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Genetic Analysis of the Promoter Region of the Serotonin Receptor 5HT2B and its Contribution to Pulmonary Hypertension Syndrome in Broiler-Type Chickens

Khaloud Awada Alzahrani
University of Arkansas, Fayetteville

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Khaloud Alzahrani
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ABSTRACT

Pulmonary Arterial Hypertension (PAH) is a sever disorder characterized by high pulmonary artery pressure and pulmonary vascular resistance along with specific histological changes. Idiopathic PAH is a human disease of unknown origin that showed common characteristics to PHS/ascites in chicken. PHS/ascites is a condition found in broilers causing right ventricular hypertrophy, valvular insufficiency, increased venous pressure, variable liver changes, accumulation of fluids in the abdominal cavity, and finally lung and heart failure. Currently, there is no acceptable animal model for human PAH. Our group has been validating the chicken as the medical animal model for human PAH, thus we have mapped chromosomal regions associated with susceptibility to ascites. The region on Gga9 contains the serotonin receptor (5HT2B) gene which showed linkage disequilibrium with respect to ascites susceptibility. Sequence data obtained from a previous research work for the 5HT2B gene from several SUS and RES lines identified a C/T SNP on the 3rd exon that result in a silent mutation and had no effect on protein production or structure. My research project was to extend our sequence analysis to the upstream promoter regions of 5HT2B to identify any sequence polymorphisms of several DNA samples from both SUS and RES lines. I identified 15 polymorphisms SNPs present in the 5HT2B promoter region. Three SNPs were found to be in a complete linkage defining alternative alleles and have a potential ability in affecting transcription factors binding sites. A TaqMan Assay was then developed for these particular SNPs used to SNPlotype a large collection of DNA samples from birds previously phenotyped for ascites susceptibility in the hypobaric chamber challenged. Statistical analyses of the SNPlotyped data support the association of particular genotypes/haplotypes of the 5HT2B gene with resistance to ascites especially in females. Identifying the underlining genetics of ascites syndrome will lead

to genetic selection to reduce ascites incidence in chicken and further establish chicken as the medical animal model for human PAH.

This thesis is approved for recommendation
to the Graduate Council.

Thesis Director:

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Dr. Douglas Rhoads

Thesis Committee:

_____

Dr. Nicholas Anthony

_____

Dr. David McNabb

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DEDICATION

This work is dedicated to my husband, Fawaz Alzahrani, for his patience and support.

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I INTRODUCTION

A. Pulmonary Arterial Hypertension

Pulmonary Arterial Hypertension (PAH) is a progressive and often a fatal disorder that involves high pulmonary artery pressure and pulmonary vascular resistance along with specific histological changes. A variety of conditions lead to the increased pressure of pulmonary arteries where the arteries are unable to carry enough blood to the lungs causing cardiac hypertrophy, failure of the right ventricle, and premature death (Barst, 2008). Imbalance in the vascular mediators that favors excessive proliferation, thrombosis and vasoconstriction (Chapman et al., 2008) and/or hypoxic conditions can also increase the incidence of PAH (Dempsie; Maclean, 2008).

PAH in humans was classified into two classes a primary and secondary pulmonary hypertension. Primary Pulmonary Hypertension is now called idiopathic pulmonary arterial hypertension (IPAH) due to its unknown etiology where secondary PAH IS known to have underlying causes (Barst, 2008; Simmonneau et al., 2004). However, some types of secondary PAH are closely similar to IPAH in their histopathology, clinical features and response to treatment. Therefore, in 1998 the World Health Organizing (WHO) reclassified the pulmonary hypertension into five groups based on the mechanism of the disease rather than associated symptoms and conditions. Group 1 is pulmonary arterial hypertension (PAH) that includes five categories: Idiopathic (IPAH), familial (FPAH), associated with risk factors or conditions (APAH), persistent pulmonary hypertension of the newborn, and associated with significant venous or capillary involvement (Barst, 2008; Simmonneau et al., 2009).

IPAH can be defined as having a sustained elevated pulmonary artery pressure of ≥ 25 mm Hg at rest or ≥ 30 mm Hg during exercise in the absence of other underlying causes such as lung or heart diseases (Gaine; Rubin, 1999). In humans, IPAH is a rare disease with an incidence of 2-3 per million worldwide per year where the median age at diagnosis is 36 years but it may occur at any age. (Rudarakanchana et al., 2001). IPAH is a sporadic disease where a family history of PAH or a risk factor can't be recognized. The mean survival after diagnosis is 2.8 years. (Simonneau et al., 2009; Rudarakanchana et al., 2001). Moreover, the ratio of male to female for IPAH in adults is 1: 1.7 (Berger, 2010), which means that females are nearly twice as susceptible for IPAH than males. Vascular remodeling in IPAH patients include hypertrophy and hyperplasia of smooth muscle cells accompanied by the formation of plexiform lesions in up to 80% of the cases (Tuder et al., 1994). Plexiform lesions are complex vascular formations originated from remodeled pulmonary arteries. Histologically, plexiform lesions are the hall mark of IPAH patients especially in advanced stages. In fact, Overbeek et al. have reported that 10 out of 11 IPAH patients' lungs had clear evidence of having plexiform lesions (Overbeek et al., 2009). When plexogenic arteriopathy obstructs the pulmonary arteries, patients tend to become unresponsive to vasodilator therapy with poor prognosis. There have been studies which addressed the linkage of IPAH with the use of anorexigens (appetite suppressant drugs). In the 1960s, the first link was identified when Primary/Idiopathic PAH was found to be associated with the use of anorexigen aminorex fumarate in Switzerland, Austria, and Germany (Kay, 1971; Gurtner, 1985). In addition, more cases of IPAH have been linked to the use of fenfluramine derivatives in the United Kingdom, France, and Belgium (Douglas, 1981; Delcroix, 1998). The increased risk of developing IPAH is associated with the increasing duration of use.

The familial form of PAH (FPAH) appears to be inherited as an autosomal dominant trait and accounts for about 6% of the total cases of pulmonary arterial hypertension. Moreover, 57% of babies born of parents who carry the gene for FPAH were females (Ghamra, 2003; Zemin, 2000). In 2000, the gene responsible for FPAH was identified as the Bone Morphogenetic Protein Receptor-2 gene (BMPR2), and a correlation has been found between FPAH and BMPR2 mutation (Deng; Morse, 2000). Approximately 9% to 26% of the IPAH patients and 75% of FPAH patients have been detected to have BMPR2 mutations. BMPR2 is a cytokine, a member of the transforming growth factor (TGF) - super family, which is important in regulating growth, differentiation and apoptosis of numerous types of cells. BMPR2 is essential in vasculogenesis and embryonic heart development (Roberts, 2004; Lane et al., 2000). BMPR2 mutations are likely to be responsible for almost 90% of FPAH. The mutation likely promotes cell division or prevents cell death, resulting in excessive growth of pulmonary arteries ultimately leading to arteries narrowed in diameter causing increased resistance to blood flow. Surprisingly, only 20% of family members with identified heterozygous BMPR2 mutations exhibit PAH phenotype indicating a need for other contributing factors in PAH pathogenesis such as environmental, genetic and pathological factors (Launay, 2002; Loyd, 1995; Newman JH, 2004)

Symptoms of PAH are non-specific and include breathlessness, fatigue, weakness, angina, syncope, and abdominal distension. Early diagnosis and treatment for PAH is always recommended because late stages of the disease may be less responsive to therapy and mostly lead to death (McDonough et al., 2011). Human PAH treatment varies depending on the causes of the disease, severity, drug interaction and personal lifestyle. The aim of treatment is to control symptoms, prevent more lung damage and slow the progress of the disease. In the last decade,

several PAH treatment therapies have been approved that target essential pathways such as prostacyclin pathway, nitric oxide pathway, endothelin pathway and serotonin pathway. Treatment of PAH patients in advanced therapy include prostanoids, endothelial receptor antagonists, phosphodiesterase 5 inhibitors or, rarely, certain calcium channels blockers. (Hopkins; Rubin, 2012). A combination of disease specific-PAH therapies and conventional drugs such as diuretics, anticoagulants, oxygen therapy and lung transplantation have improved the overall life quality and survival, increased exercise capacity, and reduced symptoms. Several studies have found also that these therapies and drugs play an important role in slow growth of smooth muscle cells, prevention of blood clots, and increase in blood and oxygen supply to the heart (Barst, 2008; Galie et al., 2004). However, PAH remains a devastating and life- threatening disease owing to the low survival rate.

In the 1960s, the serotonin hypothesis of PAH was first proposed after an outbreak of the disease was reported among patients using certain diet pills. Aminorex and fenfluramine are appetite suppressant drugs that inhibit serotonin uptake by platelets leading to increased extracellular concentration of the serotonin (Rothman et al., 1999). Under hypoxic conditions, serotonin is synthesized by tryptophan hydroxylase 1 enzyme (Tph1) and released from pulmonary endothelium cells. Serotonin can then influence pulmonary vascular smooth muscle proliferation and/or contraction via activity at the serotonin transporter (SERT) and serotonin receptors. Intracellular accumulation of serotonin may generates reactive oxygen species (ROS), which induce phosphorylation of ERK1/2 MAP kinase. Also, 5HT1B/D receptors activate Rho-kinase (ROCK). This enables the translocation of extracellular-regulated kinase (ERK) 1/2 into the nucleus. Once Inside the nucleus phosphorylated ERK1/2 can increase transcription of nuclear growth factors and mediate cellular proliferation while Rho- kinase activation promotes

hypoxic pulmonary vasoconstriction (Lee et al., 1999; Liu et al., 2004; Maclean. M. R, 2007). Serotonin (5-hydroxytryptamine, 5-HT) is a pulmonary vasoconstrictor and smooth muscle cell mitogen, and it is believed to play a prominent role in the etiology of PAH. In 1990, high levels of the plasma 5-HT was reported in patients with platelet storage disease that developed IPAH (Herve, et al., 1990). Subsequent experiments revealed that patients with IPAH have increased circulating 5-HT levels even after heart-lung transplantation (Herve, et al., 1995). Additionally, cultured pulmonary arterial smooth muscle cells (PASMCS) from pulmonary hypertensive patients demonstrate higher proliferative response to 5-HT compared to control subjects. Furthermore, isolated endothelial cells and smooth muscle cells from pulmonary arteries of PAH patients express mRNAs for 5-HT2A, 5-HT2B and 5-HT1B/D receptors (Launay, 2002; Willers et al., 2006). Serotonin induces the proliferation of not only PASMCs, but also pulmonary arterial fibroblasts through binding to the 5-HT transporter SERT (welsh et al., 2004; Eddahibi et al., 1999). Thus, SERT is believed to be responsible for the mitogenic effects of 5-HT (Willers et al., 2006). In contrast, the constricting action of 5-HT on smooth muscle cells is mediated by 5-HT receptors 5HT2A, 5HT1B/D and 5HT2B (MacLean et al., 2000). However, some research groups have shown that the mitogenc responses in pulmonary vascular cell can be achieved by using either SERT inhibitors or 5-HT receptor antagonists (Pitt et al., 1994; Welsh et al., 2004). This is attributed mainly to cross-talk between SERT and 5-HT receptors to mediate serotonin-induced contraction and proliferation of pulmonary artery smooth muscle cells. These vascular changes result in the thickening of the medial layer and narrowing the lumen of the pulmonary artery leading to the vascular remodeling observed in PAH patients. Moreover, an Indel polymorphism was described in the upstream promoter region of the SERT gene with a higher frequency of the long (L-type) polymorphism than the short one (S- type) in IPAH patients,

which is directly associated with increased SERT expression and activity in platelets and PASMCs (Eddahibi et al., 2001). However, SERT genotype neither correlated with age at diagnosis, and there was no statistical association with survival interval (Willers et al., 2006). Experimentally, mice lacking the 5-HT receptor/transporter gene or carrying mutations in the 5-HT transporter/receptor or treated with 5-HT transporter/receptor inhibitors were protected against hypoxic pulmonary hypertension *(*McLean M et al., 2000) while introduction of exogenous serotonin potentiated the development of hypoxia-induced PAH in rats (Eddahibi et al., 1997). Substantial increases in the expression of 5HT2B receptor in pulmonary arteries were seen in chronic hypoxic mice. Subsequent treatment with 5HT2B antagonists completely prevented the development of the hypoxia induced pulmonary hypertension. Additionally, genetic deficiency/mutation of the 5HT2B serotonin receptor reduced the susceptibility towards hypoxic PAH in mice (Launay et al., 2002). In this hypoxia-dependent model, elevated pulmonary blood pressure and lung remodeling was associated with increased vascular proliferation, elastate activity and transforming growth factor- β (TGF- β) levels. These three parameters were also found to be potentiated by -Dexfenfluramine- treatment and dependent on 5HT2B receptor activity (Launay et al., 2002). The 5-HT signaling pathway has been shown to play an essential role in PAH and it is believed that by targeting specific components in the signaling pathway, investigators can develop some therapeutic strategies that could contribute to PAH treatment.

PAH is a multifactorial polygenic disease which involves the interaction of multiple environmental factors, growth factors, receptors, and signaling pathways along with genetic influences, all of which can interact with each other in disease development. The National Institutes of Health proposed that animal models of pulmonary arterial hypertension are needed

to understand the etiology of this disease (Tankson et al., 2001). Currently, there are a number of animal models for PAH that has been proposed. Animals models used commonly include chronically hypoxic rats/mice, and the monocrotaline injected rats. Fawn-hooded rats are known to be the most used animal model for PAH in research because they develop more severe forms of PAH than other strains following exposure to hypoxia (Stenmark et al., 2009). Other animals that have been used as models of PAH are beagle, macaque, calf, rabbit, and piglet (Badyal, 2003; McMurtry, 2008). At present, there is no perfect animal model for human PAH since no animal model reproduces all the clinical histopathological features of human PAH. However, using animals as medical models still provides valuable insights into the numerous pathways that contribute to the development and the pathogenesis of human PAH.

B. Pulmonary Hypertension Syndrome/ Ascites

Ascites, the industrial name for PAH in chicken (also called pulmonary hypertension syndrome PHS) is a series of events that affect broiler chickens (meat-type poultry) causing right ventricular hypertrophy, valvular insufficiency, increased venous pressure, variable liver changes, accumulation of fluids in the abdominal cavity and finally lung and heart failure. In the 1950s, PHS was first noticed with commercial lines in farms that were located at high altitudes. Three decades later PHS in meat- type chickens was associated with a rapid increase in growth rate and feed conversion. Increased selection for rapid growth rate leads to increased demands for oxygen to keep up with the growing tissues putting more pressure on the pulmonary vascular system. However, there are a variety of additional or secondary causes that can increase the incidence of PHS (Julian, 1993; Maxwell et al, 1990). Over the years, many environmental and management practices have been identified for their ability to trigger ascites in chickens. The major environmental cause is hypoxia due to poor ventilation, high altitude and cool

temperatures during commercial chicken production. Furthermore, high feed intake, dietary energy content, continuous lighting, poor air quality (dust), and genetic selection for mass and muscle accumulation for many generations, are the most common management practices that increase the incidence of ascites (Julian, 2000). Some studies have shown that some microorganisms cause respiratory damage or obstruct the pulmonary airways leading to the PHS/ascites development. Gram negative bacteria such as: *Escherichia, Salmonella,* and *Campylobacter*, produce a lipopolysaccharide (LPS) which can trigger pulmonary vasoconstriction leading to pulmonary hypertension in broiler chickens (Chapman et al., 2005).

For more than 60 years, broilers have been genetically selected for high body weight and meat production to accommodate the increased human population demands. In the 1940s, it took about 16 weeks for a broiler chicken to become 2-2.5 Kg in weight. However, by 1990, the continued practice of selection improved broiler strains to reach the same market weight within 43-47 days only (Griffin et al., 1994). In 2001, broilers took only 35 days to reach the same weight. Consequently, continued selection for rapid growth improved the meat production and increased the poultry industry profits. However, as a result of unnatural rapid growth and heavy body weight, broilers are often experiencing skeletal and metabolic disorders including PHS/ascites. In addition, broilers possess insufficient vascular capacity that contributes to the initiation of PHS. Chicken lungs are relatively small, rigid, and fixed in the thoracic cavity compared to mammalian lungs. Moreover, they do not expand and contract with each breath as mammals lungs do. Birds rely on the expansion and compression of their air sacs to get air through their lungs (Julian, 1993). Normally, the right ventricle pumps only sufficient blood to overcome the increased pressure in the lungs. Also, blood capillaries of the lungs can expand only a little to accommodate the increased blood flow (Julian, 1993).

The etiology of PHS in broilers starts with the increased basal metabolic rate and oxygen supply required for rapid growth. Any increase in the blood flow because of the demands of the fast growing tissues will cause an increase in the blood pressure of the lungs (pulmonary hypertension). The right ventricle then will work harder to pump blood to the lungs in response to the increasing rate of the blood pressure leading to the thickening of the right ventricle wall (Hypotrophy). The cardiac muscles hypertrophy involves proliferation of smooth muscle and connective tissues by upregulation of some growth factors. Furthermore, red blood cell production increases, to elevate the blood's oxygen carrying capacity. This causes a 'thickening' of the blood or increase in the blood viscosity that contributes to the resistance to blood flow. Thus, increased pulmonary arterial pressure is a result of both an increase in blood flow and an increase in resistance to blood flow. The valves between the right ventricle and the right atrium become inefficient and allow some blood to flow back into the atrium as the right ventricle gets enlarged and dilated, eventually leading to right ventricular failure. The increase in the blood back flow also causes liver congestion (edema) and the high pressure causes the plasma fluids to leak out from the liver and the vessels into the body cavity. This results in accumulation of fluid in the body cavity; a condition termed “water belly” which is the most apparent sign of ascites (Wilson et al., 1988; Julian, 1993; Wideman et al., 2013).

As mentioned previously with PAH patients, ascitic chickens demonstrate an imbalance of vasoactive mediators that favors vasoconstrictors, thromboxane A2 (TxA2), serotonin (5-HT), and endothelin-1(ET-1) over vasodilators, nitric oxide (NO), prostacyclin (PGI2), and vascular endothelial growth factor (VEGF) etc. Studies have indicated that the key pulmonary vasodilator for broilers is (NO) and accumulating evidence revealed that L-arginine, the substrate for (NO) production, may be downregulated during PAH/ascites progression (Wideman et al., 2013). In

2004, Wideman et al., compared the relative balance between vasoconstrictors and vasodilators in broilers challenged by bacterial LPS and found that pulmonary hypertensive responses to LPS in broilers were limited when more vasodilators are generated by the animal during LPS challenge than vasoconstrictors. Thus, they proposed that innate differences in chemical response by the animal during LPS challenge might contribute to differences in susceptibility in broilers towered PHS/ascites (Wideman et al., 2004).

Ascites is a concern for the poultry industry worldwide because economic losses have been estimated to be more than $100 million annually in the US only (Maxwell et al, 1997). Ascites affects around 4.7% of living broiler chickens around the world, but the incidence of ascites is higher at high altitudes and colder environmental temperatures (Wideman, 1988). Morbidity usually is 1-5% and the mortality rate is 1-2% which can increase up to 30% at high altitude (McMullin, 2004). Development of ascites in chickens has resulted from genetic selection for high growth rate and efficient feed conversion to allow the accumulation of body mass. Thus, poultry industry and producers must find ways and strategies to eliminate this disorder in the near future. There are several management and environmental techniques that can be used to reduce the incidence of ascites in chicken for example, improving the air quality and ventilation, applying intermittent light and reducing the rapid growth rate (reduce metabolism requirements) by applying feed restriction (Balog, 2003). However, all these attempts to reduce ascites in chickens have a negative impact on the poultry industry which is not desirable since it will slow down the early growth rate of chickens. Genetic selection for ascites resistance could reduce this disorder around the world.

Chicken has been proposed as a preclinical animal model for human IPAH. Although all forms of human PAH exhibit arterial changes and vascular remodeling, what appear to

distinguish progressive forms of IPAH in humans from all other forms of PAH are the severity of the arteriopathy and the formation of plexiform lesions (Meyrick, 2001). Furthermore, these lesions distinguish human IPAH from other animal models, such as the hypertensive dog or fawn-hooded rat. Surprisingly, the only animal model of IPAH that spontaneously develops lesions which have similar characteristics with those of humans is chicken (Wideman, 2011). Methods for induction of PAH in chickens include rapid growth rate, LPS injection, micro-particle injection, high altitude, poor ventilation, or exposure to hypoxia such as growth in a hypobaric chamber. (Chapman et al., 2005, Pavlidis, 2007). Therefore, chicken may be a valid medical animal model for genetic and physiological studies of human PAH/IPAH.

C. Chicken Genome and Chicken Mapping

In 2004, the International Chicken Genome Sequencing Consortium published the genetic sequence of the red jungle fowl (*Gallus gallus*); the wild ancestor of domestic chicken (International Chicken Polymorphism Map Consortium, 2004). Sequence differences were only minor between the sequence of the red jungle fowl and the domestic chicken. The chicken's genome is comprised of about 1 billion DNA base pair containing 20,000-23,000 genes. The chicken genome is comprised of 38 autosomes, 8 macrochromosomes, 30 microchromosomes, and one pair of sex chromosomes.

Availability of a genome can significantly facilitate mapping of chromosomal regions that show association to ascites susceptibility or resistance in chickens. Identification of genes associated with ascites will also contribute to the development of a better treatment and understanding for human PAH. There are several genetic markers that can be used for gene detection and genotyping such as RFLP (Restriction Fragment Length Polymorphism), VNTR

marker (Variable Number Tandem Repeat), STR (Short Tandem Repeat) or microsatellites, and SNP (Single Nucleotide Polymorphism). In my research study, I used SNP polymorphism as the genetic marker.

D. Single Nucleotide Polymorphism SNPs

Single nucleotide polymorphism (SNP) is the genetic variation in the DNA sequence of a single nucleotide base (A, G, T or C) between members within the same species or paired chromosomes in an individual. SNPs can be homozygous or heterozygous and can occur within coding genes, non-coding regions of genes or in the intregenic regions. SNPs also include one or two base insertions/deletions (indels). In humans, 99.9% of the DNA sequence is identical between individuals within the same population. The remaining 0.1% is significant because it contains approximately 80-90% of all genetic variations as SNPs. This sort of DNA variation occurs at a frequent of about 1 every 100-300 bases along the human genome and less frequent in the coding regions (Rapley & Harbron 2004; Syvanen, 200). The majority of SNPs convey no biological consequence when they occur within the non-coding regions of the DNA or occur as synonymous SNPs that cause no change in the amino acid sequence of a protein. However, a fraction of the SNPs have a functional significance and are the key basis of diversity among humans (Kwok et al., 2003). SNPs and structural variants were initially discovered by the comparison of multiple genomic sequences obtained from different individuals within a population. SNP mutation mechanisms include either transitions: purine-purine (A<>G) exchange or pyrimidine-pyrimidine (C<>T) exchange, or transversions: purine-pyrimidine/pyrimidine-purine exchanges (A<>C, A<>T, G<>C, G<>T). In the human genome, the ratio of transitions to transversions is approximately 1.7, indicating that transitions occur

more frequently along the DNA sequence (Vignal et al., 2002; Kwok et al., 2003). Investigators can link a SNP with a phenotype by using Genome-Wide Association Studies (GWAS) where hundreds of thousands of SNPs can be scanned per sample.

As the demand for genetic analysis increases, many approaches and technologies for SNP genotyping have been developed. The most popular and simplest one is to perform direct sequencing of genomic PCR products from different individuals for a candidate region. The major advantages of PCR sequencing are the ability to determine the SNP type, position and the sequence context of each polymorphism (Vignal et al., 2002; Kwok et al., 2003). Fluorescently tagged PCR primers specific for a particular SNP can be used for sensitive detection of SNPs. Subsequent comparison between SNP genotypes to specific phenotype of many individuals identify the SNP genotypes that are significantly associated with the phenotype of interest. The International Chicken Polymorphism Map Consortium has identified approximately 2.8 million single nucleotide polymorphism (SNPs) based on a comparison of the jungle fowl sequence to three domestic chicken strains (a broiler, a layer and a Chinese silkie) with a mean rate of about five SNPs per kilobase (International Chicken Polymorphism Map Consortium, 2004).

E. Divergent Selection for Ascites

Since ascites was first noticed in chickens raised at high altitudes, a simulated high altitude environment was the first and simplest technique to select ascites resistant and susceptible lines of chickens (Balog, 2003). Dr. Nicholas Anthony, (University of Arkansas, Poultry Science) has developed divergently selected susceptible (SUS) and resistant (RES) lines of broiler chickens reared in a hypobaric chamber (2,900 m above sea level) to induce the disease. The hypobaric chamber simulated high altitude conditions via a partial vacuum, which thereby lowered the partial pressure of oxygen. A separate group was reared under typical

management and environmental condition, local altitude (390 m above sea level); to serve as the control subjects maintained as a randomly mated population (REL line) .The initial generation was a commercial elite breeding line obtained from a primary poultry breeding company. Ascites mortality data for birds reared in the hypobaric chamber were used for selection of siblings reared at ambient pressure. Thus, susceptible and resistant lines were generated based on sibling selection over 14 successive generations in the hypobaric chamber. This selection showed an average incidence of ascites of 95.1% in the SUS line by the 8^{th} generation, and an average incidence of ascites of 7.1% in the RES line by the 9^{th} generation, while the REL line was approximately 60% (Pavlidis et al., 2007).

A cross of the SUS and RES lines was used to generate an F2 population to map some chromosomal regions that have been linked to PAH in humans (Pavlidis et al., 2007). A genome-wide association study (GWAS) identified 7 chromosomal regions that showed linkage disequilibrium with ascites susceptibility and cardiac hypertrophy (Smith, 2009). Further genetic analysis confirmed 4 regions on 3 chromosomes that were found to be associated with ascites susceptibility. These regions contain candidate genes that might be involved in resistance/susceptibility. These genes include AGTR1 (angiotensin 2 type 1 receptor) and UTS2D, (urotensin receptor 2 D) located on Gga9:13.5–14.8 Mbp; 5HT2B, (serotonin receptor/transporter type 2B) located on Gga9:16 Mbp and ACE (angiotensinogen cleaving enzyme) located on Gga27:2 Mbp (Wideman et al., 2013). All of these genes have been shown to be involved in the development of human PAH and they are now under further investigation in our lab to determine the extent to which each is involved in ascites/PHS in chicken. My thesis work has focused on associations of the 5HT2B gene with ascites.

F. 5HT2B Receptor

The serotonin receptor 5HT2B (also designated HTR2B) is composed of 3 exons located at 16.252 Mbp on Gga9 for the 2006 assembly or 14.632 Mbp in the 2011 assembly. In broilers, 5-HT is the most potent pulmonary vasoconstrictor that triggers pulmonary hypertension (Chapman, 2002). The major effect of 5-HT is mediated through the receptors/transporter (5HT2B) and (5HT1A) on the PASMCs. In chickens, serotonin is released from thrombocytes; the equivalent to platelets in humans. It is also released by the pulmonary neuroendocrine system. The 5-HT is internalized by the PASMCs causing smooth muscle cells proliferation and vascular remodeling (Chapman et al., 2008; Wideman et al., 2013). High levels of 5-HT have been induced using various methods such as intravenous micro-particles injection, diets containing high levels of Tryptophan (serotonin precursor) or serotonin intra venuous infusion (Hamal et al., 2010a; Wideman et al., 2013). Moreover, high expression of 5HT2B receptors in the lungs of SUS broilers has been recorded suggesting their key role pulmonary vasoconstriction and PASMC proliferation. However, pretreatment with methiopthepin (serotonin receptor blocker) eliminates the increase in pulmonary vascular resistance and reduce pulmonary arterial pressure (Hamal et al., 2010a; Wideman et al., 2013). The evidence is that susceptibility to PAH in broilers is partially attributed to the serotonin and serotonin receptors overexpression.

G. The Promoter Region

The promoter region of a gene is a region that contains specific short DNA sequences called *transcription factors binding sites* where specific transcription factors bind and recruit RNA polymerase to the transcription start site. Transcription factors are proteins that positively or negatively regulate gene expression through binding to their transcription factor binding sites. Sequential assembly of the transcription factors and then RNA Polymerase II forms the pre-

initiation complex. Promoter regions can be considered important molecular tool for the regulation of the expression of genes of interest. Typical vertebrate promoters are 100-1000 base pairs long located close to and upstream of the genes they transcribe. However, some critical sequences may be located further from the transcription start site. The 5' proximal region of the 5HT2B receptor is a critical *cis*-element that should contain regulatory elements to direct the transcription process of a particular gene, and would constitute much of the promoter region. Thus, sequence analysis of the 5HT2B promoter regions may identify sequence polymorphisms that affect expression of the gene and contribute to ascites.

Previously in our laboratory, Patrick Greenburg (Honor student, University of Arkansas) sequenced the exonic regions of 5HT2B from several DNAs from SUS and RES lines He identified 4 SNPs; a SNP in the 5' untranslated region (UTR), one in an intron 2, and two in exon 3. Exon 3 contains all of the predicted protein coding sequence. The two SNPs in the 3rd exon are C/T and C/A SNPs that result in silent mutations, and thus are not likely to affect protein production or structure.

My research project was to extend our sequence analysis to the upstream promoter regions of 5HT2B to identify any sequence polymorphisms, especially those affecting predicted transcription factor binding sites. SNPs affecting binding sites would then be analyzed for linkage disequilibrium with ascites phenotype. I sequenced approximately 900 bases of 5' proximal DNA from the RES and SUS lines. I developed a TaqMan Assays for a particular group of SNPs. This TaqMan assay was then used to SNPlotype a collection of DNA samples from birds previously phenotyped for ascites susceptibility in the hypobaric chamber challenged. Statistical analysis of the SNPlotyped data helped us determine the linkage between the different alleles and their association with resistance and susceptibility of the chickens to the disease.

II MATERIALS AND METHODS

1. DNA Isolation

Genomic DNAs were previously isolated from blood from phenotyped birds and stored frozen (Krishnamooorthy, 2012).

2. Primers

The DNA region for the 5HT2B gene on Gga9 at 16.2 Mbp was downloaded from the UCSC genome browser http://genome.ucsc.edu/. Primer3 http://frodo.wi.mit.edu/ was used to design PCR primers to cover the promoter region of the gene with lengths of 20-23 base pairs and annealing temperature of 50-54^{o}C. Primers were synthesized by Integrated DNA Technologies. Primers and probes information are listed in Table 1.

3. Polymerase Chain Reaction

PCR was performed using either an MJ Research PTC-100 thermocycler or an Eppendorf Mastercycler gradient. PCR mixtures (20 µL) contained: 1X Taq Buffer (50 mMTris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA), 0.2 mM dNTPs, 1 µM of reverse and forward primers, 4 units of Taq polymerase and 2 µL (approximately 100 ng) of target DNA. PCR amplification conditions were initial denaturation at 90°C for 30 seconds, followed by 40 to 45 cycles of denaturation at 90°C for 20 seconds, 20 seconds of annealing at the indicated temperature (Table 1), and elongation at 72°C for 60 seconds, followed by a final extension at 72°C for 3 minutes.

4. Gel Electrophoresis and Gel Imaging

Following the PCR, 5 µL of purified PCR product was mixed with 5 µL of loading dye (20% Ficoll, 2 mM EDTA, and 0.02% Bromophenol Blue) and loaded into a 1.5% agarose gel in 0.5x TBE buffer (50 mM Tris, 1mM Na_2EDTA, ~25 mM Borate pH 8.3) to evaluate PCR

products for proper amplification. Gels were stained in ethidium bromide and then either scanned at 610 nm on a model 9600 Typhoon Imager (GE Health Care) or imaged using a CCD camera and an Ultraviolet transilluminator. Images were analyzed using ImageQuant software. PCR fragment sizes were determined using the molecular weight ladder pGEM5 digested with Sau3A

5. PCR Product Purification and Quantification

PCR products were purified for sequencing using RapidTip from Diffinity Genomics according to the manufacturer's instructions. DNAs then were quantified using a TKO 100 Fluorometer (Hoefer Scientific Instruments) according to the manufacturer's instructions.

6. DNA Sequencing and Analysis

DNA samples were mixed with single primers and submitted for sequencing by the University of Arkansas, DNA Resource Center. The sequence files were aligned and analyzed using SeqMan software (DNAStar ver 6.0). Specific SNPs in our region of interest were identified from the sequence data through comparison to the Jungle Fowl sequence from UCSC.

7. Real Time PCR- Taqman Assay for SNPlotyping

A Taqman assay was developed to cover specific SNPs at positions 491, 488, 484 respectively of the 5HT-2B promoter region (Table 2). The primers and probes are listed in Table 1. Real time PCR was performed in 96 well plates using a BioRad CFX 96 Real Time System. Reactions (20 μL) were as for standard PCRs (see above with addition of the two probes at 0.25 μM. The cycling protocol was: 1) 90°C for 30 seconds, 2) 90°C for 15 seconds, 3) 64°C for 30 seconds, 4) repeat steps 2 and 3 for 10 times, 5) 90°C for 15 seconds, 6) 64°C for 30 seconds + plate read, 7) Repeat steps 5 to 6 for 30 times. Amplification profiles were visually inspected to score for homozygous for either SNP pattern or heterozygous.

8. Statistical Analysis

Genotype data (SNPlotypes) for each sample were compiled and statistically analyzed in Microsoft Excel. Allelic and genotypic frequencies were calculated using observed allele counts. Expected allele and genotype frequencies were calculated based on the allele frequencies observed in the entire population. Expected genotype counts were also computed based on standard Hardy-Weinberg Equilibrium ($p^2+2pq+q^2$) to assess whether the assay was performing adequately. For each allele and genotype we calculated P- values using the ChiTest in Excel comparing the observed counts to the expected counts for the resistant and susceptible subpopulations. The level of significant ($P<0.05$) is the measurement that I used in my study. The null hypothesis can be rejected if the significance test gave a P-value lower than ($P< 0.05$) and considered as statistically significant results, and fail to reject the null hypothesis if the significance test gave a P-value higher than 0.05 ($P> 0.05$).

III. RESULTS

1. Primer Design, DNA Sequencing and SNP Detection

For this study, we designed two pairs of oligonucleotide primers 5HT2B PF1/PR1 and 5HT2B PF2/PR2 respectively to amplify an approximately 900 base region upstream of exon 1 of the 5HT2B receptor gene located on Gga9. The primer pair names, sequences, fragment sizes, annealing and melting temperatures are shown in Table 1. The primers were used to PCR amplify the upstream promoter region from DNAs from SUS and RES line birds, and from a single White Leghorn male (WL). PCR products were evaluated on agarose gels, purified; quantified and submitted for capillary sequencing. The sequences were aligned with the sequence for this same region of the 5HT2B receptor gene from the 2006 genome assembly for *Gallus gallus*, the Red Jungle Fowl (JF). The alignments identified 15 polymorphisms (SNPs) in

the promoter region of the 5HT2B receptor gene (Table 2). These SNPs are located at different distances upstream of the predicted transcription start site of the 5HT-2B gene (14, 641511bp) according to the 2011 chicken assembly (GCA_000002315.2).

We analyzed the locations of these 15 SNPs for proximity to potential transcription factor binding sites. The three SNPs at 484, 488, and 491 were found to affect potential binding sites for NF-κB and TonEBP/OREBP transcription factors. Therefore, polymorphisms for these SNPs have the potential to affect gene expression which in turn could contribute to ascites susceptibility or resistance. The SNP found in position 484 was a C/T transition with homozygous T in JF, WL and two RES birds, a C in three SUS birds, and heterozygous Y in two each of the RES and SUS birds. The rest of the SUS and RES DNA birds for this particular SNP could not be determined because of flanking insertion/deletions (indels). The SNP at position 488 was an indel with JF, WL and three RES birds homozygous T, three SUS birds were homozygous for the deletion, and heterozygotes (designated H in Table 2) were found in three RES and six SUS birds. The SNP at position 491 was also an indel with homozygous C in JF, WL and three RES birds, homozygous deletion in three SUS birds, and heterozygous H for the C/deletion in three RES and six SUS birds (Table 2). Examination of the DNA sequences shows that the three SNPs are in complete linkage and thus define two alternative alleles. Therefore, this region was targeted for SNPlotyping for a larger collection of DNA samples. Two pairs of primers were designed to amplify this specific region containing the SNPs located at positions 484, 488, and 491. The primer pair 5HT2B PF3/PR3 did not amplify a PCR product. However, the primer pair 5HT2B PF4/PR3 produced a PCR product size of 322bp based on evaluation by gel electrophoresis (data not shown).

2. TaqMan Assay for SNP Analysis

I developed a TaqMan assay to target this particular group of SNPs for genotype determination. The TaqMan assay provides a fast and simple method to genotype for specific SNP patterns in a large number of DNA samples. I designed two specific TaqMan probes for the two SNPs patterns with alternative reporter dyes (Table 1). One probe, 5HT-2Btm1 represents the JF and WL allele (C-T-T) with HEX as the fluorescent reporter dye. The other probe, 5HT-2Btm2 represents the mutant allele found in SUS and RES birds (_-_-C) with FAM as the fluorescent reporter dye. Both probes have a quencher (BQ1) on the 3' end, so as long as the probe remains intact, the quencher reduces the fluorescence emitted by the reporter dye. However, once the probe anneals with the specific target DNA sequence, it is digested during the extension step of PCR by the Taq polymerase 5'→3' exonuclease activity. The hydrolysis of the probe removes it from the target DNA strand and separates the reporter dye from the quencher, allowing detection of the reporter dye fluorescence signal in a real-time PCR machine. For my probes, if the TaqMan assay results in the generation of only the HEX signal, then the individual is homozygous wild type at that locus. If the assay results in the generation of only the FAM signal, then the individual is homozygous for the alternative allele. However, if both fluorescent signals are produced, then the individual is heterozygous. The TaqMan assay was evaluated at different anneal/extension temperatures with DNAs previously sequenced for this region to establish optimal temperatures for genotype analyses.

3. REL Line

The REL line is the unselected line descended from the original source population for the RES and SUS lines. The TaqMan assay was employed on 192 DNA samples previously obtained from birds from generation 14 that had been phenotyped for ascites susceptibility in the hypobaric chamber (Sriram Kirshnamoorthy, 2012). According to the Hardy Weinberg

equilibrium, if there are two alleles in a population, with frequencies of p and q, respectively, then the values of p and q can be used to calculate expected genotype frequencies using the formula $1=p^2+2pq+q^2$.

By analyzing the REL line for the promoter SNPs, two alleles were identified with the tm1 allele present at 59% while the tm2 allele was at 41% (Table 3). The observed homozygous frequency was relatively high (75%) compared to the expected frequency (52%) where the heterozygous frequency was lower (24%) than expected (48%). The higher number of observed homozygous and fewer heterozygous than expected is an indication of the presence of null alleles. The presence of null alleles (non-amplified alleles) could lead to false observation of excess frequency of homozygotes when segregating with other alleles and fewer heterozygotes counts than expected appeared. Thus, it may result in misleading data and cause deviation from the Hardy Weinberg equilibrium. A statistical deviation from Hardy Weinberg expectations can be detected in the REL line. However, no significant differences from expected genotype frequency in respect to ascites phenotype can be detected in this line according to Chi- square analyses.

4. REL vs. RES vs. SUS

I genotyped samples from 102 RES birds and 59 SUS birds to compare with genotype frequencies in the REL. This analysis was to determine how divergent selection for ascites affected allele and genotype frequencies at this locus. Both alleles were detected in all lines. The tm1 homozygotes showed similar high frequencies in all the three lines REL, RES and SUS (73%, 72%, and 76% respectively). The tm1/2 heterozygotes showed similar lower frequencies for the respective lines (24%, 26%, and 20% respectively). For the homozygous tm2, the REL, RES, and SUS lines had a very low frequency (2%, 1%, and 3% respectively). The

tm1homozygotes were found to be more frequent in all three lines. The tm2 homozygotes were found to be at lower frequencies in the three lines as well. Therefore, selection for ascites susceptibility or resistance does not appear to have affected the frequencies of these SNP patterns.

5. Linkage Analysis for Ascites Phenotype in REL

According to the genotype data in Table 3, the different genotypes were seeing at roughly equal frequencies in both SUS and RES birds. At the gender level, the genotypes frequencies were approximately the same in both females and males. Chi- square test showed no significant deviation from expected allele counts with respect to the two ascites phenotype. Thus, it appears that there is no association of the 5HT2B promoter region SNPs with ascites phenotype with respect to gender in the REL line.

6. Haplotype Analysis for the REL Line

A former honors student, Patrick Greenburg, had sequenced all three exons for 5HT2B and identified a C/T SNP (SNP9093) which is 9093 bases downstream of the transcription start site and early in the 3rd exon. The same REL samples had been previously genotyped for that SNP. Chi- square analysis did not detect a statistically significant association of any genotype with ascites phenotype. Table 4 represents allele and genotype data for SNP9093 for the REL line.

We combined the genotype data for SNP9093 and the promoter SNPs (tm1 and tm2) to generate haplotypes for the REL line birds (Table 5). The haplotype analysis was to determine which SNP9093 allele is in linkage with the tm1 or tm2 allele in the promoter region and whether a particular haplotype showed significant linkage disequilibrium (LD) with respect to

ascites phenotype. Surprisingly, we found that SNP9093 and the promoter proximal SNPs show a high frequency of recombination despite only being approximately 9500 base pairs separated. Whereas we expected to find a high frequency of association of homozygotes for each position we instead found that approximately half (74 out of 183) haplotypes were homozygous for one SNP position while heterozygous for the other SNP position (haplotypes 12, 21, 23, 32 in Table 5). This data suggests an unusually high level of recombination between the two SNP regions despite their close proximity. However, analysis of haplotype with respect to phenotype showed that the double heterozygote, 22, is statistically overrepresented in resistant birds (P=0.017). The association was greater in females (P=0.014) than in males (P=0.072).

7. Promoter SNP Analysis in Commercial Line Y

Genotype data for samples from commercial line Y identified that the tm1 and tm2 alleles appear to represent the major alleles with tm2 allele present at 72% and tm1 28% (Table 6). The genotype data did not deviate from Hardy Weinberg expectations so there do not appears to be evidence of additional alleles segregating. The tm1 homozygotes were overrepresented in resistant birds (16:10.9, observed to expected) with frequency of 14%, and underrepresented in susceptible birds (2:7.1, observed to expected) with frequency of 2%. Chi-square test showed that this difference was statistically significant (P = 0.014) with the deviation only significant in females (P=0.001) where all 11 homozygous tm1 females resistant. It appears that the homozygous tm1 increases resistance to ascites in females.

8. Promoter SNP Analysis of Commercial Line Z

Analysis of commercial line Z samples for the promoter SNPs identified the tm1 allele at 43% while the frequency for the tm2 allele was 56% (Table 7). Hardy Weinberg analysis

suggests no evidence for other major alleles in this line. There was no significant difference from expected genotype counts with respect to ascites phenotypes based on Chi-square analyses. However, if we compared the observed to the expected counts of resistant and susceptible female birds in this population, we found that tm2 homozygous genotype was overrepresented in resistant female birds (15:14, observed to expected) and underrepresented in susceptible female birds (6:11.8, observed to expected) but the deviation was not statistically significant (P=0.09). Thus, there might be an association to ascites in females for this line but would require analysis of additional phenotyped samples.

9. Promoter SNP Analysis of Commercial Line W

Analysis of samples from commercial line W for the promoter region SNPs identified the tm1 allele was present at 84% compared to the tm2 allele at 16% (Table 8). Hardy Weinberg analysis was consistent with these alleles being the predominant alleles in this line. Chi-square analysis of the three genotypes with respect to phenotype detected no significant deviations for any genotype with respect to ascites phenotype. There was also no association of any genotype when the line was analyzed separately with respect to gender. Thus, no strong association to ascites can be detected in this line.

IV. DISCUSSION

In previous work, 7 chromosomal regions showed linkage disequilibrium with respect to ascites susceptibility and cardiac hypertrophy (Smith, 2009). These regions were identified in the whole genome SNP analysis of an F_2 cross of the SUS and RES lines. Further genetic analysis with microsatellite loci has confirmed the involvement of 4 regions on 3 chromosomes in ascites susceptibility. The region on Gga9 contains the serotonin receptor (5HT2B) gene

located at 16.252 Mbp in the 2006 assembly or 14.632 Mbp in the 2011 assembly. Several studies showed that susceptibility to PAH in broilers involves the serotonin pathway or metabolism. Moreover, substantial increases in the expression of 5HT2B receptor in pulmonary arteries were seen in both human and mice (Launay et al., 2002). For this study, the main objective was to identify SNPs present in the promoter region of 5HT2B in SUS and RES line birds. Unequal occurrence of several SNPs between the SUS and RES lines could be attributed to possible effect of divergent selection for ascites incidence. Fifteen SNPs were identified with SNPs located in close proximity chosen for further analysis owing to their potential to affect binding sites for NF-κB and TonEBP/OREBP transcription factors. The three SNPs define two alternative alleles and therefore, I developed a TaqMan assay for further analyses of different chicken lines.

The REL line served as the founder base population for both SUS and RES lines. By analyzing the REL line genotypic frequencies, a significant deviation from Hardy Weinberg equilibrium was found where higher number of observed homozygous and fewer heterozygous than expected were present. Over representation of homozygotes suggests that we are not detecting all alleles for this region and thus null alleles might contribute to the deviation from the Hardy Weinberg equilibrium. The deviation could also derive from an inaccurate genotyping assay or the samples analyzed were not a random sampling of the population. However, the possibility of inaccuracy in genotyping performance being responsible for this deviation is not supported since we see agreement with Hardy Weinberg in the other lines. Further, the REL line samples were for all those challenged in the hypobaric chamber and weren't selected for any specific trait. Thus, there is no evidence that suggests prove that these samples were non-random. For this study, we believe that null alleles are most likely responsible for this deviation. Any

mutation that affects the DNA sequences specific for either the primers or the probes will prevent their hybridization during PCR amplification. Thus, no amplicon is generated or is not detected, and this allele would be considered as a null allele. Apparently, undetected alleles for this region were segregating with null alleles explains the high frequency of homozygotes. We could conclude from this that some of the promoter regions for 5HT2B are actually heterozygous but that mutations in either the primer or probe binding sites for one of its two alleles results in a null allele and thus it appears as a homozygote. Although null allele heterozygotes are undistinguishable from expected homozygotes, direct resequencing for the same region could further identify SNPs responsible for the null alleles. Moreover, a possible way to distinguish null alleles from any biological processes that might result in deviation from Hardy Weinberg equilibrium is by testing candidate genes of REL line for deviation across Gga9. If this deviation occurs only in the 5HT2B gene then we could assume that this deviation is a locus- specific phenomenon, possibly null alleles.

Comparison analyses between the REL, SUS, and RES lines showed the equal distribution of the allelic/genotypic frequencies in all three lines indicating that selection for ascites susceptibility/resistance had no affect on the respective frequencies. The genotype data for SNP9093 located on the 3rd exon of the 5HT2B gene and the promoter SNPs (tm1 and tm2) were combined into haplotypes for linkage determination. Generated haplotypes gave us an informative insight into possible linkage disequilibrium of specific haplotypes to ascites phenotype. Haplotype analysis in the REL line revealed an unusually high frequency of recombination between SNP90933 and the promoter proximal SNPs despite the short distance between the two DNA positions. The high recombination frequency results in individuals that are homozygous for SNP9093and heterozygous for the promoter and vice versa. There was almost

the same number of haplotypes that were homozygous for 9093SNP and heterozygous for the promoter SNPs (27 haplotypes) as for haplotypes that were heterozygous for 9093SNP and homozygous for the promoter SNPs (38 haplotypes). We may conclude from this that this recombination event could not be a result of null alleles being present in the promoter region because if it is true then we should see mostly heterozygotes for SNP9093 that are homozygous for the promoter SNPs. One possible approach that would help resolve this would be to conduct a pedigree-based study. For future research, it is very important to look at SNP genotyping data for this particular region in a large parent- offspring population to detect the segregation patterns of the SNPs for exon 3 and the promoter. If we were able to link specific deletion variants/polymorphisms to nearby SNPs in the pedigree then we could associate this region to ascites. Analysis of haplotype with respect to phenotype showed that the double heterozygote, 22, haplotype was statistically overrepresented in resistant birds and primarily a female affect. Thus, double heterozygote 22 haplotype in REL line showed a significant contribution to ascites resistance.

Commercial broilers represent the product of continuous selection for traits of economic importance. The allelic and genotypic frequencies for SNPs present in the 5HT2B promoter region were different between the three commercial lines. Probably, the reason behind that is differences in selected traits as per the company's goals. The main objective in analyzing these commercial lines was to associate overrepresented genotypes in resistant or susceptible phenotypes. In the commercial line Y, the homozygous tm1genotype was highly associated to ascites resistant phenotype in females (P= 0.001). Thus, it contributed in increasing resistance to ascites in females. Analyzing the commercial line Z showed that the homozygote tm2 genotype was overrepresented in females resistant to ascites. However, since no significant deviation was

detected by Chi-square analyses, additional phenotyped samples are required to determine whether females are strongly associated with ascites resistance or not. In the commercial line W, no strong association with ascites was detected. Haplotype analysis of these samples for the promoter region and SNP9093 would help us to further search for potential association of the 5HT2B gene to ascites in this line.

In summary, our data support the association of particular genotypes/haplotypes of the 5HT2B gene with resistance to ascites especially in females. The double heterozygote 22 haplotype and the homozygote tm1 genotype were found to be the most ascites resistant haplotype/genotype in the REL and Y line respectively, especially in females. Increasing genetic selection for resistant genotypes is a promising tool for commercial poultry improvement. Since the promoter SNPs affect potential binding sites for transcription factors it will be important to examine gene expression levels for the alternative alleles. Differential expression in particular tissues could contribute to ascites susceptibility or resistance. One way to prove the hypothesis is by detecting difference in 5HT2B gene expression levels in different pulmonary and arterial tissues from resistant and susceptible chickens. Any significant differences in the gene expression levels in the two populations can thus be correlated to specific alleles/genotypes in this gene. However, genetic selection for ascites resistance could negatively impact traits of economic importance like body weight (Pavlidis, 2007). Thus, traditional selection for traits of economic importance should go along with selection for ascites resistant genotypes to overcome undesirable economic losses. This knowledge of underlining genetics of ascites syndrome will lead to genetic selection to reduce ascites incidence in chicken and further establish chicken as the medical animal model for human PAH.

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VI. TABLES AND FIGURES

Table 1. PCR primers and probes used in this study. Primer name and sequences (presented as 5' to 3' sequence) are listed along with their fragment size, Annealing Temperature used, and Calculated Melting Temperature. For the TaqMan probes the specific 5' and 3' modifications are listed and the probes are presented in lower case with SNPs indicated in upper case. Note for indels the SNP is missing in the tm2 probe.

Name	Sequence (5'>3')	Fragment Size	Annealing Temperature	Calculated Melting Temperature
5HT2B-PF1	CTTCCTGACCCTCACCTGAT	814 bp	50^{o}C	62.4^{o}C
5HT2B-PR1	CGCACTTTGTTTCCTTGCTC		50^{o}C	60.4^{o}C
HT2B-PF2	GAAGCCCTGCTCAGATTACAA	984 bp	50^{o}C	60.6^{o}C
HT2B-PR2	GATGATCACCAGCAGGATCAG		50^{o}C	62.6^{o}C
5HT2B-PF3	AGGAAACATGGGAACTGCTG	No product	50^{o}C	55.3^{o}C
5HT2B-PR3	TGCCTTACTGGGTGTGATGA	322 bp	54^{o}C	56.0^{o}C
5HT2B-PF4	GAGGAGGAGTTGCACACACA		54^{o}C	57.2^{o}C
5HT2Btm1	HEX-tCttTgggTttttcctacagcgg-BQ1		64^{o}C	57.8^{o}C
5HT2Btm2	FAM-ttt_tt_gggCttttcctacagcggt-BQ1		64^{o}C	59.5^{o}C

Table 2. SNPs identified in the upstream promoter region of HTR2B in selected chicken DNAs. Sequences were aligned for DNAs from the RES and SUS lines (RES or SUS), White Leghorn (WL) and the published sequence for Jungle Fowl (JF). SNP positions are presented relative to the predicted transcription start site at 14641511 in the 2011 chicken assembly (GCA_000002315.2). Question mark (?) indicates that the SNP was not determined for that DNA. Underscore indicates that the base is missing relative to the JF reference, and H indicates where an individual was heterozygous for the deleted base. Standard IUPAC base nomenclature is used for heterozygous SNPs.

		Distance upstream from Transcription Start Site 14641511														
		654	642	637	502	494	491	488	484	412	383	341	289	217	123	86
	JF	G	G	C	T	C	C	T	T	A	G	G	A	C	A	T
Line	WL	G	G	Y	Y	C	C	T	T	A	A	A	_	C	G	Y
RES	8465	?	?	?	C	C	?	T	Y	M	?	?	?	?	?	?
RES	8301	?	?	?	T	C	C	T	T	A	G	A	_	?	?	?
RES	8415	G	A	C	T	C	C	T	T	A	G	A	_	C	G	T
RES	8550	?	?	?	Y	H	H	H	Y	M	G	A	_	?	?	?
RES	8553	G	G	?	Y	H	H	?	?	?	?	?	_	?	?	?
RES	8496	R	G	C	Y	H	H	H	?	?	?	?	_	C	G	?
SUS	785	G	G	T	C	_	_	_	C	C	G	A	_	C	G	T
SUS	739	G	G	Y	Y	H	H	H	Y	M	G	A	_	C	G	T
SUS	760	G	G	T	C	_	_	_	C	C	G	A	_	C	G	T
SUS	609	R	G	C	Y	H	H	H	?	?	?	?	_	?	?	?
SUS	789	?	G	Y	Y	H	H	H	?	?	?	?	_	Y	G	T
SUS	281	G	G	Y	Y	H	H	H	Y	M	G	?	_	C	G	T
SUS	852	G	G	Y	Y	H	H	H	?	?	?	?	_	C	G	T
SUS	907	G	G	T	Y	H	H	H	?	?	?	?	_	?	?	?
SUS	1090	G	G	Y	C	_	_	_	C	C	G	A	_	C	G	T

Table 3. Genotype data for the promoter region SNPs for REL line samples. Genotypes are listed in the left most column as tm1= homozygous (_-_-C); tm1/2= heterozygous; tm2= homozygous (C-T-T). Counts, Expected counts, and Frequency are presented for All, resistant (Res), and susceptible (Sus) phenotype based on hypobaric chamber challenge. Counts, expected and frequency are also presented for each gender. Counts for Res and Sus were compared to expected for computation of ChiSquare values (Chitest).

ALL	Counts			Expected		Frequency			Chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
tm1	137	74	45	74.7	43.2	0.733	0.725	0.763	0.777
tm1/2	45	27	12	24.5	14.2	0.241	0.265	0.203	0.444
tm2	5	1	2	2.7	1.6	0.027	0.010	0.034	0.272
Female									
tm1		29	19	29.3	19.0		0.725	0.731	0.954
tm1/2		11	6	9.6	6.3		0.275	0.231	0.649
tm2		0	1	1.1	0.7		0.000	0.038	0.273
Male									
tm1		45	26	45.4	42.2		0.726	0.788	0.707
tm1/2		16	6	14.9	7.9		0.258	0.182	0.457
tm2		1	1	1.7	0.9		0.016	0.030	0.599

Table 4. Genotype data for SNP9093 (C/T SNP in exon 3) for REL line samples. Genotypes are listed in the left most column. Columns and headings are as described in Table 3.

All	Counts			Expected		Frequency			chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
C	35	18	11	18.7	11.2	0.19	0.18	0.18	0.858
Y	58	37	13	31.0	18.6	0.31	0.37	0.22	0.092
T	94	45	36	50.3	30.2	0.50	0.45	0.60	0.195
Female									
C		7	3	7.9	5.1		0.17	0.11	0.335
Y		18	7	13.0	8.4		0.43	0.26	0.145
T		17	17	21.1	13.6		0.40	0.63	0.197
Male									
C		11	8	10.9	6.2		0.19	0.24	0.462
Y		19	6	18.0	10.2		0.33	0.18	0.179
T		28	19	29.2	16.6		0.48	0.58	0.529

Table 5: Combined genotype data for haplotype analysis for REL samples. Haplotype designations are 1= homozygous C or tm1; 2= heterozygous; 3= homozygous T or tm2. Genotype combinations are listed in the left most column with SNP9093 listed first. Columns and headings are as described in Table 3.

All	Counts			Expected		Frequency			Chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
11	32	16	10	17.3	10.1	0.175	0.162	0.172	0.750
12	3	2	1	1.6	1.0	0.016	0.020	0.017	0.764
13	0	0	0	0.0	0.0	0.000	0.000	0.000	
21	37	23	11	20.0	11.7	0.202	0.232	0.190	0.484
22	18	14	1	9.7	5.7	0.098	0.141	0.017	0.017
23	1	0	0	0.5	0.3	0.005	0.000	0.000	0.354
31	64	32	23	34.6	20.3	0.350	0.323	0.397	0.453
32	24	11	10	13.0	7.6	0.131	0.111	0.172	0.304
33	4	1	2	2.2	1.3	0.022	0.010	0.034	0.306
Female									
11		6	2	6.5	4.4		0.162	0.080	0.250
12		0	1	0.6	0.4		0.000	0.040	0.228
13		0	0	0.0	0.0		0.000	0.000	
21		8	6	7.5	5.1		0.216	0.240	0.645
22		8	1	3.6	2.5		0.216	0.040	0.014
23		0	0	0.2	0.1		0.000	0.000	0.561
31		12	10	12.9	8.7		0.324	0.400	0.618
32		3	4	4.9	3.3		0.081	0.160	0.352
33		0	1	0.8	0.5		0.000	0.040	0.276
Male									
11		10	8	10.8	5.8		0.161	0.242	0.336
12		2	0	1.0	0.5		0.032	0.000	0.222
13		0	0	0.0	0.0		0.000	0.000	
21		15	5	12.5	6.7		0.242	0.152	0.342
22		6	0	6.1	3.2		0.097	0.000	0.072
23		0	0	0.3	0.2		0.000	0.000	0.471
31		20	13	21.7	11.5		0.323	0.394	0.575
32		8	6	8.1	4.3		0.129	0.182	0.421
33		1	1	1.4	0.7		0.016	0.030	0.654

Table 6. Genotype data for promoter region SNPs for commercial line Y (35). Genotypes, column headings, column values are as described in Table 3

All	Counts			Expected		Frequency			chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
tm1	18	16	2	10.9	7.1	0.097	0.142	0.027	0.014
tm1/2	67	40	27	40.7	26.3	0.360	0.354	0.370	0.860
tm2	101	57	44	61.4	39.6	0.543	0.504	0.603	0.374
Female									
tm1		11	0	5.0	3.5		0.212	0.000	0.001
tm1/2		18	13	18.7	13.0		0.346	0.361	0.866
tm2		23	23	28.2	19.5		0.442	0.639	0.209
Male									
tm1		5	2	5.8	3.5		0.083	0.056	0.388
tm1/2		21	14	21.6	13.0		0.350	0.389	0.752
tm2		34	20	32.6	19.5		0.567	0.556	0.788

Table 7. Genotype data for promoter region SNPs for commercial line Z (58). Genotypes, column headings, column values are as described in Table 3

All	Counts			Expected		Frequency			Chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
tm1	37	23	13	24.3	11.9	0.20	0.19	0.20	0.42
tm1/2	92	58	34	60.4	29.9	0.49	0.47	0.53	0.21
tm2	60	43	17	39.4	22.2	0.32	0.35	0.27	0.31
Female									
tm1		9	9	8.6	6.3		0.20	0.26	0.28
tm1/2		20	19	21.4	15.9		0.45	0.56	0.40
tm2		15	6	14.0	11.8		0.34	0.18	0.09
Male									
tm1		14	4	14.9	5.5		0.18	0.14	0.50
tm1/2		36	14	37.0	13.6		0.47	0.50	0.85
tm2		26	10	24.1	8.9		0.34	0.36	0.59

Table 8. Genotype data for promoter region SNPs for commercial line W (74). Genotypes, column headings, column values are as described in Table 3

All	Counts			Expected		Frequency			Chitest
	All	Res	Sus	Res	Sus	All	Res	Sus	
tm1	135	68	67	68.2	66.6	0.70	0.70	0.71	0.956
tm1/2	52	25	27	26.3	24.5	0.27	0.26	0.28	0.572
tm2	5	4	1	2.5	3.9	0.03	0.04	0.01	0.082
Female									
tm1		34	40	30.9	39.3		0.77	0.71	0.573
tm1/2		10	15	11.9	14.4		0.23	0.27	0.565
tm2		0	1	1.1	2.3		0.00	0.02	0.169
Male									
tm1		34	27	37.3	27.4		0.64	0.69	0.589
tm1/2		15	12	14.4	10.6		0.28	0.31	0.635
tm2		4	0	1.4	1.0		0.08	0.00	0.014

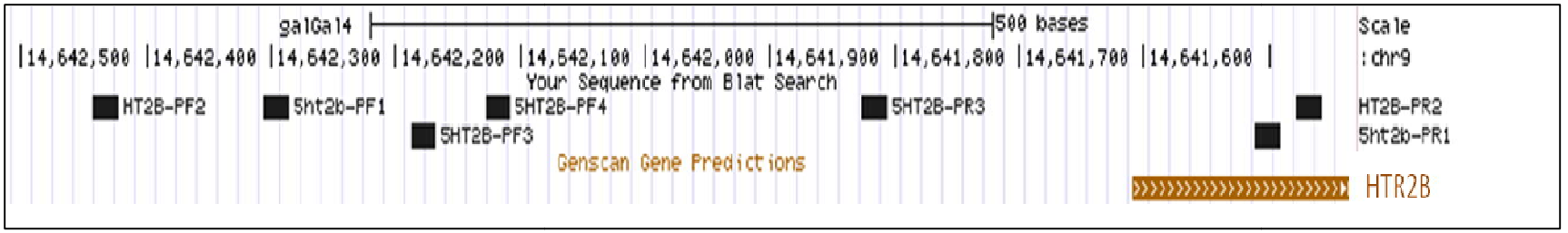

Figure 1. Genomic region analyzed and location of PCR primers used in this study. The region of chromosome 9 (2011 Assembly) as graphically depicted by the UCSC genome browser presents the negative strand. Exon 1 is indicated for the HTR2B gene with transcription proceeding to the left. Locations of PCR primers are indicated as black boxes.