock chromosome *4* (NC_015014.2).

[2]Sequence context containing SNP and flanking region, allele at the SNP locus appear in parentheses. Sequence alignments of Broad Breasted Bronze (BBB), Blue slate (SL), Spanish Black (SBL), Midget White (MW) and Royal Palm (RP).

Table 7. Characteristics of single nucleotide polymorphisms (SNPs) identified in the IL 8 gene in commercial breed

Gene	*Primer ID*	Consensus Location	Nucleotide[1] position	*Sequence Context*[2]	*Genoty*
IL 8	IL6P1	40	16138068	CCTACGTTATGCTGTGGC-T(G/A)TGGGCTTTTCGCAGTCTCTC	G/G G/A A/A

1 Position of SNP in the from IL 8 *Meleagris gallopavo* Nicholas breeding stock chromosome 4 (NC_015014.2).

[2]Sequence context containing SNP and flanking region, allele at the SNP locus appear in parentheses. Sequence alignments of Nicholas select commercial breed turkeys Aviagen (Lewisburg, WV, USA).

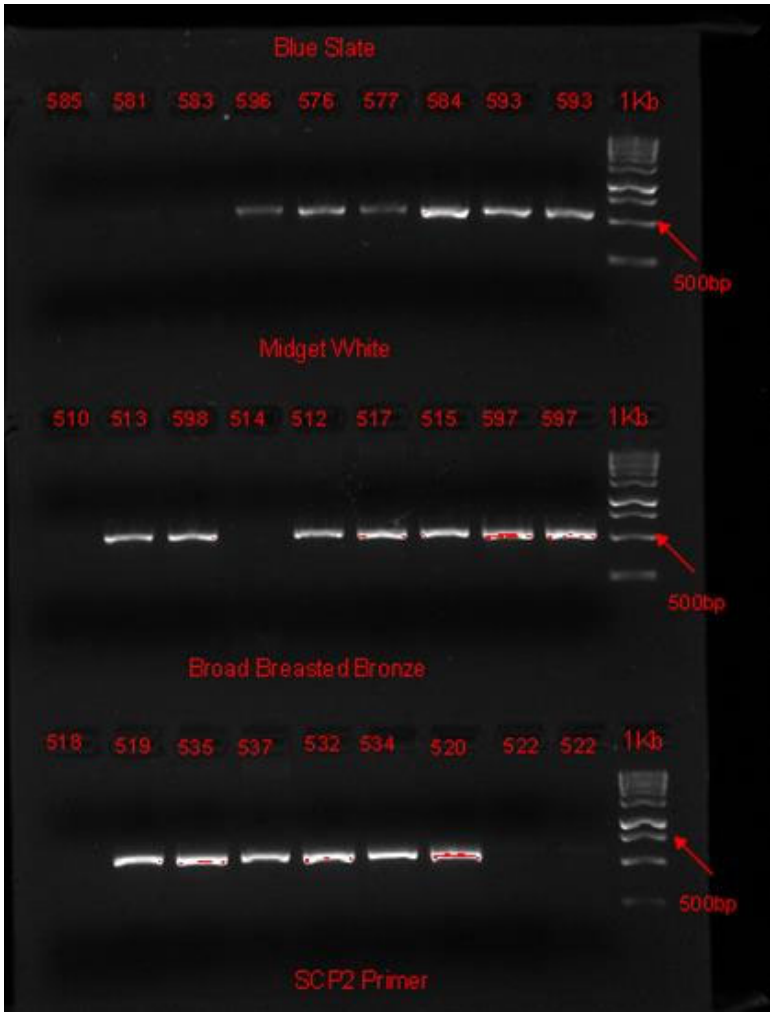


Figure 1 A: 2% Agarose gel patterns of BBB, MW, and BS turkey amplicons specific for primer SCP2 of *Gallus gallus* NLRX1 gene, Accession number NC_006111.4. The numbers on each lane represent the wing band number associated with a specific breed. The "Marker" is a 1KB DNA ladder, and the position 500 bp band is indicated.

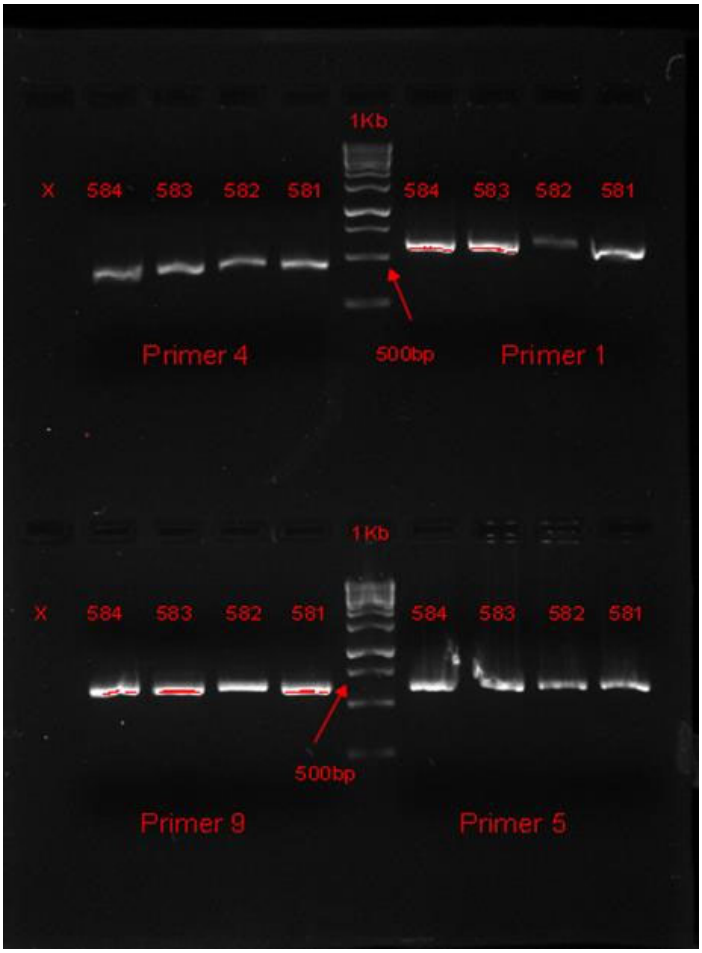


Figure 1 B: 2% Agarose gel pattern of PCR products for amplification of turkey DNA using four primers specific for IL8 gene (NC_015014.2). The three-digit number on each lane represents the wing band number of a specific bird. The marker is a 1KB ladder. The position of the 500 bp band is identified. Amplicon length: (500-600 bp).

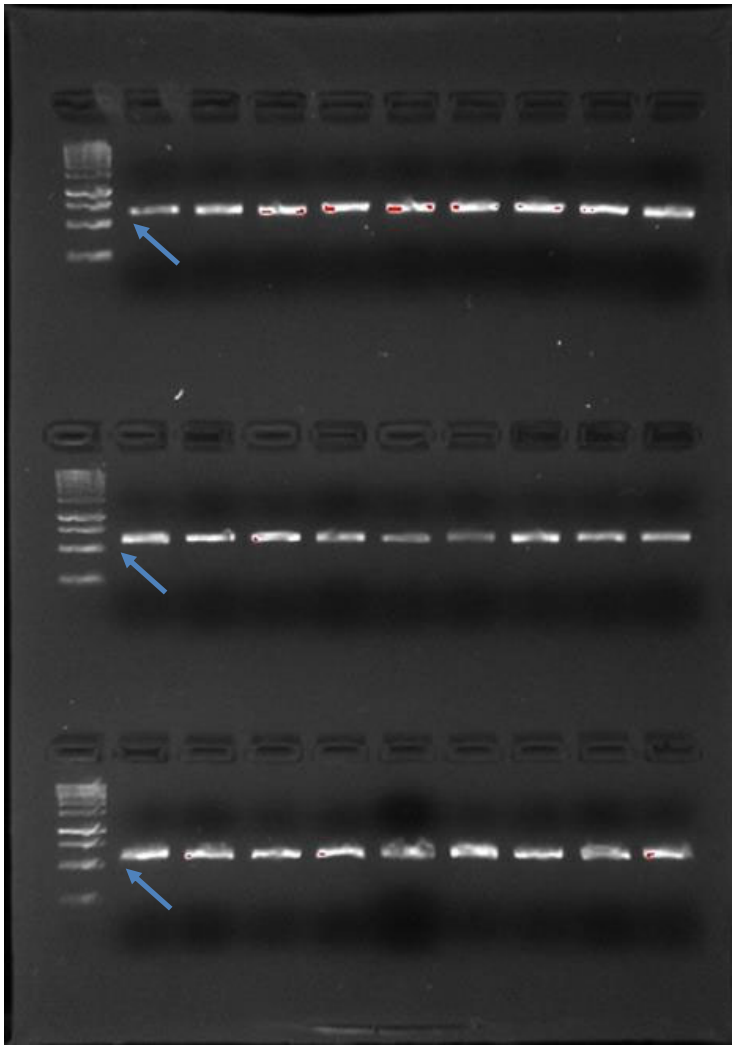
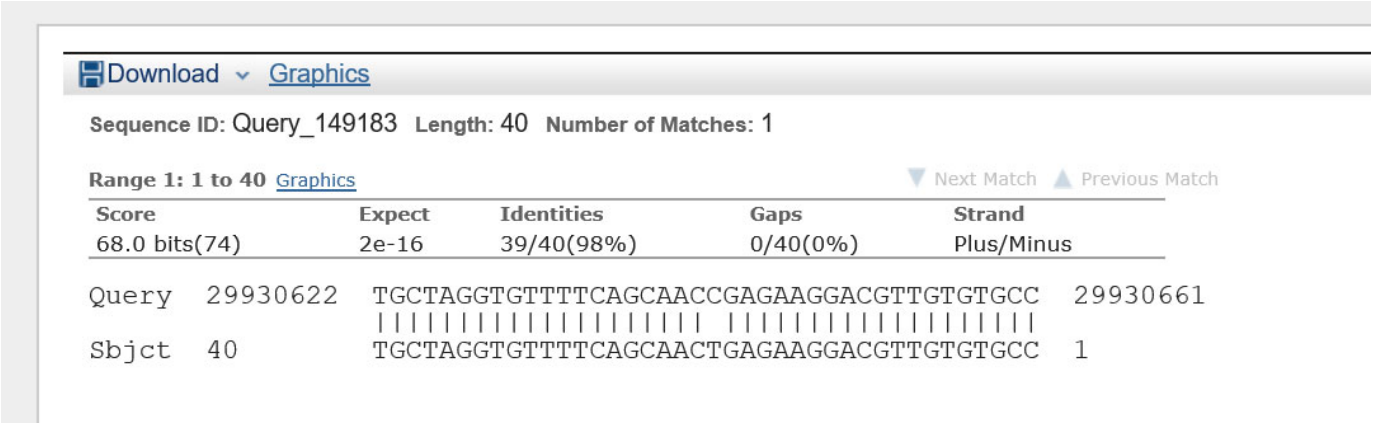


Figure 1 C: 2% Agarose gel pattern of PCR products for amplification of turkey DNA using Primer 4, specific for IL6 gene ((NC_015016.2). The three-digit number on each lane represents the wing band number of a specific bird. The marker is a 1KB ladder, blue arrow indicating the 500 bp band. Amplicon length: 571bp.

Figure 2. NCBI BLAST-2 alignment of the 40 bp flanking region containing the SNP to verify



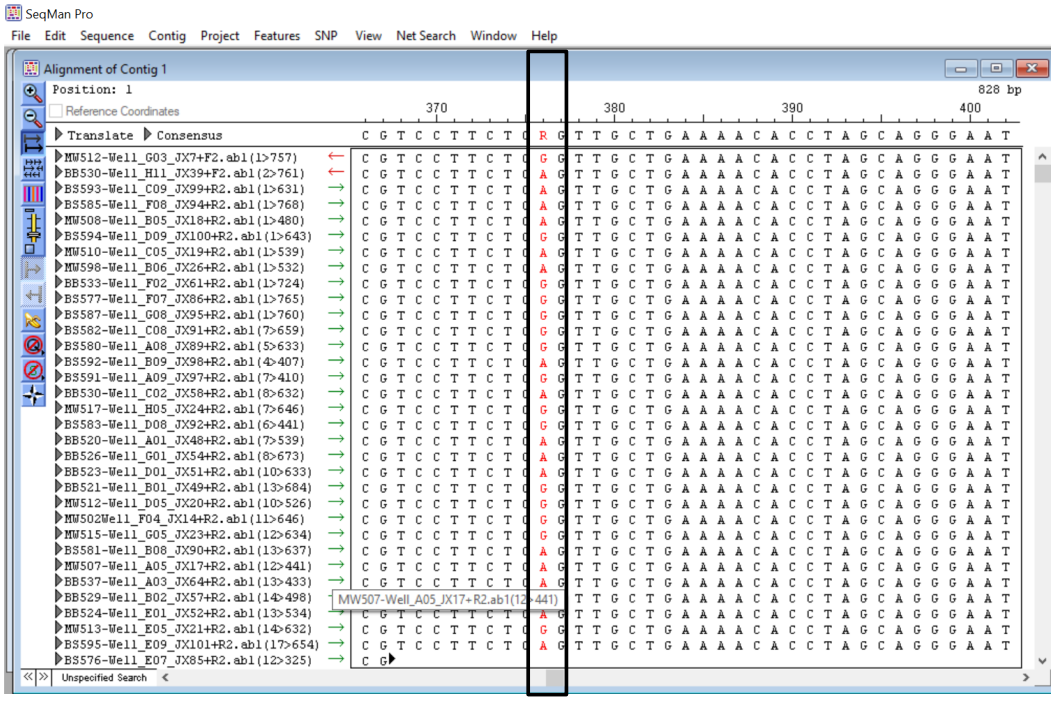
Nucleotide location 29930642 described in Table 3.1 in IL 6 *Meleagris gallopavo* Nicholas breeding stock chromosome 6 (NC_015016.2).

Query is the FASTA sequence for IL 6 *Meleagris gallopavo* Nicholas breeding stock chromosome 6 (NC_015016.2: 29929594-29932033)

Subject is the sequence of the turkey amplicon produced using primers (IL6P4) specific for 29930906-29931477 region of the IL 6 *Meleagris gallopavo* Nicholas breeding stock chromosome 6. The sequence shares a 98% sequence identity.

Figure 3. Blast-2 alignment of the sequence NCBI Blast 2. Query-NC_006111.4: 4264185-4269995. Subject: XM_010723679.2. Search NCBI database for *Gallus gallus* Red jungle fowl chromosome 24 (NC_006111.4) to validate primers designed against this gene has amplification potential of turkey genomic DNA.

A



B

Figure 4 A-B. Seqman Pro collapsed chromat of sequence of the turkey amplicon produced using primers specific for IL 6 *Meleagris gallopavo* Nicholas breeding stock chromosome 6. IL6 SNP consensus position 376 described in Table 3.1

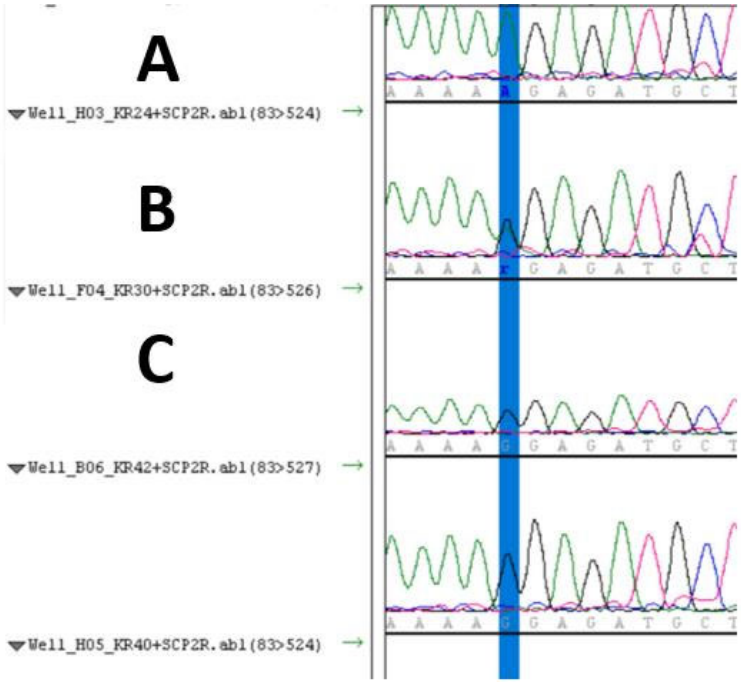


Figure 5. **SNP in NLRX1 gene (NC_006111.4) at location 4264727** Seqman Pro sequence alignment traces of BS, BBB, and MW heritage breed turkeys containing the SNP found at nucleotide position 4264727 in NLRX1 gene in the *Gallus gallus* isolate RJF whole genome shotgun sequence, Accession number NC_006111.4. G->A mutation. Located in the non-coding region.

A. SNP, A-peak observed and validated by reverse-primer sequencing of PCR product from Midget White bird 517.

B. A false positive heterozygous. The heterozygous SNP "r" was observed in the reverse trace but not observed in the forward trace of Midget White bird 515.

C. G-peak observed and validated by reverse-primer sequencing of PCR product from Midget White bird 598.

Chapter 4

SUMMARY OF THESIS

This thesis research investigated the hypothesis that DNA sequence variations exist in inflammatory response genes are can be potentially associated with phenotypic differences in the heritage turkey. The objective of this project was to search for single nucleotide polymorphisms (SNPs) in candidate inflammatory response genes IL 6, IL 8, and NLRX 1. Specific conclusions are:

1. Discovered and validated a total of 16 SNPs across three genes; IL 6, IL 8, and NLRX , characterized by nucleotide position of reference gene SNPs are as follows:
    a. IL 6 gene Heritage turkey: 29929969, 29930039, 29930307, 29930642, 29930832, 29931003
    b. IL 6 gene Commercial turkey: 29929969, 29930039, 29930307, 29930531, 29930642, 29930832, 29931003, 29931004, 29931079
    c. IL 8 gene Heritage turkey: 16138068, 161367122, 16138068, 161367122
    d. IL 8 gene Commercial turkey: 16138068
    e. NLRX1: 4264686, 4264718, 4264727

Future Work

The present study evaluated the presence of single nucleotide polymorphisms in candidate inflammatory response genes. The discovery of SNPs permit analysis of variation in both the genotypic and phenotypic capacity. Future work will include examination of mortality data.

In this specific study mortality data from the LPS challenge were not significantly different among the strains. Future research could increase the network of information known about the heritage turkey breeds, improve disease prognosis, drug efficacy, and vaccination.

1. Determine if NLRX1, IL6, and IL8 sequence and haplotype variations are associated with differences among heritage and commercial turkey strains in the inflammatory response to LPS
2. Use tissue samples collected in this study post LPS challenge to perform expression analysis in the spleen, bursa of fabricius, and lymph nodes
3. Examine the mortality effects of LPS dosage on varying heritage breeds using a larger sample size
4. Examine expected weight and determine its effects on LPS mortality

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