## Project Narrative

## Introduction:

The focus of this research proposal is to explore the genetic correlation between modern broiler selection, the accumulation of ascites incidence, and the genetic loci underlying ascites susceptibility. Background information will be presented describing the work that has been done thus far and tools developed to successfully achieve our Aim. Background information includes: 1) description of ascites as a metabolic disorder; (2) development of divergent lines for ascites susceptibility; (3) correlated response to selection for ascites susceptibility; and (4) identification of a major locus affecting ascites susceptibility. Completion of the proposed research would provide the primary breeders a better understanding of the pitfalls of traditional selection methods and why metabolic disorders become entrenched in commercial populations. In addition, we will identify whether additional loci exist and whether we can identify key pathways involved in ascites susceptibility. Key insights will be whether ascites susceptibility is correlated with selection traits of economic significance.

### *Ascites Relationship to the Poultry Industry*

The success of the poultry industry has been fueled by the ability to continue to economically produce consumer acceptable products. This accomplishment has been achieved through high selection intensities, reduced generation interval and minimized environmental influences. Body weight and rate of growth were and still remain primary traits of focus for broiler breeder selection programs. However, muscle yield and feed conversion also became efficiency traits as well, and currently receive a tremendous amount of selection pressure. The sustainability of the broiler industry is hinged on substantial genetic progress in slaughter age, body weight, muscle yields and feed conversion at specific ages.

Unfortunately, growth progress has changed the natural balance of resource allocation at the cost of general health, maintenance and reproduction. Specifically, intense artificial selection for commercially important traits has led to physiological complications such as skeletal abnormalities (Julian, 1998; Cook, 2000), increased carcass fat, atypical poultry meat production (Anthony, 1998; Barbut, 1997, 1998), reduced immunocompetence, compromised reproductive performance (Qureshi and Havenstein, 1994; Emmerson, 1997; Anthony, 1998; Julian, 1998; Cheema et al., 2003) and ascites (Julian, 2000). It is interesting that incidence of these metabolic disorders have accumulated in virtually all intensely selected meat type poultry indicating a genetic relationship with commercial traits of economic importance.

Ascites is a pathophysiological progression leading to mortality associated with fluid accumulation in the abdominal cavity that has been observed and reported worldwide in fast-growing broiler chickens reared under a wide variety of conditions. Ascites in broilers represents the terminal consequence of an initial increase in blood pressure within the pulmonary circulation, consequently the terms “ascites syndrome” and “pulmonary hypertension syndrome” (PHS) are widely accepted as synonyms (Huchzermeyer and DeRuyck, 1986; Julian, 1993; Wideman and Bottje, 1993; Balog, 2003). We have recently published an invited review on ascites covering the genetics

physiology and immunology (Wideman et al., 2013). Ascites is a two-tiered problem facing not only live production but also the processing portion of the industry. The economic loss caused by PHS has been estimated at $100,000,000 per year in the United States (Odom, 1993). In 2002, it was estimated that 8% of the 361,000,000 broiler deaths each year can be attributed to ascites. Direct economic costs include losses due to on-farm mortality, mortality during transportation to the processing plant, and processing condemnations. Ascites accounts for 0.05 % of all processing plant condemnations. The economic loss associated with ascites is high because it tends to affect the heaviest, fastest growing birds which have had a considerable amount of labor and feed invested (Lubritz and McPherson, 1994; Anthony, 1998). Indirect economic costs are associated with nutrition and management strategies that successfully reduce the incidence of PHS by reducing metabolic oxygen demand (slower growth rates, the maintenance of thermoneutral temperatures) and improving air quality (reduced bird density, fans for air circulation). These strategies prevent broiler producers from fully realizing the genetic profit potential that modern broilers offer which, under ideal circumstances, are inherently capable of extremely fast growth and excellent feed efficiency.

***Characterization of Avian IPAH***

Chickens bred for rapid growth and meat production (broiler chickens, broilers) provide an excellent model of spontaneous Idiopathic Pulmonary Arteriole Hypertension (IPAH) in humans, encompassing many of the classic symptoms and a genetically tractable system for elucidating the underlying causes. Approximately 3% of all broiler chickens spontaneously develop IPAH when reared under conditions that promote very rapid growth, and incidences of IPAH exceed 20% when broilers are subjected to conditions that further increase the cardiac output or PVR (sub- or supra-thermoneutral environmental temperatures; hypobaric hypoxia; vascular occlusion; respiratory disease; poor air quality; hyperthyroidism) (Wideman and Bottje, 1993, Wideman, 2000, 2001). The lung volume of broilers is poorly correlated with body mass, creating an incipient pulmonary insufficiency that continues to be further exacerbated by ongoing genetic selection for very rapid muscle accretion and thus increased metabolic oxygen demand (Wideman, 1999; Owen et al., 1995a,b; Julian, 1998; Silversides et al., 1997). Our research has confirmed that the pulmonary vascular capacity of broiler chickens is marginally adequate to accommodate the cardiac output required to support the metabolic demands incurred by fast growth and the extremes of environmental temperatures. The pulmonary vascular capacity can be broadly defined to encompass metabolic limitations related to the tone (degree of contraction) maintained by the primary resistance vessels (arterioles), as well as anatomical constraints related to the compliance and effective volume of the blood vessels. The pulmonary vasculature of broilers is functionally inelastic (marginally compliant) and is fully engorged with blood at a normal (resting) cardiac output. Consequently, broiler lungs do not adapt to an increasing cardiac output through compensatory mechanisms known to minimize PVR in mammals, such as capillary distention and recruitment of previously un- or under-perfused vascular channels. **Instead, susceptible individuals having the most limited pulmonary vascular capacity spontaneously develop IPAH leading to terminal pulmonary hypertension syndrome (PHS, ascites) when the right ventricle must develop an excessively elevated PAP to propel the cardiac output through the**

**lungs (Wideman and Bottje, 1993; Wideman, 2000, 2001).** Broiler chickens that otherwise appear to be clinically healthy can be demonstrated by pulmonary arterial catheterization to have PAH (Wideman et al., 2006) that precedes characteristic hypertrophy of the right ventricle (Cueva et al., 1974; Sillau and Montalvo, 1982; Hernandez, 1987; Huchzermeyer and DeRuyck, 1986; Julian, 1998; Peacock et al., 1989; Wideman, 2000). Wedge pressure measurements confirm the precapillary arterioles as the primary sites of excessive resistance to blood flow in IPAH-susceptible broilers (Chapman and Wideman, 2001; Lorenzoni et al., 2007). The distinctive pathophysiological progression following the onset of PAH includes the gradual onset of systemic arterial hypoxemia (cyanosis), polycythemia, regurgitation by the monocuspid right a-v valve, right-sided congestive heart failure, central venous hypertension, hepatic cirrhosis, and transudation of plasma from the surface of the liver into the abdominal cavity (ascites). The systemic arterial hypoxemia is attributable to a diffusion limitation initiated by erythrocytes flowing too rapidly past the pulmonary gas exchange surfaces to permit full equilibration with oxygen (Huchzermeyer and DeRuyck, 1986; Julian, 1998, 1993; Peacock et al., 1989, 1990; Wideman and Bottje, 1993; Wideman and Kirby, 1995a,b; Wideman, 1999, 2000, 2001; Wideman et al., 1996a,b, 1997, 1998, 1999, 2000, 2011; Wideman and Tackett, 2000).

### *Genetic Selection for Ascites Susceptibility*

Procedures used to reveal sub-clinical genetic susceptibility to IPAH within breeding populations of broilers include exposure to hypobaric hypoxia (hypoxic pulmonary vasoconstriction) (Ploog, 1973; Owen et al., 1990, 1995a,b,c; Anthony et al., 2001; Balog, 2003); surgical occlusion of one pulmonary artery (50% reduction in pulmonary vascular capacity + 100% increase in cardiac output to unoccluded lung) (Wideman and Kirby, 1995a,b; Wideman and French, 1999; 2000); and i.v. microparticle injections (proportional occlusion of pulmonary arterioles and initiation of focal inflammatory response) (Wideman and Erf, 2002; Wideman et al., 2002).

Exposure to sustained hypobaric hypoxia (simulated 9500 ft) has been used by Dr. Anthony and his associates at the University of Arkansas to utilize sib selection to develop IPAH-susceptible and IPAH-resistant broiler lines (Anthony et al., 2001; Balog, 2003; Pavlidis et al., 2007). Briefly, birds from a commercial elite line were reared in a hypobaric chamber that simulated high altitude by operating under a partial vacuum, which thereby lowered the partial pressure of oxygen. Ascites mortality data from birds reared under hypobaric chamber conditions were used to select siblings to be used for breeding. Twenty four sire families, 3 dams per sire, were used to reproduce each line each generation and provide sibs to generate mortality data for selection. We have also maintained an unselected, Relaxed (REL) line representing the founder population. The response to selection for the susceptible (SUS) and resistant (RES) lines of chickens was very rapid from the base population, which exhibited an incidence of ascites of 75.3%. In fact, the incidence of ascites we observed in generation 8 for the SUS line, 95.1%, and in generation 9 for the RES line, 7.1% (Figure 1). Ascites mortality for the SUS line is observed as early as 3 days post hatch and has a rapid cumulative mortality. The RES line has some ascites related mortality however it is substantially delayed as compared to the SUS line (Figure 2). Currently (generation 15) the SUS line reared at simulated 8000 ft has a cumulative mortality of 98% while the RES reared at simulated 12,000 ft

has a mortality of 15% (Wideman et al., 2013). The heritabilities for ascites were estimated to be 0.30 ± 0.05 and 0.55 ± 0.05 for the SUS and RES lines, respectively. These heritability estimates are consistent with what has been previously reported for ascites measured using other conditions (Deeb et al., 2002; Huchzermeyer et al., 1988; Lubritz et al., 1995, Moghadam et al., 2001; Navarro et al., 2001; Pakdel et al., 2002; Peacock et al., 1989; Wideman and French, 1999, 2000). The rapid selection response observed for ascites (Figure 1), coupled with the moderate to high heritabilities suggest that a few major genes may control ascites.

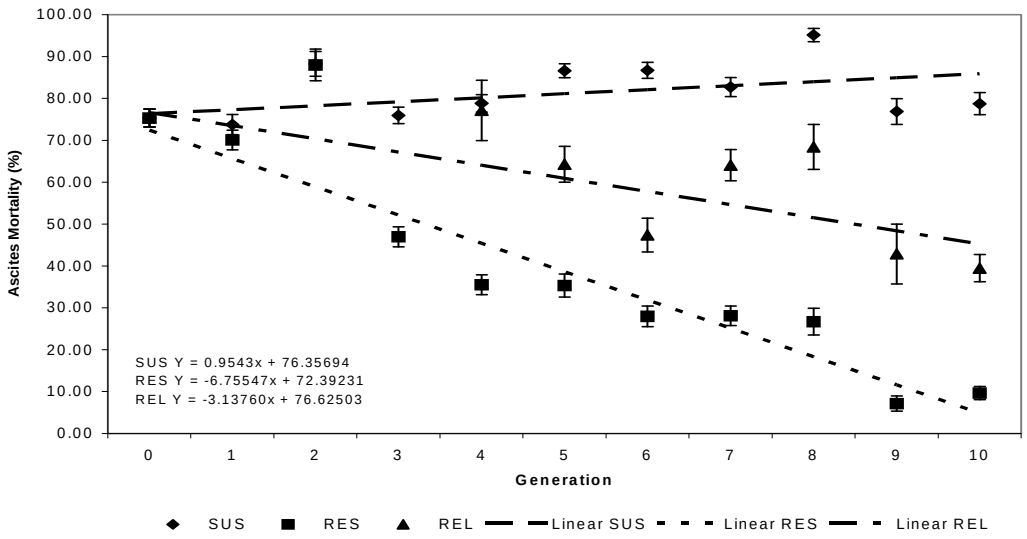



Figure 1. Incidence of ascites mortality per generation for ascites the SUS, RES and REL lines.

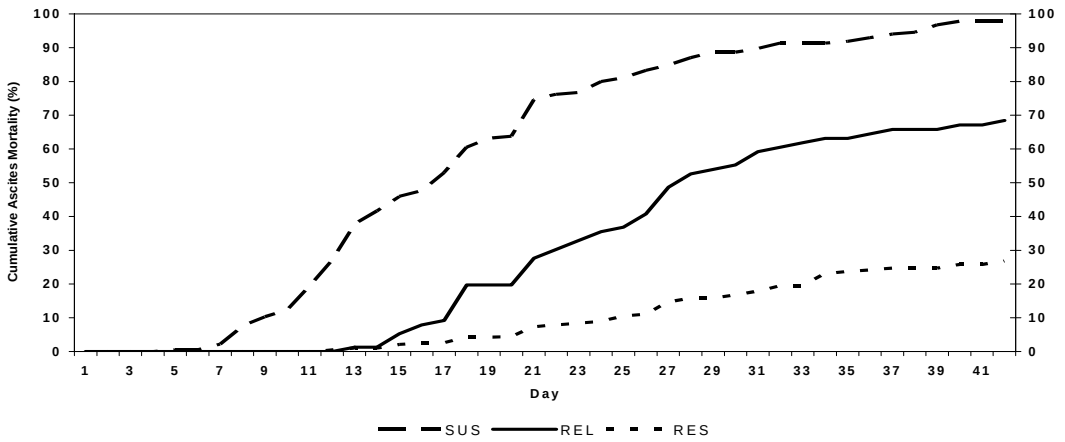



Figure 2. Ascites mortality curve for the SUS, RES and REL lines at Generation 15

Experiments were conducted to evaluate if ascites selection using hypobaric hypoxia was consistent with other methods known to induce ascites. It was observed that broilers

from the SUS line also succumb to IPAH during cool temperature exposure or when microparticles are injected i.v. to occlude pulmonary arterioles, whereas broilers from the RES line remain markedly unperturbed by these challenges (Wideman et al., 2002; Chapman and Wideman, 2006b). Clinically healthy broilers from the SUS line had higher PAP and PVR when compared with clinically healthy individuals from the RES line (Wideman et al., 2002; Bowen et al., 2006a,b; Chapman and Wideman, 2006b; Lorenzoni et al., 2007). Lung volume as a percentage of BW does not differ between the SUS and RES lines (unpublished observations). The cumulative evidence demonstrates that selection pressures rigorously focused to challenge the pulmonary vascular capacity readily expose the genetic basis for spontaneous IPAH in broilers (Wideman, 2001; Wideman et al., 2007, 2011).

### *Correlated response to Divergent Selection for Ascites*

Divergent selection exclusively for ascites has led to changes in growth related traits. For example, ascites selection has modified late embryonic growth and hatch weight with the RES line being heavier than the SUS line (Pavlidis, 2003). By Day 7 post hatch there is a complete reversal with SUS being heavier then RES. This difference is maintained through to maturity in unrestricted birds. Ascites selection has resulted in the modification of the *Pectoralis* muscle by increasing the absolute and relative percentages in the SUS line as compared to the nonselected (control) REL line. Selection for ascites resistance in the RES line has resulted in a reduction in absolute *Pectoralis* muscle, however relative *Pectoralis* muscle has not been impacted (Pavlidis, 2003). These data indicate a relationship between breast yield and ascites since breast has been modified in a population that is selected based solely on ascites mortality. The association between breast yield and ascites may provide insight into why ascites persists in heavily selected broiler populations. We also observed that the SUS line, when reared under normal environmental conditions actually shows improvement in muscle color as compared to the REL line, while not negatively impacting muscle pH, $\Delta$pH, or WHC. It is not clear if this relationship will also be a factor when considering the loci on which we have focused (see below).

Selection for ascites in the SUS line has resulted in an increase in total heart due to an increase in both right and left ventricle. The increase in right ventricle was expected due to the positive genetic correlation between RV:TV (right ventricle to total ventricular weight) ratio, pulmonary hypterension, and ascites (Lubritz et al., 1995; Wideman et al., 2007) however the change in left ventricle was not expected. Overall these modifications in total heart and lung have potentially created a cardio-pulmonary system that is not robust enough to support the rapid growth and muscle deposition and this may be the reason why the SUS line develops ascites so readily when exposed to stressors such as cold or hypobaric stress. It was clear from this work that selection for resistance to ascites without regard to body weight will result in changes to correlated traits consistent with, but not to the same magnitude as, those that would be under selection pressure by commercial geneticists.

### *Molecular Approaches to Ascites*

Clear ascites line differences, the fact that the lines came from a common base population and the development of the chicken genome project stimulated the

investigation of molecular changes that may have occurred due to selection. The initial approach was to apply a candidate gene approach. In humans, Familial Primary Pulmonary Hypertension (FPPH) is similar to PHS in broilers both genetically and physiologically. Several studies have recently shown that FPPH in humans is caused by mutations in the bone morphogenetic protein receptor type II (BMPR2) gene (De Caestecker and Meyrick, 2001). We sequenced BMPR-II mRNAs from ascitic and non-ascitic commercial broilers and compared those sequences to the published Leghorn chicken BMPR2 gene (Cisar et al., 2003a,b). Fourteen single nucleotide polymorphisms (SNPs) were identified in the commercial broiler BMPR-II mRNAs. No mutations unique to ascitic broilers were present in the coding, 5' untranslated, or 3' untranslated regions of BMPR-II mRNA. The twelve SNPs present within the coding region of BMPR-II mRNA are synonymous substitutions and do not alter the BMPR-II protein sequence. These data indicate that mutations in the BMPR2 gene are probably not responsible for susceptibility to PHS in broilers.

In 2006 we participated in a USDA supported genome wide SNP panel (Muir et al., 2008). We submitted 183 DNAs representing F1 and F2 birds from a cross between our ascites RES (resistant) and SUS (susceptible) lines (Pavlidis et al., 2007). For our samples, 970 SNPs were uninformative while 1763 were polymorphic. SNP distributions (frequencies) were compared for low vs. high RV/TV (hypertrophy) and resistance vs. susceptibility to ascites. The data were analyzed by Chi square to detect deviation of observed from expected for both ascites and RV/TV. We then selected regions where successive SNPs with minor allele frequencies greater than 0.20 deviated from expected ($P<0.01$) for both traits. This analysis identified at least 7 regions on 4 chromosomes; three regions on Gga1: 0.6-1.1, 18.3-21.5, and 127.0-128.3 Mbp; two regions on Gga9: 13.5-14.8, and 15.5-16.3 Mbp; one region on Gga27: 2.0-2.3 Mbp; and three regions on GgaZ:31.2-34, 47.1-48.9, and 65.0-66.0 Mbp on GgaZ. The most statistically significant regions were those on Gga9 and the effect was primarily in females. We the examined these regions using microsatellites in the REL line and in three commercial lines. The two regions on Gga9 were found to correlate with ascites in at least one other test population, but neither was highly significant in the REL (Krishnamoorthy et al., 2014; Wideman et al., 2013). We have identified potential candidate genes. The most likely candidate genes are: Gga9:13- AGTR1, angiontensin II type 1 receptor; and Gga9:16- 5HT2B, serotonin receptor/transporter type 2B. Each of these genes has been implicated in some aspect of human idiopathic pulmonary arterial hypertension (Chung et al., 2009; MacLean, 2007; Simonneau et al., 2004) which is analogous to ascites in birds. That we found 5HT2B is in agreement with earlier pharmacological work with our lines showing involvement of the serotonin pathway (Chapman and Wideman, 2006a, b; Wideman et al., 2007; Hamal et al., 2010).We have resequenced these candidate genes for SNP analyses. These efforts are still underway.

In 2012 we performed a new GWAS using the 60k SNP chip for 96 samples from the REL line. Statistical analyses showed no association for any of the regions from the 2006 GWAS, but did identify one significant association for a region on GgaZ around 59 to 60.5 Mbp that was only associated with ascites in males (Figure 3).

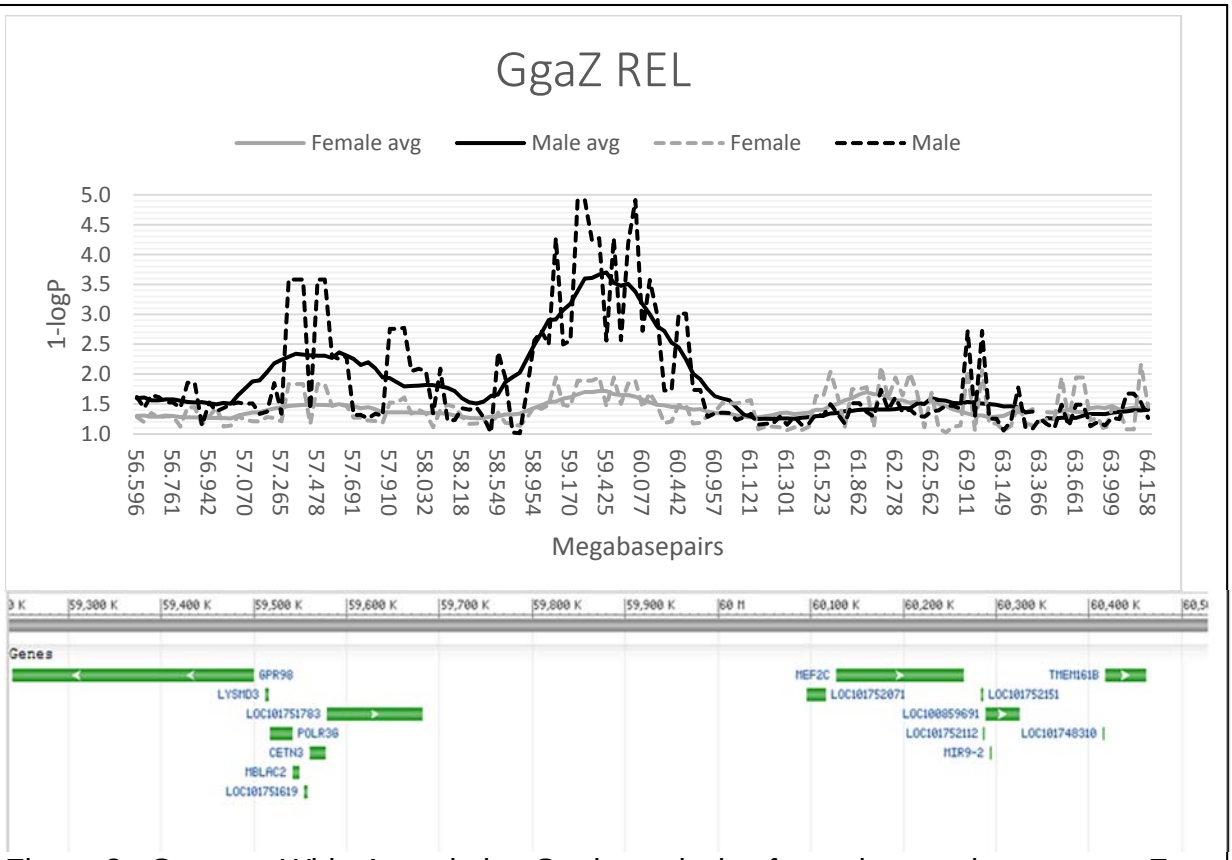



Figure 3. Genome Wide Association Study analysis of a region on chromosome Z associated with ascites phenotype in the REL. Top panel: Chisquare values are plotted as 1-logP vs. SNP position in Mbp for both genders according to the legend. Dotted lines are the values for each SNP and the solid lines are averages over a 15 SNP window. The region depicted is for 140 informative SNPs. The graph is for analysis of SNP allele counts but a similar plot was observed for genotype counts. Bottom Panel is a NCBI genome browser view of the identified genes in the region from 59.2 to 60.5 Mbp on chromosome Z.

We developed a TaqMan assay for a C/T SNP at 60.058 Mbp for use in further genotyping. Analysis in REL (n=258) showed no association in female, and in males there was a significant association with susceptibility for the heterozygous Y genotype (19% resistant vs 30% susceptible; P=0.0026). We also genotyped 192 samples from each of three elite commercial breeder lines. There was no association in 2 lines but in the third the Y genotype was associated with susceptibility in males (4% resistant vs 10% susceptible; P=0.021) and the C homozygote was associated with resistance in males (48% resistant vs 17% susceptible; P=0.015) and also in females (58% resistant vs 25% susceptible; P=0.01). We then performed initial Marker-Assisted-Selection (MAS) using this SNP in the REL line and challenged progeny in the hypobaric chamber.

When TT sires were mated to C dams (females are homogametic and hemizygous for GgaZ) to generate heterozygous males the % resistance in the male offspring is 33% whereas when TT sires were mated with T dams to produce TT male offspring the % resistant of male offspring was 74%. We are repeating this experiment using additional sires of the other two genotypes (CT and CC). However, our genotype data show that susceptibility to ascites is associated with heterozygosity for this region and the initial MAS data corroborate and suggest this is a major QTL for ascites.

Table 1. Marker assisted selection for ascites phenotype using a SNP on GgaZ at 60.058 Mbp. Parents were selected based on genotype and mated. Ascites phenotype for offspring are presented as raw counts or % reisistant overall and by gender.

| Parent Genotype | | Resistant Offspring Count | Susceptible Offspring Count | |
|---|---|---|---|---|
| Sire | Dam | Overall | | % Resistant |
| TT | C | 20 | 25 | 44 |
| TT | T | 84 | 40 | 68 |
| | | Male | | |
| TT | C | 4 | 8 | 33 |
| TT | T | 35 | 12 | 74 |
| | | Female | | |
| TT | C | 9 | 9 | 50 |
| TT | T | 19 | 11 | 63 |

The region we mapped in the GWAS contains several genes (Figure 3 lower panel) that include AARDC3 (arrestin domain containing), GPR98 (G-protein receptor), LYSMD3 (peptidoglycan-binding domain), MBLAC2 (metallo bet-lactam binding, CETN3 (centrosome protein), mir9-2 (also known as LINC0461) and MEF2C (myocyte enhancer factor 2C. Investigation of the Pubmed and OMIM databases at NCBI identifies two of these genes as likely candidate genes. MEF2C is known to regulate production of cardiac myocytes Song et al., 2012; Zweier, et al., 2010), consistent with cardiac hypertrophy as a hallmark of ascites (Wideman et al, 2013). LINC0461 has been implicated in association studies to be involved in regulating microcirculation in cardiovascular diseases (Ikram, et al., 2010), and others have reported dysregulation of several non-coding RNAs in heart disease (Yang, et al., 2014). Therefore, it is highly plausible that this region could be contributing to ascites susceptibility through effects on cardiac hypertrophy and microcirculation. What is not known is what the costs of MAS based on this region would be and whether we can identify additional components of either the MEF2C interactome/pathway or the targets of LINC0461 that are additional QTLs for ascites. We examined the chickenQTLdb at www.animalgenome.org which identifies several production traits that have been mapped to this region including abdominal fat (Ikeobi et al., 2002; Tuiskula-Haavisto, et al., 2002; Zhang, et al., 2012), chest width (Sharman, et al., 2007), body weight at 3 or 6 weeks (Podisi, et al., 2013; Sewalem, et al., 2002), and drum and thigh muscle weight (Ikeobi, et al., 2004). Therefore, MAS using this region might have negative impacts for production traits that are critical for the broiler industry.

*Rationale and Significance*

Although ascites can result from poor management it appears that the primary problem is that the modern broiler chicken grows too fast for the cardio-pulmonary infrastructure to keep up with the demands. We have shown that there is a significant genetic component to this disease and that selection can be employed to reduce the incidence. However, concomitant with our selection for resistance and susceptibility in the hypobaric challenge we noted obvious differences between the lines for growth rate, meat quality, and breast yield. Now that we have mapped a major QTL affecting ascites susceptibility we need to assess what the production costs are for genetic selection (marker assisted selection) for ascites resistance. The differences in production traits between the lines have to result from either 1) genetic drift, 2) linkage of production QTLs to ascites susceptibility loci, or 3) ascites susceptibility loci are the QTLs for production traits. We will use the relaxed line (progenitor of the resistant and susceptible lines) to measure production traits and determine whether genotype for the region on GgaZ correlates with any significant production traits. Once completed we will test the robustness of this locus in a completely unrelated random bred control line representing the commercial broiler of 1997. The experiments we propose will not discriminate between alternatives 2 and 3 but they will determine whether it is alternatives 2 and/or 3 vs. alternative 1. Owing to the number of negative production traits we have observed in the ascites resistant line we contend that alternative 1 is highly improbable. However, if positive production traits are linked to, or the result of, ascites susceptibility loci then our proposed specific aim will determine the specific production affected by each of our four ascites loci. This would allow breeders to understand the ascites susceptibility consequences for selection for particular traits. Additional aims (3 and 4) are proposed to expand our search for ascites susceptibility QTLs when we control for the effect of the QTL on GgaZ. Aim 5 is designed to begin to elucidate the underlying changes in gene expression targeting those which contribute to cardiac hypertrophy.

**IMPLEMENTATION**

**Overall hypothesis or goal:** The region we have identified on Chromosome Z can be used for marker assisted selection for increased ascites resistance and can help us identify other components of a pathway that contribute to ascites.

**Specific objectives:**

**Specific Aim 1:** Conduct Marker Assisted Selection (MAS) using the Z chromosome marker and evaluate progeny for ascites phenotype, and production traits including a) 3 and 6 week body weight, b) breast yield, c) feed conversion, and d) meat quality.

Rationale and Hypothesis

We have shown that breeding based on a single SNP marker on GgaZ can markedly affect ascites susceptibility of male progeny when challenged in the hypobaric chamber. We hypothesize that genotype for this GgaZ region is associated with one or more production traits for which the industry selects that negatively impacts ascites resistance. The gene responsible for the QTL for the production trait could be the same or tightly linked to the gene for ascites susceptibility.

Experimental Procedure

We will pool semen from heterozygous sires of the REL line for insemination onto REL dams hemizygous for each Z allele. This will produce male progeny at approximately 1:2:1 for CC, CT, and TT genotypes, whereas the females will be 1:1 C and T hemizygotes. We will collect 10 ul of blood from male and female progeny at 3 days of age for DNA preparation (Bailes et al., 2007). We will place 600 chicks in floor pens under broiler production conditions for assessment of production traits. We will also challenge 300 offspring in the hypobaric chamber at a simulated altitude of 9500 feet (Anthony and Balog, 2003). The hypobaric chamber challenge is essential to confirm that the GgaZ region is associated with ascites phenotype and to provide additional DNAs for genotyping for the additional markers.

Each DNA sample will be genotyped for confirmation of gender using a W chromosome specific PCR (Mozdziak, et al., 2005), and for 6 SNPs spanning the region of interest on GgaZ. This will include our original SNP marker and 5 additional SNPs distributed evenly across the region from 58.9 to 60.5 Mbp. The precise SNPs will be based on inspection of the regions surrounding the SNP for suitability for development of a TaqMan assay (no repeats, GC content 50-60%, no low complexity regions, etc.).

For evaluation of production traits all birds will be weighed at hatch, 2, 4, 6, 7 and 8 weeks post hatch. The chicks will be reared on full feed for the duration of the trial. Consistent with industry standards, birds will be subjected to a one week feed conversion trial at 49 days of age. Upon completion of the trial all birds will be transported to the University of Arkansas Pilot Poultry Processing Plant and processed under typical commercial conditions. Carcasses will be hand eviscerated for collection of carcass and organ data. Heart, liver (without gall bladder), spleen, lungs (pair), and abdominal fat pad will be removed and individual weights obtained. Hearts will then be split into the right ventricle and left ventricle and RV:TV (right ventricle to total ventricular weight) ratio calculated. Weights of individual carcasses without giblets (WOG) will also be obtained for calculation of parts yield. Parts yield data will be expressed as a percentage of WOG weight. Following the measurement of WOG weight, carcasses will be chilled for 4 hours at which time the carcasses will be drained and reweighed to determine carcass moisture uptake. The carcass will then be deboned and weights obtained for the Pectoralis major and minor, wings, drums, and thighs. Muscle color (L*, a*, b*) will be evaluated at 4 and 24 hours post mortem using a Minolta colorimeter. Each fillet will be placed on a white tray and three color measurements taken with the Minolta colorimeter and averaged. Muscle color will be measured on the medial side of the fillet in areas free of defects as described by Qiao et al. (2002). Muscle pH will be evaluated at 4 and 24 hours post mortem by directly inserting the spear probe of a pH/mV/temperature meter into the cranial portion of the fillet. Drip and cook loss will be determined for breast fillet (Lippens et al., 2000; Woelfel et al., 2002). Tenderness will be determined using the Allo-Kramer shear cell on an Instron Universal Testing Machine (Allen et a. 1998, Skarovsky and Sams, 1999).

Expected outcomes

We expect to find that one or more of the production traits will be associated with the GgaZ 60 Mb region. Traits of economic and physiologic importance will be measured and means statistically compared for each of the three genotypes for each of the SNPs. The most informative SNPs will be those with nearly equal allele frequencies, so we will primarily focus on those for relevance. Since some of these traits may be correlated or

dependent we may find that several production traits are associated with this region. We expect that most of the SNPs will be sufficiently linked that we can define haplotypes across the region but with a large enough population size we expect there may be some recombination within this region that would allow us to better discriminate the bounds of the QTL more so than the original GWAS which included only 48 males.

Data analyses

For Statistical analyses we will primarily focus on the tails of the distributions for each production trait. For each trait the population will be partitioned to define the birds in the upper and lower thirds of the distribution. Allele, and genotype counts in the tails will then be compared to the expected values computed from the frequencies for the entire population. Deviation from expected will be based on a Chi-Square comparison of expected to observed counts. Microsoft Excel or JMP Genomics 4.1 will be used for statistical analyses. For each locus we will evaluate the entire population and then separately for males and females, to determine whether the effect is primarily within one or both sexes. The sample size proposed in this grant is consistent with that calculated by conducting a power analysis (Bausell and Li, 2002). In that analysis it was determined what sample size would be necessary to achieve an 80 percent chance of detecting an ES (Effective Size) of 0.50 between the mutated and the normal locus (p= 0.05). For example, in order to detect a 10 g difference in body weight, which may be subtle enough for measuring a single gene effect, one would generally need a sample size of 200 birds. It is important to note that since there are so many phenotypic traits considered in this study the trait that is the least variable will require the most individuals to detect significance. Our primary focus is on body weight and breast yield, but we have expanded to a number of other traits that may not have as significant variation. Thus we have more than doubled the size of the population over that required for body weight.

Pitfalls and limitations

All of the techniques we will employ have been in use in our group. The production traits have been assessed over many generations. The molecular genotyping has been ongoing for many years. We have working developed TaqMan assays for several different projects VNTRs that will give us the associations, even if we have problems with developing SNP assays for some of the regions. Therefore, we will be able to complete this project. If we are able to find association of one or a few important production traits then we will repeat the entire production trait assessment in the subsequent generation of the REL using an additional 300 birds for production traits and genotypes to be certain that the results are repeatable. If we find no association of genotype or allele frequencies for this region with any production trait then this will have evidence that breeders may be able to select for resistance using this region without great concern for loss of production values. However, we feel this is highly unlikely or else ascites would have been eliminated with simple selection strategies.

**Specific Aim 2:** Test the same SNP markers in commercial broiler breeder parent stocks challenged in the hypobaric chamber.

Rationale and Hypothesis

The REL line is a random bred descendent of a commercial elite breeding line from the mid 1990's, and so represents an historical commercial breeding stock. The ascites susceptibility of the REL is similar in ascites susceptibility to current commercial

breeding lines that we have evaluated in our hypobaric chamber, and we have shown that the GgaZ region on which we have focused is associated with ascites phenotype in at least one elite breeding line. We hypothesize that the same GgaZ region will be associated with ascites phenotype in some broiler lines and will focus on the actual commercial product: broiler breeder parent stock.

Experimental Procedure

We will obtain commercial broiler parent stock from local sources (300 from each of 2 different commercial breeding companies) as fertile eggs and hatch the eggs in our hatchery. Chicks will placed in the hypobaric chamber at hatch and blood sampled on day 3. The hypobaric challenge will be as in Specific Aim 1, and the DNA samples from blood will be genotyped for gender, and the GgaZ SNP markers produced from Specific Aim 1.

Data Analysis

SNP genotypes will be evaluated for association as for Specific Aim 1.

Expected Outcome

One or more of the commercial products will show an association of ascites susceptibility with one or more of the SNPs for the region of GgaZ around 60 Mbp.

Pitfalls and limitations

There are no new techniques or procedures so the results will be obtained. However, there is a formal possibility that neither commercial broiler breeder will show association for this particular region. However, we will have archived DNAs from these along with phenotypes so that as we identify additional regions in Aims 3 thru 5, we can test those regions on the archived DNAs.

**Specific Aim 3:** Repeat GWAS in REL to look for other regions that are of smaller effect.

Rationale and Hypothesis

Our GWAS in the REL with 96 samples only detected one male specific QTL region in REL whereas the F2 cross of RES and SUS identified at least two female specific QTL regions. We hypothesize that there are additional male specific QTLs that are minor QTLS that we can identify by focusing on the most susceptible phenotype in males for an additional GWAS.

Experimental Procedure

DNA samples will be further purified from each of 30 resistant and susceptible males for the more susceptible CT genotype from Specific Aim1. The DNAs will be submitted to DNA Landmarks for genotyping with the 60k chicken SNP chip.

Data Analysis

The SNP data will be filtered for SNPs with minor allele frequency<0.05, unmapped SNPs for the 2011 assembly, and those which do not conform (P<0.001) with Hardy Weinberg Equilibrium (HWE). In our experience, 40 to 42k SNPs will pass these filters, and are then analyzed using Chi-Square for observed vs expected for both genotype and allele counts. Chi-Square values are then plotted as 1-logP vs chromosomal position and averaged over a sliding window of 5, 10, or 20 SNPs to identify blocks of SNPs differentially represented in phenotypic subgroups.

Expected Outcomes

We will identify one or two additional regions that show association with ascites phenotype. We can then develop TaqMan assays for these regions to test in additional samples that we have archived from past generations of the REL.

Pitfalls and limitations

The 60k SNP chip is $130/DNA sample whereas the cost for the 600k chip is $420/sample. There is insufficient room in the budget to afford the 600k chip, but we will seek internal state funding to "upgrade" to the denser chip. If we are successful we will use the 600k chip otherwise we will use the 60k chip which has worked well for us for 3 different projects, and we are able to identify 10 to 20 successive SNPs that show linkage to quantitative traits.

**Specific Aim 4:** Next-Gen Sequencing for CNVs and SNPs in REL based on gender and phenotype.

Rationale and Hypothesis

To date our analyses for QTLs affecting ascites have utilized SNP panels in GWAS. In these analyses we always discard loci that do not conform to HWE for the entire sample set. This often results in discarding 5 to 8,000 SNP locations. These SNP locations may not conform to HWE because they are part of a CNV (copy number variation). Our hypothesis is that we can identify CNVs that are differentially represented in ascites resistant and susceptible birds in the REL line.

Experimental Procedure and Data Analysis

DNA samples from resistant and susceptible birds from Aim 1 will be purified, quantified and then pooled as two pools of 10 DNAs from each of resistant and susceptible for male and female, which will generate 8 pools (2 pools x 2 phenotypes x 2 genders). Each pool will be then used to generate a library and each library will be digitally tagged. Library aliquots will be mixed and submitted for 2x150 paired end reads on the Illumina 2500 or HiSeq (University of Delaware core lab). We intend to generate about 30x genome equivalents for each pool or about 240 Gbp. The sequence reads will then be aligned with the most recent chicken genome assembly as a template using the DNAStar NGen software. The alignment will identify potential CNVs based on read depth and overlaps. We will compare the CNV report across gender and phenotype to determine whether there are particular CNVs that appear to be differentially represented in a phenotypic group.

For regions we identify we will design qPCR TaqMan assays that will allow us to screen additional DNAs for the CNVs in additional DNAs.

Expected Outcomes

We expect to identify CNVs in the REL and that a few of these will be differentially represented relative to ascites phenotype, and that some of these will be gender specific for their association.

Pitfalls and Limitations

Dr. Kong has already performed pilot studies on sequencing pools of 10 samples from the REL, RES and SUS line for identification of SNPs that are differentially represented in the three research lines. Both Dr. Kong and Dr. Rhoads have many years of experience in genome alignments and analyses using DNAStar NGen. We foresee no issues with completing this specific aim.

**Specific Aim 5:** RNAseq analyses on hypertrophic vs normal hearts.

Rationale and Hypothesis

Ascites has two significant hallmarks: accumulation of abdominal fluid, and a hypertrophied heart, that are commonly used for verification of phenotype at necropsy. Dr. Carl Schmidt at University of Delaware has been investigating cardiac hypertrophy in chickens, and has agreed to perform RNAseq on samples from our birds as a sub-award. The hypothesis is that we will identify altered expression of Mef2C as well as other genes in this pathway in hypertrophied hearts and that the altered expression will correlate with genotype for genotype for the GgaZ SNPs in the 60 Mbp region.

Experimental Procedure

From Specific Aim 1 we will archive heart samples from resistant and susceptible birds in RNA later. Based on gender and genotype we will isolate RNA that will be shipped to Delaware for library production and RNAseq. Samples will constitute: 6 resistant and 6 susceptible males from the CT and TT genotypes, and 3 resistant and 3 susceptible females from each genotype (C and T). This will constitute 36 samples for cDNA library generation with bar coding. Samples will be pooled and sequenced on lanes on at HiSeq to generate 600 million reads per lane. This will provide approximately 20 million reads from each original sample. The experiment is designed to determine differences in the response to hypobaric challenge with respect to gender, and genotype. Additionally, since we are not only looking for altered expression levels that might contribute to the causes of cardiac hypertrophy, but also looking for downstream effects we propose to also perform RNAseq on the microRNAs. There is a miRNA gene in the region on which we have focused, and MEF2C regulates transcription. Others have reported dysregulation of noncoding RNAs in heart damage and remodeling (Yang, et al., 2014) and these ncRNAs may produce miRNAs. Therefore, it is possible that hypertrophy and ascites includes dysregulation of one or more miRNAs.

Data Analysis

RNAseq reads will be analyzed in the ArrayStar/QStar modules in LaserGene. Raw reads will be converted to RPKM values and exported to an Excel spread sheet to identify statistically significant deviations between sample sets. The critical comparisons will be between the resistant and susceptible CT males for transcriptional modulators of the effect of the GgaZ genotype. This will be contrasted with the differences seen in the TT male samples. The female samples will constitute controls and may allow us to discern differences with respect to gender response, since we know that females are more susceptible in the hypobaric chamber challenge but males are more susceptible in standard grower conditions (Wideman et al., 2013). Gene IDs for those transcripts differentially represented between sample groups will be submitted to KEGG and Ingenuity Pathway for identification of pathways which are differentially represented.

Expected Outcomes

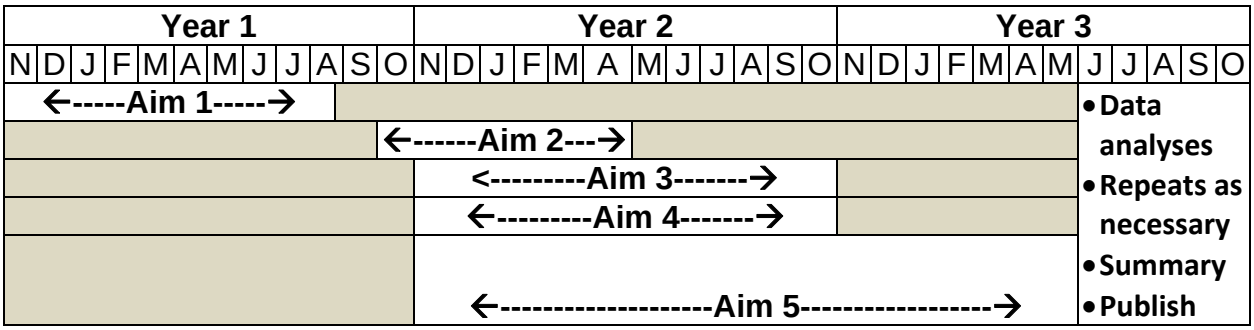
We expect to identify components of the Mef2C pathway as well as other pathways that are differentially expressed. Some will be male specific and some will be seen in both genders. Identification of other genes that are differentially regulated will allow us to expand the regions for investigation of other regions as QTLs in other research and commercial lines.

Pitfalls and Limitations

Dr. Schmidt has been performing RNAseq for many years, and Dr. Rhoads has several years analyzing RNAseq data. They are both part of an international consortium for analysis of the transcriptomes of birds (manuscript submitted).

**Potential impact and expected outcomes:** If we can determine the effect of this region on production traits then this would be the first validated marker for selection for ascites resistance and the overall cost for selection for resistance will be known. Identification of additional regions of lesser effect will allow lines to be developed that "stack" resistance in the broilers produced by selected crosses. Objectives 3 through 5 will be critical for identification and development of these additional loci for resistance.

**Time Line**




**Animal Care and Use**

All methods necessary to complete this study are approved as University of Arkansas IACUC protocol #12039 (expiration date: 06/06/2015).