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Genome-Wide Analyses of Genomic Variation Regulating Differential Stress Responses in Japanese Quail

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Genome-Wide Analyses of Genomic Variation Regulating Differential Stress Responses in Japanese Quail

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ABSTRACT

As consumer demands for poultry grow, producers work to improve bird productivity in both meat and egg production. This endeavor is multifaceted, as many different factors play a part in influencing the productivity of birds, including environmental conditions, overall health, and genetic potential. One aspect controlled for in all aspects of poultry production optimization is stress management. Mitigation of stress is of primary concern to producers, as improper stress management can have many deleterious effects on chickens. Stress management requires an understanding of poultry stress responses and their physiological mechanisms, which can be more deeply understood through genomic analysis. Chapter 1 of this thesis covers common stressors in poultry production environments, methods of genomic evaluation, and two lines of Japanese quail bred as models of stress responses in poultry.

Chapter 2 covers a genome-wide analysis of two lines of Japanese quail – a high stress (HS) and a low stress (LS) line – bred for divergent blood corticosterone levels in response to restraint stress. The goal of this analysis was to identify genetic variations that could influence differential stress responses. Single Nucleotide Polymorphisms (SNPs), specifically non-synonymous SNPs resulting in amino acid changes, were chosen as the genetic mutation for analysis, and their potential effects on physiological function were determined. Genomic resequencing was performed on 24 birds from the HS and LS lines of Japanese quail using Illumina Hiseq 2 x 150 bp end read technology. From 6,364,907 SNPs observed, 2,886 unique, non-synonymous SNPs with a SNP% ≥ 0.90 and a read depth ≥ 10 were identified. Through use of Ingenuity Pathway Analysis (IPA), we identified genes affected by SNPs tied to immune responses, DNA repair, and neurological signaling.

Chapter 3 covers a genome-wide analysis of the same two lines of Japanese quail, this time with a focus on nervous and neuroendocrine system functions, as these systems control a large part of stress responses. Focus was also placed on the hypothalamic-pituitary-adrenal (HPA) axis. This axis forms an important connection between the nervous and endocrine system, and it triggers a hormonal cascade in response to stressors that leads to the release of corticosterone hormone. Using IPA, we found that a large portion of genes affected by SNPs were found with the HPA axis, many of which have been implicated in glucocorticoid receptor-dependent gene regulatory networks. Over a third of genes and molecules in the HS line and about a third of the genes and molecules in the LS line are found within the hippocampus and hypothalamus, indicating large genetic changes within the brains and neuroendocrine systems of birds in these lines.

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DEDICATION

I would like to dedicate this thesis to my parents. My mother has provided me with endless encouragement and emotional support that has shaped me into who I am today. Without the help and direction of my father, I would not have been able to pursue further education, and I will always be grateful for the unconditional support I've received from him. Finally, I would also like to dedicate this thesis to my brother, who remains the most impressive and influential individual in my life.

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CHAPTER 1

INTRODUCTION

1.1 STRESS IN POULTRY

The poultry industry is a global one that continues to grow to meet consumer demands. In order to meet these demands, producers are always working to improve bird productivity for both meat and egg production. There are a plethora of factors that influence the productivity of birds, including environmental conditions, overall health, and genetic potential. While these factors have positive impacts on production when well controlled, they can also cause deleterious effects if not properly maintained. Furthermore, the different factors surrounding production efficiency can be connected via a single common factor. Many of the issues facing producers are centered on this factor: stress.

Stress mitigation is at the forefront of many poultry producer operations. Stress strongly influences production in poultry, as it has been shown to suppress immune function, decrease egg production and feed intake, and slow down the growth rate of chickens (Liu et al., 2020; McEwen & Seeman, 1999; Shini et al., 2010). Chronic stress also causes prolonged immunosuppression that can decrease the effectiveness of vaccines, consequently increasing the occurrence of disease within flocks of chickens (Shini et al., 2010).

Combatting stress and its deleterious effects in poultry requires an understanding of the various different sources of stress affecting flocks, as well as the genetic mechanisms underlying physiological responses to stressors.

1.2 COMMON STRESSORS

1.2.1 TEMPERATURE

Consistent control of poultry production environments is a crucial aspect of optimizing production. One of the most important factors controlled for is temperature. As temperatures increase globally as a result of climate change, the effects of heat stress become a more prevalent issue in the industry. Heat stress negatively impacts production in many ways, causing decreased feed intake, lowered body weight, decreased reproductive efficiency, decreased meat quality, decreased egg production, and decreased egg quality (Deng et al., 2012; Lara & Rostagno, 2013; Mashaly et al., 2004; Sohail et al., 2012).

Heat stress has also been observed to cause increased activity of the hypothalamic-pituitary-adrenal (HPA) axis, as well as increased levels of corticosterone (Quinteiro-Filho et al., 2010). In turn, increased corticosterone has been associated with lowered weight gain and decreased spleen weight, linking increased HPA axis activity with decreased production (Post et al., 2003; Shini et al., 2008). These observations taken alongside findings of decreased lymphoid organ weights (thymus, bursa, spleen, and liver), lowered antibody responses, and lowered percentage of macrophages in birds affected by heat stress show a direct link between heat stress and immunosuppression (Bartlett & Smith, 2003).

1.2.2 NUTRITION AND HEALTH

Modern broilers have drastically improved production compared to birds of the 1950s, mostly as a result of genetic selection (Havenstein et al., 2003). Ross 708 broilers grow at a significantly faster rate than unselected birds, putting on overall body mass at 1.8 times the rate of the unselected birds. This change is even more drastic when looking at individual parts, as the Ross birds gain breast muscle mass 3.8 times faster than unselected birds (Schmidt et al., 2009). However, this improvement has not come without a cost. These fast-growing, high-yielding lines

have brought with their selection increased rates of diseases, such as ascites, wooden breast, and white striping, as well as welfare issues such as lameness and skin lesions developed through lack of activity (Bessei, 2006; Kalmar et al., 2013; Kestin et al., 2001; Knowles et al., 2008; Kuttappan et al., 2016).

Wooden breast and white striping in particular have become focal points of research in the industry as the prevalence of these diseases has increased, causing economic losses within the industry and raising welfare concerns. Wooden breast is correlated with growth rate, indicating that in selecting for production traits, broiler chickens have become inadvertently prone to muscular myopathies. One recent study focused on the effects of these diseases on stress responses of fast-growing broilers, specifically Ross 708 chickens. This study found a 3 fold increase in circulating corticosterone levels within birds affected by wooden breast and a 2.6 fold increase within birds affected by both wooden breast and white striping when compared to control birds (Kang et al., 2020). The study did not note any significant difference between birds affected solely by wooden breast when compared to birds affected by both myopathies.

This study also analyzed the activity of DNA methyltransferases within birds affected by wooden breast and white striping. DNA methyltransferases are enzymes that catalyze DNA methylation, a process that plays a major role in epigenetics by influencing gene activity (Moore et al., 2013). Despite not resulting in alterations to the nucleotide sequence, epigenetic modifications to DNA are heritable and can affect an organism's development. The study found that DNA methyltransferases, specifically DNMT1 and DNMT3b, were differentially expressed within the birds affected by myopathies when compared to control birds. These two DNA methyltransferases were downregulated in the birds affected by myopathies, indicating a

possible change to these chickens' epigenetics as it applies to stress response genes (Kang et al., 2020).

1.3 GENETIC MARKERS AND GENOMIC SEQUENCING

All of these points of control – genetic selection, welfare changes, and temperature control – address how to decrease the amount of negative stimuli the birds have to respond to. Another tangential approach towards combatting negative effectors is to address the birds' stress responses directly, therefore ensuring that when they are exposed to stressors, the impact those stressors have can be minimized. However, in order to pursue this approach, there must be an understanding of the underlying mechanisms driving these stress responses. These physiological responses are driven by the birds' genetics. Thus, any differences between individual bird's physiological responses to stress will be the result of genetic variation.

Copy Number Variations (CNVs) are one form of genetic variation between individuals that alters the diploid status of DNA via deletions and duplications (Zarrei et al., 2015). CNVs are named such as they are regions of the genome that differ in copy number, or rather the number of copies, between individuals. Compared to Single Nucleotide Polymorphisms, CNVs are quite large with some even encompassing entire genes. The duplication and deletion events that generate CNVs create unique combinations of protein-coding genes, inevitably leading to phenotypic differences between individuals (Schrider & Hahn, 2010). CNVs can be created by multiple mechanisms that either duplicate or delete lengths of DNA, which are listed here in descending order of common occurrence: non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ), replication slippage, and retrotransposition. NAHR is a mechanism for chromosomal repair in which a break in the chromosome is repaired by a

homologous sequence located in a different chromosomal position (Hastings et al., 2009). These homologous sequences are typically previously duplicated sequences of DNA (Schrider & Hahn, 2010; Turner et al., 2008). NHEJ occurs after a double-stranded break in chromosomes, requires no sequence homology, and is less accurate than homologous recombination, often resulting in deletions or insertions within these double-stranded breaks (Hastings et al., 2009; Schrider & Hahn, 2010). Replication slippage occurs during DNA replication; during replication, it is possible for the 3' primer end to move to another homologous sequence on the exposed DNA sequence before continuing synthesis. This will skip a portion of the template, resulting in a deletion (Hastings et al., 2009). Retrotransposition is unique compared to the other mechanisms in that it results in new duplications only. These duplications are generated by mRNA being reverse-transcribed into cDNA that is inserted into a new position in the genome (Schrider & Hahn, 2010). Given the nature of CNVs is that they vary in number between individuals, identification of CNVs is contingent on the use of a reference genome. Previously, research on stress related traits in poultry has been conducted using CNVs between differing lines of Japanese quail (Khatri et al., 2019).

Single Nucleotide Polymorphisms (SNPS) are another incredibly common genetic mutation that serve as good candidate genetic markers (Emara & Kim, 2003; Shastry, 2009). SNPs may be either synonymous or non-synonymous depending on where they manifest within a chromosome (Shastry, 2009). Synonymous SNPs either manifest in noncoding regions or silently within coding regions, whereas non-synonymous SNPs manifest within coding regions. Non-synonymous SNPs are further categorized as either missense or nonsense mutations. Missense mutations refer to a substitution of one amino acid for another, while nonsense mutations are

those which stop translation entirely. Both of these mutations alter the protein produced (Petersen et al., 1998). Protein alterations resulting from these mutations may then lead to alterations in phenotype. It is through this mechanism that SNPs enact phenotypic change, and the simplicity of the mechanism has led to their employment as biomarkers in studies surrounding population genetics (Syvänen, 2001). Utilizing SNP genetic markers along with next generation sequencing (NGS) and knowledge of the chicken genome, which was sequenced in 2004, our understanding of the effects of selection on phenotypic traits of different chicken lines has deepened (Boschiero et al., 2018; Hillier et al., 2004).

NGS refers to a general method of DNA sequencing. While there are a few different practices of NGS, the general workflow remains the same. First, DNA is fragmented at random before oligonucleotide adaptors are ligated to the different fragments to generate a library. This library is then clonally amplified into clusters. Following clonal amplification, sequencing is finally performed using fluorescently labelled nucleotides (Shendure & Ji, 2008). Prior to the development of NGS, genomic sequences were generated using Sanger Sequencing methods. With Sanger Sequencing, the DNA template is first amplified. The amplified template then undergoes a process of denaturation, primer annealing, and extension. However, in Sanger Sequencing, primer extension is terminated round by round using fluorescently labelled dideoxynucleotides (ddNTPs). These ddNTPs effectively mark the nucleotide at the end of the current fragment. The many fragments generated during this process, each with different nucleotide endpoints due to the binding of different ddNTPs, are then separated and read through capillary electrophoresis, generating the full DNA template sequence in the end (Shendure & Ji, 2008). This method functions at a much smaller volume than NGS, as it sequences

one template fragment at a time; NGS on the other hand, sequences many different template fragments in parallel. NGS has proven to be a large improvement in genome sequencing speed, as it takes but a single day to sequence an entire human genome using this method (Behjati & Tarpey, 2013). For the research conducted in this thesis, the Illumina NGS method was used.

1.4 QUAIL AS A MODEL IN POULTRY

Japanese quail (*Coturnix japonica*) have served as research model organisms since 1959 (Padgett & Ivey, 1959). The embryos' ease of manipulation and visualization, as well as the birds' rapid growth cycle and quickened sexual maturity have made them a preferable model organism in fields beyond poultry, including studies of vertebrate developmental biology, physiology, aging, ethology, animal learning, and disease (Huss et al., 2008).

1.4.1 THE QUAIL GENOME

Recently, the genome of the Japanese quail was sequenced in high-quality using Illumina HiSeq method (Morris et al., 2020). The genome was generated through a male individual belonging to a partially inbred line. Sequencing of the genome revealed that quail have more compact chromosomes than chickens, possibly due to energy costs linked to migratory flight (Kapusta et al., 2017; Morris et al., 2020). This project found that 78.2% of all quail genes and 91.8% of protein-coding quail genes have one-to-one ortholog genes within the chicken genome, highlighting the high genetic conservation between the two bird species.

To showcase the benefit of the newly sequenced genome, those who sequenced it revisited several genetic studies using quail as a poultry model. Upon realignment of genomic data from previous studies to this new quail genome rather than to the chicken reference

genome, 92% of reads mapped in proper pairs whereas only 55% had mapped in proper pairs previously. Using this increase in coverage, 17 new significant selection signature regions were detected than when the chicken genome was used as a reference (Morris et al., 2020). This realignment not only confirmed previous findings regarding genes involved in social behavior or in human autistic disorders, but also revealed new genes tied to these traits.

The researchers also revisited quail as a model organism in seasonal biology by performing a genome-wide analysis focused on the effects of photoperiod and temperature on the medial basal hypothalamus (MBH) region of the brain of quail, which is seen as a critical region for avian species' recognition of photoperiod. This analysis found 269 significantly differentially expressed genes, with 127 differentially expressed genes having been regulated regardless of temperature. Three core clock genes, TSHβ, DIO2 and DIO3, were analyzed in previous studies for temperature-dependent effects; in this new analysis with the newly sequenced genome, these findings were confirmed, with the exception of the effect of cold on DIO2. Additionally, they found significant photoperiod-dependent downregulation of a new gene, SLC16A2, which is a thyroid hormone-specific transporter. CRY4 is a clock gene that has been recently been a focus in research of migratory birds, and this analysis was able to observe its activity, possibly implicating quail as a good model for this field of research (Morris et al., 2020).

Immune genes within the quail genome were also investigated using the newly sequenced genome. Two families of antimicrobial peptides, cathelicidins and defensins, were found to have orthologs in the new quail genome. Orthologs of four chicken cathelicidins and 13 chicken defensins were found, leaving only one chicken defensin without an ortholog in quail.

RIG-I, TLR, and IFITM, three genes critical to influenza resistance in birds, were analyzed within the new genome. RIG-I was found to still not have an ortholog within the quail genome, while all IFITM genes were found to have orthologs in quail. All TLRs in chickens were also found to have orthologs in the quail genome, with the exception of TLR1A (Morris et al., 2020).

Through these realignments and new analyses, the newly sequenced quail genome not only proved to be a reliable and powerful template for comparative studies, but also further cemented Japanese quail as a more than sufficient model for poultry.

1.4.2 QUAIL AS A MODEL FOR STRESS RESPONSES

There is no shortage of studies into the response of *C. japonica* to different stressors. For the purposes of genomic evaluation, however, comparing differing lines of quail is much more valuable than simply analyzing one genetic line. To this end, Drs. Satterlee and Johnson sought to create a standard Japanese quail model for stress, and they succeeded in generating two differing lines of Japanese quail as documented in Satterlee and Johnson (1988). These two lines were selected based on divergent blood corticosterone levels in response to immobilization.

Drs. Satterlee and Johnson used a randomly bred population of Japanese quail as the basis of their selection program. For the first three generations of selection, quail were selected based on divergent blood corticosterone levels in response to a social stressor: the introduction of albino quail to the population (Satterlee & Johnson, 1988). The remaining nine generations of selection were based on divergent blood corticosterone levels in response to a type of restraint stress. The quail were subjected to immobilization of varying lengths of time, ranging from four to ten minutes. This change of stressor was made for methodological purposes, as consistency was an issue when attempting to expose the quail population to the albino intruders. The

efficiency of each method was analyzed by comparing the mean blood corticosterone levels of the third generation (selected via social intrusion stress) and the fourth generation (selected via immobilization stress). This comparison revealed that immobilization appeared to be the more potent stressor when measuring blood corticosterone levels. With both stressors, Drs. Satterlee and Johnson note that selection towards a high stress response was much quicker than selection towards a low stress response, possibly indicating an unknown process creating resistance to downward selection (Satterlee & Johnson, 1988).

In this thesis, these High Stress (HS) and Low Stress (LS) lines of Japanese quail were analyzed in order to broaden our understanding of the molecular processes underlining differing stress responses.

REFERENCES

Bartlett and M.O. Smith, *Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress.* Poultry Science, 2003. 82(10): p. 1580-1588.

Behjati, S. and P.S. Tarpey, *What is next generation sequencing?* Archives of disease in childhood. Education and practice edition, 2013. 98(6): p. 236-238.

Bessei, W., *Welfare of broilers: a review.* World's Poultry Science Journal, 2006. 62(3): p. 455-466.

Boschiero, C., et al., *Genome-wide characterization of genetic variants and putative regions under selection in meat and egg-type chicken lines.* BMC genomics, 2018. 19(1): p. 83-83.

Deng, W., et al., *The probiotic Bacillus licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens1.* Poultry Science, 2012. 91(3): p. 575-582.

Emara, M.G. and H. Kim, *Genetic markers and their application in poultry breeding.* Poultry Science, 2003. 82(6): p. 952-957.

Hastings, P.J., et al., *Mechanisms of change in gene copy number.* Nature reviews. Genetics, 2009. 10(8): p. 551-564.

Havenstein, G.B., P.R. Ferket, and M.A. Qureshi, *Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets.* Poultry Science, 2003. 82(10): p. 1500-1508.

Hillier, L.W., et al., *Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution.* Nature, 2004. 432(7018): p. 695-716.

Huss, D., G. Poynter, and R. Lansford, *Japanese quail (Coturnix japonica) as a laboratory animal model.* Lab Animal, 2008. 37(11): p. 513-519.

Kalmar, I.D., D. Vanrompay, and G.P.J. Janssens, *Broiler ascites syndrome: Collateral damage from efficient feed to meat conversion.* The Veterinary Journal, 2013. 197(2): p. 169-174.

Kang, S.W., et al., *Characterization of stress response involved in chicken myopathy.* General and Comparative Endocrinology, 2020. 295: p. 113526.

Kapusta, A., A. Suh, and C. Feschotte, *Dynamics of genome size evolution in birds and mammals.* Proc Natl Acad Sci U S A, 2017. 114(8): p. E1460-e1469.

Kestin, S.C., et al., *Relationships in broiler chickens between lameness, liveweight, growth rate and age.* Veterinary Record, 2001. 148(7): p. 195-197.

Khatri, B., et al., *Copy number variation study in Japanese quail associated with stress related traits using whole genome re-sequencing data.* PLOS ONE, 2019. 14(3): p. e0214543.

Knowles, T.G., et al., *Leg Disorders in Broiler Chickens: Prevalence, Risk Factors and Prevention.* PLOS ONE, 2008. 3(2): p. e1545.

Kuttappan, V.A., B.M. Hargis, and C.M. Owens, *White striping and woody breast myopathies in the modern poultry industry: a review.* Poultry Science, 2016. 95(11): p. 2724-2733.

Lara, L.J. and M.H. Rostagno, *Impact of Heat Stress on Poultry Production.* Animals, 2013. 3(2).

Liu, L., et al., *Heat stress impacts on broiler performance: a systematic review and meta-analysis.* Poultry Science, 2020. 99(11): p. 6205-6211.

Mashaly, M.M., et al., *Effect of Heat Stress on Production Parameters and Immune Responses of Commercial Laying Hens1.* Poultry Science, 2004. 83(6): p. 889-894.

McEwen, B.S. and T. Seeman, *Protective and Damaging Effects of Mediators of Stress: Elaborating and Testing the Concepts of Allostasis and Allostatic Load.* Annals of the New York Academy of Sciences, 1999. 896(1): p. 30-47.

Moore, L.D., T. Le, and G. Fan, *DNA Methylation and Its Basic Function.* Neuropsychopharmacology, 2013. 38(1): p. 23-38.

Morris, K.M., et al., *The quail genome: insights into social behaviour, seasonal biology and infectious disease response.* BMC Biology, 2020. 18(1): p. 14.

Padgett, C.A. and W.D. Ivey, *Coturnix Quail as a Laboratory Research Animal.* Science, 1959. 129(3344): p. 267.

Petersen, G.M., G. Parmigiani, and D. Thomas, *Missense Mutations in Disease Genes: A Bayesian Approach to Evaluate Causality.* The American Journal of Human Genetics, 1998. 62(6): p. 1516-1524.

Post, J., J.M. Rebel, and A.A. ter Huurne, *Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress.* Poultry Science, 2003. 82(8): p. 1313-1318.

Quinteiro-Filho, W.M., et al., *Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens.* Poultry Science, 2010. 89(9): p. 1905-1914.

Satterlee, D.G. and W.A. Johnson, *Selection of Japanese quail for contrasting blood corticosterone response to immobilization.* Poult Sci, 1988. 67(1): p. 25-32.

Schmidt, C.J., et al., *Comparison of a modern broiler line and a heritage line unselected since the 1950s.* Poultry Science, 2009. 88(12): p. 2610-2619.

Schrider, D.R. and M.W. Hahn, *Gene copy-number polymorphism in nature.* Proceedings. Biological sciences, 2010. 277(1698): p. 3213-3221.

Shastry, B.S., *SNPs: impact on gene function and phenotype.* Methods Mol Biol, 2009. 578: p. 3-22.

Shendure, J. and H. Ji, *Next-generation DNA sequencing.* Nature Biotechnology, 2008. 26(10): p. 1135-1145.

Shini, S., et al., *Biological response of chickens (Gallus gallus domesticus) induced by corticosterone and a bacterial endotoxin.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2008. 149(2): p. 324-333.

Shini, S., et al., *Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes1.* Poultry Science, 2010. 89(4): p. 841-851.

Sohail, M.U., et al., *Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress.* Poultry Science, 2012. 91(9): p. 2235-2240.

Syvänen, A.-C., *Accessing genetic variation: genotyping single nucleotide polymorphisms.* Nature Reviews Genetics, 2001. 2(12): p. 930-942.

Turner, D.J., et al., *Germline rates of de novo meiotic deletions and duplications causing several genomic disorders.* Nature genetics, 2008. 40(1): p. 90-95.

Zarrei, M., et al., *A copy number variation map of the human genome*, in *Nat Rev Genet*. 2015: England. p. 172-83.

CHAPTER 2

Identification of SNPs Associated with Stress Response Traits within High Stress and Low Stress Lines of Japanese Quail

2.1 ABSTRACT

Mitigation of stress is of great importance in poultry production, as chronic stress can affect the efficiency of production traits. Selective breeding with a focus on stress responses can be used to combat the effects of stress. To better understand the genetic mechanisms driving differences in stress responses of a selectively bred population of Japanese quail, we performed genomic resequencing on 24 birds from High Stress (HS) and Low Stress (LS) lines of Japanese quail using Illumina HiSeq 2 × 150 bp paired end read technology in order to analyze Single Nucleotide Polymorphisms (SNPs) within the genome of each line. SNPs are common mutations that can lead to genotypic and phenotypic variations in animals. Following alignment of the sequencing data to the quail genome, 6,364,907 SNPs were found across both lines of quail. 10,364 of these SNPs occurred in coding regions, from which 2886 unique, non-synonymous SNPs with a SNP% ≥ 0.90 and a read depth ≥ 10 were identified. Using Ingenuity Pathway Analysis, we identified genes affected by SNPs in pathways tied to immune responses, DNA repair, and neurological signaling. Our findings support the idea that the SNPs found within HS and LS lines of quail could direct the observed changes in phenotype.

Keywords: stress response; SNPs; quail; ingenuity pathway analysis

2.2 INTRODUCTION

Phenotypic diversities occur due to genetic variations within and between populations. Many genotypic variations arise as the result of genetic mutations. One of these mutations is known as a Single Nucleotide Polymorphism (SNP). SNPs are a common mutation and can be considered synonymous or non-synonymous (Shastry, 2009). Synonymous SNPs will either manifest within a noncoding region of a chromosome or silently within a coding region. Non-synonymous SNPs occur within coding regions of chromosomes and may be either missense or nonsense mutations. Both alter the amino acid at the point of mutation, though in differing ways. Missense mutations substitute one amino acid for another, while nonsense mutations stop translation entirely. Each type of mutation alters the protein produced (Petersen et al., 1998). These protein alterations may lead to alterations in phenotype. Due to this simple mechanism, SNPs are employed as biomarkers in studies surrounding population genetics (Syvänen, 2001).

Mitigation of stress is one of the primary goals of poultry producers. Stress has been shown to suppress the immune system's function, decrease egg production, and slow body growth in chickens (McEwen & Seeman, 1999; Shini et al., 2010). Hormones released during stress can affect the production of cytokines and chemokines by leukocytes, which have proinflammatory functions. Immunosuppression resulting from chronic stress has also been shown to decrease the effectiveness of vaccines, which would increase the occurrence of disease with a flock. Two different approaches are used for mitigating stress in chickens: improving the production environment and genetic selection for healthier stress responses (Shini et al., 2010).

This study aimed to identify genetic variations that could influence differential stress responses using a whole genome resequencing method. Two different lines of Japanese quail

bred for differential stress responses were used. SNPs resulting in amino acid changes (nonsynonymous) were identified and their potential effects on functionalities were determined.

2.3 MATERIALS AND METHODS

2.3.1 ETHICS STATEMENT

This study was conducted following the recommended guidelines for the care and use of laboratory animals for the National Institutes of Health. All procedures for animal care were performed according to the animal use protocols that was reviewed and approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocol #14012).

2.3.2 BIRDS AND DNA SEQUENCING

For the purposes of this study, two different lines of Japanese quail—a high stress (HS) line and a low stress (LS) line—have been utilized. These two lines have been selected since the 1980s for divergent blood corticosterone response to restraint-induced stress in order to serve as models for stress responses in poultry (Khatri et al., 2019). The differences between the two lines include higher sociability, lower fear, higher body weight, increased egg production, and a reduced heterophil/lymphocyte ratio in the LS line compared to the HS line. Additionally, the LS line had decreased stress-induced osteoporosis, accelerated onset of puberty, heightened male sexual activity and efficiency, and a decrease in corticosterone levels of approximately one-third when compared to the HS line (Khatri et al., 2019).

The development of HS and LS lines of Japanese quail up to the 12th generation is documented by Satterlee and Johnson (1988) (Satterlee & Johnson, 1988). Each line was selected for its plasma corticosterone response to immobilization (Khatri et al., 2019; Satterlee & Johnson,

1988). An independent random mating condition has since been used for their maintenance (Satterlee et al., 2000; Satterlee et al., 2002; Suhr et al., 2010). These research lines were shipped to the University of Arkansas at generation 44 from Louisiana State University and maintained at the Arkansas Agricultural Experimentation Station, Fayetteville, AR (Huff et al., 2013).

Bird selection and DNA sequencing was performed in direct accordance with Khatri et al. (2019) (Khatri et al., 2019). Male HS and LS birds were chosen for this study as they have a more stable physiology as a result of having fewer hormonal changes when compared to females. Blood samples (3 ml) from 20 birds each from HS and LS lines were collected. Genomic DNA was purified from each sample using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) following manufacturer's method. DNA quality was assessed using NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis. Twelve samples showing highest quality per line were pooled to represent each line. Library preparation and Illumina sequencing for the pooled DNA samples were performed by the Research Technology Support Facility at Michigan State University (East Lansing, MI, USA) using Illumina HiSeq 2 × 150 bp paired end read technology (Khatri et al., 2019).

2.3.3 DATA QUALITY AND ASSESSMENT

We used the FastQC program (v0.11.6) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to assess the quality of raw reads obtained after sequencing in form of FASTQ files. After quality assessment, the low quality reads were trimmed out using Trimmomatic tool (v0.32) (Bolger et al., 2014). The clean reads were then mapped onto the Japanese quail reference genome obtained from NCBI (Bioproject PRJNA292031; https://www.ncbi.nlm.nih.gov/genome/113) (Khatri et al., 2019).

For the reference-based genome alignment, the Seqman NGen genome sequence assembly program of the Lasergene software package (DNAStar, Madison, WI, USA) was used. Assembly parameters were as follows: File format, Binary Alignment Map (BAM); mer Size, 21; mer skip query, 2; minimum match percentage, 93; maximum gap size, 6; minimum aligned length, 35; match score, 10; mismatch penalty, 20; gap penalty, 30; SNP calculation method, diploid Bayesian; minimum SNP percentage, 5; SNP confidence threshold, 10; minimum SNP count, 2; minimum base quality score, 5. After assembly, the SeqMan Pro program of the Lasergene package (DNAStar) was used for further analyses (Jang et al., 2014; Khatri et al., 2018).

2.3.4 SNP DETECTION AND ANALYSIS

Detection and analysis of SNPs followed the methods of Khatri et al. (2018) (Khatri et al., 2018). The JMP genomics (SAS Institute Inc., Cary, NC, USA) program was used for filtering unique SNPs between the HS and LS lines of Japanese quail. Single nucleotide polymorphism (SNPs) occurring in both HS and LS lines were removed, leaving behind the unique SNPs for each line. To identify highly fixed and homozygous SNPs, the SNPs were filtered based on SNP percentages (SNP%) of SNP% ≥0.90 (for example, number of SNP = 9 of read depth = 10) were chosen. SNPs that induce non-synonymous changes in CDS (coding DNA sequences; protein coding) regions were chosen and unique SNPs in either HS or LS showing ≥10 read depths were selected as reliable SNPs. To reduce false positives, reliable SNPs chosen by criteria described above were confirmed by double-checking the initial assembly results with alignment view in SeqMan Pro program of Lasergene package (DNAStar) (Khatri et al., 2018).

2.3.5 BIOINFORMATIC PATHWAY ANALYSIS

Genes containing unique SNPs from the HS and LS lines were entered into Ingenuity Pathway Analysis (version 60467501; Ingenuity Systems; Qiagen, Redwood City, CA, USA) for variant effect analyses for functional interpretation of genes including SNPs. The top 5 canonical pathways were chosen based on p-value calculated by the Fisher's exact test, which was provided by the IPA algorithm.

2.3.6 SORTING INTOLERANT FROM TOLERANT (SIFT) PREDICTION

The SIFT prediction for amino acid changes to be deleterious was conducted following the instruction (https://sift.bii.a-star.edu.sg/; accessed on 1 March 2021). Briefly, since SIFT database for Japanese quail (Coturnix japonica) is not available, a new database for Japanese quail genome was generated using sequences in FASTA format for genome and GFF file retrieved from NCBI (https://www.ncbi.nlm.nih.gov/genome/?term=txid93934[orgn]) following instruction (https://sift.bii.a-star.edu.sg/sift4g/SIFT4G_codes.html). The newly generated database was, then, annotated with .VCF file containing SNP list causing non-synonymous mutations. The SIFT score ≤ 0.05 is considered as deleterious amino acid substitution according to the instruction (https://sift.bii.a-star.edu.sg/sift4g/AnnotateVariants.html).

2.4 RESULTS

2.4.1 GENOME RE-SEQUENCING AND DISTRIBUTION OF SNPS

In accordance with Khatri et al. (2019) (Khatri et al., 2019), we performed whole genome resequencing of pooled DNA samples from 12 birds each from HS and LS lines of the quail and produced ~250 and ~257 million reads of 150 bp respectively. Of those, ~85 and ~84 million reads

were mapped to the reference genome (NCBI/C. japonica) and their respective depth of coverage reached to ~41× and ~42× for HS and LS (Table 1) (Khatri et al., 2019).

Totally, 10,364 SNPs were uniquely found across the HS and LS line of Japanese quail with SNP% ≥ 0.90 and present within the CDS region. The HS line contained 6551 SNPs, and the LS line contained 3813 SNPs. These SNPs were further filtered to remove synonymous SNPs, leaving only those within the CDS region that would induce changes in amino acid sequences. This brought the number of SNPs to 1831 within the HS line and 1055 within the LS line. This list of SNPs was further filtered to include genes that occurred uniquely within each line. There were 980 and 539 uniquely affected genes (which were used for Ingenuity Pathway Analysis) in the HS and the LS line, respectively. The SIFT prediction tool predicted 707 and 374 amino acid changes to be deleterious substitutions in HS and LS lines, respectively. The 20 SNPs from each line with the highest read depths can be found in Table 2.2 and Table 2.3, while the occurrence of each type of impact of the unique SNPs can be found in Table 2.4. A previous study of SNPs within a chicken autoimmune vitiligo model identified similar mutations in coding regions, causing changes in amino acid sequences (Jang et al., 2014).

2.4.2 BIOINFORMATICS AND PATHWAY ANALYSIS

IPA was used to analyze connections of genes containing SNPs to canonical pathways and changes in molecular interactions.

Glycoprotein VI (GP6) Signaling, Signaling by Rho Family GTPases, Rac Signaling, Amyotrophic Lateral Sclerosis Signaling, and Synaptogenesis Signaling were the top 5 canonical pathways found to be significantly affected within the HS line (p-value < 0.01) (Figure 2.1). In the LS line, the canonical pathways found to be significantly affected (p-value < 0.05) include GP6

Signaling, Activation of Interferon-Regulatory Factor (IRF) by Cytosolic Pattern Recognition Receptors, Th17 Activation, Role of BRCA2 (BRCA2, DNA repair associated) in DNA Damage Response, and IL-15 Production (Figure 2.2).

The Synaptogenesis signaling and Amyotrophic Lateral Sclerosis signaling pathways found in the HS line are both tied to nervous system development and signaling. Affected genes within the Amyotrophic Lateral Sclerosis signaling pathway are involved in regulation of neuronal damage and degeneration, as well as motor neuron apoptosis, while affected genes within the synaptogenesis signaling pathway are tied primarily to cytoskeletal development mechanisms, such as microtubule stabilization and synaptic spine organization and development (Beffert et al., 2012; Chernoff et al., 2000; Gonzalez-Billault et al., 2001; Lee et al., 1994; Martin et al., 2005).

The GP6 Signaling pathway is found to contain SNPs in both lines of quail (Figure 2.3a,b). Each line shows SNPs within collagen, laminin, phosphoinositide 3-kinase (PI3K), and protein kinase C (PKC) gene families. In the HS line, Protein kinase C delta (PKCδ) is also affected. PKCδ is part of a signaling pathway with proinflammatory functions within the nervous system (Pierchala et al., 2004). In the LS line, talin 1 (TLN1) and inositol 1,4,5-triphosphate receptor type 1 (ITPR1) are affected. TLN1 expression mediates leukocyte adhesion to platelets (Bromberger et al., 2018). ITPR1, also known as IP3R1, is a regulator of B cell apoptosis (Assefa et al., 2004). GP6 is a member of the immunoglobulin superfamily. It is expressed within platelets and their precursor megakaryocytes, and it serves as the major signaling receptor for collagen, which leads to platelet activation and thrombus formation (Clemetson et al., 1999; Gibbins, 2004). GP6 is activated by a few different factors, one of which being low shear stress (Nieswandt et al., 2001; Roberts et al., 2004; Sakariassen et al., 1998). Shear stress is one of many factors that can drive gene expression

within endothelial cells of the circulatory system, and low shear stress is often an indication of improper blood flow (Papaioannou & Stefanadis, 2005).

The Th17 Activation pathway, which includes genes containing SNPs in the LS line, is an integral part of cellular immune response (Figure 2.4). In addition to controlling differentiation of Th17 cells, this pathway produces IL-17A, IL-17F, IL-21, and IL-22 cytokines (Chen et al., 2007; Wilson et al., 2007). Both IL-17 cytokines have pro-inflammatory functions, are important regulators of neutrophil activity, and help mount antifungal immune responses alongside IL-22 (Eyerich et al., 2010; Gaffen et al., 2006). IL-21 induces differentiation of B cells and T cells (Leonard & Wan, 2016). Similarly, the activation of IRF pathway is also associated with an immune response. This pathway is primarily involved in a host's response to viral infection, but its activation is nonetheless a result of a stimulated innate immune response (Weaver et al., 1998).

The canonical pathways observed within the HS line of quail show significant overlap, most of which is attributed to the presence of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PIK3CD), phosphatidylinositol-4,5-bisphosphate 3-kinase regulatory subunit 3 (PIK3R3), and phosphatidylinositol-4,5-bisphosphate 3-kinase regulatory subunit 6 (PIK3R6), which are genes found to be active in all five pathways listed in Figure 2.1. These three genes code for a family of molecules known as phosphoinositide 3-kinases (PI3Ks). PI3Ks are part of the immune response. These kinases carry out phosphorylation within leukocytes and regulate the activity of Rho GTPases, which are important controllers of leukocyte motility (Rickert et al., 2000).

Found within the Rac signaling, ALS signaling, signaling by Rho GTPases, and synaptogenesis signaling pathways is the gene p21-activated kinase 1 (PAK1), which encodes a

p21-activated kinase (PAK) protein. PAK proteins form the bridge between Rho GTPases and the cytoskeleton, leading to changes in cell motility and growth. This bridge is necessary for successful thrombin-mediated platelet activation (Aslan et al., 2013).

Mitogen-activated protein kinase 8 (MAPK8), neutrophil cytosolic factor 1 (NCF1), and nuclear factor kappa B subunit 1 (NFKB1) genes are found within both the signaling by Rho GTPases and Rac signaling pathways. MAPK8, also known as c-Jun N-terminal protein kinase (JNK1), belongs to the MAP kinase family and reacts to cellular stimuli to trigger early gene expression responses (Figure 2.5). In mice, this specific kinase family has been linked to T cell proliferation, differentiation, and apoptosis (Arbour et al., 2002). NCF1 is a subunit of NADPH oxidase, an enzyme complex that drives production of superoxide during an immune response (Vignais, 2002). NFKB1 is part of a protein complex known as nuclear factor kappa B (NF-κB) that acts as a transcriptional regulator integral in both innate and adaptive immune response. Issues with NF-κB activation can lead to inflammatory diseases (Liu et al., 2017).

Within the LS line, only one gene, Janus kinase 2 (JAK2), is found within multiple pathways. JAK2 is found within both the Th17 Activation and IL-15 production pathways (Figure 2.4). JAK2 codes for a kinase that is part of the JAK family of kinases. This kinase is integral for cell responses to interferon γ (IFNγ), a cytokine that is an important part of both innate and adaptive immune responses, thus cementing this gene within the immune response (Ghoreschi et al., 2009).

The LS line of quail contained multiple SNPs at various stages of a DNA damage response that responds to damage caused by ionizing radiation (Figure 2.6). Both a gene involved in the initial activation complex, Fanconi anemia complementation group F (FANCF), and genes involved in each of the end points of the pathway were found to contain SNPs. In the initial activation,

FANCF was identified. BRCA2, Bloom syndrome RecQ-like helicase (BLM), SWItch/Sucrose Non-Fermentable (SWI/SNF), and E2F were genes identified at end points of the repair process. This DNA damage response mediates homologous recombination and chromatin remodeling, while also playing a role in tumor suppression (Bochar et al., 2000; Wang et al., 2000; Yu & Baer, 2000).

Diseases and bio functions that were found to be significantly affected within the HS line (p-value < 0.05) include cancers, neurological disease, organismal injury and abnormalities, psychological disorders, and developmental disorders. In the LS line, the significantly affected (p-value < 0.05) diseases and biofunctions include cancers, and organismal injury and abnormalities, neurological disease, hematological disease, and metabolic disease.

The top physiological systems found to be significantly affected (p-value < 0.05) in development and function within the HS line include embryonic development, nervous system development and function, organismal development, tissue development, and cardiovascular system development and function. The top significantly affected (p-value < 0.05) systems within the LS line include nervous system development and function, tissue development, embryonic development, organismal development, and organ development .

As the nervous system plays an integral role in stress response signaling, further analysis of its affected developmental functions was performed. Significantly affected (p-value < 0.01) nervous system developmental functions in the HS line include branching, growth and outgrowth of neurites, neuritogenesis, dendritic growth and branching, proliferation of neuronal cells, polarization of hippocampal neurons, transport of synaptic vesicles, migration of cerebellar granule cells, and development of neuron. Significantly affected (p-value < 0.02) nervous system developmental functions in the LS line include function, maturation, and development of

neurons, development of central nervous system, myelination of nerves and the sciatic nerve, formation of inhibitory synapse and the brain, long-term potentiation of mossy fibers, and quantity of dendrites.

2.5 DISCUSSION

Our analysis found unique SNPs in pathways tied to a shear stress induced immune response, a DNA damage repair response network, and several other immune response pathways, including cytokine production and regulation and differentiation of immune cells. Additionally, pathways related to nervous system development were found to be significantly affected by SNPs.

Differing mutations within GP6 Signaling pathway could be an indication that the two lines of quail have different immune responses to changes in blood pressure (Stegner et al., 2014). It is possible these two lines may experience changes in blood pressure when exposed to stressors, but the LS line could more efficiently minimize the effects that would have affected endothelial cells. The location of SNPs in the BRCA2 DNA Damage Response is evidence that the LS line may have become better adapted at repairing cellular damage caused by stress. These two factors taken in tandem indicate that the two lines of quail primarily differ in how their bodies have adapted to control the impact of stress at the cellular level. The LS line, when exposed to the same stressors as the HS line, would not show the same negative impacts to productive traits should its physiology sufficiently compensate.

The LS line was found to have SNPs affecting multiple different pathways tied to the immune response. In particular, the presence of SNPs in the Th17 activation pathway and IL-15 production indicate a possibly direct effect on cytokine production. IL-17A, IL-17F, IL-21, and IL-22 are considered signature cytokines within the immune system with various functions each, including

regulation and differentiation of immune cells. Changes in regulatory T-cell activity may protect the cells of the LS line of quail, as these cells suppress many harmful effects of immune responses (Shini et al., 2010).

The HS line also contained SNPs in genes related to immune function across multiple canonical pathways. GP6 signaling, Rho GTPase signaling, and Rac signaling pathways were found to be directly connected transitively through SNP mutations of PI3K genes and Rho GTPases. The connections of these kinases to leukocytes, as well as each pathway's connection to inflammatory responses, once again show an association between the immune system and the SNP mutations in these birds.

Both lines of quail were selected based on their response to restraint stress, which is a psychological stressor. Psychological stressors are detected by the nervous system, which can, in turn, activate hormonal responses within the endocrine system. The hypothalamo-pituitary-adrenal (HPA) axis is one example of this type of response. The HPA axis is a driving force behind the endocrine response to stress, as it initiates a hormone cascade leading to the release of corticosterone hormone (Kuenzel & Jurkevich, 2010). The HS and LS lines of Japanese quail show divergent blood corticosterone levels, indicating a lasting effect of genetic selection on a significant change in the response of the neuroendocrine system to stress. Our findings show that SNPs are present in genes throughout several nervous system developmental networks, though the extent to which these SNPs could alter nervous system function still remains unclear. Further studies are needed to understand the relationship of these genes containing SNPs to nervous system development associated with the differential stress responses observed in the

HS and LS lines of Japanese quail. Comparison of SNPs from the brain transcriptome could also provide more insight into the effect of stress on neuronal gene expression.

2.6 CONCLUSIONS

Overall, these findings seem to support the idea that the higher body weight and increased egg production observed in the LS line of Japanese quail may be attributed to differing neuronal development and a stronger protection from stress induced immune responses. When paired with recent findings that have found CNVs affected genes within nervous and endocrine system development, humoral and cell-mediated immune responses, and various metabolisms, our results support the theory that genotypic variation leads to the observed phenotypic diversity between the HS and LS lines of Japanese quail (Khatri et al., 2019). Additionally, more research directed towards the immune responses and cellular activity of each line of quail could deepen our understanding of the effects chronic stress may have on differently adapted production birds.

REFERENCES

Arbour, N.; Naniche, D.; Homann, D.; Davis, R.J.; Flavell, R.A.; Oldstone, M.B. c-Jun NH2-terminal kinase (JNK)1 and JNK2 signaling pathways have divergent Roles in CD8+ T Cell–mediated antiviral immunity. *J. Exp. Med.* 2002, *195*, 801–810.

Aslan, J.E.; Baker, S.M.; Loren, C.P.; Haley, K.M.; Itakura, A.; Pang, J.; Greenberg, D.L.; David, L.L.; Manser, E.; Chernoff, J.; et al. The PAK system links Rho GTPase signaling to thrombin-mediated platelet activation. *Am. J. Physiol. Cell Physiol.* 2013, *305*, C519–C528.

Assefa, Z.; Bultynck, G.; Szlufcik, K.; Kasri, N.N.; Vermassen, E.; Goris, J.; Missiaen, L.; Callewaert, G.; Parys, J.B.; De Smedt, H. Caspase-3-induced truncation of type 1 inositol trisphosphate receptor accelerates apoptotic cell death and induces inositol trisphosphate-independent calcium release during apoptosis. *J. Biol. Chem.* 2004, *279*, 43227–43236.

Beffert, U.; Dillon, G.M.; Sullivan, J.M.; Stuart, C.E.; Gilbert, J.P.; Kambouris, J.A.; Ho, A. Microtubule plus-end tracking protein CLASP2 regulates neuronal polarity and synaptic function. *J. Neurosci.* 2012, *32*, 13906.

Bochar, D.A.; Wang, L.; Beniya, H.; Kinev, A.; Xue, Y.; Lane, W.S.; Wang, W.; Kashanchi, F.; Shiekhattar, R. BRCA1 is associated with a human SWI/SNF-related complex: Linking chromatin remodeling to breast cancer. *Cell* 2000, *102*, 257–265.

Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 2014, *30*, 2114–2120, doi:10.1093/bioinformatics/btu170.

Bromberger, T.; Klapproth, S.; Rohwedder, I.; Zhu, L.; Mittmann, L.; Reichel, C.A.; Sperandio, M.; Qin, J.; Moser, M. Direct Rap1/Talin1 interaction regulates platelet and neutrophil integrin activity in mice. *Blood* 2018, *132*, 2754–2762.

Chen, Z.; Laurence, A.; O'Shea, J.J. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. *Semin. Immunol.* 2007, *19*, 400–408.

Chernoff, J.; Vadlamudi, R.K.; Adam, L.; Wang, R.-A.; Mandal, M.; Nguyen, D.; Sahin, A.; Hung, M.-C.; Kumar, R. Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. *J. Biol. Chem.* 2000, *275*, 36238–36244.

Clemetson, J.M.; Polgar, J.; Magnenat, E.; Wells, T.N.; Clemetson, K.J. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to fcαr and the natural killer receptors. *J. Biol. Chem.* 1999, *274*, 29019–29024.

Eyerich, S.; Eyerich, K.; Cavani, A.; Schmidt-Weber, C. IL-17 and IL-22: Siblings, not twins. *Trends Immunol.* 2010, *31*, 354–361.

Gaffen, S.L.; Kramer, J.M.; Yu, J.J.; Shen, F. The IL-17 cytokine family. In *Vitamins and Hormones*; Academic Press: Cambridge, MA, USA, 2006; pp. 255–282.

Ghoreschi, K.; Laurence, A.; O'Shea, J.J. Janus kinases in immune cell signaling. *Immunol. Rev.* 2009, *228*, 273–287.

Gibbins, J.M. Platelet adhesion signalling and the regulation of thrombus formation. *J. Cell Sci.* 2004, *117*, 3415–3425.

Gonzalez-Billault, C.; Avila, J.; Cáceres, A. Evidence for the role of MAP1B in axon formation. *Mol. Biol. Cell* 2001, *12*, 2087–2098.

Huff, G.R.; Huff, W.E.; Wesley, I.V.; Anthony, N.B.; Satterlee, D.G. Response of restraint stress-selected lines of Japanese quail to heat stress and Escherichia coli challenge. *Poult. Sci.* 2013, *92*, 603–611.

Jang, H.-M.; Erf, G.F.; Rowland, K.C.; Kong, B.-W. Genome resequencing and bioinformatic analysis of SNP containing candidate genes in the autoimmune vitiligo Smyth line chicken model. *BMC Genom.* 2014, *15*, 707.

Khatri, B.; Hayden, A.M.; Anthony, N.B.; Kong, B.C. Whole Genome Resequencing of Arkansas Progressor and Regressor Line Chickens to Identify SNPs Associated with Tumor Regression. *Genes* 2018, *9*, 512.

Khatri, B.; Kang, S.; Shouse, S.; Anthony, N.; Kuenzel, W.; Kong, B.C. Copy number variation study in Japanese quail associated with stress related traits using whole genome re-sequencing data. *PLoS ONE* 2019, *14*, e0214543.

Kuenzel, W.J.; Jurkevich, A. Molecular neuroendocrine events during stress in poultry. *Poult. Sci.* 2010, *89*, 832–840.

Lee, M.K.; Marszalek, J.R.; Cleveland, D.W. A mutant neurofilament subunit causes massive, selective motor neuron death: Implications for the pathogenesis of human motor neuron disease. *Neuron* 1994, *13*, 975–988.

Leonard, W.J.; Wan, C.-K. IL-21 Signaling in Immunity. *F1000Research* 2016, *5*, 224.

Liu, T.; Zhang, L.; Joo, D.; Sun, S.-C. NF-κB signaling in inflammation. *Signal Transduct. Target. Ther.* 2017, *2*, 17023.

Martin, L.J.; Chen, K.; Liu, Z. Adult motor neuron apoptosis is mediated by nitric oxide and fas death receptor linked by dna damage and p53 activation. *J. Neurosci.* 2005, *25*, 6449–6459.

McEwen, B.S.; Seeman, T. Protective and damaging effects of mediators of stress: Elaborating and testing the concepts of allostasis and allostatic load. *Ann. N. Y. Acad. Sci.* 1999, *896*, 30–47.

Nieswandt, B.; Bergmeier, W.; Eckly, A.; Schulte, V.; Ohlmann, P.; Cazenave, J.-P.; Zirngibl, H.; Offermanns, S.; Gachet, C. Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. *Blood* 2001, *97*, 3829–3835.

Papaioannou, T.G.; Stefanadis, C. Vascular wall shear stress: Basic principles and methods. *Hell. J. Cardiol.* 2005, *46*, 9–15.

Petersen, G.M.; Parmigiani, G.; Thomas, D. Missense mutations in disease genes: a bayesian approach to evaluate causality. *Am. J. Hum. Genet.* 1998, *62*, 1516–1524.

Pierchala, B.A.; Ahrens, R.C.; Paden, A.J.; Johnson, E.M. Nerve growth factor promotes the survival of sympathetic neurons through the cooperative function of the protein kinase C and phosphatidylinositol 3-kinase pathways. *J. Biol. Chem.* 2004, *279*, 27986–27993.

Rickert, P.; Weiner, O.D.; Wang, F.; Bourne, H.R.; Servant, G. Leukocytes navigate by compass: Roles of PI3Kgamma and its lipid products. *Trends Cell Biol.* 2000, *10*, 466–473.

Roberts, D.E.; McNicol, A.; Bose, R. Mechanism of collagen activation in human platelets. *J. Biol. Chem.* 2004, *279*, 19421–19430.

Sakariassen, K.S.; Holme, P.A.; Ørvim, U.; Barstad, R.M.; Solum, N.O.; Brosstad, F.R. Shear-induced platelet activation and platelet microparticle formation in native human blood. *Thromb. Res.* 1998, *92*, S33–S41.

Satterlee, D.G.; Cadd, G.G.; Jones, R.B. Developmental instability in japanese quail genetically selected for contrasting adrenocortical responsiveness. *Poult. Sci.* 2000, *79*, 1710–1714.

Satterlee, D.G.; Johnson, W.A. Selection of japanese quail for contrasting blood corticosterone response to immobilization. *Poult. Sci.* 1988, *67*, 25–32.

Satterlee, D.G.; Marin, R.H.; Jonest, R.B. Selection of Japanese quail for reduced adrenocortical responsiveness accelerates puberty in males. *Poult. Sci.* 2002, *81*, 1071–1076.

Shastry, B.S. SNPs: Impact on gene function and phenotype. *Methods Mol. Biol.* 2009, *578*, 3–22.

Shini, S.; Huff, G.R.; Shini, A.; Kaiser, P. Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. *Poult. Sci.* 2010, *89*, 841–851.

Stegner, D.; Haining, E.J.; Nieswandt, B. Targeting glycoprotein vi and the immunoreceptor tyrosine-based activation motif signaling pathway. *Arter. Thromb. Vasc. Biol.* 2014, *34*, 1615–1620.

Suhr, C.L.; Schmidt, J.B.; Treese, S.T.; Satterlee, D.G. Short-term spatial memory responses in aged Japanese quail selected for divergent adrenocortical stress re-sponsiveness. *Poult. Sci.* 2010, *89*, 633–642.

Syvänen, A.-C. Accessing genetic variation: Genotyping single nucleotide polymorphisms. *Nat. Rev. Genet.* 2001, *2*, 930–942.

Vignais, P.V. The superoxide-generating NADPH oxidase: Structural aspects and activation mechanism. *Cell. Mol. Life Sci.* 2002, *59*, 1428–1459.

Wang, Y.; Cortez, D.; Yazdi, P.; Neff, N.; Elledge, S.J.; Qin, J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.* 2000, *14*, 927–939.

Weaver, B.K.; Kumar, K.P.; Reich, N.C. Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. *Mol. Cell. Biol.* 1998, *18*, 1359–1368.

Wilson, N.J.; Boniface, K.; Chan, J.R.; McKenzie, B.S.; Blumenschein, W.M.; Mattson, J.D.; Basham, B.; Smith, K.J.; Chen, T.; Morel, F.; et al. Development, cytokine profile and function of human interleukin 17–producing helper T cells. *Nat. Immunol.* 2007, *8*, 950–957.

Yu, X.; Baer, R. Nuclear localization and cell cycle-specific expression of CtIP, a protein that associates with the BRCA1 tumor suppressor. *J. Biol. Chem.* 2000, *275*, 18541–18549.

APPENDIX

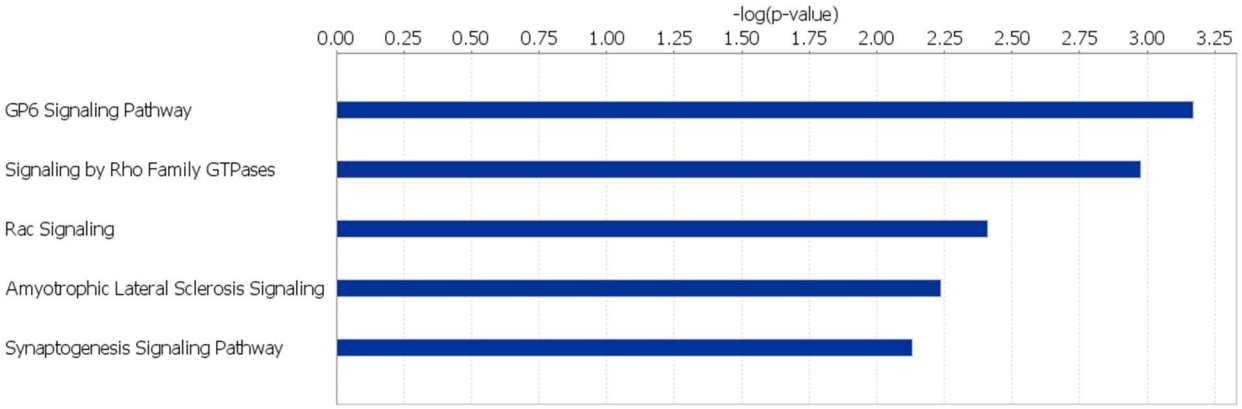


Figure 2.1 Canonical pathways affected by unique SNPs in HS line of Japanese quail.

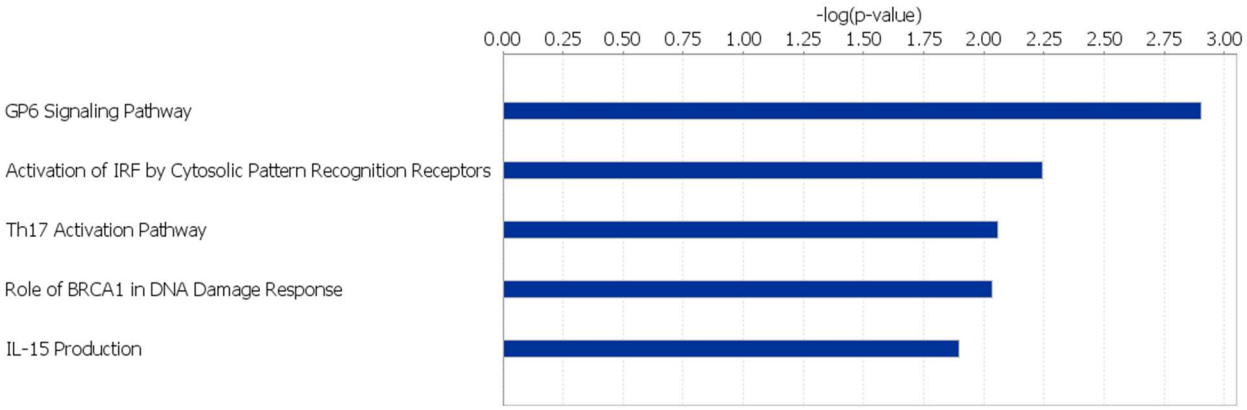
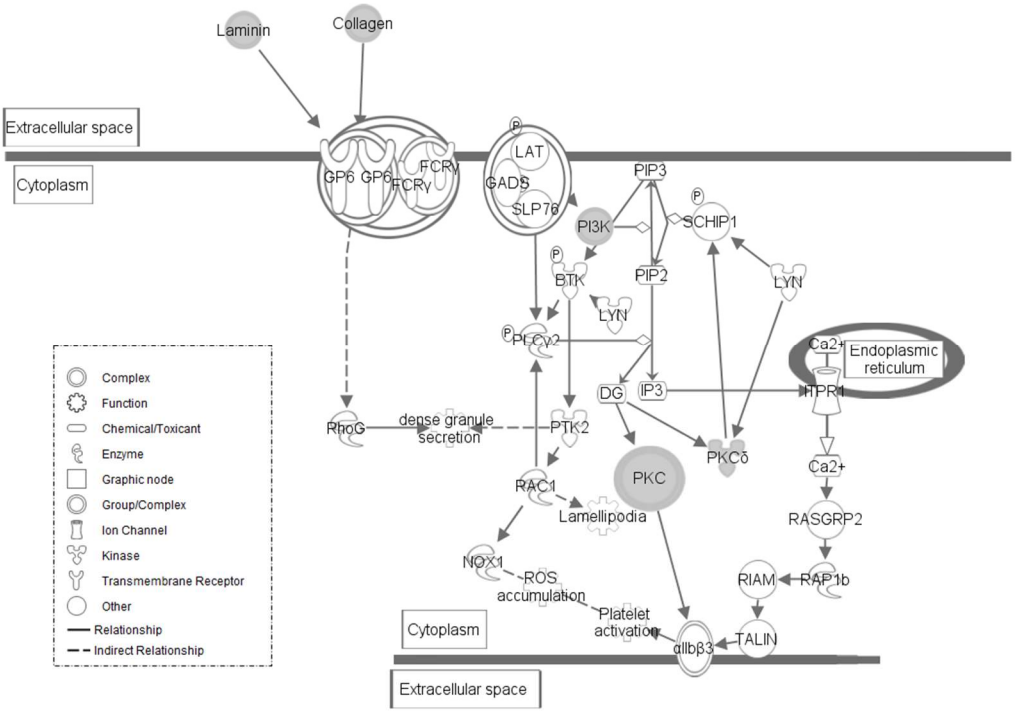
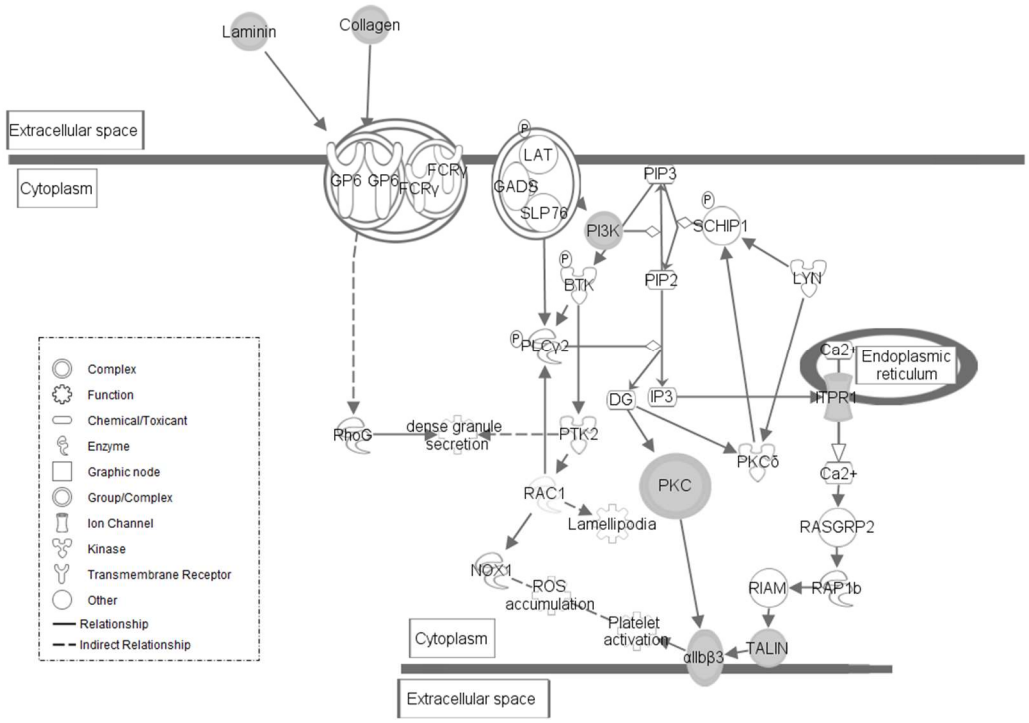


Figure 2.2 Canonical pathways affected by unique SNPs in LS line of Japenese quail.



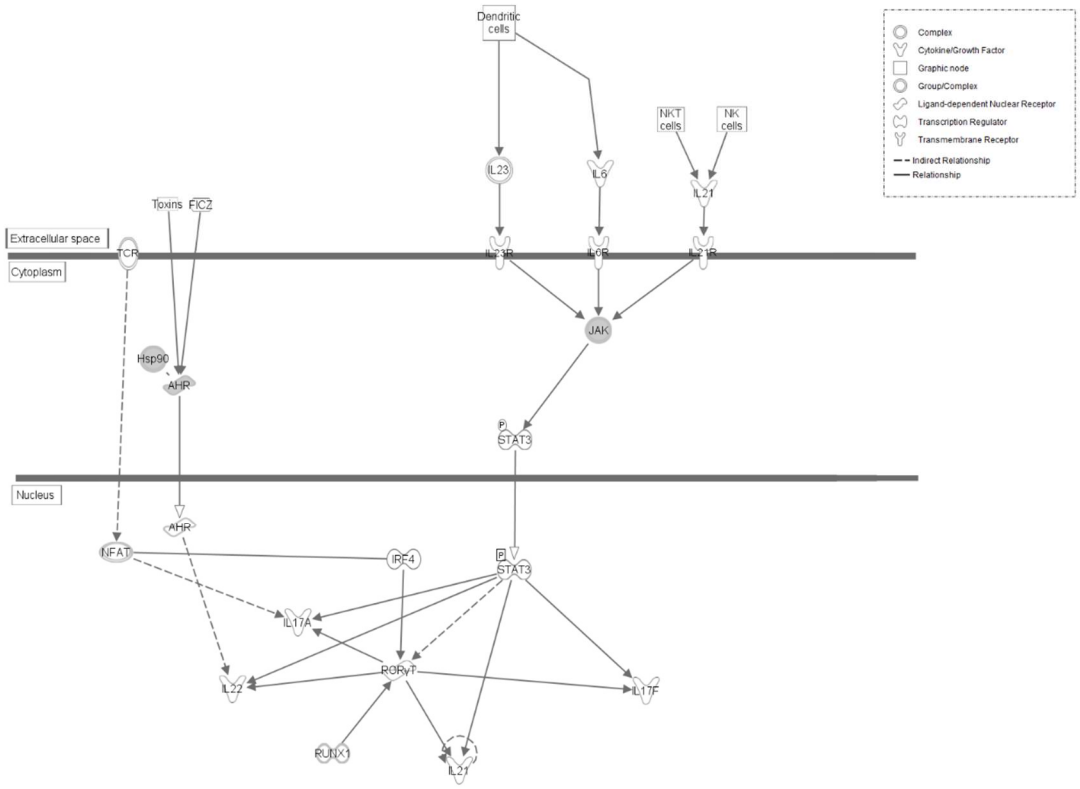
(a)

Figure 2.3 (a) GP6 Signaling Pathway in HS Line of Japanese Quail. Molecular interactions within the given pathway are shown. Grayed symbols indicate genes containing SNP mutations while white symbols indicate genes that do not contain SNPs but are functionally associated within the pathway. Each symbol represents the given molecule's function.



(b)

Figure 2.3 (b) GP6 Signaling in LS Line of Japanese Quail. Molecular interactions and symbols within the pathway are the same as described in Figure 2.3a.



a

Figure 2.4 Th17 Activation Pathway in LS Line of Japanese Quail. Molecular interactions and symbols within the pathway are the same as described in Figure 2.3.

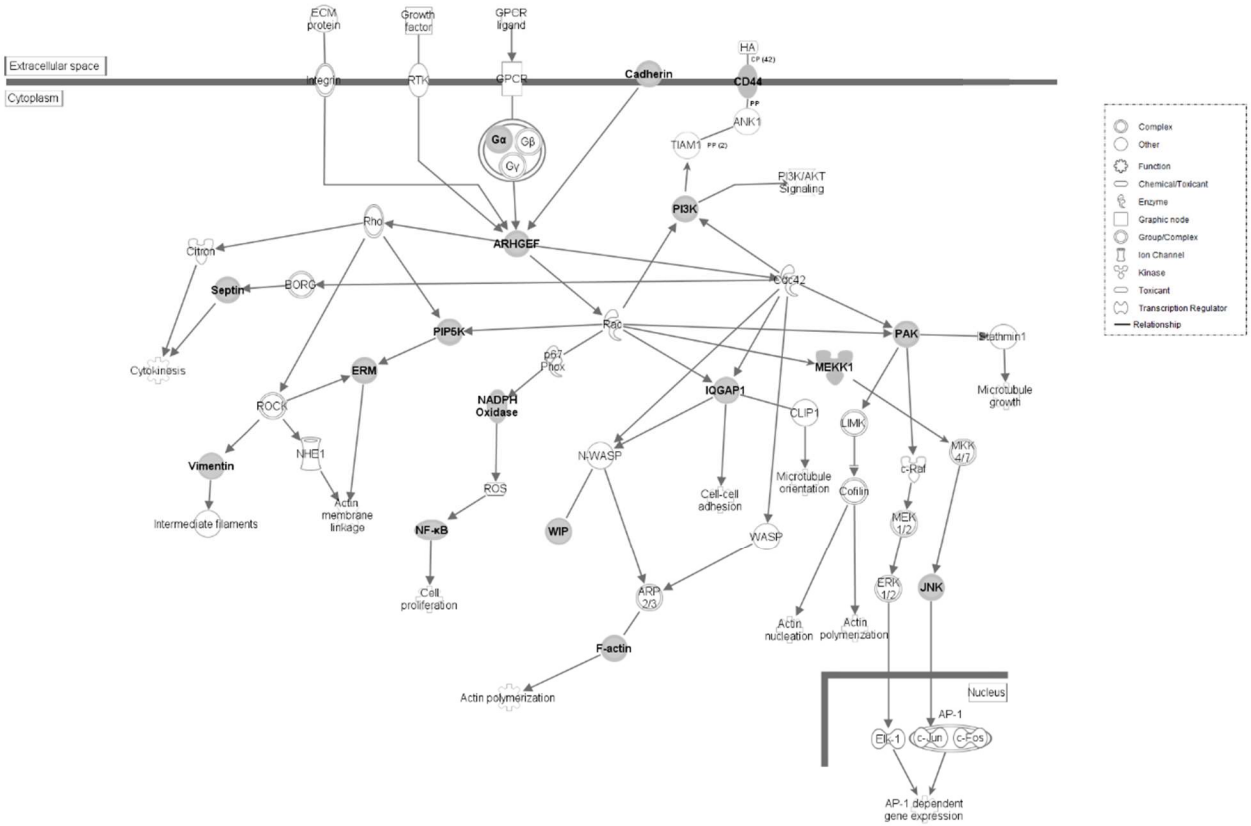


Figure 2.5 Signaling by Rho Family GTPases and Rac Signaling Pathways in HS Line of Japanese Quail. Molecular interaction and symbols are the same as described in Figure 2.3.

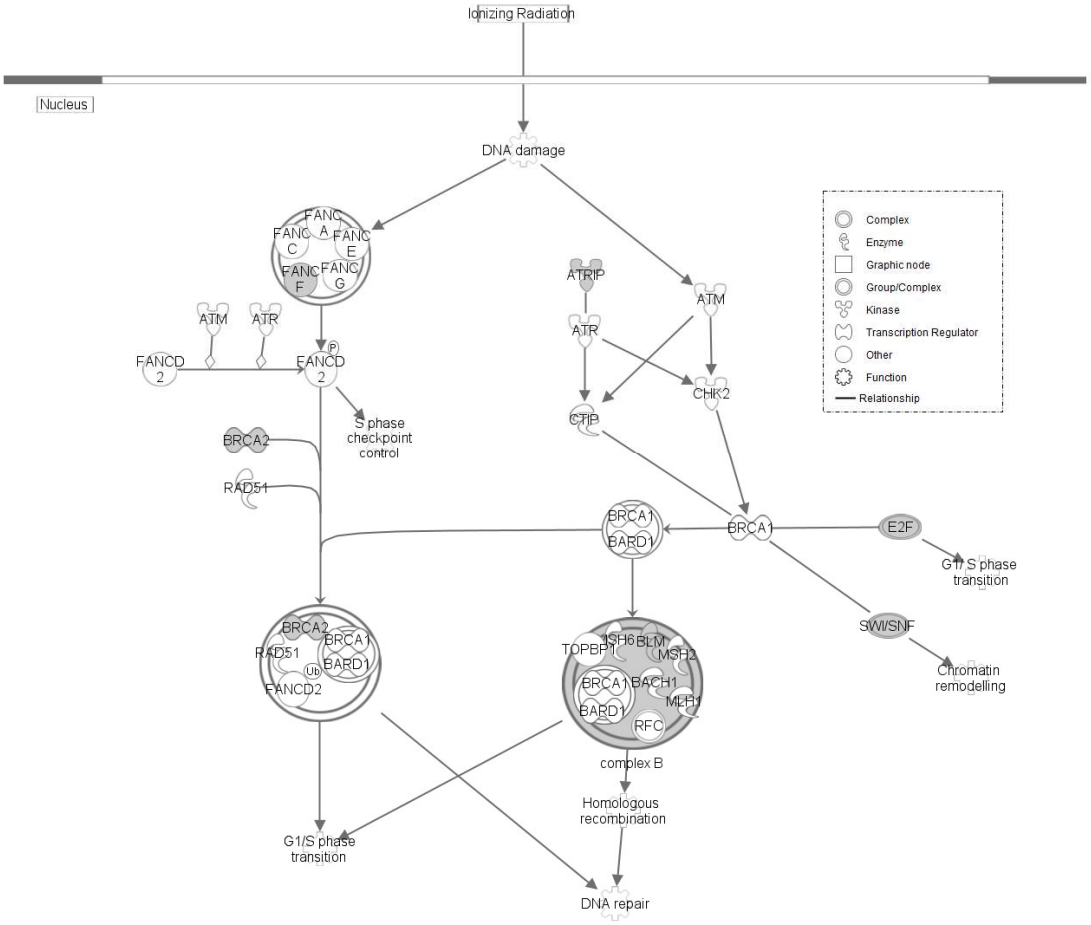


Figure 2.6 Role of BRCA in DNA Damage Response within LS Line of Japanese quail. Molecular interaction and symbols are the same as described in Figure 2.3.

Table 2.1 Sequencing and mapping data of high and low stress lines of Japanese quail.

Line	Number of SNPs	Coverage
HS	3,492,469	41.45x
LS	2,872,438	42.59x

Table 2.2 Top 20 SNPs with the highest read depths in HS Line.

Line	Chr	Ref Pos	Ref Base	Called Base	Impact	SNP %	Feature Name	Amino Acid Change
HS	21	5131174	AC	GT	Non-synonymous	0.99	LOC107323251	p.W177R
HS	22	2387923	G	A	Non-synonymous	1	R3HCC1	p.P613S
HS	8	9560343	-	GCTCAAACAC	Frameshift	1	RNPC3	p.N316fs
HS	5	34813334	AT	GC	Non-synonymous	1	TTLL5	p.S882P
HS	8	21795021	A	T	Non-synonymous	1	ZCCHC11	p.F759L
HS	12	14314737	A	C	Non-synonymous	0.94	CNTN3	p.H322Q
HS	1	1.5E+08	A	G	Non-synonymous	1	LACC1	p.T367A
HS	3	91429204	T	A	Non-synonymous	0.94	LOC107312240	p.N682Y
HS	7	15772589	A	G	Non-synonymous	1	LOC107316692	p.S386G
HS	4	56633029	CC	TT	Non-synonymous	1	PCM1	p.G617N
HS	2	31086012	T	C	Non-synonymous	0.91	PLEKHA8	p.L435S
HS	1	70128156	C	G	No-stop	1	EPHB6	p..816Sext.?
HS	21	2172350	T	C	Non-synonymous	1	LOC107323499	p.Q240R
HS	3	60212158	T	C	Non-synonymous	1	REV3L	p.S1447P
HS	12	8367256	C	G	Non-synonymous	1	XPC	p.V104L
HS	7	18690075	T	G	Non-synonymous	0.97	C7H2orf76	p.L4F
HS	11	9853879	G	A	Non-synonymous	0.93	CCDC79	p.A274T
HS	3	43357143	A	C	Non-synonymous	1	KATNA1	p.D237E
HS	2	68425475	A	G	Non-synonymous	1	KIF13A	p.W1936R
HS	17	6369984	C	G	Non-synonymous	0.95	INPP5E	p.F248L

Table 2.3 Top 20 SNPs with the highest read depths in LS Line.

Line	Chr	Ref Pos	Ref Base	Called Base	Impact	SNP %	Feature Name	Amino Acid Change
LS	2	32838673	A	G	Non-synonymous	1	PP2D1	p.V539A
LS	9	8491817	C	T	Nonsense	1	TRIP12	p.W1658.
LS	7	9557668	G	A	Non-synonymous	1	C7H2orf69	p.V322I
LS	4	2143170	T	C	Non-synonymous	0.91	ACRC	p.D81G
LS	1	110870115	T	C	Non-synonymous	1	EGFL6	p.K134R
LS	1	53603348	C	A	Non-synonymous	0.96	PKP2	p.V614L
LS	1	64715297	GC	AT	Non-synonymous	0.93	LOC107317569	p.R134H
LS	6	13674979	A	G	Non-synonymous	0.98	USP54	p.E1083G
LS	18	3018090	-	TTG	Inframe insertion	0.93	HEXDC	p.S392del
LS	1	120607492	A	C	Non-synonymous	0.98	LOC107306797	p.K125T
LS	11	8576282	C	T	Non-synonymous	0.93	TDRD12	p.S265F
LS	4	30750477	C	G	Non-synonymous	0.95	FRAS1	p.R550T
LS	Z	44293804	G	A	Non-synonymous	1	PDZPH1P	p.A46T
LS	1	19982878	A	T	Non-synonymous	0.98	IQUB	p.S416T
LS	1	72163526	A	G	Non-synonymous	1	LOC107319872	p.I597V
LS	7	29069125	A	T	Non-synonymous	1	LRP1B	p.D2179E
LS	13	4497924	A	G	Non-synonymous	1	RUFY1	p.T553A
LS	3	69683334	A	T	Non-synonymous	1	SMIM8	p.I10N
LS	LGE64	150066	T	C	Non-synonymous	1	LOC107325885	p.V16A
LS	4	66305765	C	T	Non-synonymous	0.97	RBPJ	p.R10Q

Table 2.4 Occurrence of unique SNP impacts in HS and LS lines of Japanese quail.

Impact	Number of SNPs	Percentage
HS Line:		
Frameshift	46	0.702%
Inframe deletion, conservative	5	0.076%
Inframe deletion, disruptive	5	0.076%
Inframe insertion, conservative	2	0.031%
Inframe insertion, disruptive	4	0.061%
Nonsense	8	0.122%
Non-synonymous	1746	26.652%
No-start	11	0.168%
No-stop	11	0.061%
Synonymous	4720	72.050%
Total	6551	
LS Line:		
Frameshift	33	0.87%
Inframe deletion, conservative	4	0.11%
Inframe deletion, disruptive	3	0.08%
Inframe insertion, conservative	0	0.00%
Inframe insertion, disruptive	4	0.11%
Nonsense	5	0.13%
Non-synonymous	1004	26.56%
No-start	2	0.05%
No-stop	0	0.00%
Synonymous	2758	72.96%
Total	3780	

CHAPTER 3

Genome-Wide SNPs Regulating Nervous System Functions Associated with Stress Response Traits in High and Low Stress Lines of Japanese Quail

3.1 ABSTRACT

Stress management is an important part of maintaining production efficiency in poultry, and improving management techniques is reliant on our understanding of physiological stress responses. The nervous and neuroendocrine systems play important roles in these stress responses. Single Nucleotide Polymorphisms (SNPs), common genetic mutations responsible for genotypic and phenotypic diversity in animals, can be used as biomarkers for detection of genetic alterations. In this chapter, Ingenuity Pathway Analysis was used to more deeply understand neural development, neurological signaling, and the neuroendocrine system using mutant genes retaining non-synonymous mutations.

Keywords: stress response; SNPs; quail; nervous system; ingenuity pathway analysis

3.2 INTRODUCTION

In poultry production environments, birds are subjected to varying levels of different stressors that ultimately negatively impact many production traits including body weight gain, feed intake, and egg production, as well as leading to inhibition of the immune system's function (Liu et al., 2020; Shini et al., 2010). Consequently, controlling the stress response of these birds is of primary concern within the industry. One approach is through genetic selection, though this is reliant on an understanding of the genetic mechanisms driving the physiological responses to stress.

One common mutation that can lead to genetic variation is a Single Nucleotide Polymorphism (SNP). SNPs may be either synonymous or non-synonymous (Shastry, 2009). Synonymous SNPs either occur within noncoding regions of chromosomes or as silent mutations within coding regions. Non-synonymous SNPs are those that alter protein production, as they occur within coding regions as missense or nonsense mutations. Missense mutations lead to amino acid substitutions, while nonsense mutations halt translation by introducing stop codons or new splice-sites (Petersen et al., 1998).

A large part of stress responses is controlled by the nervous and endocrine systems. The hypothalamic-pituitary-adrenal (HPA) axis, which is composed of the hypothalamus, pituitary gland, and the adrenal gland, forms a bridge between the nervous and endocrine systems (Smith & Vale, 2006). In response to stressors, this axis triggers a hormonal sequence that leads to the release of corticosterone hormone (Kuenzel & Jurkevich, 2010).

The two lines of Japanese quail used for this study were previously selectively bred for divergent blood corticosterone responses when exposed to restraint stress. In order to identify

and characterize the genetic variations within the nervous and neuroendocrine systems that could be driving these different responses, whole genome resequencing was performed on each line of quail. We identified non-synonymous SNPs within the nervous system that were unique to each line to determine their potential roles within the stress response.

3.3 MATERIALS AND METHODS

Methods for animal use, birds and DNA sequencing, data quality and assessment, SNP detection and analysis, and bioinformatics pathway analysis are described in Chapter 2.

3.3.1 ETHICS STATEMENT

This study was conducted following the recommended guidelines for the care and use of laboratory animals for the National Institutes of Health. All procedures for animal care were performed according to the animal use protocols that was reviewed and approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC Protocol #14012).

3.4 PATHWAY ANALYSIS WITH MUTANT GENES IN NEUROENDOCRINE SYSTEM

IPA was used to analyze connections of SNPs to canonical pathways, molecular networks and changes in molecular interactions.

The top 5 canonical pathways in the HS line were Amyotrophic Lateral Sclerosis signaling, GP6 signaling, signaling by Rho Family GTPases, Ephrin A signaling, and Semaphorin Neuronal Repulsive signaling pathways, while the top 5 canonical pathways in the LS line were GP6 signaling, telomere extension by telomerase, axonal guidance, role of BRCA1 in DNA damage response, and activation of IRF by cytosolic pattern recognition receptors (Figure 3.1; Figure 3.2).

Of the top 25 molecular networks identified in the HS line, 13 networks are tied to nervous system development and function, behavior, or neurological disease, while only 6 of the top 25 molecular networks in the LS line showed these same connections (Table 3.1; Table 3.2). However, 2 networks in the LS line are connected to endocrine system development and function and disorders. Genes within these two molecular networks include several molecules that regulate the release and metabolism of thyroid hormones. The thyroid is part of the hypothalamic-pituitary-thyroid (HPT) axis, which is another bridge between the nervous and endocrine systems. This axis, like the HPA axis, is a part of the neuroendocrine stress response. Thyroid hormones are multifunctional and contribute towards embryonic development, growth, and metabolic traits (Darras et al., 2011; Helmreich & Tylee, 2011). Dysregulation of thyroid hormones has been found to be associated with anxiety and depressive symptoms (Gulseren et al., 2006).

The top 20 significantly affected functions in the HS line overwhelmingly relate to nervous system signaling (Table 3.3). Additionally, MAPT and its associated molecules are found across most of these developmental functions. MAPT is a tau protein, and tau proteins have been found to be associated with changes in physical activity, anxiety, and development of Alzheimer's (Roberson et al., 2007).

The top 20 significantly affected functions in the LS line are more diverse, though several functions relate to early development of the nervous system (Table 3.4). Many of the genes present in these developmental networks are implicated in nervous system diseases, such as Alzheimer's, mental retardation, intellectual disorder, and autism spectrum disorder (Ba et al., 2016; Iqbal et al., 2013; Rudenko, 2019; Sáez et al., 2019).

Within the HS line, 328 of the significantly affected genes are expressed within the hippocampus, and 349 of the significantly affected genes are expressed within the hypothalamus. The hypothalamus is responsible for one of main hormonal cascades of the stress response. Within the hypothalamus corticotropin-releasing hormone (CRH) is produced, which leads to the release of adrenocortiotropic hormone (ACTH) in the anterior pituitary. In turn, ACTH then triggers corticosterone release in the adrenal gland of birds (Kuenzel & Jurkevich, 2010). Corticosterone is a glucocorticoid, which are hormones that will halt production of CRH via negative feedback mechanisms (Miller & O'Callaghan, 2002). Glucocorticoid receptors are primarily found within the hippocampus, and gene transcription of glucocorticoid-dependent genes has been observed in the hippocampus of rats after exposure to acute stress (Mifsud & Reul, 2016). Several genes identified within this group have also been identified within glucocorticoid receptor-dependent gene regulatory networks in mice, including ACTG1, PTPN12, RNF10, SLC31A2, and VIM (Phuc Le et al., 2005). We also identified genes two genes, HNRNPH1 and HNRNPR, that are not found directly within these pathways but are found to bind to genes that are present within them (Lee et al., 2018).

Within the LS line, 193 of the significantly affected genes are expressed within the hippocampus, and 207 of the significantly affected genes are expressed within the hypothalamus. Unlike the HS line, no genes were identified that have previously been found to directly act within glucocorticoid receptor-dependent gene regulatory network. However, several molecules were found that have been known to either bind or regulate molecules found in the network. These molecules are COL4A1, DPYSL4, F9, F10, HOXA11, HOXD9, ITGB3, LAMA1, NDUFS1, NDUFV2, PDLIM5, PPP2R2D, and PSMB1 (Cao et al., 2008; Chastney et al., 2020; Choi et

al., 2015; Fukada et al., 2000; Guarani et al., 2014; Hutchins et al., 2010; Jiang et al., 2003; Lapan & Fay, 1997; Niakan et al., 2010; Sarma et al., 2008; Uechi et al., 2014). The molecule F8 from the glucocorticoid receptor-dependent gene network was also identified within our SNP analysis, but it is not found within the HPA or HPT axis data.

3.5 DISCUSSION

In this analysis, we found that a vast number of genes that were found to contain SNPs in the HS and LS lines of quail are associated with the nervous and neuroendocrine systems, as well as within signaling pathways tied to hormonal cascades and nervous system development and function. A large portion of these SNPs were found within the HPA and HPT axes with affected genes and molecules having been implicated in glucocorticoid receptor-dependent gene regulatory networks.

Across both lines of quail, many genes were found to have connections with nervous system disorders, including anxiety, Alzheimer's, mental retardation, intellectual disorder, and autism spectrum disorder (Ba et al., 2016; Iqbal et al., 2013; Roberson et al., 2007; Rudenko, 2019; Sáez et al., 2019). A few of the traits exhibited by the HS line when compared to the LS line include lower sociability and heightened fear (Khatri et al., 2019). Collectively, these observations may be an indication of potential psychological disorders.

As identified in IPA, over a third of genes and molecules in the HS line are found within the hypothalamus or hippocampus, while about a third of genes and molecules in the LS line are found within the hypothalamus or hippocampus. This seems to show that the genetic changes that occurred between these lines of birds were largely focused within the brain, particularly within the regions tied to the neuroendocrine system as it applies to the organism's stress

response. When considering the various molecular networks implicated in the nervous and neuroendocrine systems, as well as the presence of genetic mutations in glucocorticoid signaling networks, these findings suggest that genetic variation within the nervous and neuroendocrine systems has a large impact on the phenotypic variation observed between the HS and LS lines of Japanese quail.

These lines of quail were selected based on their response to immobilization stress that lasted no longer than 10 minutes, meaning the lines were subjected to acute stress (Satterlee & Johnson, 1988). Further research into chronic stress selection and stress responses would provide an even more comprehensive picture of the genetics underlying stress responses in poultry.

REFERENCES

Ba, W., et al., TRIO loss of function is associated with mild intellectual disability and affects dendritic branching and synapse function. Human molecular genetics, 2016. 25(5): p. 892-902.

Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. 30(15): p. 2114-20.

Cao, R., et al., Role of hPHF1 in H3K27 methylation and Hox gene silencing. Molecular and cellular biology, 2008. 28(5): p. 1862-1872.

Chastney, M.R., et al., Topological features of integrin adhesion complexes revealed by multiplexed proximity biotinylation. The Journal of cell biology, 2020. 219(8): p. e202003038.

Choi, S.J., et al., Human coagulation factor VIII domain-specific recombinant polypeptide expression. Blood research, 2015. 50(2): p. 103-108.

Darras, V.M., et al., Thyroid Hormone Receptors in Two Model Species for Vertebrate Embryonic Development: Chicken and Zebrafish. Journal of Thyroid Research, 2011. 2011: p. 402320.

Fukada, M., et al., Molecular Characterization of CRMP5, a Novel Member of the Collapsin Response Mediator Protein Family *. Journal of Biological Chemistry, 2000. 275(48): p. 37957-37965.

Guarani, V., et al., TIMMDC1/C3orf1 functions as a membrane-embedded mitochondrial complex I assembly factor through association with the MCIA complex. Molecular and cellular biology, 2014. 34(5): p. 847-861.

Gulseren, S., et al., Depression, Anxiety, Health-Related Quality of Life, and Disability in Patients with Overt and Subclinical Thyroid Dysfunction. Archives of Medical Research, 2006. 37(1): p. 133-139.

Helmreich, D.L. and D. Tylee, Thyroid hormone regulation by stress and behavioral differences in adult male rats. Hormones and behavior, 2011. 60(3): p. 284-291.

Huff, G.R., et al., Response of restraint stress-selected lines of Japanese quail to heat stress and Escherichia coli challenge. Poult Sci, 2013. 92(3): p. 603-11.

Hutchins, J.R.A., et al., Systematic analysis of human protein complexes identifies chromosome segregation proteins. Science (New York, N.Y.), 2010. 328(5978): p. 593-599.

Iqbal, Z., et al., Homozygous and heterozygous disruptions of ANK3: at the crossroads of neurodevelopmental and psychiatric disorders. Human Molecular Genetics, 2013. 22(10): p. 1960-1970.

Jang, H.M., et al., *Genome resequencing and bioinformatic analysis of SNP containing candidate genes in the autoimmune vitiligo Smyth line chicken model.* BMC Genomics, 2014. 15(1): p. 707.

Jiang, G., et al., Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. Nature, 2003. 424(6946): p. 334-337.

Khatri, B., et al., Copy number variation study in Japanese quail associated with stress related traits using whole genome re-sequencing data. PLOS ONE, 2019. 14(3): p. e0214543.

Khatri, B., et al., *Whole Genome Resequencing of Arkansas Progressor and Regressor Line Chickens to Identify SNPs Associated with Tumor Regression.* Genes (Basel), 2018. 9(10).

Kuenzel, W.J. and A. Jurkevich, Molecular neuroendocrine events during stress in poultry1. Poultry Science, 2010. 89(4): p. 832-840.

Lapan, K.A. and P.J. Fay, Localization of a Factor X Interactive Site in the A1 Subunit of Factor VIIIa *. Journal of Biological Chemistry, 1997. 272(4): p. 2082-2088.

Lee, Y.J., Q. Wang, and D.C. Rio, Coordinate regulation of alternative pre-mRNA splicing events by the human RNA chaperone proteins hnRNPA1 and DDX5. Genes & development, 2018. 32(15-16): p. 1060-1074.

Liu, L., et al., Heat stress impacts on broiler performance: a systematic review and meta-analysis. Poultry Science, 2020. 99(11): p. 6205-6211.

Mifsud, K.R. and J.M.H.M. Reul, Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus. Proceedings of the National Academy of Sciences, 2016. 113(40): p. 11336.

Miller, D.B. and J.P. O'Callaghan, Neuroendocrine aspects of the response to stress. Metabolism - Clinical and Experimental, 2002. 51(6): p. 5-10.

Niakan, K.K., et al., Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal. Genes & development, 2010. 24(3): p. 312-326.

Petersen, G.M., G. Parmigiani, and D. Thomas, Missense Mutations in Disease Genes: A Bayesian Approach to Evaluate Causality. The American Journal of Human Genetics, 1998. 62(6): p. 1516-1524.

Phuc Le, P., et al., Glucocorticoid Receptor-Dependent Gene Regulatory Networks. PLOS Genetics, 2005. 1(2): p. e16.

Roberson, E.D., et al., Reducing Endogenous Tau Ameliorates Amyloid ß-Induced Deficits in an Alzheimer's Disease Mouse Model. Science, 2007. 316(5825): p. 750.

Rudenko, G., Neurexins - versatile molecular platforms in the synaptic cleft. Current opinion in structural biology, 2019. 54: p. 112-121.

Sáez, M.E., et al., Genome Wide Meta-Analysis identifies common genetic signatures shared by heart function and Alzheimer's disease. Scientific reports, 2019. 9(1): p. 16665-16665.

Sarma, K., et al., Ezh2 requires PHF1 to efficiently catalyze H3 lysine 27 trimethylation in vivo. Molecular and cellular biology, 2008. 28(8): p. 2718-2731.

Satterlee, D.G. and W.A. Johnson, Selection of Japanese quail for contrasting blood corticosterone response to immobilization. Poult Sci, 1988. 67(1): p. 25-32.

Satterlee, D.G., G.G. Cadd, and R.B. Jones, Developmental instability in Japanese quail genetically selected for contrasting adrenocortical responsiveness. Poult Sci, 2000. 79(12): p. 1710-4.

Satterlee, D.G., R.H. Marin, and R.B. Jonest, Selection of Japanese quail for reduced adrenocortical responsiveness accelerates puberty in males. Poult Sci, 2002. 81(7): p. 1071-6.

Shastry, B.S., SNPs: impact on gene function and phenotype. Methods Mol Biol, 2009. 578: p. 3-22.

Shini, S., et al., Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes1. Poultry Science, 2010. 89(4): p. 841-851.

Shumaker, S., et al., *Identification of SNPs Associated with Stress Response Traits within High Stress and Low Stress Lines of Japanese Quail.* Genes, 2021. 12(3).

Smith, S.M. and W.W. Vale, The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in clinical neuroscience, 2006. 8(4): p. 383-395.

Suhr, C.L., et al., Short-term spatial memory responses in aged Japanese quail selected for divergent adrenocortical stress responsiveness. Poult Sci, 2010. 89(4): p. 633-42.

Uechi, H., J. Hamazaki, and S. Murata, Characterization of the testis-specific proteasome subunit α4s in mammals. The Journal of biological chemistry, 2014. 289(18): p. 12365-12374.

APPENDIX

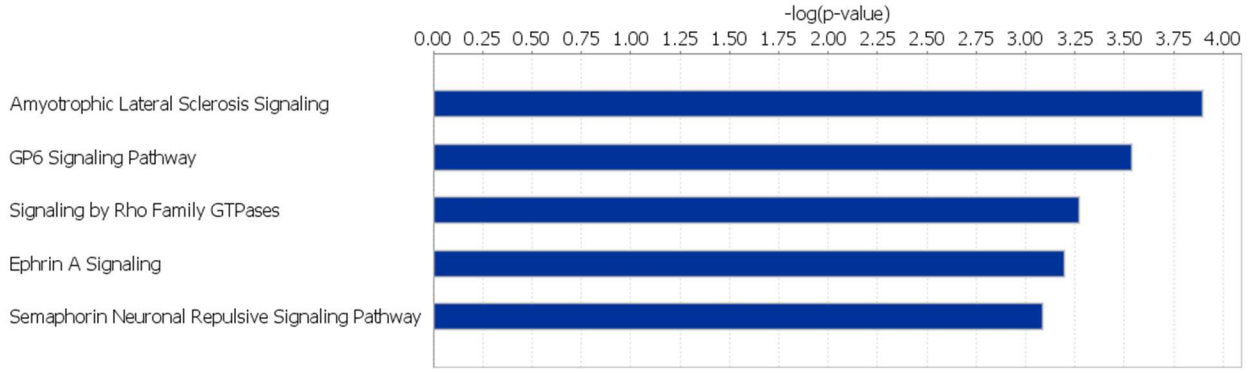


Figure 3.1 Top 5 canonical pathways in the HS line of Japanese quail.

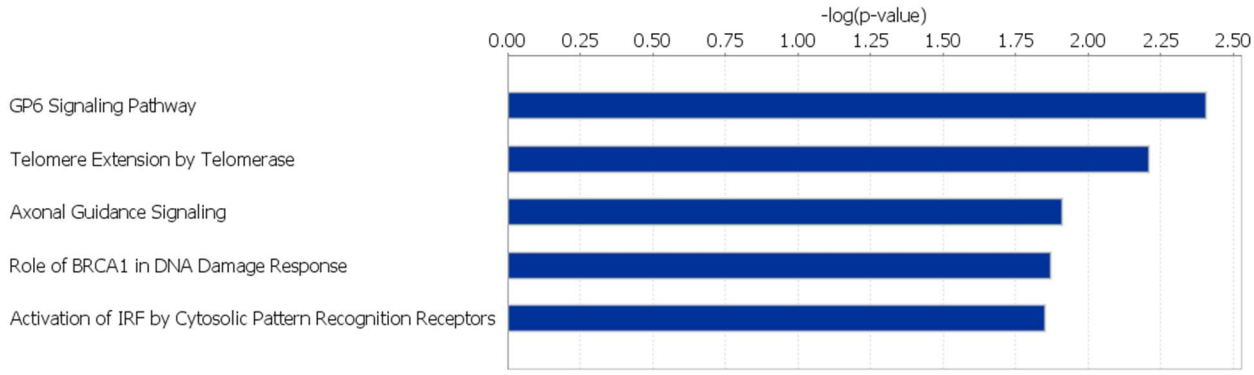


Figure 3.2 Top 5 canonical pathways in the LS line of Japanese quail.

Table 3.1 Top 25 significantly affected molecular networks in the nervous system of the HS line of Japanese quail.

Top Diseases and Functions	Molecules in Network
Cellular Assembly and Organization, Cellular Compromise, Cellular Function and Maintenance	AAK1, ABCG2, ACAT1, ACTB, ACTG1, ADAMTS3, C1R, CHRNA4, CLASP2, DDX60, DMXL2, DYNC1H1, HDAC9, HPCA, KATNA1, LATS1, MAP1A, MAP1B, MAP2, MAPK8, MAPT, MSN, MTHFR, OTOF, PAK1, PCLO, PI4KA, PPME1, PRKCD, SERPINF2, SHROOM3, SLC1A2, SRGAP3, UNC13C, USP18
Cellular Development, Cellular Growth and Proliferation, Nervous System Development and Function	ACTB, ACTN2, APBA2, APBB1, APP, CAMK2B, CDK5RAP2, CLSTN1, CRB2, CYFIP1, DBN1, ELAVL4, EPHA4, FOXP2, FXYD1, GAB1, GABBR2, INA, JUNB, MECP2, NCSTN, NXPE3, PSENEN, RANBP9, RPS6KB1, RSAD2, SYN1, SYNCRIP, SYT4, TPM1, UBE3A, VCAN, WASF1, WNK1, YWHAZ
Cardiovascular System Development and Function, Embryonic Development, Organismal Development	APP, ARSG, CDH23, CGA, CITED2, E2F1, EFHC2, EPHB3, FHL1, Fus, GALK1, HERPUD1, HNRNPH1, IGSF1, IKBIP, KMT2A, KMT2D, LRRN4, MBNL2, NNAT, NRP1, OPTN, PHC1, PIGZ, PRDM2, RAB38, RAN, REV3L, RGS4, RIOX2, SCN1A, STBD1, TP53INP1, TUBA4A, WDR33
Cellular Development, Nervous System Development and Function, Tissue Morphology	AHR, ANK3, ARHGDIB, ARX, CERS1, CNKSR3, CSPP1, CTSS, CX3CL1, DLX1, Dlx1as, DLX2, ELOVL1, EN2, GLDN, GSN, GSX1, HEY2, KDM1A, LPAR1, MYT1L, NEFL, NFASC, PHF21B, PIK3R3, PPP4R3A, PPP4R3B, RYK, SEMA5A, SOX2, SPTBN4, TLE1, TNS3, TUBB3, WIPF1
Cell Death and Survival, Lymphoid Tissue Structure and Development, Tissue Morphology	ADAMTS9, AKT1, ASCL1, BCL2, BCL2L1, CASP2, CASP3, CASP4, CASP8, CD44, CDKN1C, DST, FAS, GMPR, IGF1, LEP, mir-155, MTA1, NEDD9, NOS2, NOTCH2, PAK5, PDCD11, PIK3CD, PIK3CG, PRKCG, PRNP, RICTOR, RPS6KA1, SURF6, TCF4, TGFB1, TNFRSF1A, UBE3A, ZNF652
Cancer, Cell Cycle, Organismal Injury and Abnormalities	ABCB1, ABCC2, ABCG2, ADAMTS12, BMI1, CDKN2A, CHD7, CRKL, EEF1D, FAIM, FAS, FREM1, ITGA2, ITGA3, ITGA8, ITGB1, MDM2, MRE11, MTUS1, MYCN, NACA, NBN, NPM1, RAPGEF1, RELN, RPL24, RPS8, TGM2, TP53, TP73, TRAP1, UBE2V2, WDR5, XPC, XRCC3
Cell-To-Cell Signaling and Interaction, Organismal Injury and Abnormalities, Psychological Disorders	ACVR1C, ADRA1B, ANXA2, BCL2, CASP8AP2, CASP9, Cdc42, COL5A1, CRH, EPB41L1, EPO, FBH1, FEV, GRIA3, GRIA4, HTR1A, IGSF21, Ins1, IQGAP1, KCTD20, MAOA, NPFFR2, NPY, NR3C1, NR3C2, NUDT14, NUMB, SDCBP, SLC22A3, SLC6A15, TCOF1, TRPC7, TSHZ3, VCAM1, VIM

Table 3.1 (Cont.)

Top Diseases and Functions	Molecules in Network
Cancer, Cell Death and Survival, Gene Expression	ADCYAP1R1, AURKA, BRWD1, CAST, CC2D1A, CCND1, CDC25A, CDK5, DDIAS, EGR2, GAS2L3, GHR, GHRH, GOLGB1, IL6R, JUN, KIF13B, LAMP3, MAP3K1, MAPK10, MAPK8IP1, MAPK8IP3, NF1, PCSK5, PIP5K1C, PLAGL1, PRKDC, PTPRF, RALGAPA1, RB1, SPAG9, SUMO2, TNC, XRCC5, ZIC2
Cell Morphology, Cellular Development, Cellular Growth and Proliferation	ADCYAP1, AMIGO2, ARC, ARNT2, AVP, BACE1, CACNA1H, CSHL1, CYP19A1, DLG4, EGF, EGR1, EGR2, FGF2, GDNF, GRIN1, GTF3A, HLA-A, HOMER1, ITPR1, ITPR3, KIF3A, LAMA2, LAMB3, LEP, LRRFIP1, MC3R, NOS1, PTPN22, RYR2, SIM1, SIN3B, SST, SSTR3, WNT7B
Cellular Development, Cellular Growth and Proliferation, Nervous System Development and Function	Abca8a, ADAMTS13, AGBL3, AKAP13, APOLD1, ATP1A1, CAPRIN1, CREB1, DIO2, EGR4, EPG5, FSTL1, GAD2, GFRA1, GPNMB, KDR, LGALS3, LPL, MARCKSL1, NEUROD6, NPAS4, NR4A2, PGM2L1, PLCH2, REM2, SEMA7A, SERINC2, SGSM1, SMPDL3A, SRF, STAC2, TAGLN, TRPC6, TSC2, YPEL1
Behavior, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function	APBA1, APC, CAMK2A, CASK, CAV1, CDK8, DLG1, DLG3, DLGAP1, DSCAM, EGR3, FMR1, FZD9, GABRA1, GABRG2, GNAO1, GRIA2, GRIN2A, GRM2, GRM3, GRM4, HTR2A, HTR2C, KALRN, LIN7C, PKP4, PLXND1, QPCT, SHANK1, SNAP25, SPTB, SYT1, TRIM9, TTC3, WNT5A
Cell Morphology, Cell-To-Cell Signaling and Interaction, Nervous System Development and Function	ABL1, ADAM19, AP2A1, APP, ARC, ASTN2, BACH2, BDNF, CAMK2A, CHRM3, EFNB2, EPHB1, EPHB2, GRIN1, GRIN2A, GRIN2B, GRIN3A, GRM1, GRM5, GULP1, ITGB1, KCND2, KCNH5, KIDINS220, MAPT, MMP9, MTHFD1L, NOS1, OLFM3, PLCXD3, RGS3, ROBO2, SNAP47, SOD1, THSD7B
Cell-To-Cell Signaling and Interaction, Neurological Disease, Organismal Injury and Abnormalities	ADRA2C, AGT, CDH6, EGR3, F2, HEG1, HES1, HNRNPR, HSP90B1, HSPA5, KIF1B, KLHL1, LAMP2, LMO7, LRRC47, MAP4, NCF1, NFE2L2, NOTCH1, OFD1, OPRK1, PAX6, PBX1, PDZRN3, PLK2, RET, SMARCA2, SNCA, SSH1, TBC1D9, TBR1, TH, TUBB3, VPS35, YWHAZ
Neurological Disease, Organismal Injury and Abnormalities, Psychological Disorders	ARHGAP32, BCL11B, CASP8, CAV1, CPEB4, DDX20, EDNRB, ETNPPL, FASN, FGF1, Foxp1, H2AX, HCN1, HMGB1, HTT, KCNK2, KCNQ2, MAPK14, mir-132, MTCL1, MYT1L, NGEF, NPTX1, PAX7, PPARD, RASGRP2, RBFOX1, SCN4B, SCN8A, SLC6A9, SQSTM1, STX1A, STXBP1, TLR4, TSPO

Table 3.1 (Cont.).

Top Diseases and Functions	Molecules in Network
Cellular Development, Nervous System Development and Function, Tissue Development	APC, APOD, ATP6V1G1, BACE1, BIN1, CACNA1G, CCP110, CTNNB1, ELMO2, FGFR2, GAP43, GLI1, GLI2, HES1, IL1RAP, IL23A, IL6, KAT2B, MBP, MOG, NDE1, NOTCH1, PHACTR4, PLEKHG1, PLP1, QKI, RHOA, RHOG, SH3GL3, SLC12A2, SOX17, TCF7L2, TRAF3IP2, WNT3A, ZNF536
Cancer, Cellular Development, Organismal Injury and Abnormalities	AKAP12, AKAP5, BACE2, BCHE, BDNF, BHLHE40, CADPS, CDKL5, CHAT, CP, DOK5, ETV5, FARP1, FLNB, FOSL1, HGF, HIVEP1, HNRNPA2B1, NAMPT, NCKAP1, NR4A3, PCDHA4, PCDHGB5, PCDHGB7, PRSS12, RAC1, RAD21, RET, S100A9, SHC1, SLC18A3, SULF1, TENM4, TRAF3, TXNIP
Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Neurological Disease	ALS2, ATP1A2, ATP1A3, CDH2, CKB, CNTNAP2, CUEDC1, EPB41, ERN1, ETS2, FASLG, GNB1, GSTP1, KCNA2, KCNAB2, LRRTM4, MT-CO1, Mt3, NFKB1, PCP4, PRDX6, PRKACA, PSEN1, PTGR1, RBM3, REL, RIMS1, SLC11A2, SOD1, SQSTM1, STAB1, STIP1, STMN1, TIAL1, ULK1
Kidney Failure, Organismal Injury and Abnormalities, Renal and Urological Disease	ADAM8, ASPM, AVL9, CACNA1E, CCL19, CXCR4, DNM1L, EPB41L5, GABRA1, GABRA2, GABRA5, GABRB2, GABRB3, GABRG2, GOLGA3, GRK2, IPO9, IRF1, ITCH, LAMB1, LCN2, MIGA2, MMUT, MOG, PTGER2, SPP1, SQSTM1, SRR, TARDBP, TBL3, TLR4, TNF, TREM2, TRIM32, UBQLN2
Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry	ACAT1, ADGRA1, Agtr1b, AGTRAP, AKAP10, ATP1A2, ATP1B1, ATP2B1, ATP5F1A, ATP5F1B, ATP5F1C, CNTN1, CNTN2, CNTNAP2, COPG2, CRY1, CRY2, DLG4, EPB41L3, FOS, FRMPD3, GNAQ, GPX4, IL16, LYN, PLAT, PRKACA, PRKAR2A, PRKAR2B, PTPRZ1, RAB3GAP2, SDHA, SEPTIN5, YWHAE, ZFYVE27
Nervous System Development and Function, Organ Morphology, Organismal Development	ACO2, ADCYAP1, ADCYAP1R1, ANGPT1, APP, ARNT, CCND2, CRABP1, DAAM1, DNAH9, ENPP2, EPHA8, GRK5, IGFBP2, LRP2, MAGI1, MGAT5B, MME, NDUFA5, NDUFA8, NEUROD1, OCLN, OGT, PAPPA2, PCM1, PIEZO2, POU4F1, SERPINE2, SHH, SMPDL3B, SUCLG1, TET3, THY1, TJP1, ULK4
Cellular Movement, Hair and Skin Development and Function, Renal and Urological System Development and Function	ARHGEF12, PLXNB1

Table 3.1 (Cont.)

Top Diseases and Functions	Molecules in Network
Behavior, Cell Death and Survival, Hematological System Development and Function	ANKRD12, CLOCK
Auditory Disease, Dermatological Diseases and Conditions, Developmental Disorder	AP1B1, KIF13A
Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Neurological Disease	BAZ1B, MYCBP2
Cell Cycle, Cell Signaling, Post-Translational Modification	DAB2, DAB2IP

Table 3.2 Top 25 significantly affected molecular networks within the nervous system of the LS line of Japanese quail.

Top Diseases and Functions	Molecules in Network
Developmental Disorder, Neurological Disease, Organismal Injury and Abnormalities	ADAM22, AKAP5, ARHGAP24, BCL11B, CACNG7, CYRIB, DLG3, EPB41L1, GABRA2, GABRG3, GRIA1, GRIA4, GUCY1A2, HTR2C, IKZF2, KCNQ3, KIF16B, KMT2D, LTK, MECP2, MEF2A, MMUT, MPDZ, NDUFS1, NEDD4L, PAK5, PHB2, PPP1R9A, PTPRS, REST, SIN3A, STIM2, SUMO1, SYT4, WIZ
Embryonic Development, Organismal Development, Tissue Development	ABCC2, ACSL1, ANK3, ATP5F1D, CACNA1G, CAP1, CCP110, CTNNB1, DAPK1, DHRS7, EPB41L5, EPHA5, FBH1, FEV, FMN2, KAT2B, LRP1B, MAL, MAP4K4, NCOR2, NDUFA5, NFASC, OTUD7B, PSEN1, PSMB1, RNF2, SLC6A9, SOX17, STAB1, STX1A, TCF7L2, TP53, TPPP, TSHB, UBQLN2
Cellular Movement, Hematological System Development and Function, Inflammatory Response	ADRA2A, AHR, AKAP12, BACE1, BCAN, CCL11, CCL19, Ccl7, CCR4, CD14, CTSD, CXCR3, DDC, ELN, GJA5, GJB2, GPR158, GPR85, IFNG, IL18, IL33, IL6ST, IRS1, IRS2, ITGAM, JAK2, NLRP3, NRG1, PSD2, PTGES, PTPN1, PTPN11, RGS10, S100A9, TLR4
Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities	ADAMTS9, AGO2, ARHGEF6, ATP2B2, BCAS1, BHLHE40, CLSTN2, CLSTN3, CORO2B, CYFIP1, ENO1, FMR1, HCN2, HTT, HUWE1, IGFBP4, IGFBP5, LEFTY1, mir-128, MYT1L, OPRL1, PIK3CA, PIK3CG, PITPNM2, PKP4, RAB3A, RAB3GAP1, RBFOX1, RPH3A, RXRG, SCN8A, STX1B, TCF4, TIA1, ZDHHC17
Behavior, Cellular Assembly and Organization, Connective Tissue Development and Function	ACTN2, AKAP13, AKAP7, APP, BMF, CACNA1C, CADPS2, CD9, CREB1, ECM1, ELK3, ESD, FGF13, GAA, GRN, LITAF, NFKBID, NNAT, PPP3CA, PRDX5, PTGER2, Ptprd, RALYL, RUNX1T1, SDC4, SMPD3, SPTY2D1, SYNJ2, TBL3, TLR5, TMEM59, TOM1, TP53INP2, TPT1, TUFM
Cellular Development, Cellular Growth and Proliferation, Nervous System Development and Function	ADCYAP1R1, BDNF, CDK5R2, CRY1, CRY2, DRD3, ELK1, EPHA8, FAT3, FOS, GABRG2, GDA, GNAQ, GRIN3A, HIPK2, HMGCR, IFRD1, IL16, IL1R1, KCNA4, MAPK8IP2, MET, NCDN, NSUN7, PDLIM5, PER2, POU4F1, PRKD1, RUNX1, SCAP, SCN7A, SCN9A, SEMA3E, SORL1, TLL1
Cell Morphology, Gene Expression, Tissue Morphology	ANGPT2, APP, ARID1A, ARNT2, AVP, BDNF, CDON, HLA-A, HTT, IGF1, IGFBP4, ITGB3, KMT2A, LRRFIP1, MC3R, NFATC2, NGFR, NMBR, PAQR8, PAX6, PIGZ, POLR2I, POMC, POSTN, PTPN22, RGS4, SIM1, SLIT3, SST, TRH, TUBB3, WNT7B, ZFHX3

Table 3.2 (Cont.)

Top Diseases and Functions	Molecules in Network
Cancer, Cellular Movement, Organismal Injury and Abnormalities	ABCA1, ABCC3, ADAMTS12, BMI1, CASP8, CDK5, CHD7, COL4A1, E2F2, EEF1D, EGFR, FN1, HSP90AB1, HTT, ITGA2, ITGA3, ITGB1, LAMA1, MAP4, MIR17HG, MMP9, MTOR, MYCN, NEFH, PTK2, RBL1, RPL12, RPS8, RUSC2, SOX2, TGM2, TNF, UGGT1, VIM, ZYX
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	ACVR1C, AIFM1, ALS2, AURKAIP1, BID, BRWD1, CASP2, CASP9, CCND1, Cdc42, CDC45, CDC6, CDK6, DDIAS, ELAVL1, FAM204A, FARP1, FN1, HELB, HIVEP3, MC4R, MYB, NPY, OFD1, PAX6, RAC1, RB1, SCG5, SOD1, TENM4, THNSL1, TRAPPC2, TRIO, UTRN, VGF
Endocrine System Development and Function, Small Molecule Biochemistry, Tissue Morphology	ACTC1, ARC, BANK1, BHLHA15, CDH9, CGA, CNTFR, DIO2, EDEM2, EGR1, EGR2, EGR4, EPG5, ETV5, FLT1, Foxp1, GOLGB1, GUSB, HR, HSPA5, KDR, LAMA3, MAG, MAPK3, MSX1, MTCL1, MYOC, NCMAP, Nefm, SLC1A3, SRF, TG, TLR7, TRH, ZNF804A
Cell Morphology, Nervous System Development and Function, Tissue Morphology	ARHGEF11, ATXN2, BRCA2, CAMK2D, CHRNA7, CLTC, DNM1L, DPYSL2, EP300, GPR37, ITPR1, KLC1, LCP1, MAP1A, MAP1B, MAPT, Mt3, MYO5A, NDUFV2, NEFL, PCP4, PLXNA2, PPP1CA, PPP2CA, PPP2R1A, PRKCD, RGS8, RTN4R, SHROOM3, SLC6A3, SP4, STUB1, SYNGR3, TNIK, VAMP2
Cell-To-Cell Signaling and Interaction, Inflammatory Response, Organismal Injury and Abnormalities	APOD, CACNA1A, Ccl2, Ccl7, CD200, CTTN, EIF2AK3, GH1, GHR, GHRHR, GPX1, IFIH1, IL2, IL23A, IL4R, IL6, IRF3, LAMP3, MALAT1, MAS1, mir-130, mir-15, mir-21, PPIA, PRKDC, SUMO2, TICAM1, TLR3, TLR8, TNFAIP8L2, TRAF3IP2, TREM2, TRPM2, XRCC5, ZIC2
Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry	RIMS1, TSPOAP1
Cardiac Arrhythmia, Cardiovascular Disease, Hereditary Disorder	CTNNA3, TREM2

Table 3.2 (Cont.)

Top Diseases and Functions	Molecules in Network
Carbohydrate Metabolism, Cellular Compromise, Inflammatory Response	PI4K2A, WASHC2A/WASHC2C
Cell Cycle, Cell Death and Survival, Cell-mediated Immune Response	SIRT6, SMARCA5
Cell Death and Survival, Gene Expression, Respiratory Disease	Fus, RAD9B
Amino Acid Metabolism, Endocrine System Development and Function, Endocrine System Disorders	DIO3, GPC3
Developmental Disorder, Hereditary Disorder, Neurological Disease	LRRC4, NTNG2
Cardiovascular System Development and Function, Cell Morphology, Developmental Disorder	HS6ST1, SPRY2
Cell Signaling, Cell-To-Cell Signaling and Interaction, Dermatological Diseases and Conditions	GAL, GALR3

Table 3.2 (Cont.)

Top Diseases and Functions	Molecules in Network
Cancer, Dermatological Diseases and Conditions, Developmental Disorder	EFNB2, KIDINS220
Cancer, Skeletal and Muscular Disorders, Tissue Morphology	HOXA10, HOXD9
Auditory and Vestibular System Development and Function, Auditory Disease, Cellular Development	TCOF1, TERF2
Cell Morphology, Cell-To-Cell Signaling and Interaction, Ophthalmic Disease	FSCN2, PLS1

Table 3.3 Top 20 significantly affected functions within the nervous system of the HS line.

Disease or Function	p-value	Molecules
Neuritogenesis	0.000428	ACTB, ADAMTS3, ALS2, ANK3, ANKRD27, APBA1, ARHGAP32, CACNA1H, CCL19, CD44, CELSR1, CHAT, CLASP2, CYFIP1, DAB2IP, DLG1, DSCAM, EPB41L3, EPHA4, EPHA8, EPHB1, EPHB3, FARP1, FARP2, FASN, FGFR2, FMN1, GNAO1, GRM4, HMGB1, IL1RAP, KDR, KIDINS220, LAMB1, MAP1B, MAP2, MAPK8, MAPT, MICALL1, MSN, NEFL, NFKB1, NOS1, NRP1, OPTN, PAK1, PKP4, PTPRF, PTPRK, PTPRZ1, RIMS1, ROBO2, RYR2, SCN4B, SLITRK6, SRGAP3, SSTR3, STAU2, SULF1, TENM4, TIAM2, TRIM9, TRPC6, ULK1, VEZT, WNT7B
Development of neurons	0.00128	ACTB, ADAMTS3, ALS2, ANK3, ANKRD27, APBA1, ARHGAP32, ARHGEF5, CACNA1H, CCL19, CD44, CELSR1, CHAT, CLASP2, CX3CL1, CYFIP1, DAB2IP, DLG1, DSCAM, EPB41L3, EPHA4, EPHA8, EPHB1, EPHB3, FARP1, FARP2, FASN, FGFR2, FMN1, GNAO1, GRM4, GSN, HCN1, HMGB1, IGSF21, IL1RAP, KDR, KIDINS220, LAMB1, LRRTM4, MAP1B, MAP2, MAPK8, MAPT, MICALL1, MSN, NEFL, NFKB1, NOS1, NRP1, OPTN, PAK1, PKP4, PLXND1, PTPRF, PTPRK, PTPRZ1, RIMS1, ROBO2, RYR2, SCN4B, SDCBP, SLITRK6, SRGAP3, SSTR3, STAU2, SULF1, TENM4, TIAM2, TRIM9, TRPC6, ULK1, VEZT, WNT7B
Branching of neurites	0.00158	ADAMTS3, APBA1, ARHGAP32, CCL19, CELSR1, CLASP2, DAB2IP, DLG1, DSCAM, EPHB1, EPHB3, FARP1, FGFR2, GNAO1, HMGB1, IL1RAP, KIDINS220, MAP1B, MAPT, NEFL, NFKB1, NOS1, OPTN, PAK1, PKP4, PTPRF, RIMS1, ROBO2, RYR2, SCN4B, SRGAP3, SSTR3, STAU2, SULF1, TRIM9, ULK1, VEZT
Proliferation of neuronal cells	0.00238	ACAN, ACTB, ALS2, ANK3, APBA2, ARHGAP32, ASPM, CELSR1, CLASP2, CX3CL1, CYFIP1, DAB2IP, DSCAM, E2F1, ELMO2, EPHB3, FAIM, FARP2, FGFR2, GNAO1, GRK5, HMGB1, IQGAP1, KATNA1, LAMA2, MAP1B, MAP2, MAPK8, MAPT, NDE1, NFKB1, NOS1, NRP1, PAK1, PAK5, PKP4, PLAGL1, PTP4A2, PTPRF, PTPRZ1, SCN4B, SGPP2, SLC39A12, SMARCD3, SRGAP3, SSTR3, ULK1, VCAN, VIM
Outgrowth of neurites	0.00329	ALS2, APBA2, ARHGAP32, CELSR1, DAB2IP, DSCAM, E2F1, ELMO2, EPHB3, FAIM, FGFR2, GNAO1, GRK5, HMGB1, IQGAP1, KATNA1, LAMA2, MAP1B, MAP2, MAPK8, MAPT, NFKB1, NRP1, PAK1, PAK5, PKP4, PTPRF, PTPRZ1, SCN4B, SLC39A12, SRGAP3, SSTR3, ULK1, VCAN, VIM
Growth of neurites	0.00336	ACAN, ACTB, ALS2, APBA2, ARHGAP32, CELSR1, CLASP2, CYFIP1, DAB2IP, DSCAM, E2F1, ELMO2, EPHB3, FAIM, FARP2, FGFR2, GNAO1, GRK5, HMGB1, IQGAP1, KATNA1, LAMA2, MAP1B, MAP2, MAPK8, MAPT, NFKB1, NOS1, NRP1, PAK1, PAK5, PKP4, PTP4A2, PTPRF, PTPRZ1, SCN4B, SGPP2, SLC39A12, SRGAP3, SSTR3, ULK1, VCAN, VIM

Table 3.3 (Cont.)

Disease or Function	p-value	Molecules
Polarization of hippocampal neurons	0.00389	MAP1B, MAP2, MAPT
Migration of cerebellar granule cell	0.0057	ASTN1, CX3CL1, KATNA1, RGS3
Dendritic growth/branching	0.00633	ADAMTS3, APBA1, ARHGAP32, CCL19, CELSR1, CLASP2, DAB2IP, DLG1, DSCAM, EPHB1, EPHB3, FARP1, FGFR2, GNAO1, IL1RAP, KIDINS220, MAPT, NEFL, NOS1, OPTN, PAK1, PTPRF, ROBO2, RYR2, SCN4B, SRGAP3, SSTR3, STAU2, VEZT
Development of nerves	0.00696	ADAM19, CD44, CHD7, EPHA4, EPHB1, FGFR2, HOXB3, NEFL, NRP1, SLITRK6
Migration of neurons	0.00759	ASPM, ASTN1, CDH23, CX3CL1, EPHA4, ITCH, KATNA1, MAP1B, MAP2, MAPT, NDE1, NRP1, PLAGL1, PRKCD, PTPRZ1, RGS3, TIAM2
Transport of synaptic vesicles	0.0106	ACTB, ACTG1, APBA1, APBA2, FGFR2, OTOF, PCLO, RIMS1, SPG11, UNC13C
Axonal transport	0.0138	DST, MAPK8, MAPT, RANBP9

Table 3.3 (Cont.)

Disease or Function	p-value	Molecules
Myelination of axon bundle	0.0144	LAMA2, LAMA5
Fast axonal transport	0.0144	MAPK8, MAPT
Neurotransmission of axons	0.0144	MAPT, RIMS1
Neurogenesis of subventricular zone	0.0165	ANK3, CX3CL1, FLT4, FOXP2
Long-term potentiation of cerebellar cortex cells	0.0231	CHRM3, RIMS1
Cell overmigration of neurons	0.0231	ITCH, NDE1
Loss of granule cells	0.0231	CX3CL1, MAPT

Table 3.4 Top 20 significantly affected functions within the nervous system of the LS line.

Disease or Function	p-value	Molecules
Development of central nervous system	0.00652	AGO2, ANK3, ARID1A, BBS2, BCAN, BPTF, BRCA2, CASP2, CDON, CELSR1, CHD7, COL4A1, DRD3, ELP1, EPHA5, FTO, GHRHR, IRS2, ITPR1, LLGL1, MAL, MET, MYO5A, MYT1L, NCMAP, NCOR2, NDST1, PRKDC, PTPRS, RAD9B, REST, RUNX1, SLIT3, SOX13, STK36, TACC2, TENM4, TG, TP53BP2, TRAPPC9, TRIO, WNT7B
Myelination of sciatic nerve	0.00974	BACE1, MAL, SCAP
Formation of inhibitory synapse	0.0101	EPHA3, GRIA1
Formation of brain	0.0119	AGO2, ANK3, ARID1A, BBS2, BCAN, BPTF, BRCA2, CASP2, CDON, CHD7, COL4A1, ELP1, EPHA5, FTO, GHRHR, IRS2, ITPR1, LLGL1, MET, MYO5A, MYT1L, NCOR2, NDST1, PRKDC, PTPRS, RAD9B, REST, SLIT3, STK36, TACC2, TG, TRAPPC9, TRIO
Action potential of central nervous system cells	0.0119	ANK3, DPYSL2, SLC6A9
Myelination of nerves	0.0125	BACE1, MAL, MYO5A, SCAP

Table 3.4 (Cont.)

Disease or Function	p-value	Molecules
Morphology of nervous system	0.0268	AHR, AKAP12, AKAP13, AKAP5, ALS2, ANK3, BACE1, BBS2, BCAS1, BRCA2, CADPS2, CASP2, CCP110, CD9, CDON, CLSTN3, CLTC, CNTFR, CTSD, DLG3, DPYSL2, DPYSL4, EPG5, FTO, GCM2, GPR37, GPR85, GRIA1, HIVEP2, ITPR1, KIDINS220, LLGL1, MAL, MET, MMUT, MYB, MYO5A, NCOR2, NDST1, OPRL1, PHB2, PKD1, PLXNA2, PPP1R9A, PTPRS, REST, RUNX1, RXRG, TENM4, TLR4, TMEM59, TRIO, UTRN
Abnormal morphology of telencephalon	0.0284	BBS2, CDON, HIVEP2, ITPR1, LLGL1, NCOR2, NDST1, PKD1, PTPRS, RXRG, TRIO
Development of neurons	0.0289	AHR, AKAP5, ALS2, ANK3, ANKRD27, ARHGEF5, BACE1, BCAN, BCL11B, CADPS2, CELSR1, CLSTN3, CYFIP1, DLG3, DPYSL2, DPYSL4, ELP1, EPHA3, EPHA8, FARP1, FTO, GRIA1, HIVEP2, HSP90AB1, ITGB3, ITPR1, KIDINS220, LAMA1, LLGL1, MET, MYO5A, NCDN, NCMAP, NTNG2, PKP4, PPP1R9A, PTPRM, RAB3A, SCAP, SLIT3, SP4, TENM4, TMEM59, TRIO, WNT7B
Quantity of Purkinje cells	0.0321	ALS2, ANK3, CADPS2, SP4
Activation of brainstem	0.0328	SLC6A9
Delay in initiation of maturation of dendritic spines	0.0328	PPP1R9A

Table 3.4 (Cont.)

Disease or Function	p-value	Molecules
Long term synaptic depression of mossy fibers	0.0328	BACE1
Differentiation of inhibitory synapse	0.0328	CLSTN3
Differentiation of excitatory synapses	0.0328	CLSTN3
Action potential of CA3 neurons	0.0328	SLC6A9
Synaptic transmission of inhibitory synapse	0.0328	CLSTN3
Retraction of parallel fiber-Purkinje cell synapses	0.0328	GRIA1
Formation of facial nucleus	0.0328	TRIO

Table 3.4 (Cont.)

Disease or Function	p-value	Molecules
Semantic memory	0.0328	BACE1

CONCLUSION

The studies found within this thesis sought to deepen our understanding of the genetic mechanisms underlying the physiological responses to stress in poultry by identifying genetic variations between two selectively bred lines of Japanese quail bred for their divergent responses to stress. Genomic resequencing was performed in order to identify non-synonymous Single Nucleotide Polymorphisms within each line of quail.

In Chapter 2, the immune system and immune responses within these quail were implicated through various differing mutations between the HS and LS lines. SNP identification within the GP6 Signaling pathway and BRCA2 DNA Damage Response seems to indicate one way that these two lines of quail differ is through ability to control the impact of stress at the cellular level. Improved damage control in this manner could suppress the negative impacts of stress to production traits. SNPs located within the Th17 Activation, GP6 Signaling, Rho GTPase Signaling, and Rac Signaling pathways seem to indicate effects on cytokine production and further connections to inflammatory responses, showing a possible genetic divergence in immune system function between the HS and LS lines of quail.

Chapter 3 contained findings of a large number of SNPs located within the nervous and neuroendocrine systems of the HS and LS lines of Japanese quail, as well as SNPs located within signaling pathways connected to hormonal cascades and nervous system development and function. A vast number of these SNPs were located within the HPA and hypothalamic-pituitary-thyroid (HPT) axes, and many of these affected genes and molecules have been previously implicated in glucocorticoid receptor-dependent gene regulatory networks. Further connections were found with genes involved in nervous system disorders, and these connections were found within both the HS and LS lines of quail. Paired with observed differences in behavior between

the lines, these connections seem to indicate potential psychological disorders. Over a third of genes and molecules implicated in the HS line and about a third of genes implicated in the LS line are found within the hippocampus or hypothalamus. This concentration of variation seems to indicate that large amounts of genetic change occurred within the brain of the two lines of quail, more specifically within regions tied to the neuroendocrine system and its role in the stress response.

Altogether, this research on HS and LS lines of Japanese quail has identified genetic variations affecting nervous and endocrine system development, glucocorticoid signaling, psychological and nervous system disorders, humoral and cell-mediated immune response, stress-induced immune responses, and various metabolisms. These findings support the theory that the observed phenotypic diversity between the HS and LS lines of Japanese quail results from underlying genotypic variation. As the quail lines used in these studies were selected according to their responses to acute stress, further research conducted on chronic stress selection and stress responses would serve to help complete our understanding of the genetics underlying stress responses in poultry.