DISSERTATION

CALCIUM SIGNALING GENES IN ASSOCIATION WITH ALTITUDE-INDUCED PULMONARY HYPERTENSION IN ANGUS CATTLE

Submitted by

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ABSTRACT

CALCIUM SIGNALING GENES IN ASSOCIATION WITH ALTITUDE-INDUCED PULMONARY HYPERTENSION IN ANGUS CATTLE

This research used multi-omics technology (i.e., RNA-seq, qPCR for gene expression, SNP discovery and validation) to understand the influence of a particular subset genes on altitude-induced pulmonary hypertension susceptibility in Angus cattle. Three research aims were established to test the hypothesis that calcium-related genes may be associated with pulmonary hypertension in beef cattle. Data and samples utilized for the research came from the Colorado State University Beef Improvement Center Angus herd managed at 2,150 m of altitude.

Transcriptome data from 6 tissues and 14 hypertensive and normotensive Angus steers were utilized for differential expression and pathway analyses. The objectives of the first aim were to: 1) to estimate and identify differentially expressed genes from RNA-Seq and pathway analyses, and 2) select putative candidate genes to analyze with qPCR (gene expression level). The largest number of DE genes was revealed in aorta (n = 631) and right ventricle (n = 2,183) samples. Top canonical pathways related to calcium signaling or utilization included: synaptic long-term depression, signaling by Rho family GTPases, and oxidative phosphorylation. Genes regulating calcium availability and utilization were expressed differently (log_2 fold change > 0.589, < -0.589; $P < 0.05$) in Angus cattle with and without pulmonary hypertension.

Isolated RNA from cardiac muscle (n = 9) and control muscle (n = 2) tissues from hypertensive and normotensive Angus steers were utilized to estimate gene expression using quantitative reverse transcription PCR in the candidate genes from Chapter 3. The objectives of

this chapter were: 1) to establish the most appropriate reference genes in cardiac muscle tissues, and 2) to estimate and validated relative gene expression of calcium-related genes in cardiac muscle tissues using qPCR methods. Differences ($P < 0.0055$) among hypertensive and normotensive steers were estimated for right papillary muscle and right cardiac ventricle tissues (top, middle, and bottom) in candidate genes: *ASIC2*, *EDN1*, *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*. Results of the current study validate the expression differences previously established of genes that regulate the availability and utilization of calcium with PH status in Angus steers at high altitude.

Variant detection and association analyses were completed with 2 sets of available -omics data to identify opportunities for development of selection tools for reduced susceptibility to PH. The objectives of the third aim were to: 1) detect single nucleotide polymorphisms (SNP) in the transcriptome of 6 tissues, and 2) identify functional consequences of those variants associated with validated candidate genes from qPCR analyses. Pooled Angus sample analysis revealed 68 SNP in the 6 candidate genes: *ASIC2*, *EDN1*, *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*. Thirty-eight SNP were revealed in the hypertensive group and 8 SNP in the normotensive steer group. Ten of the 68 identified SNP are utilized on large density commercially available bovine SNP chips (Illumina BovineHD BeadChip; GeneSeek Genomic Profiler HD; GeneSeek Genomic Profiler HDv2; Affymetrix Axiom Bovine). Analysis of transcriptome data identified SNP within genes regulating calcium availability and utilization, enhancing our understanding of sequence polymorphisms that may be involved in regulating pulmonary hypertension in Angus cattle raised at high altitude. These SNP are available for additional validation and potential use in genetic improvement programs.

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CHAPTER 1

INTRODUCTION

A disease of importance in high altitude beef production systems is pulmonary hypertension (PH) and heart failure. At high altitude, risk of heart failure as a consequence of pulmonary hypertension is defined by abnormal pulmonary arterial pressures (PAP; > 41 mmHg). Pulmonary hypertension develops through remodeling of the vasculature of the heart and lung and an inability of the animal to overcome the necessary force to eject the blood through the pulmonary artery. This remodeling leads to hypertrophy of the right ventricle, and eventually heart failure (Neary et al., 2015; Pugliese et al., 2015; Ryan et al., 2015). In cattle, PH has been widely recognized and referred to as high mountain disease (HMD) or brisket disease. The term HMD was established due to the reaction of some cattle to changes in elevation, usually > 1,500 m (Pauling et al., 2018; Thomas et al., 2018).

Calcium is a mediator of the physiology of the heart, including myocardial function (Hasenfuss and Pieske, 2002; Stanfield, 2011). Rhodes (2005) suggested a role of Ca^{2+} sensitization in myocytes in hypoxic PH to distinguish hypertensive from normotensive cattle. Evidence revealed that altered Ca^{2+} homeostasis was of importance for the pathophysiology of myocardial dysfunction and heart failure (Hasenfuss and Pieske, 2002). Unintentionally, previous omics approaches (i.e., GWAS and RNA-sequencing) with cattle determined quantitative trail loci (QTL) windows and differentially expressed genes related to calcium homeostasis and metabolism (Newman et al., 2011; Newman et al., 2015; Zeng, 2016). However, there have been no research studies designed to specifically address the influence of calcium on genetic susceptibility of cattle to PH. Therefore, we hypothesized that genes regulating intracellular availability and utilization of calcium would be of importance to differentiate beef cattle with pulmonary hypertension.

CHAPTER 2

REVIEW OF LITERATURE

SECTION 1: PULMONARY HYPERTENSION

Pulmonary arterial pressures (PAP) define pulmonary hypertension (PH) status in beef cattle and pressures greater than 41 mmHg at high altitude signify risk of heart failure. Pulmonary hypertension has been classified in many different ways over the last 50 years, many of which are from World Health Organization symposiums (Tuder et al., 2009). More recently, PH has been classified into groups based on etiology: 1) Pulmonary arterial hypertension (PAH), 1') Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis, 2) Pulmonary hypertension due to left heart diseases, 3) Pulmonary hypertension due to lung diseases and/or hypoxemia, 4) Chronic thromboembolic pulmonary hypertension, and 5) PH with unclear multifactorial mechanisms (Simonneau et al., 2013). Typically, cattle fall within Group 3, associated with chronic exposure to high altitudes and alveolar hypoventilation disorders, amongst others (Krafsur et al., 2016).

In cattle, PH has been widely recognized and referred to as high mountain disease (HMD) or brisket disease. The term HMD was established due to the reaction of some cattle to changes in elevation, usually > 1,500 m. High mountain disease has been observed in cattle at high altitude since the early 1900s (Glover and Newsom, 1914; Glover and Newsom, 1917). The term brisket disease was derived from the pronounced appearance of edematous fluid in the dependent tissues covering the parasternal muscles, known as the brisket. Ranchers have used the terms brisket disease and HMD synonymously since the early 1900s.

1.1 PATHOPHYSIOLOGY & EPIDEMIOLOGY

Pulmonary hypertension develops through remodeling of the vasculature of the heart and lung and an inability of the animal to overcome the necessary force to eject the blood through the pulmonary artery, leading to hypertrophy of the right ventricle, and eventually heart failure (Neary et al., 2015b; Pugliese et al., 2015; Ryan et al., 2015).

In most cases of PH, the hallmark signs are sustained vasoconstriction and vascular remodeling (Shimoda and Laurie, 2013). This vascular remodeling includes thickening of the intimal, medial, and adventitial layer of muscular vessels, and the appearance of muscle-like cells in the walls of arteries (Stenmark et al., 2009). These effects can get progressively worse with time, resulting in lesions that obstruct pulmonary arteries and arterioles, limiting the blood flow through the pulmonary arteries (Stenmark et al., 2009).

Vascular remodeling from PH can result in increased artery stiffening, which leads to increased distal resistance of those arteries. The increased resistance has an effect on blood pressure and flow as the resistance creates more difficulty for blood to move. The heart must compensate for this increase in resistance, and must increase afterload, to overcome the force opposing the myocardial contraction and necessary to eject the blood. The edema build up in the dependent tissues covering the parasternal muscles is a result of increased hydrostatic pressure and subsequent loss of fluid in the extravascular spaces (Louis and Fernandes, 2002). Symptomatic and physical changes in animals suffering from PH include: intrathoracic edema, pulmonary edema, plural effusions, passive linear congestion, intraabdominal and mesenteric edema, and ascites (T. N. Holt, personal communication). The muscles of the right ventricle of the heart enlarge to compensate, and as the impedance increases, the heart could eventually fail. Right heart

failure (RHF) is the resulting action that can take place in cattle with PH, if the heart succumbs to these pathophysiological changes (Voelkel et al., 2006).

The cardiac cycle can be summed up in 4 main phases: 1) inflow, 2) isovolumetric contraction, 3) outflow, and 4) isovolumetric relaxation. The processes of systole and diastole are encompassed within these phases (Figure 1-1). Each phase can be explained from the perspective of physiology of the right side of the heart leading into the pulmonary artery and lungs. The left side of the heart has the same mechanisms, however different valves are involved.

Systole begins at the second phase, isovolumetric contraction. During this stage, the tricuspid valve is closed, in which there is no flow of blood in or out of the heart. During this time the ventricular pressure is increasing. Systole continues into phase 3, the outflow phase (also known as the ejection phase), where the pulmonary semilunar valve opens, while the tricuspid valve remains closed. Upon contraction and opening of the pulmonary semilunar valve, blood is then ejected from the right ventricle into the pulmonary artery and to the lungs (Boron and Boulpaep, 2012).

Diastole begins in the fourth phase of isovolumetric relaxation. Like isovolumetric contraction, the tricuspid valve is closed creating no blood flow in or out. The ventricular pressure begins to decrease. Diastole continues with the first phase, inflow phase, in which the tricuspid valve opens, the pulmonary semilunar valve is closed, and blood then flows into the ventricle (Boron and Boulpaep, 2012).

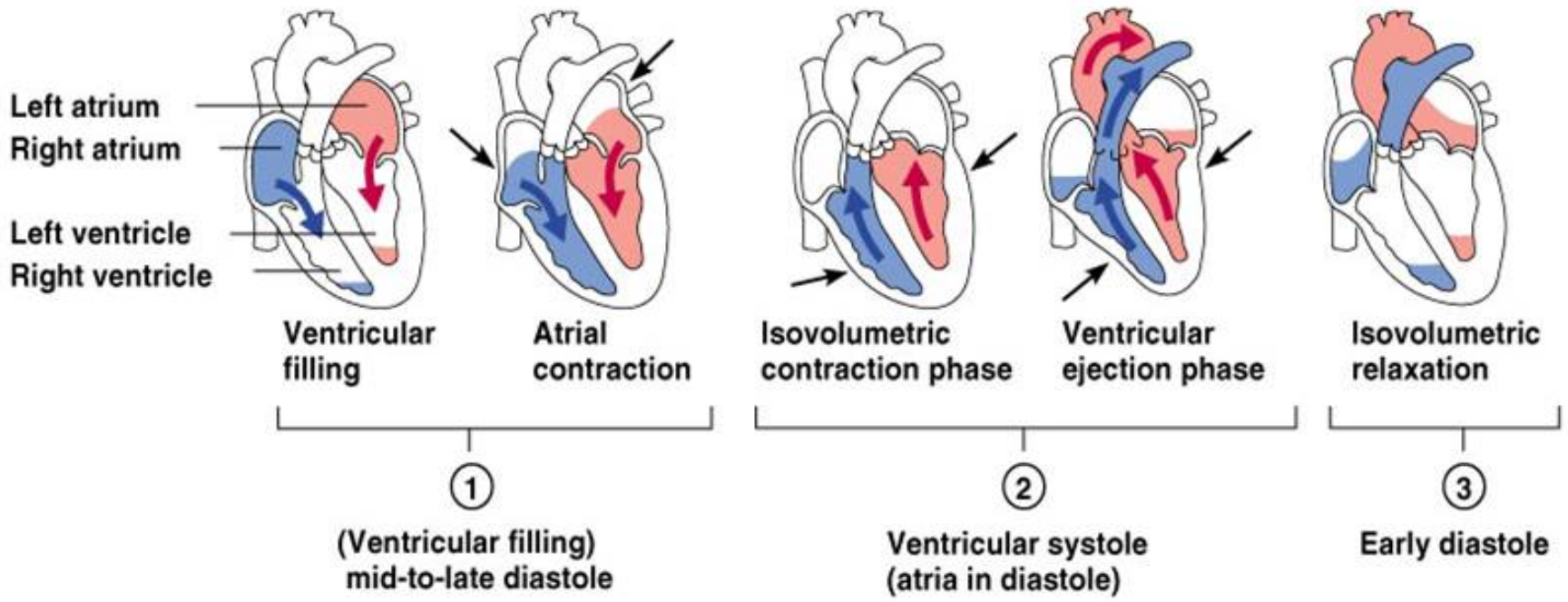


Figure 1-1. Cardiac cycle. Systole and diastole as blood is pumped from the systemic and pulmonary systems through the atria and ventricles of the heart.
https://www.austincc.edu/apreview/PhysText/Cardiac.html

1.2 INCIDENCE

Historically, heart failure as a result of PH most commonly occurred in herds at high altitude (> 1,500 m), with incidence rates of 3 to 5% in cattle native to high altitude (Holt and Callan, 2007). Hohenboken et al. (2005) stated that the adaptation of animals to a specific environment declines when outside or non-native animals are used for breeding purposes. The use of non-native cattle in high altitudes has the potential to increase incidence rates to 10 to 40% (Will and Alexander, 1970). However, despite selection procedures that have been implemented, incidence of PH and death resulting from RHF has not appeared to decrease over the years. Additionally, a similar phenomenon is becoming more prevalent at lower altitudes and in late fed cattle in the feedlot (Neary et al., 2015; Krafsur et al., 2016; Neary et al., 2016a). Approximately 15 cases of PH appeared in every 10,000 cattle, with highest incidences in feedlots at high altitudes (Neary et al., 2016a). Exact incidence rates are currently unknown and many cases could be mistaken for respiratory disease or vice versa (Malherbe et al., 2012; Neary et al., 2013).

1.3 MEASURING PH

The pathophysiological condition of PH is defined by a mPAP above a certain level (i.e., humans mPAP ≥ 25 mmHg at rest; Badesch et al., 2009). In yearling cattle, risk of PH and potential RHF was previously categorized as low (< 41 mmHg), moderate (41 to 49 mmHg), or high (> 49 mmHg; Holt and Callan, 2007). The true phenotype we seek to understand is death due to PH or at least a reduction in performance, however PAP measures are currently our best indicator of susceptibility.

Systolic, diastolic, and mean arterial pressures (sPAP, dPAP, mPAP, respectively) can be measured through different regions of the heart. Figure 1-2A outlines the process necessary to receive these pressures, where a catheter navigates the different sections of the heart and pressures

are measured with a transducer on the end of the catheter. Figure 1-2B illustrates the systolic (sPAP; top), diastolic (dPAP; bottom), and mean pressures (mPAP; calculated average) based upon waveform.

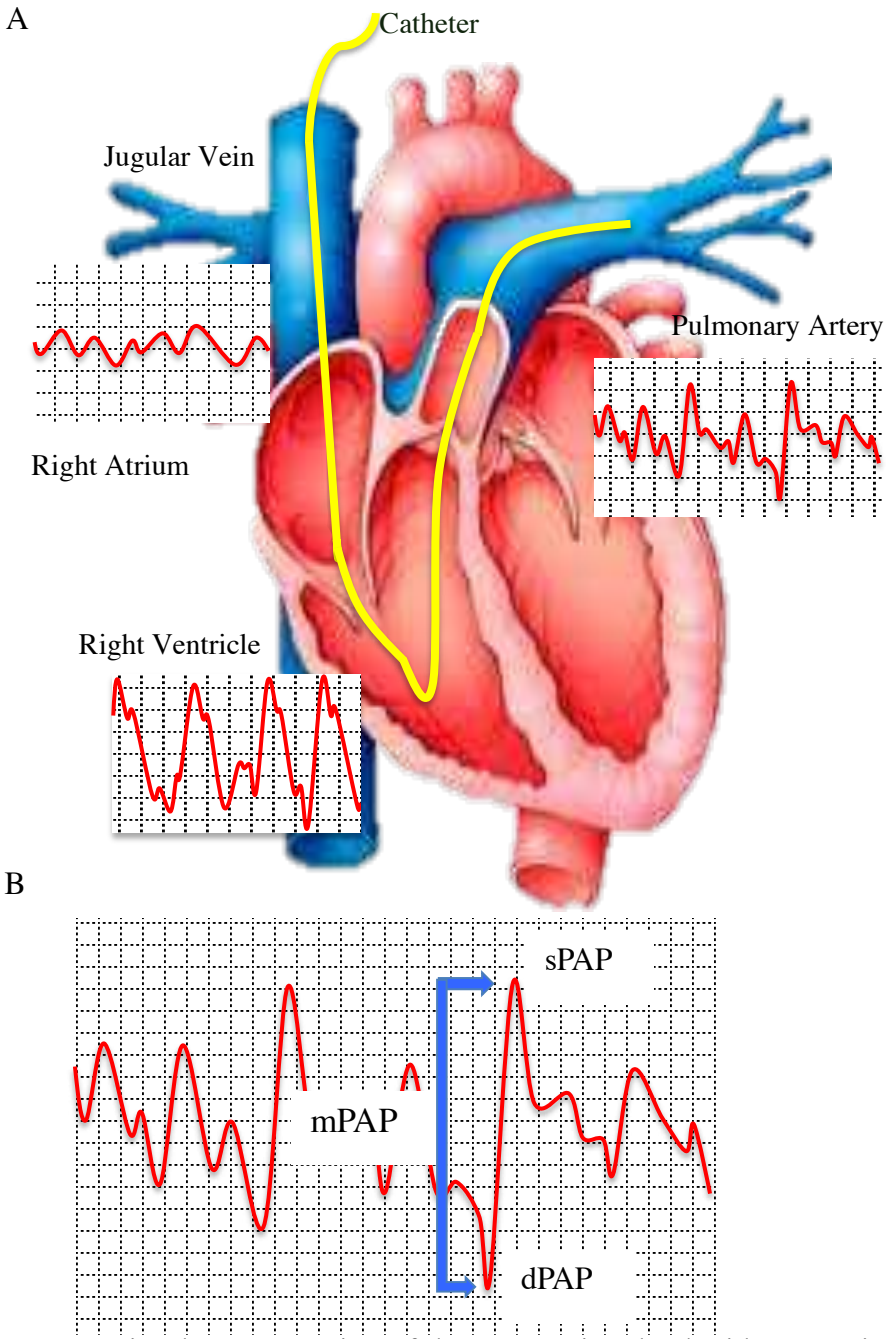


Figure 1-2. Visual representation of the process involved with measuring arterial pressures. (A) A catheter (yellow) if fed through the compartments of the heart (right atrium, right ventricle, pulmonary artery) via jugular venous puncture. Waveforms depict changes in amplitude and frequency of blood pressure in the different compartments. (B) Illustration of systolic (sPAP; top), diastolic (dPAP; bottom), and mean pressures (mPAP; calculated average) based upon wave form (Overall image created by author).

Afterload represents the force opposing the myocardial contraction and the necessary force to eject the blood. Systole (contraction and outflow) is an indicator of afterload (Norton, 2001). These pressures indicate dynamic resistance and are characterized by ventricular blood ejection and proximal arterial stiffness (Neary et al., 2016b). The right ventricle is attempting to overcome the resistance, therefore creating more afterload and increased pressures. Lower sPAP suggests that the contraction and outflow of blood from the right ventricle is sufficient and the heart is working effectively.

Diastole can represent the sufficient or insufficient mechanism of the heart as well. Increased dPAP can be a reflection of the insufficient workings of the left heart during the passive and active ventricular filling phase (phase 1) through the mitral valve. Either the mitral valve has a dysfunction (i.e., doesn't open completely), or the left atrium is not being active (contracting) sufficiently during the active phase of filling. This increase in dPAP is observed in the pulmonary artery prior to entering the lung. This is due to backpressure from blood not progressing forward into systemic circulation. Likewise, insufficient left ventricle function can create residual blood in the pulmonary vein (Lee et al., 1989). Measuring a wedge pressure is a method to measure this "back-pressure" of the blood. If the pressure in the pulmonary vein is high, then there is a necessity for the pressure on the right side to equal or exceed that back-pressure at all times. If not, this would create negative pressure and blood would be drawn back into the ventricles. Therefore, dPAP and sPAP must to be higher than the wedge pressure at all times to prevent backflow of blood (F. Garry, personal communication).

Mean PAP represents the average of sPAP and dPAP measures. This measure is not a direct average of the 2 other measures, but a calculated mean of the sPAP and dPAP measures, produced by the pressure transducer. Traditionally, mPAP has been calculated as:

$$mPAP = \frac{1}{3}sPAP + \frac{2}{3}dPAP$$

However, research by Razminia et al. (2004) developed a more accurate calculation of mPAP through the incorporation of heart rate (HR):

$$mPAP = dPAP + \left[\frac{1}{3} + (HR * 0.0012)\right] * (sPAP - dPAP).$$

Mean PAP is determined by static resistance that can be attributed to distal pulmonary arterial stiffness (Neary et al., 2016b). We have utilized mPAP measures for selection decisions on herds at high altitude because it has been thought to be an accurate reflection of the occurrence of PH in cattle. However as discussed previously, the incidence of death due to RHF has not decreased, therefore there is opportunity to better understand the impact sPAP and dPAP measures have on incidence of PH and subsequent RHF in cattle.

A genome-wide association study (GWAS) by Zeng (2016) utilized estimated breeding values (EBV) from mPAP phenotypes (raw and transformed continuous, and 2 or 3 trait categorical) as the response variables. The research found limited re-ranking of animals when the different mPAP phenotypes were compared, and therefore the raw continuous mPAP phenotype was determined to be the best choice for further analysis, given the ease of estimation and interpretation as compared to the other mPAP phenotypes.

Collecting PAP measure is an invasive and expensive procedure. Ahola et al. (2006) studied potential alternative methods to predict PAP scores in cattle. Three blood parameters packed cell volume (r = 0.31), hemoglobin concentration (r = 0.33), and red cell distribution width

(r = -0.36) were moderately correlated with PAP. The results suggested that PAP measuring was still the best indicator of PH in cattle.

1.4 INFLUENTIAL FACTORS

1.4.1 Hypoxia Exposure & Adaptation

Hypoxia is deprivation of adequate oxygen supply to the body or specific parts, which can elicit unfavorable responses of the pulmonary system. Multiple studies determined that the incidence of PH is lower in cattle born at high altitudes (native) than cattle born at lower altitudes (non-native) and moved to higher altitudes later in life (Will and Alexander, 1970; Weir et al., 1974; Holt and Callan, 2007). Will et al. (1975) found that PAP increased with increasing altitudes of residence and the magnitude of changes in PAP was much less in native cattle than in cattle originating from low altitude production systems. Therefore, it may not be advantageous to test cattle that are not native to high altitude without an acclimation period (Tucker and Rhodes, 2001). Neary et al. (2015a) confirmed this result in more recent research findings and observed that calves born at high altitudes had the greatest increase in mPAP with age.

Altitude and decreased oxygen availability has been the main factor discussed in the occurrence of PH, specifically in cattle (Alexander et al., 1960; Will et al., 1962). It is important to recognize how this decrease in oxygen is affecting cattle. The primary risk factor for PH and pulmonary vascular remodeling is alveolar hypoxia and cattle exposed to hypobaric hypoxia have a greater baseline risk of alveolar hypoxia (Neary et al., 2016a). There is a response of pulmonary arterial constriction to hypoxemia, which results in increased vascular resistance. As discussed prior, vessel narrowing increases the resistance to blood flow and increases mPAP (Neary et al., 2016a). The increased vascular resistance will direct blood flow away from the hypoxic region to maintain the ventilation-perfusion balance (Kuriyama and Wagner, 1981). This would imply that

some cattle did not have an erythrocytic response to hypoxia, which could infer a survival adaptation to those predisposed to hypoxia-induced PH by preventing an increase in arterial resistance.

Neary (2013) administered supplemental oxygen to calves suffering from PH, however oxygen-diffusing capacity of the lung did not improve, suggesting the issue to be low ventilation to perfusion mismatch. Results from a study by Gulick et al. (2016) found that calves adapted to high-altitude hypoxia by increasing their alveolar ventilation rate, as indicated by a decrease in partial pressure of carbon dioxide in the arterial blood (p_aCO_2). Additionally, impairment of fluid clearance from the alveoli may be involved in the pathophysiology of high-altitude PH. Under normal conditions, reabsorption of sodium through sodium channels and exchangers generates an osmotic gradient within the lung, allowing the reabsorption of water. Hypoxia inhibits the activity of sodium exchangers, which decreases transport of sodium, ultimately reducing fluid reabsorption in the lung (Bärtsch et al., 2003).

Both cattle and pigs lack collateral ventilation, meaning that ventilation of alveolar structures through passages or channels that bypass the normal airways does not exist in these species. It has been proposed that if hypoxia occurs in different parts of the lung due to the lack of collateral ventilation, then vasoconstriction in response to the hypoxia would lead to hypertrophy of the vascular smooth muscle (Kuriyama and Wagner, 1981). This hypertrophy of the vascular smooth muscle would justify why cattle have thick-walled pulmonary arteries both at low and high altitudes (Kuriyama and Wagner, 1981; Tucker and Rhodes, 2001). It was suggested that animals with more vascular smooth muscle (i.e., thicker arteries) would respond more vigorously to chronic exposure to hypoxic conditions, thus their PAP would be higher. Results of a study by Tucker et al. (1975) confirmed this thought when positive correlations were found between medial

thickness and the degree of PH (r = 0.88) and right ventricular hypertrophy (r = 0.97). Calves exhibited the greatest medial thickness of small pulmonary arteries and also exhibited medial thickening in response to altitude exposure (Naeye, 1965; Tucker et al., 1975). Increased medial thickness ultimately increases the resistance of blood flow and pulmonary pressures above normal.

Research has shown that there is re-ranking of cattle for mPAP measures as cattle transition between low and high altitudes, suggesting a genotype by environment interaction (Pauling, 2017). Additionally, there are many other environmental factors, such as microclimate, season, weed or vegetation exposure, and management, that influence the onset of the HMD in cattle (Busch et al., 1985; Panter et al., 1988; Holt and Callan, 2007).

1.4.2 Age

Incidence of HMD was estimated based upon age and the majority of cases (approximately 75%) occurred between birth and 2 years of age (Pierson and Jensen, 1956; Blake, 1968). Incidence was estimated to decrease to 3% or less in cattle between 2 to 5 years of age, and increase up to 20% in cattle over 5 years of age (Rhodes, 2005). Evidence of this statement can be found in a Utah Agriculture Experiment Station circular, where 397 cases of HMD were reported, of which 269 (approximately 68%) were in calves (Blake, 1968). No explanation has been presented to explain the higher susceptibility in calves as opposed to adult cattle.

Pressures (specifically mPAP) have been shown to change over time. Age was estimated as a significant factor in predicting mPAP ($P < 0.02$) and mPAP increased with increasing age ($b = 0.0387$ mmHg▪d^{-1}; Enns et al., 1992). In a more recent study, mPAP was regressed on yearling age yielding an estimate of 0.03 ± 0.01, indicating that with each day increase of yearling age, a 0.03 mmHg increase in mPAP was expected ($P < 0.001$; Crawford, 2015). In a study of cattle over an extended time frame, sPAP and pulmonary arterial pulse pressure increased more uniformly

with age (Neary et al., 2015a). Dr. Timothy Holt explained that age of the animal should always be considered when PAP testing and the accuracy of a PAP measure is dependent upon the age at which it is measured. Typically, PAP measures are less accurate and more variable with cattle younger than 12 months of age, whereas cattle 16 months and older have PAP measures that are more consistent and accurate (Holt and Callan, 2007). Other than the study by Neary et al. (2015a), research examining PAP changes over time via repeated records on individual animals over multiple time points throughout their lives is lacking.

The most abundantly recorded PAP measures are from weaning and yearling age in cattle. This lends minimal insight into how PAP changes over time, as well as how weaning PAP is correlated to yearling PAP measures. However, research by Zeng et al. (2015) analyzed the correlation between weaning mPAP and yearling mPAP measures and found the relationship to be 0.67 ± 0.18, suggesting that the two traits are different. These results reiterated the conclusions of Holt and Callan (2007) that pressures measured at ages prior to one year old are different from those measured at older ages. In cattle, overt signs of the progression of PH to RHF are not easily observed. The vascular remodeling discussed previously occurs over a longer period of time, therefore the animal won't exhibit these signs until later. The time necessary to observe overt signs is dependent upon many of the factors listed in this section. This may explain why we see increasing PAP measures with age and disease incidence later in life.

1.4.3 Genetics

Due to its moderate heritability (0.26 to 0.34), genetic selection has been conducted using mPAP phenotypes and breeding values on cattle at high elevations (> 1,500 m; Shirley et al., 2008; Crawford et al., 2016). Pauling (2017) estimated two separate heritabilities for mPAP by splitting the phenotype into high elevation mPAP (0.34 ± 0.03; >1,620 m) and moderate elevation mPAP

(0.29 ± 0.09; ≤1,620 m). Estimated progeny differences (EPDs) for mPAP have been developed and implemented into selection procedures for cattle at high altitude. A low percentage of cattle succumb to PH and RHF, therefore making it difficult to understand the effectiveness of genetic selection with mPAP EPDs. Pauling (2017) additionally estimated Spearman rank correlations of sire estimate breeding values (EBVs) and observed re-ranking of sires across low and high elevation, suggesting an influence of genetics at the different altitudes.

Research by Crawford et al. (2017) estimated breed differences in mPAP in bulls from the San Juan Basin Research Center 4-Corners Bull Tests from 1983 to 2005. Angus-Gelbvieh crossed bulls were estimated to have the lowest mPAP, whereas Simmental bulls were estimated to have the highest mPAP, adjusting for a fixed effect of pen and birth year contemporary group. Holt and Callan (2007) reported high mPAP measures in all breeds of cattle, therefore suggesting no breed has resistance to altitude-associated PH. However, not all cattle will die due to RHF. Increased knowledge of why genetically some cattle tolerate and why others do not tolerate high altitudes is necessary. This is important because of those cattle that do not tolerate high altitudes, some cattle with hypertension do not exhibit physiological changes, while others with hypertension and succumb to RHF (Krafsur et al., 2015).

1.4.4 Sex

Research by (Chu et al., 2005) outline differences in cardiac performance and pathology attributed to sex differences from multiple studies. Differences include: increased difficulty to induce cardiac hypertrophy and failure in females, slower progression of heart failure in females, increased likelihood of females to develop impaired relaxation, and a survival advantage of females with heart failure. Research by Zeng (2016) estimated the genetic correlation between performance traits and yearling mPAP phenotypes in different sex categories (i.e. bull, heifer and

steer). The results suggested that, other than sex, different management environments may contribute to the genetic differences observed between the yearling mPAP measures. Heritabilities were also estimated as 0.19 ± 0.03, 0.37 ± 0.07, and 0.33 ± 0.06 for heifers, bulls, and steers, respectively. Likewise, Cockrum et al. (2014) estimated the heritability of mPAP in yearling Angus cattle as 0.21 ± 0.04, 0.38 ± 0.08, and 0.20 ± 0.15 for heifers, bulls, and steers, respectively. Shirley et al. (2008) estimated mPAP increased 0.022 ± 0.008 mmHg per day increase in age for females and decreased 0.004 ± 0.01 mmHg per day increase in age for males. Results suggested sex is an important source of variation when investigating PH and RHF susceptibility. Results of these studies are logical as heifers, steers, and bulls are managed different from each other. Heifers are fed and managed in general to maximize reproduction traits. For the males, steers produce lower amounts of testosterone because of castration, which in turn favors more fat thickness compared with bulls (Owens et al., 1993). Nutrient requirements for overall maintenance differs between sexes (National Research Council, 2000).

1.4.5 Predisposing Conditions

There are many infectious and noninfectious agents of respiratory diseases that can predispose cattle to PH (Holt and Callan, 2007). Gram-negative sepsis can also cause elevation in mPAP measures and affect an animal's susceptibility to PH (Tikoff et al., 1966; Reeves et al., 1972; Reeves et al., 1973). Research by Neary et al. (2016a) suggested that cattle treated for bovine respiratory disease (BRD) were approximately 3 times more likely to die from RHF than those that were not treated. However, the causal relationship between BRD incidence and RHF incidence is still unknown.

There are many plausible reasons that research has found to explain how PH could be caused by obesity. Neary et al. (2016a) speculated that PH could be a result of reduced effective

alveolar ventilation due to compression of the lungs of cattle after eating. Neary et al. (2015b) estimated a positive association between sPAP and pulmonary pulse pressure with age and high pulmonary arterial wedge pressures in steers at 18 months of age. He attributed this to the body fat accumulation during the feeding period. This study also found significant increases in mPAP, sPAP, and pulse pressures across time points in cattle 4 months to 18 months of age as they were growing.

Pulmonary hypertension is present in a high percentage of morbidly obese individuals, estimated as high as 80% in a single study (Valencia-Flores et al., 2004). And a positive association was made between body mass index and systolic PAP, which may be attributed to increased cardiac output (McQuillan et al., 2001). Alpert et al. (2014) reported increases in cardiac output due to stroke volume (and stoke rate) from obese patients as a result of a reduction in peripheral vascular resistance. Cardiac output increases in concordance with oxygen demand; consequently, increasing PAP in calves with a high oxygen demand will likely have negative effects on cardiac workload, creating greater risk for RHF (Neary et al., 2016b). Little changes in cardiac output are observed due to heart rate differences. Likewise, increases in central blood volume in obese patients can augment the venous return to the right heart and increase left ventricular preload (at the end of the filling; Alpert et al., 2014). De Divitiis et al. (1981) discovered that ventricular function, more specifically the contractile response of the ventricle, is impaired in obese patients. This research found that despite the increased ventricular filling pressure and volume, contractile response of the left ventricle did not allow adequate systolic work. As a result, patients with left ventricular dysfunctions have a high likelihood (up to 70%) to have PH (Galiè et al., 2009a).

1.5 PULMONARY HYPERTENSION: OTHER SPECIES

As alluded to above, PH occurs in many species, not solely in cattle at high altitudes. Within the poultry industry, a PH-related phenomenon has been observed and called sudden death syndrome (SDS), also known as flip-over or ascites (Wideman et al., 2013; Afolayan et al., 2016). High mortality occurs in turkeys due to ruptured aortas, spontaneous cardiomyopathy (i.e., round hearts), resulting in sudden death. What was believed to be mediating these occurrences was rapid growth and with that, the metabolic imbalances that can be induced from the high nutrient intakes of these birds (Julian, 1998). Through measuring systolic blood pressure, peak incidence of SDS has been shown to occur at the end of the growing period and has been observed in fast growing birds (Varmaghany et al., 2015).

Humans exhibit many different classifications of disorders that cause PH, as briefly defined in the introduction (Simonneau et al., 2013). PH in humans is defined as a mPAP > 25 mmHg. Prevalence of pulmonary arterial hypertension (Group 1) is 15 cases per one million people (Humbert et al., 2006).

1.6 TREATMENT

Despite genetic selection procedures for reduced incidence of PH utilizing mPAP measures, researchers have examined the use of pharmaceutical agents to combat PH in cattle, humans, and other species. These agents include: beta-blockers, diuretics, angiotensin-converting enzyme inhibitors, and calcium-channel blockers, amongst others. Each is uniquely utilized to interact or interrupt functionality of the heart or other organs influential to PH susceptibility. Additionally, transporting cattle from high altitude to a lower altitude of residence can be an effective way of reducing incidence of PH by eliminating the stress decreased atmospheric oxygen.

However, in U.S. beef production systems, transporting cattle from one location to another is not always feasible.

According to Dr. Timothy Holt, a treatment protocol of PH in cattle could involve: diuretics, limits of water and salt intake, antibiotic therapy (minimize bacterial infection), external environmental control, oxygen therapy/move to lower elevation/hyperbaric chamber use, or thorocentesis. The thorocentesis is utilized to remove excess fluid from the pleural space to ease breathing (T. N. Holt, PAP Seminar).

1.6.1 Pharmaceutical agents

Angiotensin-converting enzyme (i.e., ACE-1) enhances the proliferation and migration of pulmonary artery smooth muscle cells, which contributes to the pathogenesis of hypoxic PH, and hypoxia has been found to up-regulate ACE expression (Zhang et al., 2009). ACE inhibitors are used for local cleavage of the vasoconstrictor octapeptide Ang II from its inactive decapeptide precursor, Ang I. At the same time, ACE inhibitor inactivates the vasodilator bradykinin generated in peripheral tissues. As a result, bradykinin is almost completely removed in a single pass through the lung, eliminating its vasodilation properties. ACE-1 is found in most tissues but the highest concentrations are found in the kidney and lung (Izzo Jr and Weir, 2011). However, the renin-angiotensin system is highly species-specific, in which some opposing results in the effectiveness of ACE inhibitors have been observed in animals and humans (Izzo Jr and Weir, 2011). These ACE inhibitors are most notably used for patients with left heart failure, as opposed to RHF.

Beta-blockers or beta-adrenergic blockers are another form of treatment widely used to support the treatment PH, more specific in humans. Beta-adrenergic receptors are found in the heart, blood vessels, and the lungs. These receptors can be stimulated by catecholamine binding to increase the activity of cells in the body. Beta-adrenergic receptor stimulation causes an increase

in heart rate, heart muscle contraction, blood pressure, and relaxation of smooth muscle in the bronchial tubes in the lung (Frishman, 2003). However, beta-blockers are used as a vasodilator to slow the heart rate and lowers blood pressure by blocking receptor site for adrenaline and noradrenaline. Like ACE inhibitors, beta-blockers are most notably used for patients with left heart failure, as opposed to RHF.

Calcium-channel blockers (CCB) were introduced for use in PH patients in the 1980's as a class of antihypertensive/vasodilator agent. The major control mechanism of calcium influx are long-lasting calcium (Ca^{2+}) channels in the cell membrane, which can be modulated by CCB (Medarov and Judson, 2015). They act by preventing calcium entry into cells through voltage gated calcium channels (L-type) that would result in relaxation of vascular smooth muscle (Kanno et al., 2015). Treatment using CCB is recommended only for patients with idiopathic pulmonary arterial hypertension (IPAH), heritable pulmonary arterial hypertension (HPAH) or drug-induced PH, all of which fall under the Group 1 classification of PH (Simonneau et al., 2013; Galiè et al., 2015). Patients are classified as "responders" and "non-responders" if they do or do not show a significant immediate hemodynamic response to this pulmonary vasodilator.

Treatments such as the use of diuretics and digoxin have been used as supportive therapies for PH and heart failure. Diuretics have been found to be effective when fluid retention begins to occur in decompensated RHF. Digoxin has been shown to improve cardiac output and slow ventricular rate. Additionally, other specific drug therapies include: endothelin-1 receptor antagonists, phosphodiestherase-5 inhibitors, and prostacyclin-derivatives (Galiè et al., 2009b).

SECTION 2: IMPORTANCE OF CALCIUM IN PH AND CARDIAC FUNCTION

Calcium (Ca^{2+}) is an intracellular messenger and regulator of cell function, and essential for actions such as: excitation–contraction coupling in muscle, neurotransmission, cell division, hormonal release, and phagocytosis. Calcium also regulates processes such as digestive enzyme activation, cytokine release, inhibition of ATP synthesis, and vasoconstriction (Marik, 2010). As stated above, calcium is a mediator of the physiology of the heart, including myocardial function (Hasenfuss and Pieske, 2002; Stanfield, 2011). Rhodes (2005) suggested a role of Ca^{2+} sensitization in myocytes in hypoxic PH to distinguish hypertensive and normotensive cattle; however, this statement was made from inference from studies conducted on mice/rat models. Hasenfuss and Pieske (2002) outlined predisposing factors or the potential for modifier genes to have a role in the manifestation and progression of RHF. Evidence revealed that altered Ca^{2+} homeostasis was of importance for the pathophysiology of myocardial dysfunction and heart failure. Additionally, Hasenfuss and Pieske (2002) also stated that, "altered calcium handling becomes apparent as altered systolic and/or diastolic myocardial function and triggered arrhythmias and is most obvious at high heart rates".

2.1 INTRACELLULAR VS. EXTRACELLULAR CALCIUM

Intracellular calcium refers to calcium found specifically within cells and cellular organelles. Extracellular calcium refers to calcium found in the blood, bone, and extracellular space. Additionally, calcium in the blood can be bound to proteins, free (also known as ionized), or chelated, which restricts its use by tissues (Marik, 2010). There are many different ways to assess elements such as calcium, intracellularly, extracellularly, and on a total basis. Total or serum calcium $[Ca^{2+}]_t$ represents all calcium in the blood that is bound to proteins, calcium in the cytoplasm, as well as calcium in cellular organelles. One method, inductively coupled plasma mass

spectrometry (ICP), can be utilized to determine elements (i.e., total calcium) in samples such as tissue. Other methods such as total reflection X-ray fluorescence spectrometry can be utilized to determine more specifically intracellular elements (Klockenkämper and Von Bohlen, 2014). Approximately half of plasma Ca^{2+} is bound, mostly to blood proteins/ligands such as albumin (Bronner, 2001). Intracellular ionized or free calcium concentration $[Ca^{2+}]_i$ is typically between 50 and 100 nM, about 104 times lower than the ionic calcium concentration outside the cell membrane, indicating mechanisms to keep Ca^{2+} out of the cell. These mechanisms include calcium-sensing receptors that modulate cell function via its response to extracellular calcium (Bronner, 2001). This becomes important as we understand hypo- vs. hypercalcemic status of an individual. Hypocalcemia is the state of abnormally low $[Ca^{2+}]_i$, whereas hypercalcemia is defined as an increase in serum or total calcium $[Ca^{2+}]_t$ above a normal range (Marik, 2010). Calcium-sensing receptors and calciotropic hormones (discussed in a later section) are key regulators of calcium availability. It is important to distinguish between intracellular and extracellular calcium as it will be regulated differently depending upon where it is located and if it was free or bound.

2.1.1 Intracellular Calcium and PH

Increased intracellular calcium in pulmonary arterial smooth muscle cells (PASMC) is a primary and necessary element for hypoxia induced pulmonary vasoconstriction and associated PH (Wang et al., 2007; Shimoda and Laurie, 2013). Intracellular Ca^{2+} release, extracellular Ca^{2+} influx, and pulmonary vascular tone are all associated with the activity and inhibition of potassium channels (Yuan et al., 1998; Wang et al., 2007). Additionally, endothelin, a vasoconstrictor that not only affects vascular tone but also promotes vascular remodeling, was reported to lead to a rapid increase in intracellular Ca^{2+} (Humbert et al., 2004). Voltage-gated calcium channels (VGCC), that regulate the influx and efflux of Ca^{2+} to a cell, can be activated by agonists and may

participate in the remodeling process in PH, particularly in the presence of excessive growth factors (Shimoda and Laurie, 2013). Results have suggested that elevated basal PASMC $[Ca^{2+}]_i$ occurs primarily via the upregulation of canonical transient receptor potential (TRPC) proteins, which comprises Ca^{2+}-permeable non-selective cation channels (NSCC). Unlike VGCC, NSCCs are not activated by depolarization (discussed in a section below) but can be controlled by other actions such as phosphorylation, receptor activation, or storage depletion. Increased abundance of TRPC proteins was observed in PASMCs derived from rats subjected to chronic hypoxia and in PH patients. Decreasing the activity of NSCCs, either pharmacologically or by RNA silencing, was reported to reduce $[Ca^{2+}]_i$ and proliferation in PH (Lin et al., 2004; Wang et al., 2006).

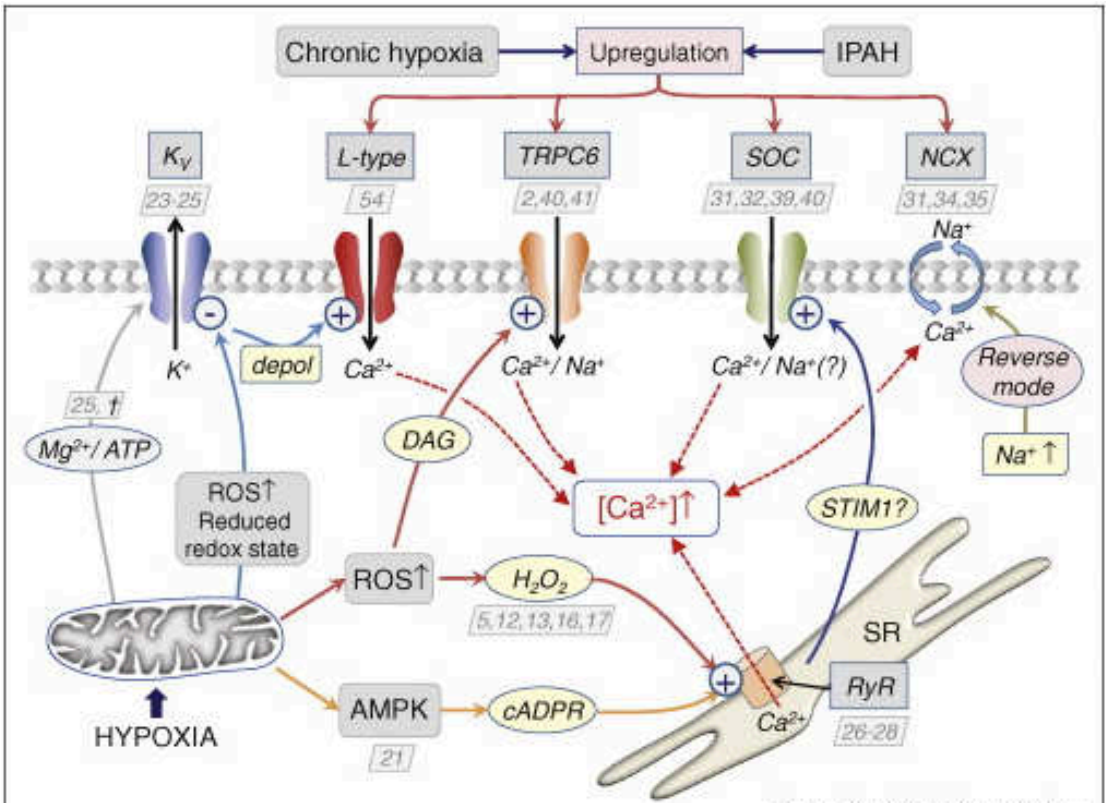


Figure 1-3. Intracellular calcium metabolism in hypoxia-induced pulmonary vasoconstriction and their potential signaling pathways. Abbreviations: DAG, diacylglycerol; cADPR, cyclic ADP ribose; depol, depolarization; KV, voltage-gated K+ channels; L-type, voltage-gated Ca^{2+} channels; NCX, Na^+–Ca^{2+} exchanger; RyR, ryanodine receptors; SOC, store-operated channels (Ward and McMurtry, 2009).

2.1.2 Extracellular calcium and PH

A biochemical blood analysis was used to analyze specific substances and underlying chemical reactions for Angus calves with PH (n = 10) and normotensive calves (n = 10). Hypertensive calves had significantly lower $[Ca^{2+}]_t$ (2.36 ± 0.06 mmol/l; $P < 0.01$) circulating in their blood than normotensive, healthy calves (Neary et al., 2013). Additional work by Neary in 2014 (results unpublished) examined $[Ca^{2+}]_i$ in blood in 18-month old Angus cattle during fattening. Comparing $[Ca^{2+}]_i$ in these cattle based on PH risk categories (low < 41 mmHg, moderate 41-49 mmHg, high > 49 mmHg) revealed no differences ($P > 0.05$) in blood $[Ca^{2+}]_i$ between groups.

A study by Olanrewaju et al. (2014) utilized venous blood samples and a blood gas electrolyte analyzer to determine specific lines of broiler chickens that had significantly different blood $[Ca^{2+}]_i$ when compared to other lines. Normal blood values could be established for commercial broilers grown to heavy weights. These results also suggest a potential genetic predisposition of certain genetic lines to be more or less susceptible to differing blood $[Ca^{2+}]_i$ and development of PH. Olanrewaju et al. (2014) reported that increasing partial pressure of CO_2, resulted in acidosis (lowered blood pH), which decreased Ca^{2+} binding to albumin, and subsequently could increase blood $[Ca^{2+}]_i$.

Within the poultry industry, a PH-related phenomenon has been observed and called sudden death syndrome, also known as flip-over or ascites (Wideman et al., 2013; Afolayan et al., 2016). High mortality occurs in turkeys due to ruptured aortas, spontaneous cardiomyopathy (i.e., round hearts), and sudden death. Research by Scheideler et al. (1995) examined dietary calcium and phosphorus based upon National Research Council (NRC) recommendations in certain strains of broiler chickens. Results suggested that slight deviations in dietary calcium and phosphorus both above (40%) and below (15%) NRC recommendations created a metabolic imbalance in

certain strains of broilers, specifically Ross x Ross, which possibly increased susceptibility to sudden death syndrome. To our knowledge, no research has been conducted assessing the relationship between dietary calcium levels and PH in cattle. These studies in poultry lend to the idea of potential opportunities to mitigate PH in beef cattle through dietary calcium regulation. However, selection for large breast size in poultry may be an extreme example not applicable to cattle.

2.2 CALCIOTROPIC HORMONES

Parathyroid hormone and vitamin D primarily maintain $[Ca^{2+}]_i$ (Marik, 2010). In addition to parathyroid hormone, other hormones involved in the maintenance of circulating calcium are said to be calciotropic and include parathyroid hormone-related protein and calcitonin. Each calciotropic hormone has a specific biochemistry and functional properties that make them similar, but also different from one another. These properties involve synthesis, secretion, metabolism, target cell activation, and cellular actions, that can have an effect on the cardiovascular system and hypertension (Crass III and Avioli, 1994).

2.2.1 Parathyroid Hormone

Parathyroid hormone (PTH) is produced by the parathyroid glands and its primary responsibility is the maintenance of circulating Ca^{2+} levels and inorganic phosphate (Crass III and Avioli, 1994). The primary action of PTH is on bone and kidney to maintain extracellular Ca^{2+} levels. The hormone is circulated in the blood (serum), and is secreted in response to low extracellular Ca^{2+} or elevated extracellular phosphate (Gensure et al., 2005).

The earliest study of PTH by Collip and Clark (1925) revealed its ability to lower systemic blood pressure in dogs. The effects of the administration of PTH as a hypotensive or vasodilator, and its influence on cardiac function, have thoroughly been described (Mok et al., 1989). Result

of research by Akmal et al. (1995) revealed that excess PTH during renal failure in dogs adversely affects both the left and right ventricles of the heart. Additionally, a correlation was reported between blood levels of PTH and left ventricular hypertrophy in patients with hypertension and normal renal function (Bauwens et al., 1991). Schlüter and Piper (1998) determined the PTH had high effects on Ca^{2+} currents and Ca^{2+} influx on cardiomyoctyes, amongst others.

2.2.2 Parathyroid Hormone-related Protein

Parathyroid hormone-related protein (PTHrP) is biologically similar to PTH, but is abundantly produced by tumors and released into circulating blood (Clemens et al., 2001). The two hormones (PTH and PTHrP) bind to the same receptor in bone and kidney target cells (Jüppner et al., 1988). This protein was expressed in cardiomyoctyes in the atria and to a small extent in the ventricles, and its expression pattern resembles that of atrial natriuretic peptide (Burton et al., 1994; Stanfield, 2011). Although they are biologically similar and affect the same receptors, the cardiac effects of PTHrP are distinctly different from PTH (Clemens et al., 2001).

In a comparison of the cardiovascular actions of PTH to PTHrP, Schlüter and Piper (1998) found PTHrP had a very high effect on vasodilation. This may be due to its interaction and inhibition of endothelin-1, a known vasodilator (Jiang et al., 1996). The protein has also been found to be a positive stimulus on heart rate, and indirect positive stimulus on speed or contraction of cardiac muscle (Ogino et al., 1995; Strewler, 2000).

2.2.3 Calcitonin

Calcitonin is a hormone synthesized by the thyroid gland and is secreted in response to elevated circulating $[Ca^{2+}]_i$ (Crass III and Avioli, 1994). It is classified as a vasoactive hormone, where its effects (i.e., inhibit or stimulate) seem to depend upon the distribution of Ca^{2+} between intracellular and extracellular spaces and the specific tissue membrane potential (Crass III and

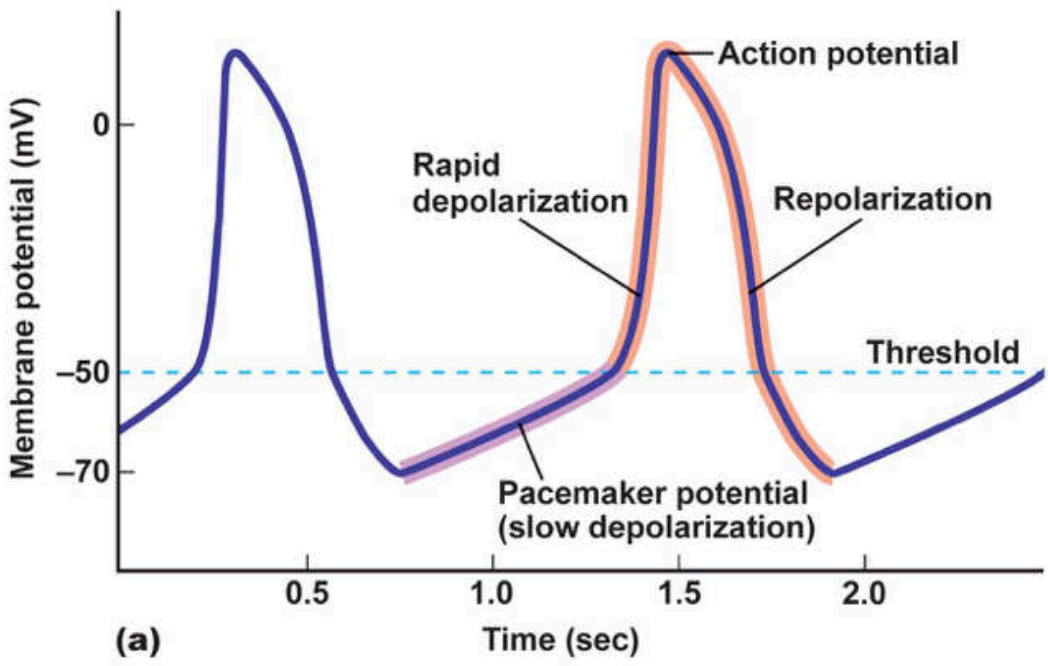
Avioli, 1994). Calcitonin alters calcium uptake distribution and release in certain tissues, as it can block some calcium channel blockers such as verapamil in the liver (Yamaguchi and Yoshida, 1985).

A gene and specific protein have been identified for calcitonin, called calcitonin gene-related peptide. A deletion or inhibition of this gene results in increased vulnerability of the heart to hypertension-induced organ damage (Supowit et al., 2005). These results are echoed in multiple species, as the vasodilator mechanism of calcitonin can be altered (Gangula et al., 2000; Márquez-Rodas et al., 2006).

2.3 CONTRACTION & RELAXATION MECHANISMS OF THE HEART

From a physiological standpoint, the mechanisms affecting the performance of the heart include: heart rate, preload, afterload, and contractility (Varon and Fromm Jr, 2014). Both excitation and relaxation of the heart are managed by the electrical activity (action potentials) of pacemaker and cardiac contractile cells through the regulation of specific ions (Figure 1-4B). Influx and efflux of calcium (Ca^{2+}), potassium (K^{+}), and sodium (Na^{+}) ions are essential to the pacemaker of the heart (Faber and Rudy, 2000). Permeability of each of ions into the cell creates the pacemaker action potential through depolarization and repolarization, which regulates the firing rate of the cell, the main determinant of heart rate.

A)



B)

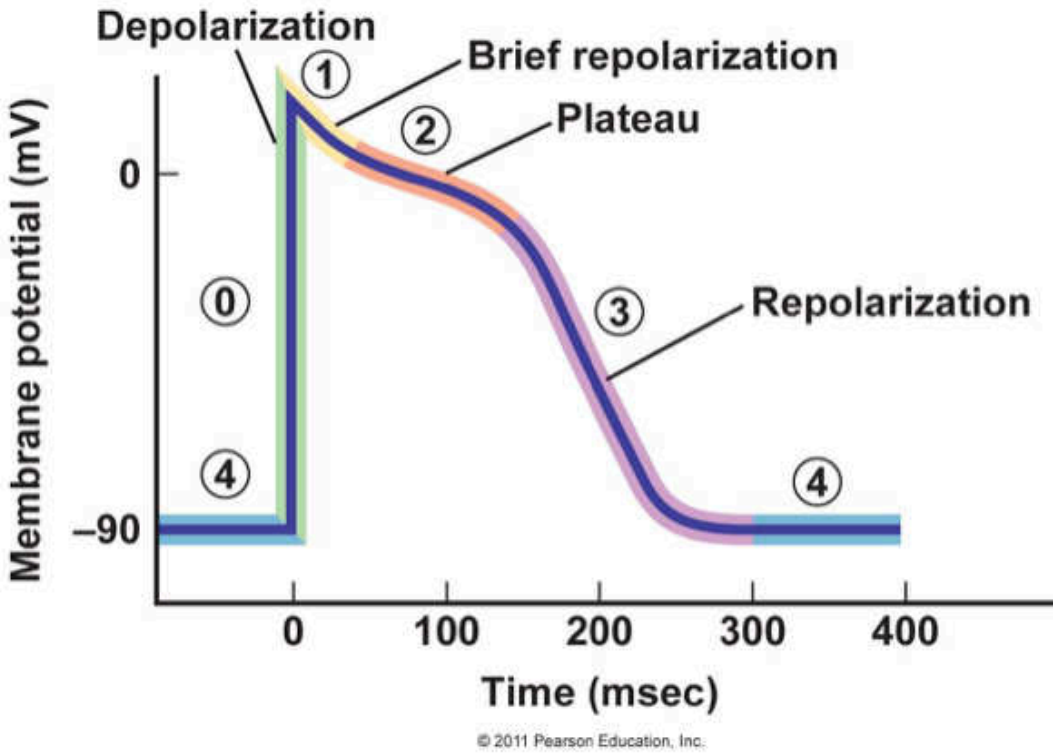


Figure 1-4. Electrical activity of cells regulating depolarization and repolarization, where permeability of the ions drives the firing rate of the cell, determining heart rate. A) Pacemaker cells, with a slow depolarization (no 'resting'). B) Ventricular muscle cells, with a stable resting potential stage . K^+ = potassium, Na^+ = sodium, Ca^{2+} = calcium
http://droualb.faculty.mjc.edu/Course%20Materials/Physiology%20101/Chapter%20Notes/Fall%202011/chapter_13%20Fall%202011.htm

Depolarization is defined as a shift in the electrical charge within a cell, in which there is a less negative (more positive) charge within the cell. Repolarization involves establishing a negative resting potential of the cell through electrical charge manipulation. Hyperpolarization is the opposite of depolarization, in which the charge within the cell is more negative (Nerbonne and Kass, 2005).

Influx of calcium triggers depolarization of the cell and moves the membrane potential towards equilibrium. There are two types of calcium channels that permit this to occur: T-type (transient) and L-type (long-lasting). The T-type calcium channels open, allowing for quick depolarization of the sinoatrial and atrioventricular nodes of the heart, and additionally triggers L-type channels to open. The L-type channels stay open longer, resulting in a rapid depolarization phase. This influx of Ca^{2+} induces Ca^{2+} release from the sarcoplasmic reticulum. This depolarization allows muscle contraction through binding of the released Ca^{2+} to troponin. The membrane is then allowed to repolarize and muscle fibers relax when the Ca^{2+} channels close and K^{+} permeability increases (Stanfield, 2011).

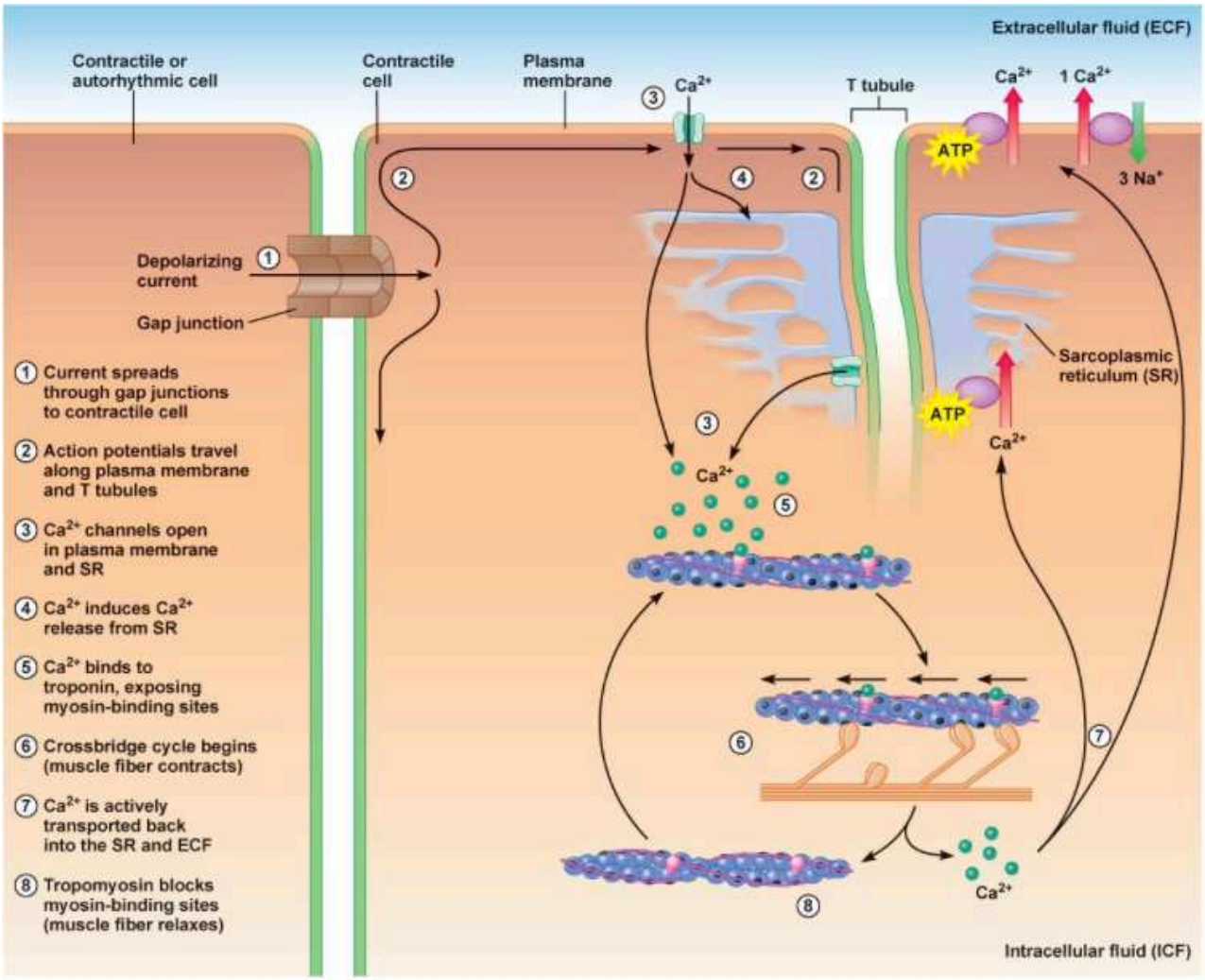


Figure 1-5. Signaling and utilization of calcium in contractile cells.
2011 Pearson Education, Inc.

2.4 CALCIUM SIGNALING GENES AND THEIR AFFECT ON PH SUSCEPTIBILITY

There are multiple studies that have surveyed genes related to PH in various species (Amberg et al., 2010; Newman et al., 2011; Zeng, 2016). However, none of those studies have intentionally examined calcium-associated genes in cattle. The results of these studies provide evidence of genes as significant predictors of PH susceptibility. There is potential for the use of genomic selection procedures based on the candidate genes and SNP discovered in those studies given differences in gene expression.

A previous GWAS was conducted by Zeng (2016) using SNP referenced to the UMD3.1.1 bovine assembly. Genotyped animals were born from 1997 to 2015 and genotypes were completed in three groups (i.e. 2013-50K chip, 2013-HD chip, and 2015-50K chip) and from two labs (Zoetis and GeneSeek). In addition, 65 Angus cattle in this herd were genotyped in 2013 using Illumina Bovine HD chip through the lab work of GeneSeek. From the GWAS, SNP windows from PAP phenotypes were aligned to the *Bos taurus* genome (UMD3.1.1) to identify the genes within these windows using Ensembl. The study yielded 22 quantitative trait loci (QTL) windows detected from nearly 36,000 SNP markers. Within these windows, genes such as ADGRVI (GRP98), ROCK2, MYH6 and MYH7, and BMPR2 were identified as either lead-genes or genes with high model frequencies associated with yearling mPAP. Many of these genes were associated with calcium regulation. A pitfall of that study was the use of an outdated bovine genome assembly (UMD3.1.1). Due to the recent debut of the ARS-UCDv1.2 bovine assembly, we now know of a significant number of gaps in the bovine genome UMD3.1.1 will be resolved through the scaffolding of the new assembly. Therefore, the results of Zeng (2016) likely contain errors which is exacerbated with animal ID and genotype errors in her data. A re-analysis of these data will improve interpretation of the GWAS results and could imply an association of genes regulating or regulated by calcium to PH.

Research by Newman et al. (2011) described the top 15 up-expressed genes (+1.77 to +4.93) and bottom 15 down-expressed genes (-0.65 to -2.03) in peripheral blood mononuclear cells from cattle at high altitude based upon fold change. Of these genes, 6 had functions within cells related to calcium. A subsequent paper by Newman et al. (2015) examined genetic differences in cattle related to PH and uncovered 3 additional calcium-associated genes differentiating cattle that likely had PH and those that did not. The 9 genes in total included: AFAP1, CD8A, CLGN, DNER,

EMR1, FLVCR2, RYR1, S100A4, and TGM3. Combining the ideas and efforts of the latest research regarding PH in cattle, there is still a large gap in knowledge of the role calcium availability and utilization may have in bovine PH. This alludes to the necessity of hypothesis-based research to understand this association, if one does exist.

SECTION 3: 'OMIC' TECHNOLOGIES FOR DISEASE SUSCEPTIBILITY

The suffix of –omics has been used to describe many biological fields, including but not limited to genomics, transcriptomics, proteomics, metabolomics, and lipidomics (Barh et al., 2013; Hasin et al., 2017). Each of which are a comprehensive or global assessment of a particular discipline (i.e., genetics, transcripts, proteins). Emerging omic technologies (i.e., RNA-seq, GWAS, whole genome sequencing, candidate gene identification, and SNP discovery) are tools that could help the beef industry reduce disease susceptibility through their utilization in breeding value estimations. Disease traits are typically a hard to measure traits, in addition to typically have low heritabilities, making genetic selection difficult. However, the field of omics has the ability increase our accuracy of selection through the identification and use of causal variants in selection methodology, while decreasing the generation interval of our animals, ultimately leading to genetic progress, specifically in disease traits of interest.

Determining gene expression allows one to begin to understand associations between physiological states (i.e., sickness, behavior changes) and the potential genes regulating those states. One can measure whether particular genes are expressed or not, as well as the relative amount of expression that exists as compared to a standard or reference. Knowing the gene transcript abundance in various tissues, developmental stages, and under various conditions is important. Although messenger RNA (mRNA) is not the ultimate product of a gene, transcription is the first step in gene regulation, and information about the transcript levels is needed for understanding gene regulatory networks. Nevertheless, the correlation between the mRNA and protein abundance in the cell are often variable and difficult to assess (Brazma and Vilo, 2000).

3.1 WHAT IS RNA?

Ribonucleic acid (RNA) is one form of nucleic acid that carries genetic information that can be inherited from one generation to the next. This single strand structure is transcribed from the DNA sequence of individuals through transcription. Transcription utilizes enzymes and proteins to read the DNA genetic code that will be translated into proteins to serve a physiologic function in the body. Processing RNA involves capping, splicing, polyadenylation, editing, export, localization, translation, and turnover. Each of these steps is necessary and can have an effect on how genes are expressed. Typical methods to extract RNA utilize samples such as: blood (i.e., white blood cells), cultured cells, plants, but most widely used is tissue (i.e., heart, lung, muscle).

3.2 TRANSCRIPT REGULATION

Determining differentially expressed genes as well as transcript abundance is dependent upon transcript length (Oshlack and Wakefield, 2009). As discussed above, there are many intracellular processes that effect transcript abundance. Many of the challenges that limit the effectiveness of determining gene expression include: purity (sample contamination), quantity, quality (degradation), abundance and expression level, and alternative splicing (Ozsolak and Milos, 2011). We can identify single nucleotide polymorphisms or mutations, alternative splicing, and post-transcription modifications with RNA-seq. Understanding each of these in greater detail will require a certain level of sequencing depth, as well as knowledge regarding RNA processing.

The purity (level of contamination) and integrity of a sample will affect RNA sequencing results by introducing ambiguity of the RNA present (Fleige and Pfaffl, 2006). This means that the isolated RNA must be free of impurities or inhibitors, such as proteins, DNA, ribosomal RNA (rRNA; most abundant RNA), or transfer RNA (tRNA). Quantity and quality of the RNA relate to the amount or extent of degradation. Ideally, we want completely intact, non-degraded RNA. The

RNA isolation method will play a role in its quantity and quality. Abundance of RNA will limit the determination of expression level of genes. If RNA for a gene of interest is more abundant than another gene, then sequencing of the more abundant gene will allow for the expression of the gene to be better captured by the reads.

Splicing is a major regulatory factor in gene expression by promoting mRNA 3'-end formation, nuclear export, and translation to stimulate expression. As an overview, splicing removes non-coding sequence regions (introns) and ligates the neighboring coding sequencing (exons; Figure 1-6). This mechanism occurs by 2 transesterfication reactions: cis- and trans-splicing (Lewin, 1990). Three or more exons together have some form of alternative splicing that occurs. The process of alternative splicing involves the removal of different exons and introns for a specific pre-mRNA, which could result in different isoforms of a protein. Many genes have more than 2 splicing patterns. Likewise, alternative splicing can vary within a cell, be developmentally controlled, vary between cells or tissues, vary in response to external stimuli, and can vary with the speed of RNA polymerase II elongation (Heyd and Lynch, 2011).

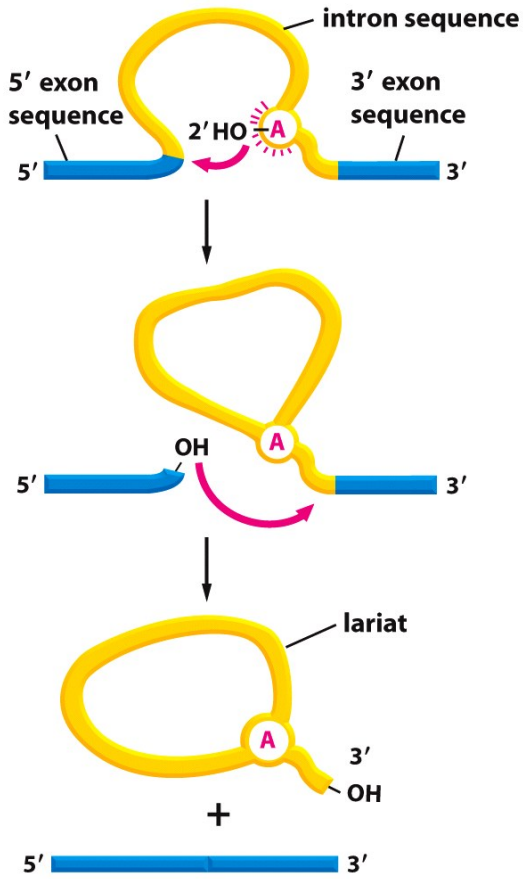


Figure 1-6. Diagram of RNA splicing. Creation of a mature messenger RNA, through splicing out intron sequences, resulting in only exons.
http://oregonstate.edu/instruction/bi314/fall11/geneexpression.html

When comparing RNA-seq expression to mass spectrometry results, the abundance of RNA does not accurately reflect the abundance of proteins in cells. The production and maintenance of proteins is dependent upon processes of: transcription, processing and degradation of mRNA, translation, localization, modification, and programmed destruction of the proteins (Vogel and Marcotte, 2012). Within each of these processes, a number of additional factors will regulate transcript and protein abundances (i.e., re-initiation, ribosome shunting, leaky scanning in translation). The resulting abundance of protein reflects the balance among these processes. Given rise in knowledge and capabilities of mass spectrometry, we are now able to use this technology to understand more about proteomics. In concordance with qPCR, RNA-sequencing, or other Next Generation Sequencing (NGS) techniques, we can begin to understand protein-

expression regulation. It is essential to understand each of the limitations listed above, amongst others influencing expression when sequencing RNA for transcript abundance, differential expression analysis, and alternative transcript usage. Standardizations and quality control measures are necessary during RNA-seq and qPCR analyses, as well as differential expression analysis to understand how these factors more clearly to determine or explain the gene expression differences observed between samples.

3.3 METHODS OF MEASURING GENE EXPRESSION

The most appropriate method of measuring gene expression is dependent upon many factors. These factors include the number of genes evaluated, accuracy of the method, sensitivity to detection, discovery, data interpretation, and cost. Brazma and Vilo (2000) outlined some important questions to answer through expression studies. These include: 1) what are the functional roles of different genes and in what cellular processes do they participate; 2) how are genes regulated; 3) how do genes and gene products interact; 4) what are these interaction networks; 5) how does gene expression level differ in various cell types and states; 6) how is gene expression changed by various diseases or compound treatments. Methods to detect expression differences amongst samples include: northern blots, microarrays, RNA-sequencing, and quantitative real-time polymerase chain reaction.

3.3.1 Northern Blots

Northern blots are a method to measure gene expression from RNA of a particular tissue or cell type. Its name was coined from the similarities of the technique to Southern blots, which are used to identify DNA sequences. A Northern blot reveals both the abundance of the gene transcript, as well as the size of the mRNA gene product. In brief, the process of creating Northern blots involves: 1) collect RNA from tissue (or other sample type), 2) electrophorese the RNA to a

gel and blot to a suitable support, 3) hybridize the blot to a labeled cDNA probe, 4) detect and measure labeled band with x-ray film (Weaver, 2011).

An advantage of the use of Northern blots for gene expression is the procedure is relatively fast, low-tech, and inexpensive. Additionally, conclusions can be drawn from a single experiment, as blotting provides a direct relative comparison of RNA abundance between samples (Scientific). There are major limitations to the use of Northern blots for gene expression studies. One limitation is its sensitivity of detection as compared to other methods. Northern blots need approximately 100,000 copies of DNA or RNA sequence for detection by blot hybridization. In contrast, techniques such as qPCR (discussed in a later section) can amplify single copies of DNA or RNA to readily detectable levels. Another limitation of the use of Northern blots for gene expression is abundance of RNA is determined in each sample. Abundance is determined by quantifying the darkness of the labeled band, and this is done through measuring the amount of light it absorbs in a densitometer or quantifying the amount of label in the band directly by phosphorimaging. These measurements will reveal the relative amounts of specific RNA in each sample (Weaver, 2011). Northern blots can only measure steady state mRNA accumulation levels; therefore, the technique fails to measure transcription rates or RNA stability, making other methods more applicable. Lastly, Northern blots of inferior to other gene expression methods in that it only can only measure expression of a single gene at a time. This method consequently increasing both the time and the workload as compared to other methods.

3.3.2 Microarrays

Microarray methodology allows for the identification of genes that are expressed or not, this is based upon if mRNA is present. This technology was the first tool available for transcriptomic studies. Microarrays can be used to 1) investigate a single change in gene

expression that may be significant in understanding the phenotypic variation that may exist (i.e., local approach), and 2) to look at the overall patterns of gene expression in order to understand the architecture of genetic networks that regulate gene transcription (i.e., global approach; Schulze & Downward, 2001). The two most commonly used systems according to array material are: complementary DNA (cDNA) and oligonucleotide microarrays (Schulze & Downward, 2001). The array materials utilized in microarrays are probes, which is equivalent to that of northern blots, albeit a northern blot is used for a single gene. Limitation

A strength of the use of microarrays is that thousands of transcripts (genes) can be detected and quantified simultaneously (Schulze & Downward, 2001). Therefore, microarrays can be useful if the genes you desire to analyze are unknown. This technology will allow for freedom in determining gene expression. Microarrays are also most cost effective on a small-scale (sample) basis than RNA-seq for gene expression.

Despite the positive aspects of microarrays, there are also limitations to the utilization of this technology. Microarrays do not account for post-transcriptional and post-translational modifications. These modifications can limit our understanding of the diverse RNA molecules and expression differences that may exist from genomes (Ozsolak and Milos, 2011). In addition, the amount of data received with microarrays in the form of spot intensities and intensity rations creates a challenge to sieve through the data to find meaningful results (Schulze & Downward, 2001). Significant variability also exists in microarray results; which is especially true for genes with low expression levels. Replication or validation is therefore necessary to instill confidence in results (Schulze & Downward, 2001). This semi-quantitative methodology would then require qPCR as a means of verification of results. Another limitation of microarrays is the necessity of having a minimum of 25 to 100 μg of total RNA. This can be an issue in the experimental process

to be able to receive this minimum amount of total RNA from all samples tested. Lastly, short oligonucleotides that are utilized for microarray gene expression may result in less specific hybridization and reduced sensitivity. Cross-hybridization (i.e., formation of double-stranded DNA, RNA, or DNA/RNA hybrids) is possible if certain DNA elements on the array to fail to detect the correct transcript species (Malone and Oliver, 2011).

3.3.3 RNA-sequencing

Since 2008, NGS through sequencing RNA (RNA-seq) has provided knowledge in the areas of both quantitative and qualitative aspects of the transcriptome (Ozsolak and Milos, 2011). The methodology involves the reverse transcription of cDNA from mRNA and sequencing of the cDNA. Millions of sequence reads can be identified through RNA-seq to reveal both if, and how much, of a transcript is present (Chu and Corey, 2012). As discussed previously, challenges can arise and limit the effectiveness of RNA-seq. Processing RNA involves capping, splicing, polyadenylation, editing, export, localization, translation, and turnover. Each of which will have a downstream effect on the results and application of RNA-seq. Kumar et al. (2012) provides an extensive overview of sequencing applications.

3.3.3.1 Advantages and Limitations

The use of RNA-seq to determine gene expression differences has an advantage in that RNA-seq provides absolute values (i.e., RPKM, total counts), as opposed the relative values that are received from other gene expression methods (i.e., on vs. off signals). As compared to microarrays, RNA-seq is a more efficient and therefore less costly method to determine gene expression (Wilhelm and Landry, 2009). Additionally, an advantage of RNA-seq is the ability to explore both known and novel transcripts. There is not a requirement of *a priori* knowledge of

transcribed regions to survey the entire transcriptome, allowing for more opportunities for research exploration (Wilhelm and Landry, 2009).

A limitation to RNA sequencing data is sequencing depth. Determining differentially expressed genes as well as transcript abundance is dependent upon transcript length. For SNP discovery purposes, larger sequencing depth is necessary to understand the differences or observe the genetic mutations between samples. To discover novel transcribed elements (i.e., related to calcium utilization), it would be necessary to increase sequence depth to a deeper (higher) level (i.e., 31 million reads to 200 million reads). One can use homology between species in more robust assemblies to help determine any newly transcribed elements. As mentioned, it is important to understand the abundance levels of the genes of interest. More highly abundant transcripts will need less sequencing depth, whereas or lowly abundant transcripts will need a higher sequencing depth to be effective at quantifying expression and determining differentially expressed genes. If the transcripts of interest are lowly abundant, a lower sequencing depth will not be sufficient.

3.3.3.2 Importance of Sequencing Depth

Sequencing depth is largely dependent upon the question or goals of a study, as well as the characteristics of the sample. From a quantitative standpoint, one can assess the amount of expression present, if the expression of one product is produced more than another. From a qualitative standpoint, gene expression studies can be used to reveal different variants of a particular transcript. If we are trying to understand gene expression differences, sequencing depth may not need to be as deep and may not have a great impact on the assessment of the gene expression. If the goal is to identify specific mutations (i.e., SNP discovery), a greater sequencing depth will be necessary to understand those differences. Coverage of the genome is dependent upon the size of the genome, where smaller genomes require less sequencing depth. Transcripts

that are low in abundance will need greater sequencing depth since more abundantly expressed genes may consume the majority of the reads being sequenced. Likewise, short RNAs will also need more sequencing depth. Both the goal of the study as well as the available funds for the research will drive the depth of sequencing.

A study by Tarazona et al. (2011) examined the trade-offs of sequencing depth for gene expression data. Their research revealed that for investigating the regulation of rare transcripts, although deep sequencing effectively enhances understanding on the diversity of the transcriptome, the identification of true differential expression at a low count range is more difficult to achieve. The study also estimated that more reads implied the detection of more genes, but would subsequently result in noisier data, making differential expression increasingly difficult (Tarazona et al., 2011). If the goal is to detect similarities between transcriptional profiles, a modest depth (i.e., 30 million paired end reads, > 30 nucleotides, with 20 to 25 million reads mapped to the genome/transcriptome) is sufficient. If the goal were to discover novel transcribed elements in a tissue sample with strong quantification to known isoforms, a more extensive sequencing depth (i.e., minimum 100 to 200 million reads of 2x 76 base pairs or longer) would be necessary (ENCODE-Consortium, 2011).

3.3.4 Quantitative Reverse-Transcription Polymerase Chain Reaction

Reverse Transcription (RT)-quantitative Polymerase Chain Reaction (qPCR) has become a versatile technique used to examine expression changes of one or more genes of interest in various pathological states. Reverse-transcription PCR is used for absolute and relative quantifications of DNA and RNA template molecules and for genotyping in a variety of applications. It can be used to determine viral loads, gene expression, titers of germs and contaminations, allele imbalances and the degrees of amplification and deletion of genes (Jacob et

al., 2013). The technique is applied for the analysis of age-dependent diseases, cytokine and tissue-specific expression, forensic samples, epigenetic factors like DNA methylation and for food monitoring (Jacob et al., 2013).

Real-time PCR was invented in 1996, and the number of research publications utilizing this technology has increased exponentially since then, with over 163,000 publications (PubMed). Due to its specificity, sensitivity, simplicity, costs and high-throughput, RT-qPCR offers a broad range of advantages over standard methods such as Northern blot and semi-quantitative PCR (Jacob et al., 2013). Real-time PCR compared with other methods has its advantages for the quantifying nucleic acids, which include having a wide dynamic range and significantly higher reliability of the results compared with conventional PCR. This is because with RT-qPCR, the whole amplification profile is known. RT-qPCR is more precise than end-point determinations, and reactions deviating in their amplification efficiency can be identified easily (Jacob et al., 2013).

There are many different formats to detect and measure gene expression, including: SYBR Green, hybridization probes, hydrolysis probes (i.e., TaqMan), molecular beacons, sunrise primers, scorpion primers, and light-up probes (Wilhelm and Pingoud, 2003). The PCR product amplification is observed through the fluorescence of dsDNA-specific dyes (i.e., SYBR Green I) or sequence-specific probes. Each curve consists of three distinct phases: 1) an initial lag phase, 2) an exponential phase, and 3) a plateau phase (Wilhelm and Pingoud, 2003).

3.3.4.1 Necessities of qPCR

To perform a qPCR assay, the required elements include: primers (Oligo(dT)s, random hexamer, sequence specific), template (mRNA), reverse transcriptase, DNA polymerase, buffer & dNTPs, and controls (No R.T. control, no template control, endogenous control).

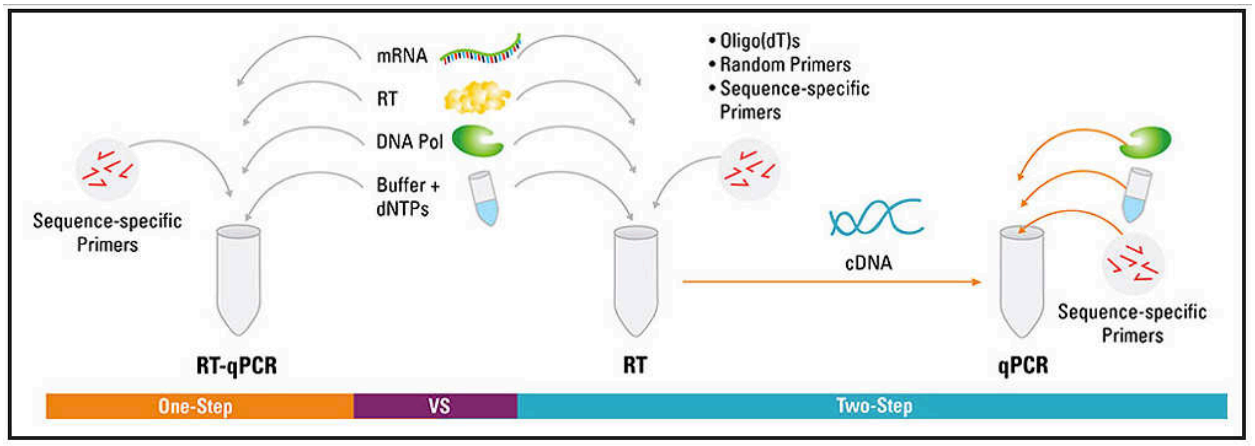


Figure 1-7. Elements needed to conduct qPCR for gene expression
https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

There are 3 types of primers can be utilized for qPCR research: Oligo(dT)s, random hexamer, or sequence specific primers (Figure 1-7). These primers anneal to the template mRNA strand and provide reverse transcriptase enzymes a starting point for synthesis. There are advantages and disadvantages to the use of each of the types of primers (ThermoFisher Scientific, 2017). Template, in the form of RNA for gene expression studies, will be utilized for reverse transcription. The reverse transcriptase enzyme is utilized to create cDNA from our RNA template. Upon completing the reverse transcription step, cDNA will then be available and utilized for the qPCR for gene expression step.

Standardizations and quality control measures must be put in place both during RNA sequencing analysis, as well as differential expression analysis to understand how these factors more clearly to determine or explain the gene expression differences observed in our samples. One form of standardization is endogenous controls. Endogenous control genes (a.k.a. housekeeping genes) can be utilized to analyze the quality of the transcript with qPCR. These genes can be applied as a normalization factor for the amount of template used. Also, the level of expression of endogenous control genes should not change between samples. Therefore, a truly highly expressed gene should correlate to a highly expressed endogenous control. Non-template controls (NTCs)

are necessary in detecting the presence of contaminating DNA. Additionally, non-reverse transcription controls (-RTC) are used to assure the absence of contaminating genomic DNA for qPCR-based gene expression analysis (D'haene and Hellemans, 2010).

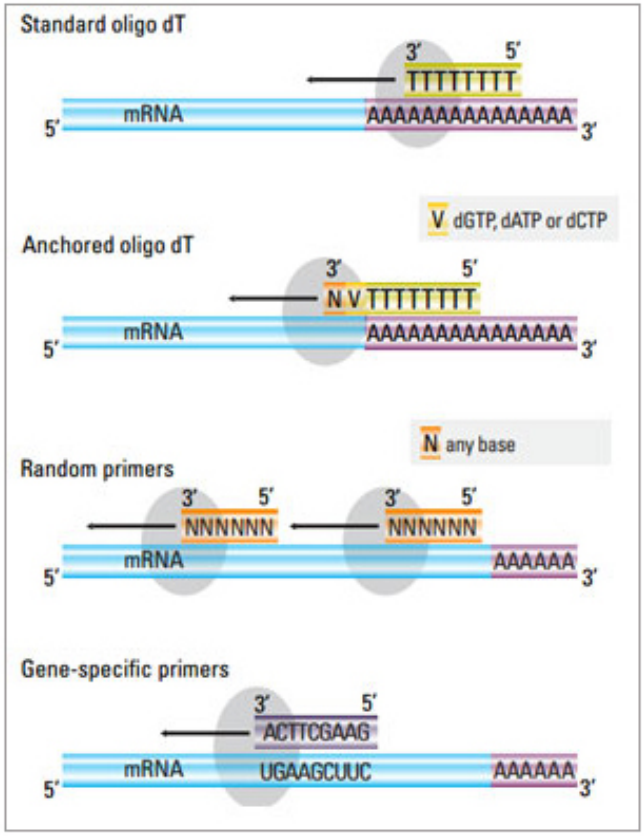


Figure 1-8. Different types of primers to use for qPCR for gene expression
https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

Primers are essential to ensure specific and efficient amplification of the products. Therefore a critical step in a PCR is the annealing of the primers to their target sequences, while preventing nonspecific annealing and primer–dimer formation (Taylor et al., 2010). The design of primers involves utilizing FASTA files that can be retrieved from the National Center for Biotechnology Information (NCBI) gene database, which are available for each of the genes of interest. The files contain the sequence information for the gene. These FASTA files can then be uploaded to UCSC Genome Browser to check alignment from cDNA. Next, the FASTA files can be uploaded to Primer BLAST software through NCBI GenBank, setting primer specifications. Some of the most

important specifications include: the primers must span an exon-exon junction, forward and reverse primers must not be in an intron region, the primer product must be < 200 bp, and near the 3' end (Figure 1-9). This primer design software will provide multiple primer options to choose. These primers usually cost between $3 to $5 each and can be used for multiple samples. This is a critical aspect for qPCR analysis. The primers are what will allow us to capture specific sequences to replicate. If the primer is not specific or accurate enough, this could result in poor amplification and expression of the gene of interest.

Additionally, there is the option to use previously designed primers from literature. Each primer is assigned a unique GeneBank Accession Number, which can to adopted and utilized in additional studies. A benefit of utilizing previously designed primers is having the knowledge that the primers were successful at amplifying the gene product and in determining expression differences. Therefore, this could help to eliminate a step in the process by having confidence in the chosen primers and eliminating the potential of having to redesign primers.

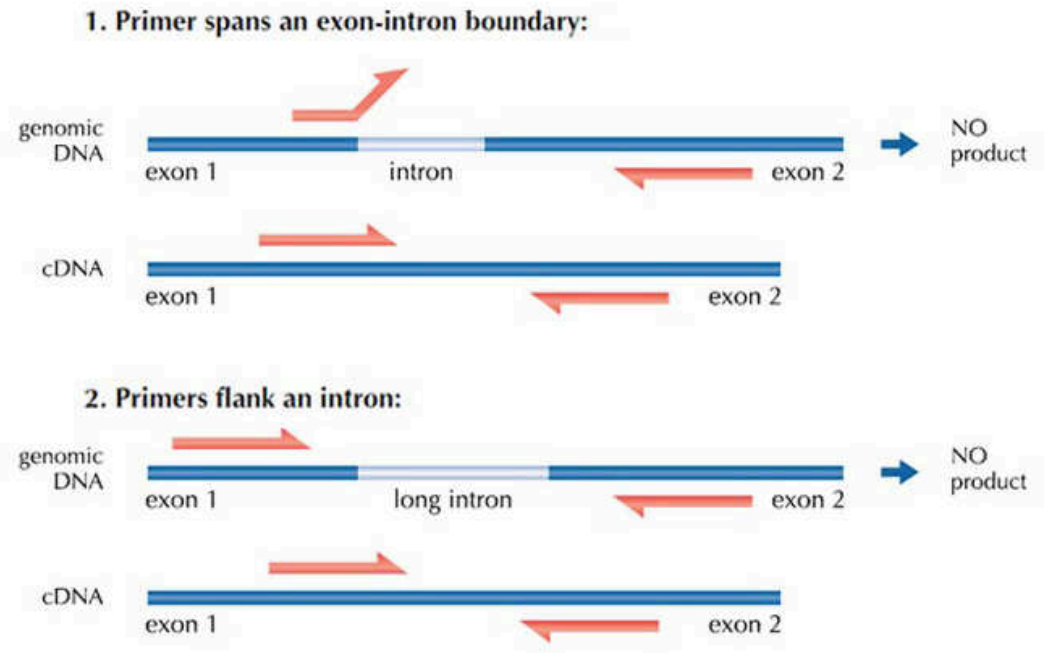


Figure 1-9. Examples of designing specific primers.
https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

3.3.4.2 Primer Efficiency Testing

Testing primer efficiency is important as it provides an indication of the effectiveness of primers you have designed in amplifying your product of interest. The consistency in PCR efficiency is determined by examination of each sample standard curve (Hui and Feng, 2013). Serial dilutions are routinely utilized to determine linearity and amplification efficiency based upon the template material. Serial dilutions of nucleic acids are used to demonstrate that observed decreases in quantification cycle (McQuillan et al.) or copy numbers are consistent with the anticipated result (Bustin et al., 2009).

A standard curve can be generated to plot the results of testing primer efficiency (Figure 1-10). This entails plotting the log of the starting concentration of the genetic material against the cycle threshold (C_t) values generated from the PCR reaction. If the quantity of genetic material is unknown, plotting the C_t value against the log of the dilution factor is an option. Testing the samples in triplicate is necessary when generating these curves to ensure reproducibility (Maddock and Jenkins, 2017). The major benefit of the dilution-replicate design strategy is that it yields data that simultaneously measures both PCR efficiency and DNA quantity for all samples (Hui and Feng, 2013).

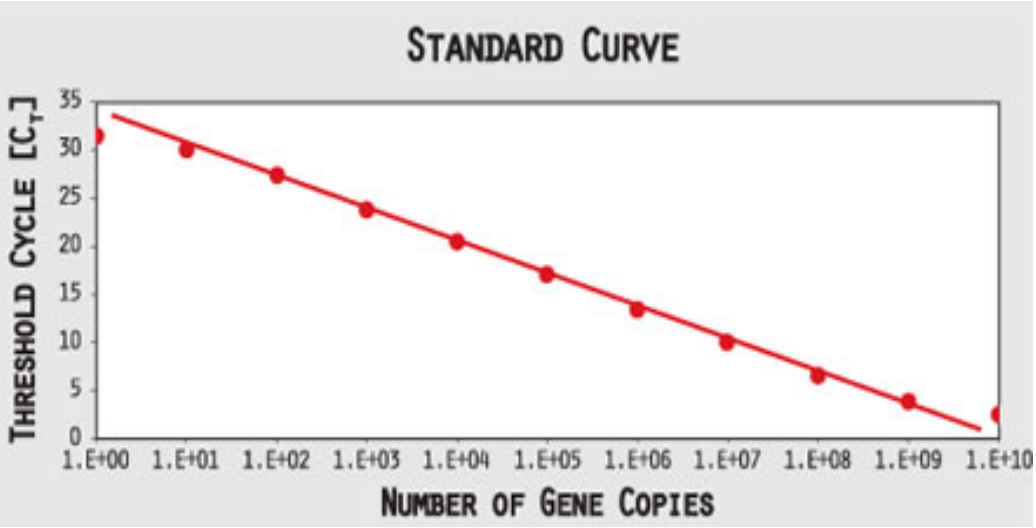


Figure 1-10. Standard curve for testing primer efficiency
http://www.sabiosciences.com/pathwaymagazine/pathways7/designing-validating-real-time-pcr-primers.php

Once the standard curve is generated, estimating the r-value (Pearson's correlation coefficient) or the R^2 value (coefficient of determination) will give an indication of the linearity of the data. If you have a low value (for either r of R^2), it indicates that you have variability across the dilutions you prepared, suggesting that the amount of starting material is affecting the amplification (Maddock and Jenkins, 2017).

Determining PCR amplification efficiency can be estimating utilizing the slop of the standard curve line. It is normally expressed as a percentage and indicates the rate at which your PCR product is generated. Ideally, the product should double with each round of amplification, reflected in an efficiency value of 100%. The closer to 100% efficiency you can get the more robust your PCR will be but generally any figure between 90% and 105% is considered acceptable (Maddock and Jenkins, 2017). Amplification efficiency outside of this range may indicate: badly designed primers, contamination, or pipetting dilutions inaccurately.

Additionally, it is important to check the specificity of the reaction by analyzing the PCR product. A melting curve analysis, performed at the end of the PCR cycles, can be used to confirm the specificity of primer annealing to the template (Taylor et al., 2010).

3.3.4.3 Considerations for Differential Expression Analysis

A few considerations must be made when determining which genes are differentially expressed: fold change, p-value, and false discovery rate. A statistical analysis (*t*-test) is conducted for each gene for the different conditions (i.e., sick vs. healthy), and therefore the problem of multiple testing arises in which p-values become more significant than they are in actuality. Therefore, a corrected p-value should be estimated accounting for multiple testing via multiple statistical methods (i.e., Benjamini-Hochberg). Additionally, a fold change greater that 2.0 and a

false discovery rate of 5% are parameters that will help to narrow down the list of candidate genes to be used in additional bioinformatic research (i.e., qPCR).

As explained in the calculations above, fold change is based upon expression of the specified gene as compared to the expression of the endogenous control genes (i.e., ACTB, B2M, GAPDH, HPRTI, HMBS, RPLP0, 18S rRNA). The expression results for each of the samples and each of the chosen genes can then be analyzed using pairwise comparisons of means. Pairwise comparisons can be conducted with statistical analyses such as, Tukey or the Bonferroni methods. Tukey is typically better than Bonferroni methods because it controls for maximum experiment-wise error rates and has better power. Additionally, Bonferroni adjustments tend to be too conservative. A problem that arises from analyzing multiple SNP genotype data is multiple comparisons.

A common goal in these studies is to identify genes that are differentially expressed among the biological conditions, which involves performing a hypothesis tests on each gene (Storey, 2011). False discovery rate (FDR) was coined by Benjamini and Hochberg (1995) as a means of controlling for the expected proportion of errors among rejected hypotheses (i.e., Type I errors or false positives). Real-time qPCR can be utilized to identify genes that are differentially expressed among the biological conditions. This involves performing a hypothesis tests on each gene. This could result in incurring false positives, but also failing to identify truly differentially expressed genes (Storey, 2011).

SECTION 4: SNP DISCOVERY & GENETIC IMPROVEMENT

4.1 WHAT ARE SNP?

Single nucleotide polymorphisms or SNP can be defined as single base pair positions in genomic DNA, where different sequence alternatives or alleles exist in individuals in a population. The frequency of the least abundant or recessive allele is 1% or greater (Brookes, 1999). About 90% of naturally occurring sequence variations are SNP (Collins et al., 1998). There are bi- tri- and tetra-allelic SNP, with the majority being bi-allelic.

There are many different applications of SNP technologies, including gene discovery, genetic mapping of QTL, GWAS, diagnostics and risk profiling, physiological genomics (i.e., gene function), use of SNP in EBV calculations, and marker-assisted selection (Schork et al., 2000; Kumar et al., 2012). Additionally, SNP can help decipher pedigree relationships, identify genomic divergence of species for the purposes of elucidating speciation and evolution, and associate genomic variations to phenotypic traits (McNally et al., 2009).

The goal of these different applications is to understand the unique aspects of SNP and the advantages and disadvantages of SNP to population-based analyses. Some of the advantages of SNP over other types polymorphisms for understanding the genetics of complex traits and diseases include: abundance, position, origins and haplotypic patterns, ease of genotyping, allele frequency drift, less mutable, and recombination oddities (Schork et al., 2000). Single nucleotide polymorphisms: occur in high frequency, are found throughout the genome (i.e., in exons, introns, intergenic regions), can be in linkage disequilibrium (LD) with other alleles creating haplotypic diversity, have a simple structure to genotype, and are more stable than other polymorphisms.

The functional consequences of SNP include synonymous and non-synonymous SNP. Synonymous SNP are those that are ‘silent’, as the allele polymorphisms do not change the

associated codon sequence and encoded amino acids. Non-synonymous SNP do change the encoded amino acid and can be further classified into missense and nonsense SNP (Hunt et al., 2009). Missense mutations result in an amino acid change, whereas nonsense mutations enact a stop codon resulting in a truncated amino acid change. Additionally, SNP can reside both within and outside of coding regions. Those residing outside of coding sequences can occur within intergenic regions, 5'- or 3'-untranslated regions, intronic regions, and other non-coding regions such as promoter and transcription factor binding sites. Although synonymous SNP result in the no change of amino acid, the previous belief of these 'silent' or trivial SNP is flawed due to factors such as mRNA splicing, mRNA stability, mRNA structure, protein translation and co-translation protein folding (Hunt et al., 2009).

It is important to understand, not only the functional consequence of SNP, but also what can phenotypically result from these polymorphisms. In understanding the functional consequence of the SNP, one can make inferences on the regulation or control of that gene. SNP may be responsible for the phenotypic diversity among individuals, genome evolution, common familial traits, complex and common diseases (i.e., hypertension; Hunt et al., 2009). Therefore, the identification of gene variation and their effects may lend greater understanding of their impact on gene function and health of an individual. Ultimately, know of these SNP provide opportunities to develop new SNP markers tests for traits such as disease susceptibility.

4.2 SNP DISCOVERY & VALIDATION PROCESS

The aim of SNP discovery (a.k.a. SNP calling or variant calling) is to determine the positions where polymorphisms exist, or where at least one base differs from the reference sequence (Nielsen et al., 2011). There are many software options available to perform SNP calling: CLC Genomics Workbench, GenomeStudio Software, Samtools, SNVer, and SOAPsnp, amongst

others (Nielsen et al., 2011; Kumar et al., 2012; Quail et al., 2012). Both RNA-seq and SNP discovery are dependent upon the assembled and mapped reads to a reference genome (Kumar et al., 2012). The new bovine reference genome is an improvement over the previous versions (UMD3.1.1 and Btau5.0.1), which contain large gaps between scaffolds (> 2,800) and 75,618 contigs and 42,267 contigs, respectively. The new assembly (ARS-UCDv1.2) has only 460 gaps, with just over 2,800 contigs (NCBI). Therefore, the new assembly is a better representation of the bovine genome. The results from previously conducted analyses utilizing these insufficient reference assemblies should warrant reanalysis, to more accurately identify genomic regions of interest in relation to PH susceptibility.

As mentioned above, determination of the functional consequences of SNP allows one to make inferences on the regulation or control of a gene. The discovery process includes determining functional consequences of SNP as a means of understanding of their impact on gene function.

Validation of discovered SNP is necessary to distinguish true SNP and eliminate false positive SNP regulating the trait of interest (Kumar et al., 2012). The process of validation can also serve as means to adjust and optimize the SNP filtering criteria. This optimization improves variant calling accuracy by identifying repetitive fractions leading to misalignment and erroneous homoeologous read mapping (Kumar et al., 2012). A biparental segregating population or a diverse panel of genotypes can be utilized to accomplish SNP validation (Kumar et al., 2012). In addition, the most important factor determining the variation in validation is sequencing accuracies. Accuracies vary by the NGS platform, at 71%, 85.4%, and 88.2% for Roche 454, Illumina, SOLiD platforms, respectively (You et al., 2011).

4.3 SELECTION AND GENETIC IMPROVEMENT

The ultimate goal of any operation in the beef industry is to make genetic improvement in traits of interest. Historically, breeding strategies focused on breeding for appearance; however, these strategies have since shifted to breeding based upon performance (Harris and Newman, 1994). The traits that are most desirous to improvement are dependent upon the breeding objectives and goals of the operation. Genetic improvement is important as it allows for the increase in performance for specific traits in a herd or within an operation. Genetic improvement is dependent upon many factors, but in addition to these factors, the amount or level of genetic control on the trait(s) influences genetic improvement. How heritable a trait is will affect the amount of genetic improvement that can be made. The higher the heritability, the more genetics influences overall phenotype.

4.3.1 Influential Factors

There are 4 factors that influence genetic progress in a particular trait. These four factors include: accuracy of selection, selection intensity, genetic variation, and generation interval. For simplicity purposes, the trait weaning weight (WW) can be utilized to define these terms in greater detail. Accuracy of selection defines how accurate we are at deciding which animals in our herd are 'best' for making genetic progress in our trait of interest WW. The more accurate we are in our selection of these animals, the more genetic progress will be made (i.e., increased accuracy = increased genetic progress). Accuracy is a measure of the correlation between the true values for WW and our estimates (index) values for WW. Heritability can range from 0 to 1 and the closer our estimates are the true values, the higher the accuracy.

A second factor in determining genetic progress for WW is selection intensity of animals for the trait of interest. This encompasses our selection for both males and females. Females are

chosen as replacements to maintain the size of the herd when culling is implemented. The proportion of animals saved will equate to a given selection intensity value. Typically, if you are utilizing superior or prominent sires for WW for your breeding program, culling more cows and keeping more replacements would be ideal for increasing genetic progress.

Genetic variability defines the amount of variation in genetics of our herd for WW. As we have a more diverse population of animals, the rate of genetic progress will increase. Heritability tells us how much of the variation we observe in WW performance can be attributed to variation in breeding values (genetics) of that population. As described by Bulmer (1971), change in genetic variance is due to the correlation between the pairs of loci, of which is induced by selection. With increasing loci, the magnitude of change is decreased, and with increased selection, a limit is reached in which genetic variance ceases to decrease (Gomez-Raya and Burnside, 1990).

Lastly, the fourth factor that contributes to genetic progress is generation interval. The factor defines the average age of males and females in the herd used for production. Typically, the most prominent sires (AI or herd sires) are used in the breeding program. Therefore, the typical age ranges for males in between 2 and 5 years old. Dependent upon many factors (i.e., cow longevity, heifer pregnancy rates, cow productivity), the age of females in the herd typically ranges from 2 to 12 years old. As I alluded to above for selection intensity, introduction of the newest genetics by increasing culling of older cows and keeping more replacements will increase selection intensity. In this case, the generation interval will go down, as a larger number of 2 year olds will enter production. Ideally for increasing rate of genetic progress, generation interval should go down, as we want to exploit the newest genetics for WW in our herd (i.e., decreased generation interval = increased genetic progress).

There are trade-offs amongst each of these factors. One trade-off involves selection intensity and generation interval. We desire to be more selective in choosing the best animals (best genetics) for WW, while also decreasing our average age of males and females in the herd. However, if the females in our herd produce superior calves for WW, it may be advantageous to be more selective with the particular sires and replacements used for your operation while retaining more old females. In this scenario, generation interval may increase while selection intensity also increases, meaning less genetic progress may be made. Another trade-off involves selection intensity and accuracy of selection. To increase rate of genetic progress, both intensity and accuracy should increase. Accuracy of selection should increase as your selection intensity increases for a trait of interest. However, if too much focus is on a that trait, the accuracy of selection could be hindered in other traits. A third trade-off involves accuracy of selection and generation interval. We desire to be most accurate in our choice of best animals to utilize, while keeping our population young with the newest genetics. There is risk associated with utilizing younger or the newest genetics. If knowledge of performance of these animals for WW is limited and the resulting performance is poor, then we didn't accurately choose the best animals for that trait. Subsequently generation interval decreased as well as accuracy. The last trade-off is between selection intensity and risk. Risk is not one of the 4 factors mentioned above but is important for us to understand what can result from our decision to be more or less selective with the animals in our herd. The less selective we are with which sires to use our intensity decreases and the risk in selection is lessened. The risk of selection in females is less than that of males because typically more females are retained as replacements in the herd.

4.3.2 Use of SNP for Genetic Improvement and Selection

The increased awareness and understanding by beef cattle producers of the field of genomics will perpetuate the desire and need for tools to improve selection. Genomics utilizes DNA based technology (i.e., northern blots, microarrays, SNP chips) to map the genome of an animal. The newest and currently most widely used of these are SNP chips, which can map 54,000 to 800,000 SNP in the bovine genome (Matukumalli et al., 2009). As the technology advances, the use of this technology will be more affordable and implementation of genomics in the beef industry will increase.

Another benefit of the application of genomic technologies for genetic improvement is increasing our understanding of the underlying genetic influence of performance, therefore understanding more of the genetic variability of the population. The genetic model can be explained through the equation: P = G + E; where P is our phenotype or performance of a trait, G is the genetics of an animal for that trait, and E is the external (non-genetic) environmental influence or residual effects imposed on that trait (Bourdon, 1999). Genomic technologies have the ability to help with lowly heritable traits, such as fertility and survivability. The use of genomics can aid in supplying causal information. Understanding of the underlying genetics of a population allows for an increase in selection intensity. This means that we can be more decisive and selective about the genetics that we introduce into the herd.

4.3.2.1 Marker Assisted and Genomic Selection

An application of SNP data to the genetic improvement of beef cattle is through the use of marker-assisted selection (MAS) techniques. Molecular breeding values (MBV), represent the sum of the effects of all significant SNP for a specific phenotype. The identification and incorporation of SNP data into traditional estimated breeding values (EBV) or expected progeny differences (EPD) on animals produces genomically-enhanced EBV (geEBV) or EPD (geEPD; Van

Eenennaam et al., 2014). Three approaches can be taken to incorporate the MBV information: 1) augment with a genomic relationship matrix as opposed to the pedigree-based relationship matrix, 2) fit the MBV as a correlated trait in the model, 3) a blending of EBV and MBV after estimation, or 4) a hybrid model of the above (Kachman, 2008; Legarra et al., 2009; Spangler, 2012).

The incorporation of genetic information into genetic evaluations provides opportunity to increase the accuracy of selecting the best performing animals for a trait of interest through increasing the accuracy of the current EBV/EPD. The generation interval of the herd can also be reduced through the use of SNP to estimate the genetic value of animals at a younger age, thereby increasing our rate of genetic improvement (Meuwissen and Goddard, 1996; Goddard and Hayes, 2009). Genome-wide association studies can be utilized to reveal the specific genomic regions that contribute to the genetic variation in our phenotypes of interest (Matukumalli et al., 2009).

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CHAPTER 3

STUDY ANIMALS AND USE

This chapter describes the animals and samples utilized in all of the following chapters. Data and samples were derived from the same cohort of animals. Refer to the individual chapters for methodology differences being experiments.

Animals & Selection Criteria

The 2012 calf-crop from Colorado State University Beef Improvement Center (CSU-BIC; Encampment, WY, elevation 2,170m) Angus herd were utilized in this experiment. Yearling (12 to 15 months of age) calf-fed steers were selected based upon specific criteria. Selected steers came from a diverse pedigree, as the CSU-BIC conducts a progeny testing program through the use of new sires into the herd each year via artificial insemination. A total of 19 sires were represented in the 2012 calf-crop. Steer calves were fed a bull-development ration (target gain of 1.5 kg/d) with the desire to understand the potential application of the results to yearling bull performance. Steers were also selected based upon their mean pulmonary arterial pressures (PAP). In yearling cattle, pulmonary hypertension (PH), represented by PAP measures, was previously categorized as low (< 41 mmHg), moderate (41 to 49 mmHg), or high (> 49 mmHg; Holt and Callan, 2007). Figure 3-1 provides the entire herd distribution of PAP (n = 6,606; years 1993 to 2016), as well as the distribution of PAP for the 2012 calf-crop (n = 370). Selected steers of the 2012 calf crop came from the tail ends of the distribution and were categorized as either normotensive or hypertensive and 13 different sires were presented in the selected animals.

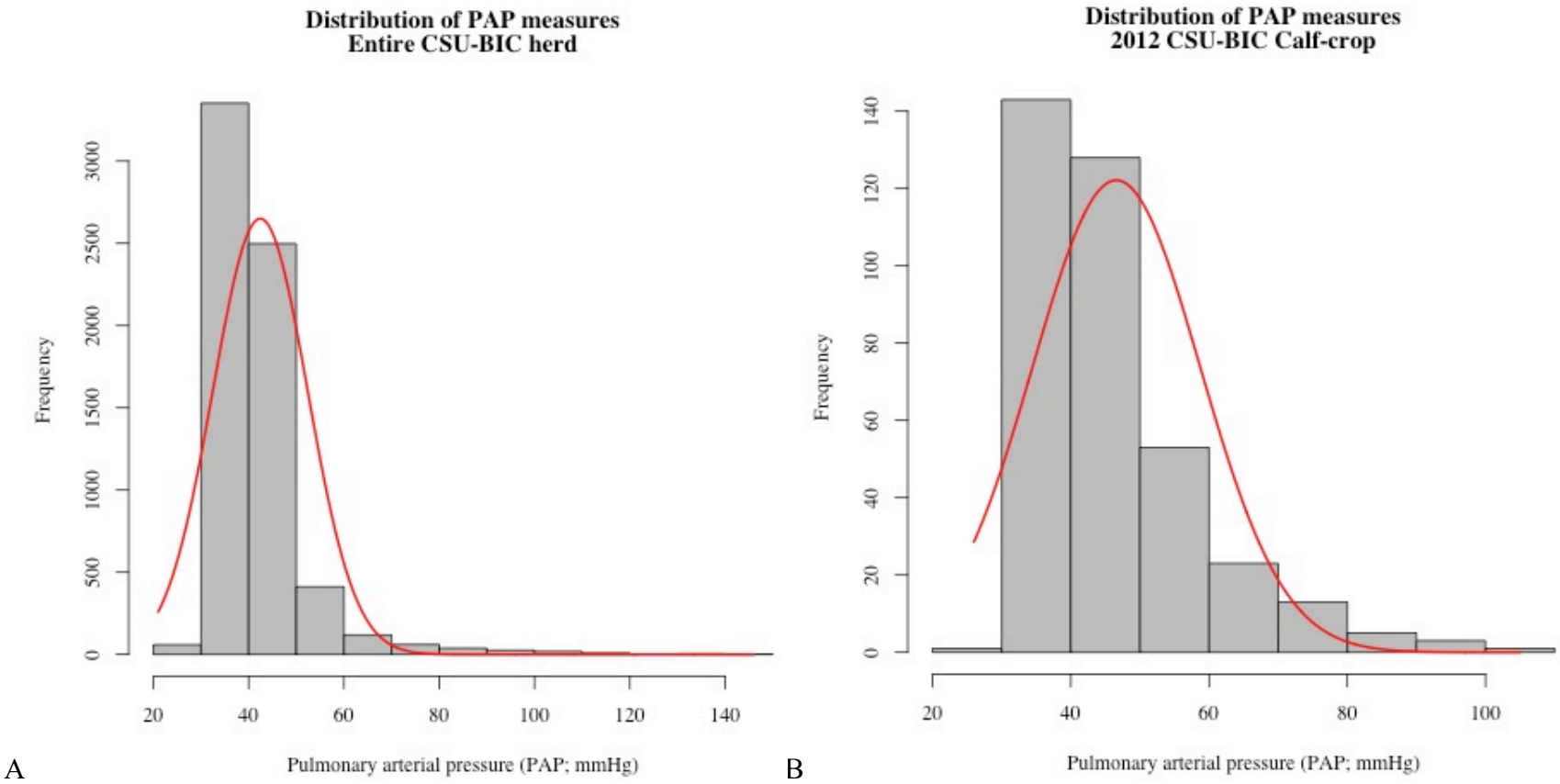


Figure 3-1. Distribution of mean pulmonary arterial pressures (PAP; mmHg) for the Colorado State University Beef Improvement Center (CSU-BIC) Angus herd. A) Entire herd distribution (n = 6,606; years 1993 to 2016); B) 2012 calf-crop distribution (n = 370). Steers came from diverse sires represented in the 2012 calf-crop distribution tails.

Pulmonary arterial pressures were measured over 3 time points. Figure 3-2 provides plots of normotensive (n = 10) and hypertensive (n = 10) selected steers. Three of the 10 selected hypertensive steers were confirmed or symptomatic for heart failure (IDs: 2108, 2162, 2355). Antemortem and postmortem symptoms of potential heart failure in cattle included: lethargy, jugular vein distension, tachypnea, brisket edema or ascites, exophthalmia, and an enlarged, dilated right heart. It is also important to note that the PAP of 3 steers categorized as normotensive (IDs: 2156, 2299, 2407), transitioned to hypertensive at the second and third measure dates. These steers are shown in blue in Figure 3-2. Additional information on sample and data utilization by chapter is provided below.

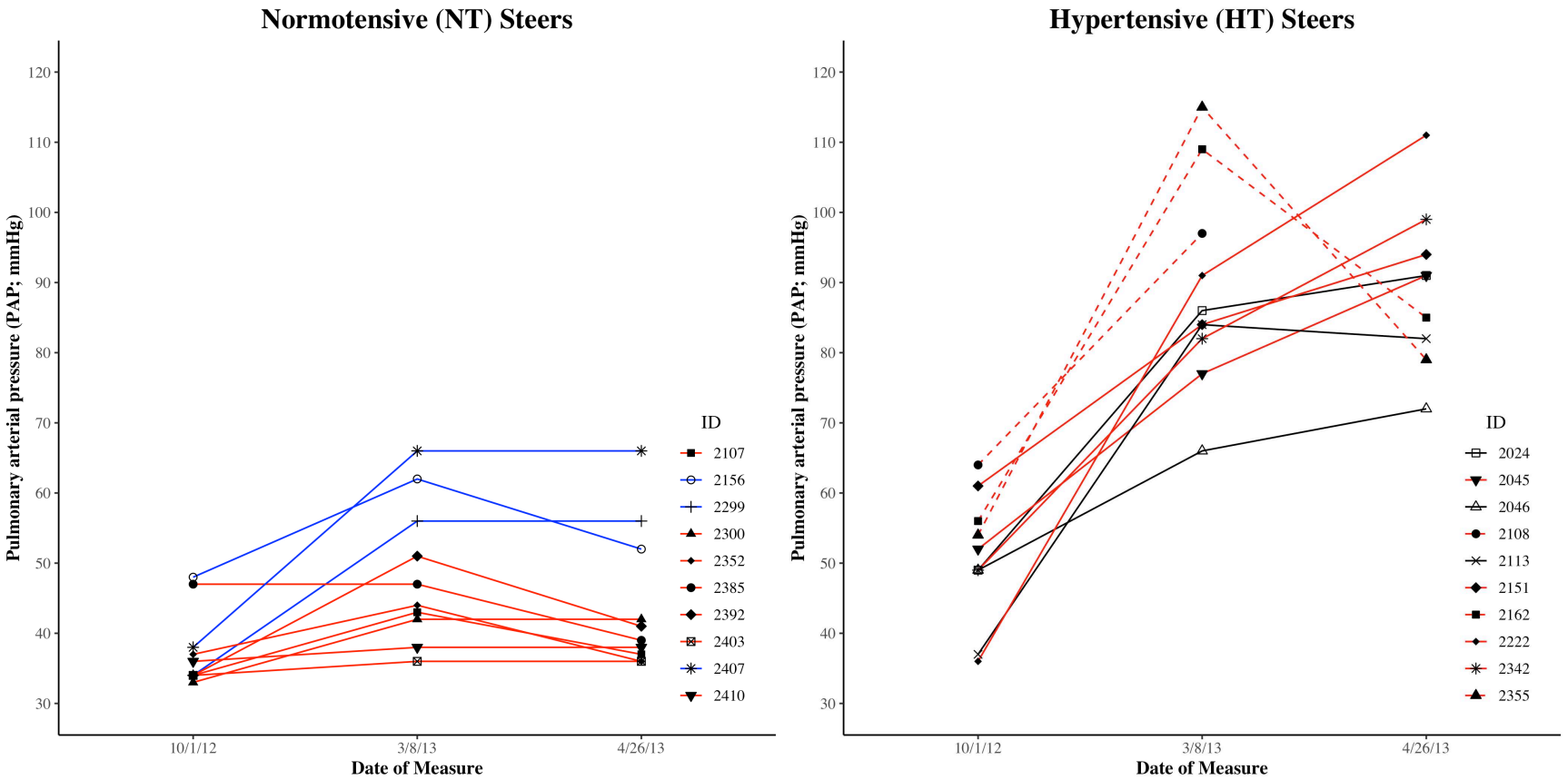


Figure 3-2. Graphical representation of normotensive and hypertensive mean pulmonary arterial pressures (PAP) for across 3 time points on steers from the Colorado State University Beef Improvement Center Angus herd. Normotensive (n = 7; excluding 3 steers in blue), average 39.9 ± 5.6 mmHg; Hypertensive (n = 10), average 76.0 ± 21.9 mmHg. Red lines represent samples with transcriptome data available (IDs: 2045, 2107, 2108, 2151, 2162, 2222, 2300, 2342, 2352, 2355, 2385, 2392, 2403, 2410). Blue lines represent steers excluded due to non-normotensive status at second and third measured PAP (IDs: 2156, 2299, 2407). Dashed lines represent steers confirmed or symptomatic for heart failure (IDs: 2108, 2162, 2355).

Tissues samples

Following finishing, the fed steers were harvested at the University of Wyoming Meat Laboratory. Upon harvest, performance data on the steers was collected (Table 3-1).

Table 3-1. Performance data of the calf-fed steers (n = 20) utilized for study.

Trait[1]	Mean	Standard deviation	Minimum	Maximum
Pulmonary arterial pressure (mmHg)[2]	68.3	28.2	36.0	115.0
Live weight (kg)	399.9	25.4	332.9	437.7
Hot carcass weight (kg)	234.8	24.0	172.8	269.4
Rib eye area (cm^2)	62.6	9.0	35.5	72.9
Yield grade[3]	2.4	0.5	1.6	3.7
Quality grade[3]	High select	-	Low select	Low choice

[1]Performance data available for all but one steer (ID #2108) due to early harvest, therefore no carcass data was available for this animal.

[2]Values included both hypertensive and normotensive steers across all three measured PAP.

[3]Categorically assigned based upon specific parameters; No units

Thirty-two tissues and blood were collected as previously designed by Canovas et al. in 2016 (Table 3-2). Samples were snap frozen in liquid nitrogen and stored over nitrogen vapor (-140°C to -180°C) at the U.S.D.A. National Animal Germplasm Preservation unit until further processing.

Chapters 4 & 6

A subset of tissues (n = 6) was utilized for sequencing, as these were most appropriate relative to the objectives of the larger study. These tissues included: aorta, left ventricle (middle), *longissimus dorsi*, main pulmonary artery, right lung (main lobe), and right ventricle (middle). Of the 20 total steers, only 14 steers were selected to be sequenced. These steers included: #2045, #2107, #2108, #2151, #2162, #2222, #2300, #2342, #2352, #2355, #2385, #2392, #2403, and #2410. Seven of the 14 were normotensive and 7 were hypertensive (Figure 3-2). Chapters 4 and 6 of this dissertation utilized the transcriptome data generated with these 6 tissues and 14 steers.

Table 3-2. List of tissues (n = 32) and blood collected in the 2012 High Altitude Steer Project.

Organ	Tissue	Location
Blood		
	Whole Blood	Jugular
	Serum	Jugular
Brain		
	Hypothalamus	
	Medulla	Caudal
	Medulla	Rostral (4th ventricle)
	Pituitary	
	Pons	
Diaphragm		
	Diaphragm	
Heart		
	Aorta	
	Apex	
	Left ventricle	Bottom
	Left ventricle	Middle
	Left ventricle	Top
	Moderator band	
	Right papillary muscle	
	Right ventricle	Bottom
	Right ventricle	Middle
	Right ventricle	Top
Kidney		
	Fat	Left
	Kidney	Left (middle)
Liver		
	Caudate	
Lung		
	Left Lung	Bottom lobe
	Right lung	Lower lobe (bottom)
	Right lung	Lower lobe (top)
	Right lung	Middle lobe
	Right lung	Upper lobe
	Left pulmonary artery	
	Main pulmonary artery	
	Right pulmonary artery	
Muscle		
	Brisket	
	Longissimus dorsi	
Spleen		
	Spleen	

Transcriptome data were provided for all 14 steers for right ventricle and lung tissues, whereas less sequence data was provided on the remaining 4 tissues (Table 3-3). Sequence information was limited on the other tissues because of poor nucleic acid isolation of the samples resulting in insufficient sequence quality. Sequence quality of transcriptome analyses was identified as low in two aorta tissue samples (APPENDIX A). One of the two samples was re-sequenced, and the transcriptome data was included in further analyses. A limitation with determining differentially expressed genes with RNA-seq is the biasedness associated with length of the transcript. The ability to detect differentially expressed genes is dependent upon the length of the transcript and such be adjusted or accounted for through methodology (Oshlack and Wakefield., 2009).

Table 3-3. Sequence information available for transcriptomic analyses by tissue and separated by pulmonary hypertension status.

Tissue	n (Total)	n (HT)[1]	n (NT)[1]
Aorta[2]	9	5	4
Left ventricle	13	7	6
Longissimus dorsi	10	5	5
Lung	14	7	7
Pulmonary artery	8	5	3
Right ventricle	14	7	7
Total	68	36	32

[1]HT = Hypertensive, 76.0 ± 21.9 mmHg; NT = Normotensive, 39.9 ± 5.6 mmHg)
[2]Failed quality control for two samples (one for each PH group)

Figure 3-3 displays the distribution of PAP measures for the 14 steers with transcriptome data. The distributed values are of the second measured PAP on March 8, 2013. This measure date was chosen as steers normotensive and hypertensive steers were clearly differentiated at this timepoint (Figure 3-2). As expected, the measures are non-normally distributed. Remedial

procedures were taken in an attempt to correct for normality in further analyses. These procedures are described in more depth in Chapter 6.

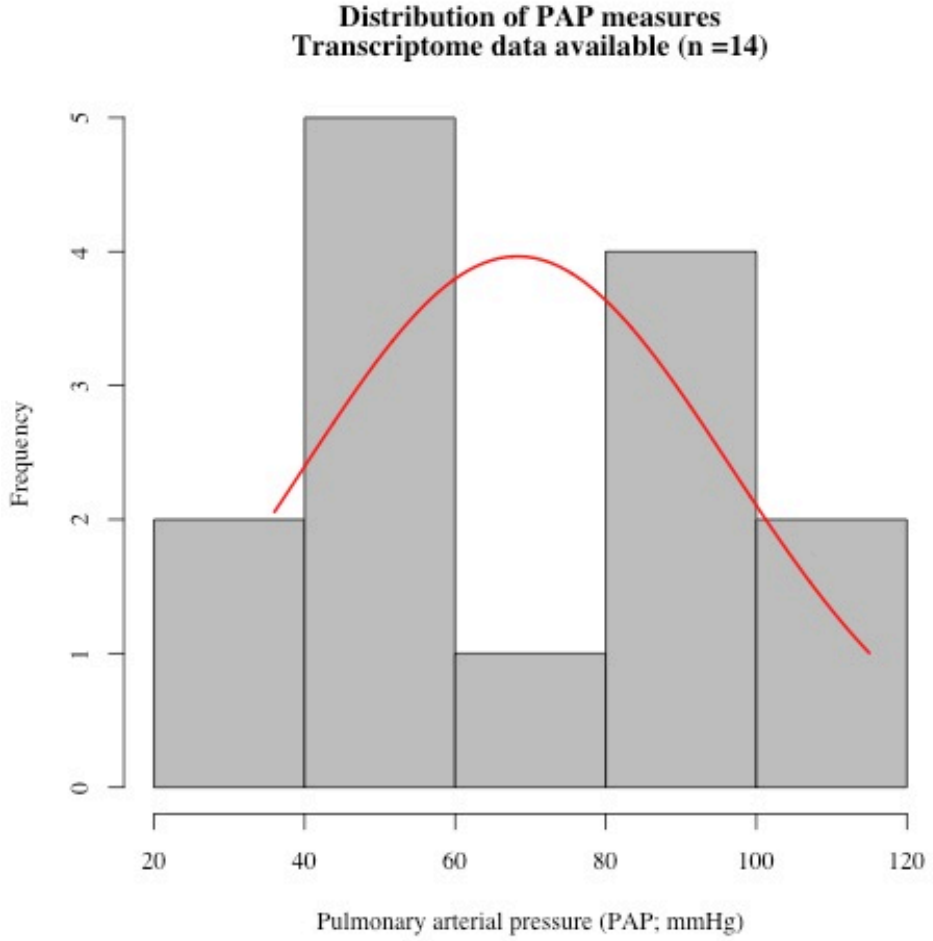


Figure 3-3. Transcriptome data from calf-fed yearling Angus steers (n = 14). Distribution of pulmonary arterial pressure (PAP) measures from the second timepoint measure on 3/8/13 (Figure 3-2).

Chapter 5

From the 32 available tissues (Table 3-2), a subset of muscle tissues were utilized, as these were most appropriate relative to the hypothesis of the study. Cardiac muscle tissues included: apex, left ventricle, moderator band, right papillary muscle, and right ventricle. Three sections of the ventricles (top, middle, bottom) were collected and analyzed separately to identify if differences existed in gene expression in the different locations of the muscles. In addition, muscle tissues *Longissimus dorsi* and brisket were utilized as control tissues. The selected muscle tissues from all of the harvested steers (n = 20) were utilized in the qPCR gene expression analyses in Chapter 5. The qPCR analyses in Chapter 5 were performed both with and without the three steers that transitioned form normotensive to hypertensive (Figure 3-2). Any changes in the results upon exclusion of theses steers was discussed within the chapter. Figure 3-4 displays the distribution of second measured PAP on March 8, 2013 for all of the selected steers (n = 20). This measure date was chosen as steers normotensive and hypertensive steers were clearly differentiated at this timepoint (Figure 3-2).

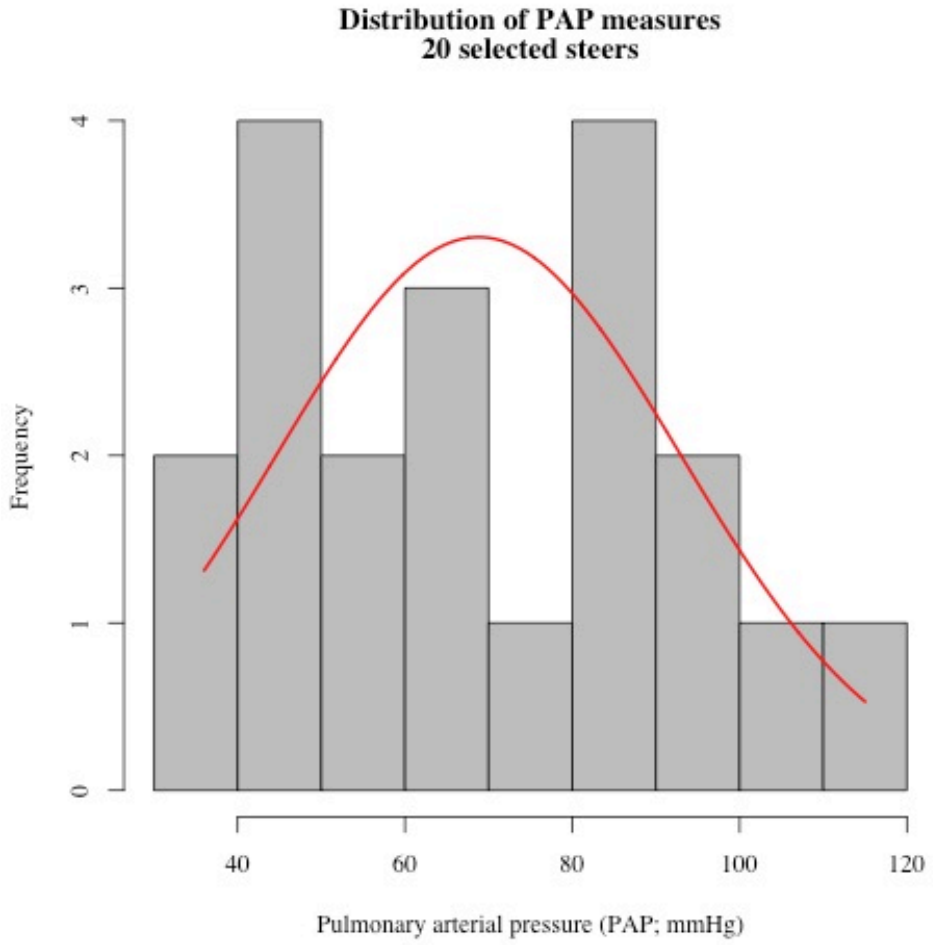


Figure 3-4. Distribution of pulmonary arterial pressure (PAP) measures of all selected steers (n = 20) from the second timepoint measure on 3/8/13 (Figure 3-2).

CHAPTER 4

IDENTIFICATION OF CANDIDATE GENES FROM RNA-SEQ AND INGENUITY PATHWAY ANALYSIS

SUMMARY

Pulmonary hypertension (PH) in cattle raised at an altitude above 1,800 m is defined by increased pulmonary arterial pressures (PAP), develops because of hypoxia-induced remodeling of the vasculature of the heart and lung leading to hypertrophy of the right ventricle, and eventually heart failure. Calcium is a mediator of the physiology of the heart, including myocardial function. We hypothesized that genes regulating the availability and utilization of calcium would be of significant importance and can be utilized to differentiate beef cattle with, and without, PH. The objectives of this research were: 1) to estimate and identify differentially expressed genes from RNA-Seq and pathway analyses, and 2) select putative candidate genes to analyze with qPCR (gene expression level). Transcriptome data provides opportunity to understand differences in gene expression in disease related traits such as pulmonary hypertension. Differential expression (DE) and pathway analyses were utilized to perform a hypothesis-based selection of candidate genes (n = 10) for subsequent expression validation. Transcriptome data of 6 different tissue samples (aorta, left ventricle, *longissimus dorsi*, lung, pulmonary artery, and right ventricle) from yearling Angus steers (n = 14) were utilized in the experiment. Samples were separated into groups, hypertensive (n = 7) and normotensive (n = 7) based upon their mean pulmonary arterial pressures (PAP). Differential expression was estimated with DESeq2 in R statistical software, and resulting DE genes were inputted and filtered into an Ingenuity Pathway Analysis to reveal influential genes within the context of biological systems. The largest number of DE genes was revealed in aorta (n

= 631) and right ventricle (n = 2,183) samples. Top canonical pathways (P < 0.0001) related to calcium signaling or utilization included: synaptic long-term depression, signaling by Rho family GTPases, and oxidative phosphorylation. The number of DE genes was reduced by relevance to the study hypothesis and previous literature, and in close proximity (upstream and downstream regulation) to calcium responsive elements to narrow the focus on influential genes on PH. Genes regulating calcium availability and utilization were expressed differently in Angus cattle with and without pulmonary hypertension. Candidate genes included: *ASIC2*, *EDN1*, *FBN1*, *KCNMA1*, *P2RY6*, *NOX4*, *PLA2G4A*, *RCAN1*, *TGS4*, and *THBS4*.

INTRODUCTION

Bovine pulmonary hypertension (PH) is defined by abnormal pulmonary arterial pressures (PAP; > 41 mmHg), and develops through remodeling of the vasculature of the heart and lung and an inability of the animal to overcome the necessary force to eject the blood through the pulmonary artery, leading to hypertrophy of the right ventricle, and eventually heart failure (Neary et al., 2015; Pugliese et al., 2015; Ryan et al., 2015). Calcium is a mediator of the physiology and mechanisms of the cardiac muscle, including myocardial function, through the utilization of Ca^{2+} ions intra- and extracellularly (Hasenfuss and Pieske, 2002; Stanfield, 2011). Rhodes (2005) suggested a role of Ca^{2+} sensitization in myocytes in hypoxic PH to distinguish hypertensive cattle from normotensive. Evidence revealed that altered Ca^{2+} homeostasis was of importance for the pathophysiology of myocardial dysfunction and heart failure (Hasenfuss and Pieske, 2002). We hypothesized that genes regulating the availability and utilization of calcium would be of significant importance and can be utilized to differentiate beef cattle with pulmonary hypertension. The objectives of this research were: 1) to estimate and identify differentially expressed genes from RNA-Seq and pathway analyses, and 2) select putative candidate genes to analyze with qPCR

(gene expression level). Results of this study will provide a focused approach to validating expression of calcium-associated genes and pulmonary hypertension status.

MATERIALS AND METHODS

Animal Care and Use Committee approval was not obtained because data were acquired from an existing sample database (Protocol # 13-4111). Figure 4-1 is a flow chart of the methodology for this chapter from sample retrieval to candidate gene selection.

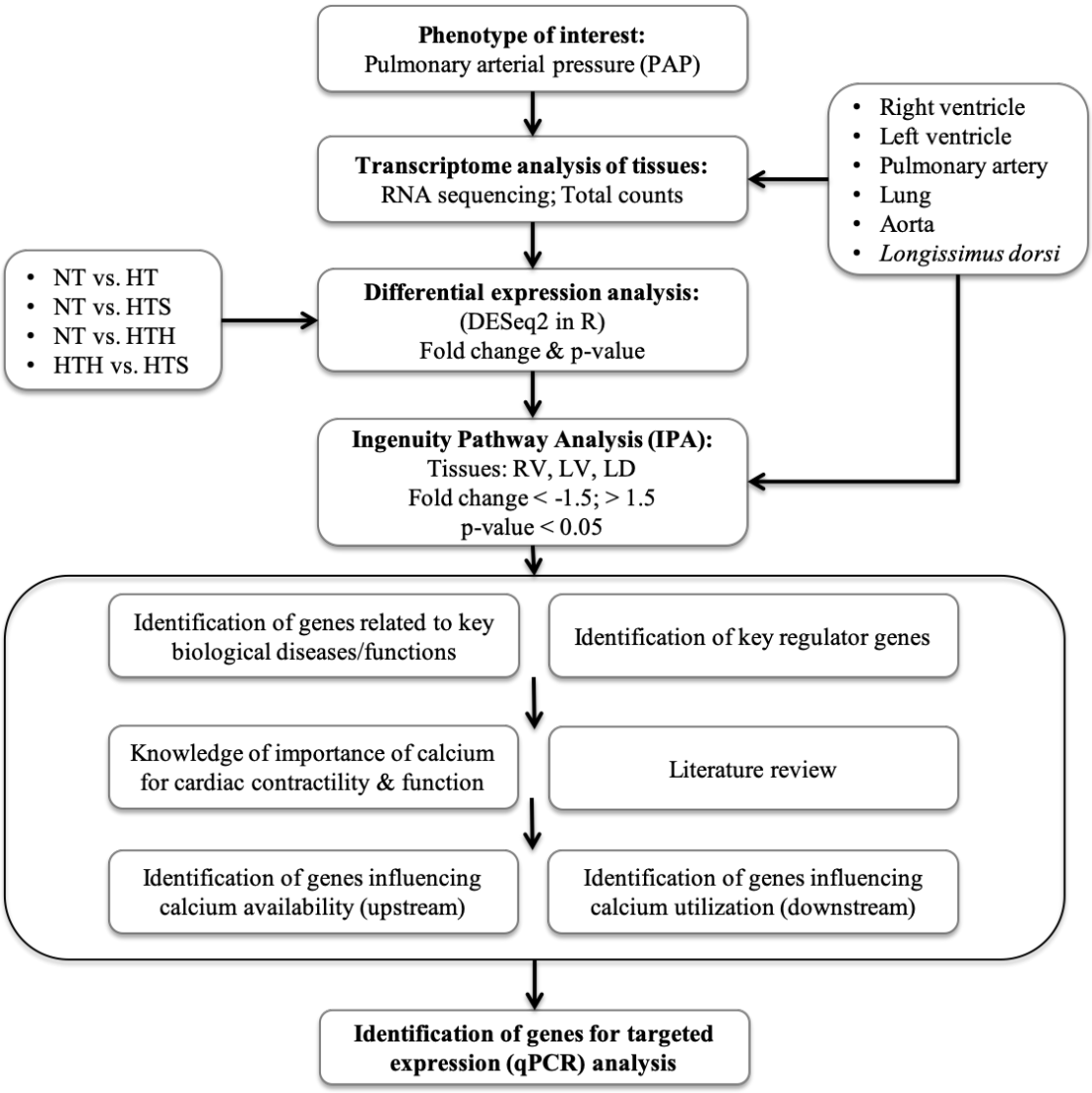


Figure 4-1. Flowchart of analytical steps from sample retrieval to candidate gene selection. HT = Hypertensive, 76.0 ± 21.9 mmHg; NT = Normotensive, 39.9 ± 5.6 mmHg); Healthy (HTH) and symptomatic (HTS) are physiological status.

Tissues/Samples

Chapter 3 of this dissertation outlines the animal population and samples utilized in this study. In short, 20 Angus steers from the Colorado State University Beef Improvement Center (CSU-BIC) were harvested in a controlled study. These steers were categorized chosen based upon their mean PAP measures (Figure 3-2).

RNA-sequencing

The subset of available tissues from 14 steers were utilized for RNA-sequencing (Table 3-2 & 3-3). These tissues included: aorta, middle left ventricle, *longissimus dorsi*, lung, pulmonary artery, and middle right ventricle. Three portions of the ventricles were retrieved, top, middle and bottom; however, the middle portions were chosen for transcriptome analyses. The Illumina HiSeq analyzer (Illumina, San Diego, CA) yielded 100 bp single read sequences. Sequence reads from approximately 19,500 genes were further analyzed using CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark) with the new bovine reference genome ARS-UCD1.2. Quality control procedures were conducted to determine the quality of the sequence reads provided.

Total count expression data was generated per sample. Count data was compared for steers based upon their PAP (normotensive/hypertensive) and physiological status (healthy/symptomatic). The three groupings were as follows: normal or normotensive (NT), hypertensive healthy (HTH), hypertensive symptomatic (HTS), or hypertensive (HT; both healthy and sick). Comparison groups included: 1) NT vs. HT, 2) NT vs. HTH, 3) NT vs. HTS, and 4) HTH vs. HTS. Groups provided identification of expression differences from one group to the other. A principle component analysis (PCA) was conducted with the total count data to determine if tissue-specific and/or animal-specific clustering existed.

Differential expression analysis

Differential expression (DE) analysis was conducted to determine which genes were differentially expressed when comparing a treatment versus a control. In our study, samples from normotensive steers were categorized as controls, while hypertensive steers were categorized as treatments. In the case of comparison between HTH and HTS steers, HTH steers were utilized as controls.

Some of the most appropriate DE analysis methods are edgeR, DESeq, and NBPSeq. These methods have been found to be most appropriate for small sample sizes (2 samples per condition; Soneson and Delorenzi, 2013). DESeq2 was implemented in R statistical software for the DE analysis. Data was filtered by the number of features present in each group, where at least 2 samples per group had to have count data. After filtering, an average of 18,509 ± 1,529 genes were utilized to determine differential expression. A significance value was estimated for every gene using a Wald t-test, where the p-value indicated the probability that the difference between treatment and control is observed, even though there was no true treatment effect.

A p-value, fold change, and false discovery rate are parameters that can be utilized to narrow down the list of putative candidate genes to be used in additional bioinformatic research. The Wald *t*-test was conducted for each gene (n = 9) for the different conditions (treatment vs. control). Therefore, p-values are inflated or become more significant than in actuality due to the parallel or multiple testing. A corrected p-value was estimated using a Benjamini-Hochberg method to control or decrease for false discovery rate (Type 1 errors).

Ingenuity Pathway Analysis

Differentially expressed genes within each tissue was entered to Ingenuity Pathway Analysis (IPA). Gene IDs were mapped to multiple common references, including human and mouse. Specification of the bovine reference genome was not available. Data filtering criteria included: $\log_2$ fold change > 0.589 and < -0.589, significance (p-value) < 0.05. Not all differentially expressed genes were mapped, as they could have corresponded to several loci or more than one gene. These identifiers were left unmapped due to the ambiguity of their identity (Qiagen). Core expression analyses were implemented to calculate z-scores (directionality of regulation; up- and down-regulated expression) of each of the mapped genes. Pathway analyses

were completed in collaboration with the Hansen laboratory (Colorado State University, Department of Biomedical Sciences).

Putative Candidate Gene Selection

The selection of putative candidate genes for additional gene expression studies encompassed a review of previous literature, results of the DE analysis and IPA, in addition to searches through the National Center for Biotechnology Information (NCBI) gene database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Ten genes were chosen for the additional gene expression studies.

RESULTS AND DISCUSSION

Principle component analysis of the transcriptome data revealed tissue-specific differentiation (Figure 4-3). Left ventricle, right ventricle, and *longissimus dorsi* muscle tissues tended to cluster together. Blood vessels (pulmonary artery and aorta) clustered together, and the lung was differentiated by itself. This separation of transcriptome data by tissue was expected as each has a distinct function within the body. Tissue-specific gene regulation is well understood and must be considered in -omics based analyses (GTEx Consortium, 2015; Barbeira et al., 2018). Further the principle component analyses revealed some differentiation of hypertensive and normotensive steer groups (APPENDIX B).

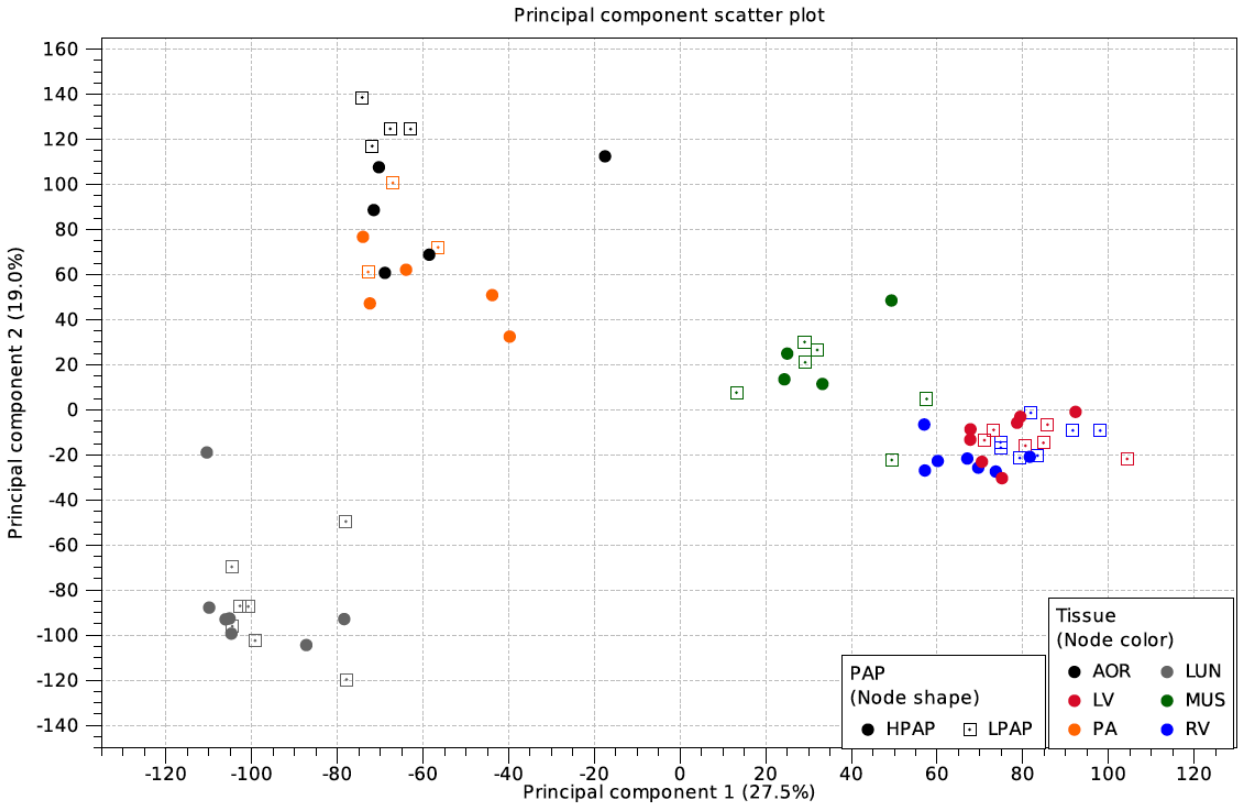


Figure 4-2. Principle component analysis (PCA) plot representing the gene level expression. Tissues are distinguished by color. Physiological state of the animal is differentiated by the plot point. HPAP = Hypertensive; LPAP = Normotensive

Differentially expressed genes

As described above, 4 comparison experiments were conducted within each tissue. Figure 4-4 is a histogram displaying the number of differentially expressed genes in each group and within each tissue. Aorta and right ventricle had the most differentially expressed genes, more specifically in the all experiments except HTH vs. HTS. These results suggested that the genetic regulation of PH is more evident between normotensive steers to hypertensive steers, as fewer gene differences existed in the HTH vs. HTS comparison.

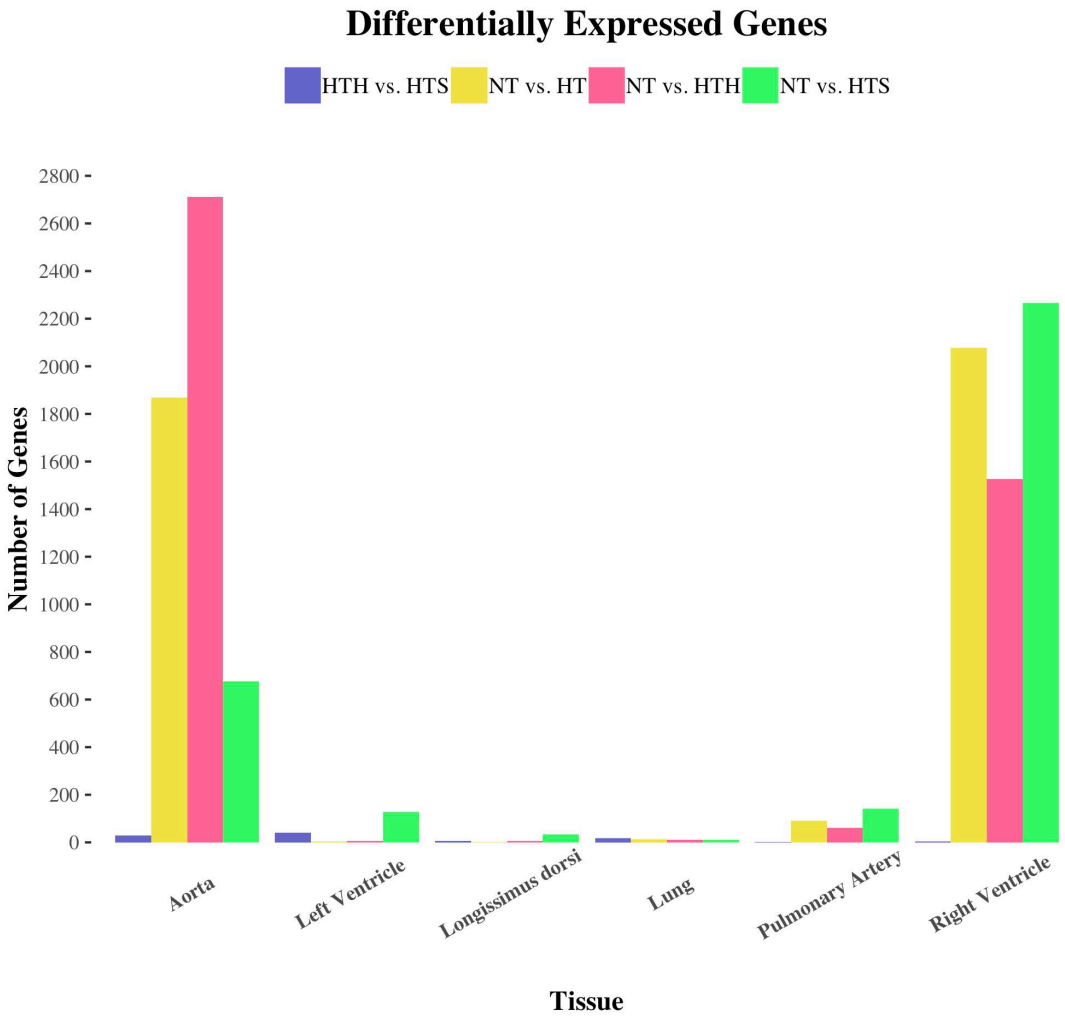
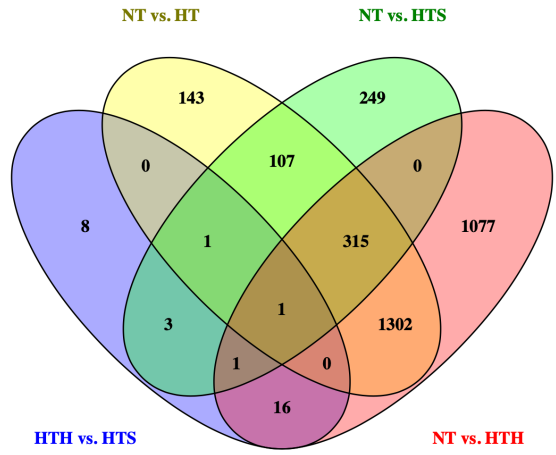


Figure 4-3. Bar plot of the number of differentially expressed genes in each of the 6 tissues and across comparison groups. HT = Hypertensive, 76.0 ± 21.9 mmHg; NT = Normotensive, 39.9 ± 5.6 mmHg; HTH = hypertensive healthy; HTS = hypertensive symptomatic, confirmed or symptomatic for heart failure. Normotensive steers used a control, hypertensive steers used as the treatment group. Between HTH v. HTS, HTH group utilized as the control group.

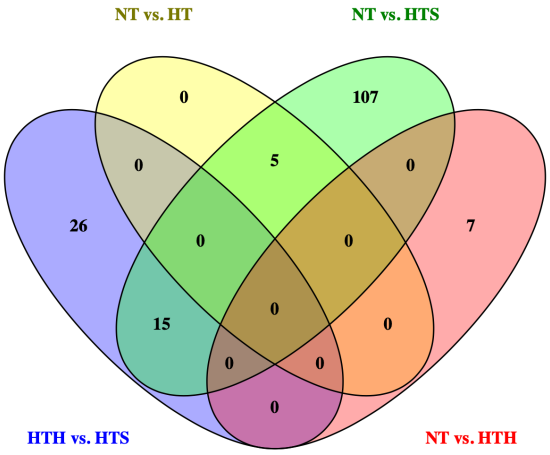
The major function of the aorta is the carry and distribute oxygen rich blood to all arteries, which then can be distributed to various regions of the body. Aortic diseases, including aortic aneurysm or aortic dissection, may limit the function of the aorta and result from issues including high blood pressure (London and Guerin, 1999; Forsdahl et al., 2009). The majority of identified DE genes were between NT and other groups, with the most between NT and HTH steers (Figure

4-3). A single uncharacterized gene overlapped between the 4 experiments: LOC783730 (a.k.a. Retrotransposon Gag-like protein 8). Although the function of this gene is uncharacterized, retrotransposons' function is to self-amplify in a genome, increasing their copy numbers and ultimately genome size. This can potentially induce mutations by inserting themselves near or within genes (Wang et al., 2006; Cordaux and Batzer, 2009). Further research is needed to elucidate the exact genetic functions of this gene and its potential role in PH and heart failure. These results suggest the potential role of key regulators in the aorta in association to PH and heart failure.

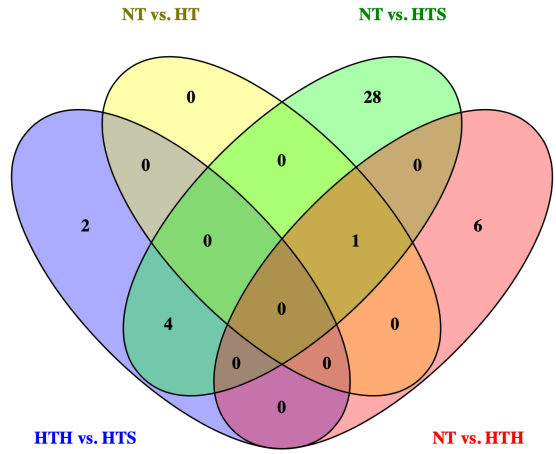
Typical high-altitude induced PH has been associated with right ventricle morphology, more specifically hypertrophy of the muscle (Pugliese et al., 2015; Ryan et al., 2015). Therefore, estimating a large number of differentially expression in this muscle was anticipated. Another interesting aspect of this research was to examine the number of DE genes overlapping in the right ventricle between the experiments. This is provided in the Venn diagram in Figure 4-5F. Between all analytical comparisons, no genes overlapped. Excluding the HTH v HTS analysis, 1,099 genes overlapped in the other three experiments. This suggested the majority of the gene expressions differences observed is due to PH, and less attributed to heart failure status. Most important to an objective of this study was to determine the differentially expressed genes in the experiment of NT versus HTS group. A total of 650 unique genes were differentially expressed between these groups of steers. These genes could provide the information needed to distinguish those animals that develop heart failure as a result of increased mPAP. A Venn diagram depicting these comparisons was created using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).



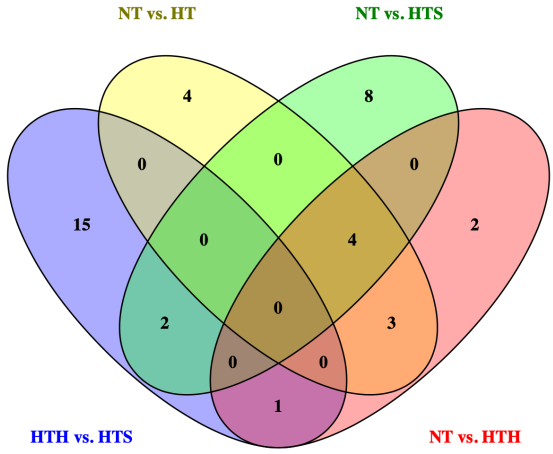
A) Aorta



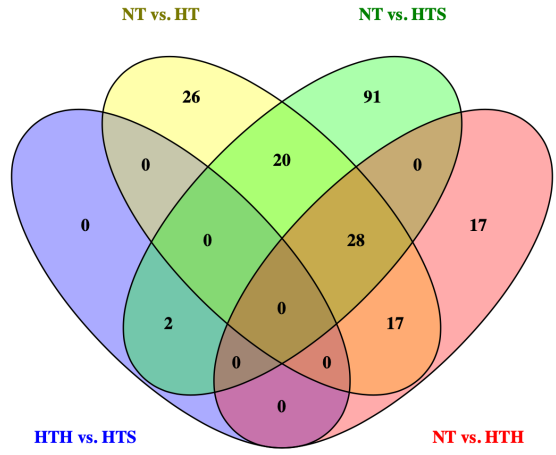
B) Left Ventricle



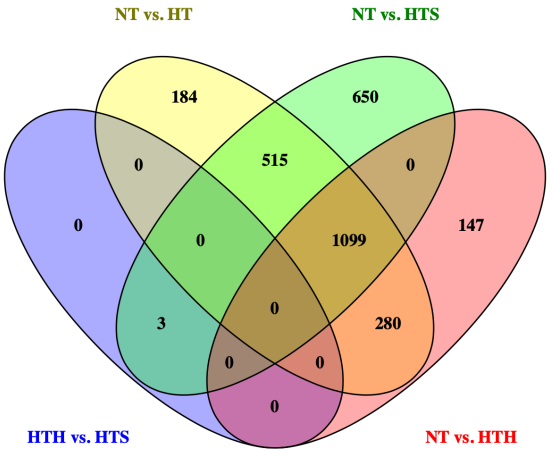
C) *Longissimus dorsi*



D) Lung



E) Pulmonary artery



F) Right Ventricle

Figure 4-4. Venn diagrams of the number of genes overlapping between the four different experimental groups in 6 tissues. HT = Hypertensive, 76.0 ± 21.9 mmHg; NT = Normotensive, 39.9 ± 5.6 mmHg; HTH = hypertensive healthy; HTS = hypertensive symptomatic, confirmed or symptomatic for heart failure. Normotensive steers used a control, hypertensive steers used as the treatment group. Between HTH v. HTS, HTH group utilized as the control group. Created using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Ingenuity pathway analyses

Ingenuity Pathway Analysis has the ability to reveal influential genes from RNA-Seq data within the context of biological systems. These tools allowed for a strategic search of genes with particular functions or disease pathways of interest. Table 4-3 provides an overview of the inputted expression data to IPA, in addition to the top canonical pathways for each tissue. In general, canonical pathways refer to the idealized or generalized pathways that represent common properties of a particular signaling molecule or pathway. In contrast, non-canonical pathways are those that deviate from or derive from alternative biogenesis pathways.

Important to note is the number of differentially expressed genes mapped in IPA is less than that identified with the differential expression analysis. This was due to the mapping. Ingenuity Pathway Analysis utilizes the most commonly used and annotated references (i.e., human and mouse) to map the genes. Therefore, genes with more than one identifier, or a gene ID not common to these references will not map, resulting in less utilized genes for the analysis. It is important to note that a minimum number of differentially expressed genes (n = 500 to 600) is needed for a proper pathway analysis to be conducted. Results suggested aorta and right ventricle pathways analyses have a sufficient number of genes (Table 4-2).

Table 4-1. Expression overview of differentially expressed (DE) genes inputted to Ingenuity Pathway Analysis for each tissue.

Tissue	Total DE genes[1]	Down regulated[2]	Up regulated[2]	Top canonical pathway
Aorta	631	287	344	Synaptic long-term depression
Left ventricle	65	27	38	Hepatic fibrosis/hepatic stellate cell activation
Lung	10	4	6	Iron homeostasis signaling pathway
Longissimus dorsi	25	7	18	Death receptor signaling
Pulmonary artery	120	67	53	Signaling by Rho family GTPases
Right ventricle	2,183	863	1,320	Oxidative phosphorylation

[1]Corresponding to only mapped gene IDs

[2]Regulation based upon expression differences from controls to treatments. Normotensive steers were used as controls and hypertensive steers used as treatments. Down regulated corresponds to genes downregulated in the hypertensive population.

Although calcium signaling is one of many canonical pathways, none of the top canonical pathways in any of the tissues were related to calcium signaling. Of the 6 tissues, calcium was recognized as a canonical pathway in aorta, *longissimus dorsi*, and right ventricle tissues. Calcium signaling is an important function of muscle contraction and relaxation, therefore it was not surprising to find this pathway in the *longissimus dorsi* and right ventricle muscles. This signaling pathway in the aorta may warrant further study to elucidate important aspects to PH status.

The top canonical pathway in the aorta was synaptic long-term depression. In brief, synaptic long-term depression is a process involving a decrease in the synaptic strength between parallel fiber and Purkinje cells induced by activation of parallel fibers and climbing fibers. Activation of these fibers opens voltage-gated calcium channels and causes a generalized influx of calcium (Hoxha et al., 2016). Therefore, this canonical pathway may be relative to our suggested hypothesis.

Hepatic fibrosis, the top canonical pathway for the left ventricle, is characterized by changes in the subendothelial space as fibrosis develops in response to liver injury. These changes include alterations in both cellular responses and extracellular matrix composition (Friedman, 2000). Left ventricular fibrosis in cattle is a current focus of study in obesity-associated PH in beef cattle (Krafsur et al., 2019). However, cattle in that study were late-fed steers, differentiated from the steers in our current study by both age and weight of finishing. This canonical pathway appears to be of influence on PH status and warrants continued exploration.

Iron homeostasis signaling pathway was the top canonical pathway identified for the lung tissue samples. Research by Cotroneo et al., (2015) revealed pulmonary vascular remodeling associated with raised pulmonary arterial pressure and right ventricular hypertrophy in iron deficient mice. This remodeling was reversed with iron treatment, suggesting the role of iron in pulmonary vascular homeostasis. In a review by Robinson et al., (2014), research on the role of iron in processes pertaining to pulmonary arterial hypertension are explored in depth.

The top canonical pathway of differentially expressed genes in *longissimus dorsi* muscle samples was death receptor signaling. In general, death receptor signaling (a.k.a. apoptosis, or programmed cell death) is modulated by a number of different factors and is characterized by approximately 8 different members (Lavrik et al., 2005). In the mammalian model, caspase activation not only controls cell death signaling, but additional vital processes such as cell differentiation, immunity, learning and memory (Fuchs and Steller, 2011). Preliminarily, it does not appear that the death receptor signaling pathway has significant correlation to either calcium regulation or pulmonary hypertension status.

Signaling by Rho family GTPases was the top canonical pathway identified in the pulmonary artery differentially expressed genes. Rho family GTPases regulate many important

processes including organization of the actin cytoskeleton, gene transcription, cell cycle progression, and membrane trafficking (Kjøller and Hall, 1999). The includes the movement of calcium across the cell membrane. The availability and signaling of calcium can have downstream regulation on Rho family GTPase activity (Saneyoshi and Hayashi, 2012)

The top canonical pathway identified for the right ventricle was oxidative phosphorylation. The process involving the formation of ATP as a result of the transfer of electrons, oxidative phosphorylation is interrupted during times of oxidative stress. In this, a cascade of events involves the influx of calcium into the cell, mitochondria, and nucleus, regulating phosphorylation/dephosphorylation of proteins and modulating signal transduction pathways (Ermak and Davies, 2002; Clapham, 2007). This canonical pathway appears to influence or be influenced by the candidate genes selected through the regulation of calcium availability and utilization.

Candidate gene selection

The selection of putative candidate genes for additional gene expression studies encompassed a review of previous literature, results of the DE analysis and IPA, in addition to searches through the National Center for Biotechnology Information (NCBI) gene database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Ten genes were chosen as candidates for qPCR (Table 4-4). Bioinformatic analyses can reveal a large number of potentially influential genes within the data. This creates difficulty in narrowing to a list of approximately 10 genes through the selection criteria methodology outlined in Figure 4-1. A literature review yielded multiple studies evaluating the influence the 10 candidate genes on both pulmonary hypertension as well as calcium signaling and regulation (Table 4-5). A more in-depth evaluation of these studies is provided in Chapter 5 of this dissertation.

Table 4-2. List of candidate genes for qPCR gene expression validation.

Gene	Name	Gene ID	Chr[1]	Location[2]	Average Log_2 FC[3]	Function
ASIC2	Acid sensing ion channel subunit 2	617930	19	16022575..17228963	-3.5395	Membrane ion channel; Activator of the calcineurin/NFAT signaling pathways
EDN1	Endothelin 1	281137	23	44156440..44163423	1.3272	Vasoconstrictor
FBN1	Fibrillin 1	281154	10	61654541..61919167	1.0283	Extracellular matrix glycoprotein
KCNMA1	Potassium calcium-activated channel subfamily M alpha 1	282573	28	32610164..33387551	1.5703	Large conductance ion channel
NOX4	NADPH oxidase 4	378474	29	6120515..6303004	2.2042	Catalytic subunit the NADPH oxidase complex; Acts as an oxygen sensor
P2RY6	Pyrimidinergic receptor P2Y6	539703	15	52754742..52790314	-1.1681	G-protein coupled receptor; Mediates inflammatory responses
PLA2G4A	Phospholipase A2 group IVA	525072	16	67907024..68081280	1.1096	Catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid
RCAN1	Regulator of calcineurin 1	539640	1	882073..1002231	1.7262	Calcium/calmodulin-dependent phosphatase
RGS4	Regulator of G protein signaling 4	617437	3	6290862..6297919	1.8992	Regulator/inhibitor of G-protein signaling
THBS4	Thrombospondin 4	541281	10	11006321..11060136	2.5556	Adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions

[1]*Bos taurus* Chromosome

[2]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[3]Average log_2 fold change estimated from differential expression analysis (hypertensive compared to normotensive) of total counts from RNA-sequencing.

Table 4-3. Candidate genes utilized in previous research associated with cardiopulmonary disease traits.

Gene	References
ASIC2	de Campos Grifoni et al., 2008; Lu et al., 2009; Abboud, 2015; Zhou et al., 2017
EDN1	Schiffrin, 2005; Murphy and Eisner, 2006; Castro et al., 2007; Deacon et al., 2010; Calabro et al., 2012; Bkaily et al., 2015
FBN1	Powell et al., 1997; Shen et al., 2011; Jeppesen et al., 2012; Chen et al., 2014
KCNMA1	Tomas et al., 2008; Barnes et al., 2016; D. Brown (Beef calf model; results unpublished)
NOX4	Mittal et al., 2007; Li et al., 2008; Chen et al., 2012; Zhao et al., 2015; He et al., 2017
P2RY6	Hou et al., 1999; Nishida et al., 2008; Tovell et al., 2008; Nishimura et al., 2016; Sunggip et al., 2017
PLA2G4A	Osanai et al., 1998; Handlogten et al., 2001; Magne et al., 2001; Ait-Mamar et al., 2005
RCAN1	Bush et al., 2004; van Rooij et al., 2004; Canalder et al., 2006; Grammer, et al. 2006
RGS4	Owen et al., 2001; Mittmann et al., 2002; Hyeseon et al., 2003; Gu et al., 2009; Opel et al., 2015
THBS4	Stenina et al., 2005; Gabrielsen et al., 2007; Mustonen et al., 2012

Figure 4-5 is a network of the candidate genes and their relation to calcium and each other both intracellularly and extracellularly. Gene regulation is depicted by the coloration of the molecules, where green is upregulation and red is downregulation. The intensity of the coloration defines the extent of regulation, where the darker the color, the more regulated the molecule in our data. The figure is a visual representation of both the upstream regulation of calcium availability, as well as the downstream utilization of calcium relative to our candidate genes. Important to note that is that not all of our candidate genes have a direct connection to calcium but may be channeled through additional pathways.

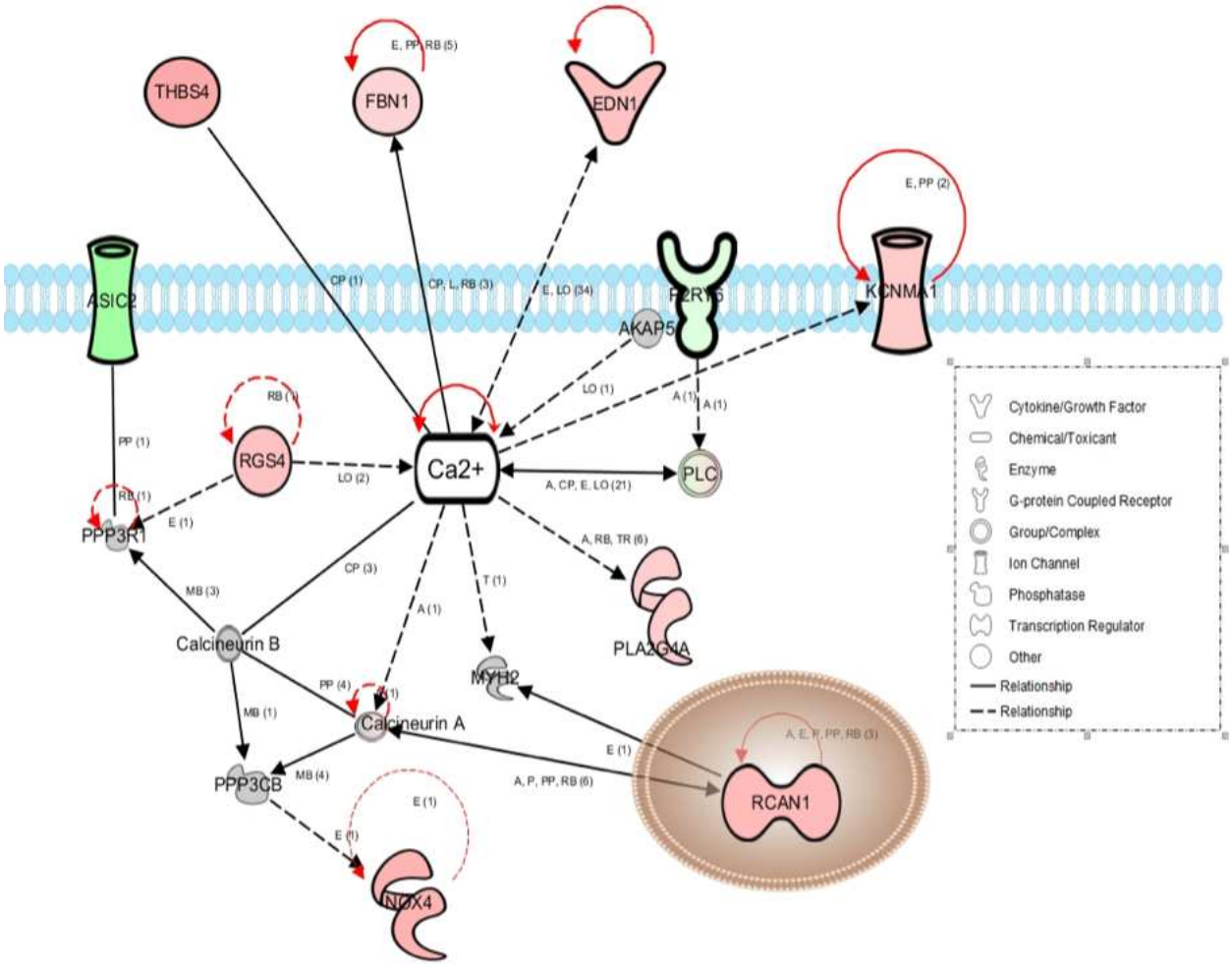


Figure 4-5. Network presenting the extracellular and intracellular relationship between calcium the candidate genes. Candidate genes include: *ASIC2*, *EDN1*, *FBN1*, *KCNMA1*, *P2RY6*, *NOX4*, *PLA2G4A*, *RCAN1*, *TGS4*, and *THBS4*. Coloration of candidate genes shows degree of regulation in hypertensive samples as compared to normotensive samples, where the darker the color the higher the regulation; Green = downregulated, Red = upregulated. Black arrows represent the relationship or regulation between genes, where solid lines are direct relationships and dotted lines are more distant relationships. Red arrows represent the regulation of the gene expression on itself.

The main objective in identifying putative candidate genes was to reduce the number of genes to validate with qPCR methodology. There are key differences between RNA-Seq and qPCR results in gene expression data. Typically, qPCR is utilized instead of RNA-Seq for a small set of genes with known sequence. These analyses have the widest dynamic range of determining expression differences, lowest quantification limits, and leased biased results, as compared to

RNA-Seq and microarray analyses (Nonis et al., 2014). Additionally, often qPCR is used to confirm the results of RNA-Seq, which is usually only conducted on a small number of animals. A limitation to the use of qPCR, as compared to RNA-Seq is the broad dynamic range of expression that RNA-Seq provides. RNA-Seq is an analysis of genome-wide expression, but we are limited to the candidate genes chosen for qPCR. Quantitative reverse transcription PCR has its advantages if the number of genes is low to reduce costs, but for a large number of genes analyzed, RNA-Seq is more cost-effective

CONCLUSIONS

Transcriptome data provides opportunity to understand differences in gene expression in disease related traits such as pulmonary hypertension. Differential expression and pathway analyses can be utilized to narrow the focus on influential genes on these traits. Genes regulating calcium availability and utilization were expressed differently in Angus steers fed at high altitude with and without pulmonary hypertension. Candidate genes included: *ASIC2*, *EDN1*, *FBN1*, *KCNMA1*, *P2RY6*, *NOX4*, *PLA2G4A*, *RCAN1*, *TGS4*, and *THBS4*. Validation of these candidate genes with additional gene expression methods is necessary.

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CHAPTER 5

QUANTITATIVE REVERSE-TRANSCRIPTION PCR VALIDATION: EXPRESSION DIFFERENCES OF CALCIUM RELATED GENES IN ASSOCIATION WITH PULMONARY HYPERTENSION IN ANGUS STEERS RAISED AT HIGH ALTITUDE

SUMMARY

Disease traits are typically polygenic, and knowledge of the genetic mechanisms regulating pulmonary hypertension (PH) in high altitude (> 1,500 m) beef production systems is somewhat limited. Pulmonary arterial pressures (PAP) are a measure of PH, where cattle can be categorized typically as hypertensive (HT; > 49 mmHg) or normotensive (NT; < 41 mmHg) for their risk of heart failure. A large number of genes were differentially expressed in cardiopulmonary tissues of steers with and without PH, many of which have biological functions regulating the availability or utilization of calcium. It is well documented that myocellular calcium is the primary determinant of normal cardiac contractile function. Quantitative real-time PCR (qPCR) was utilized to estimate stability of endogenous controls and expression of candidate genes in cardiac muscle tissues (n = 11) from Angus steers (n = 20). Ten candidate genes were studied: *ASIC2*, *EDN1*, *FBN1*, *KCNMA1*, *NOX4*, *PLA2G4A*, *RCAN1*, *RGS4*, and *THBS4*. The most stable endogenous control, *ACTB*, was utilized for expression normalization (Qbase^{+} stability M = 0.23). Gene expression differences ($P < 0.0055$) between HT and NT steers were estimated for right papillary muscle in *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*. Additionally, right cardiac ventricle tissues (top, middle, and bottom) exhibited differences ($P < 0.0055$) with candidate genes *ASIC2*, *EDN1*, *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*. Directional expression changes in right ventricle middle tissues were validated between RNA-seq and qPCR analyses, with less abundant expression in *ASIC2*, and more abundant expression for all other genes in hypertensive steers as compared to steers with

normotensive. Results of the current study provide evidence of the dynamics of expression of genes that regulate the availability and utilization of calcium with PH status in Angus steers at high altitude. The identification and utilization of causative variants in the validated candidate genes provides opportunity for selection strategies to reduce susceptible to PH and potential heart failure in beef cattle.

INTRODUCTION

Pulmonary hypertension (PH) in beef cattle is characterized by abnormal pulmonary arterial pressures (PAP; > 41 mmHg) at high elevations. This disease phenotype develops through ventricular remodeling of the heart due to an inability to overcome the contractile mechanisms needed to pump blood through the pulmonary vasculature to be oxygenated (Neary et al., 2015; Pugliese et al., 2015; Ryan et al., 2015). Calcium functions as an intracellular messenger and cycling of calcium in muscle cells is important for normal cardiac contraction, with perturbations associated with heart dysfunction and disease (Houser et al., 2000; Hasenfuss and Pieske, 2002). Previously conducted transcriptome analyses revealed differentially expressed genes between hypertensive (76.0 ± 21.9 mmHg) and normotensive (39.9 ± 5.6 mmHg) Angus steers (unpublished data; N. Crawford). Many of these genes were in pathways that regulated either the availability (upstream) or utilization (downstream) of intracellular calcium. Validation of RNA-seq results is dependent upon factors such as study design and statistical analyses, where the rigor of these, help reveal the need for validation with methods such as qPCR, in addition to answering the question of biological reproducibility of the results (Wise et al., 2007). We hypothesized that candidate genes (*ASIC2*, *EDN1*, *FBN1*, *KCNMA1*, *NOX4*, *PLA2G4A*, *RCAN1*, *RGS4*, and *THBS4*), as determined with preliminary RNA-seq differential expression analyses, will be validated with quantitative reverse transcription PCR (qPCR) methods in Angus steers at high altitude. The

objectives of this research were: 1) to establish the most appropriate reference genes in cardiac muscle tissues, and 2) to estimate and validate relative gene expression of calcium-related genes in cardiac muscle tissues using qPCR methods. Results of the current study will help to delineate calcium-associated genes involved in PH and heart failure susceptibility in Angus cattle at high altitude.

MATERIALS AND METHODS

All procedures for animal care, handling, and sampling were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol # 13-4111).

Tissue/Samples

Chapter 3 of this dissertation outlines the animal population and tissue samples available and utilized in this study. In short, cardiac muscle (n = 9) and muscle control tissues (n = 2) from yearling Angus steers (n = 20) were used.

RNA isolation

Total RNA was isolated from bovine muscle tissues with TRIzol Reagents and methodologies (APPENDIX C). In brief, the steps of the protocol included: homogenization, phase separation, RNA precipitation, wash, and solubilization. On average, 90 ± 15 mg (fresh mass) of each sample was cut into 3-6 small pieces and added to a 2 mL zirconium bead tube (D1033-30G) with 1 mL TRIzol reagent. Samples were homogenized with a Bead Bug Microtube Homogenizer (D1030). The DNAse treatment was conducted with a TURBO DNA-free Kit (Ambion; AM1907).

RNA quantification and purity

Concentrations and purity were estimated with a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). These volumes were estimated both prior to a DNAse treatment, and then again after. All samples were normalized to a concentration of 500

ng/µL. Total RNA concentrations post DNAse treatment averaged 548 ± 62 ng/µL across the 11 tissues and 20 animals (Table 5-1). Table 5-1 presents the average RNA concentrations (ng/uL), 260/280 and 260/230 absorbance for each of the 11 tissues across 20 Angus steers. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA in a sample. The ratio of absorbance at 260 nm and 230 nm is used to assess the purity nucleic acids in a sample (ThermoFisher Scientific, 2017). Brisket and *Longissimus dorsi* muscle tissues had lower average 260/230 absorbance values, suggesting the presence of contaminants in the samples. This was likely because of the fatty and fibrous nature of these muscle tissues, as RNA purity and integrity could be affected. Alternative isolation methods could be implemented to increase RNA purity and integrity in these types of muscle tissues (Bio-Rad Laboratories, 2019).

Table 5-1. Average concentrations, 260/280, and 260/230 absorbance of isolated RNA from cardiac and control muscle tissues from yearling Angus steers.

Tissue	Concentration (ng/uL)	260/280 Absorbance[1]	260/230 Absorbance[2]
Apex	563.64	1.99	1.90
Brisket[3]	576.42	2.00	1.67
Longissimus dorsi[3]	570.08	2.00	1.62
Left ventricle, bottom	567.62	2.02	2.02
Left ventricle, middle	561.81	1.98	2.04
Left ventricle, top	537.41	1.99	1.88
Moderator band[4]	475.77	1.99	1.89
Right papillary muscle	536.65	2.01	1.98
Right ventricle, bottom	574.08	1.95	1.94
Right ventricle, middle	532.56	1.99	1.99
Right ventricle, top	535.34	1.97	1.79
Average	548.31	1.99	1.88

[1] Used to assess purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA.

[2] Used as a secondary measure of nucleic acid purity. Expected 260/230 values are commonly in the range of 2.0-2.2.

[3] Utilized as a control muscle

[4] Very low yields (< 270 ng/uL) for two of the samples (#2385, #2392)

Reference and candidate genes

Reference genes, commonly used in study of cattle, were a normalization factor for the candidate genes, as the level of expression or stability of endogenous control should change little between both animal and tissue. Of those available, 8 reference genes were chosen for the current study. Table 5-2 presents all controls, including their name, location in the *Bos taurus* genome, and function.

Previous research described in Chapter 3 resulted in 10 candidate genes for validation, also referred to as putative candidate genes. Table 5-3 lists of the genes chosen for quantitative real-time PCR gene expression validation, including their name, location in the *Bos taurus* genome,

estimated Log_2 fold change from RNA-seq expression analyses, and function. In brief, candidate genes were chosen based upon their Log_2 fold change (> 1.5, < -1.5) and significance ($P < 0.05$) from RNA-seq, their function as an upstream regulator of calcium availability or downstream utilizer of calcium, and their influence on cardiac function and contractility. The 10 candidate genes were not an all-encompassing list of those genes that fit within the above criteria.

Table 5-2. List of reference genes (endogenous controls) evaluated for qPCR gene expression normalization.

Gene	Name	Gene ID	Chr[1]	Location[2]	Function
18S rRNA	Ribosomal RNA, 18 subunit	493779	27	6224662..6226184	Protein biosynthesis
ACTB	Actin beta	280979	25	38799230..38802643	Cytoskeletal structural protein
B2M	Beta-2-microglobulin	280729	10	103095452..103110775	Protein binding; Immune response/defense
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	281181	5	103870384..103874667	Oxidoreductase in glucose metabolism
HMBS	Hydroxymethylbilane Synthase	515614	15	29666771..29674064	Involved in porphyrin metabolism
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1	281229	X	18177361..18208571	Purine synthesis in salvage pathway.
RPLP0	Ribosomal protein, large, P0	286868	17	62557773..62561795	Protein biosynthesis; Structural constituent of ribosome.
RPS9	Ribosomal Protein S9	533892	18	63196977..63204297	Protein biosynthesis; Structural constituent of ribosome.

[1] Chromosome

[2]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

Table 5-3. List of candidate genes for qPCR gene expression validation.

Gene	Name	Gene ID	Chr[1]	Location[2]	Average Log_2 FC[3]	Function
ASIC2	Acid sensing ion channel subunit 2	617930	19	16022575..17228963	-3.5395	Membrane ion channel; Activator of the calcineurin/NFAT signaling pathways
EDN1	Endothelin 1	281137	23	44156440..44163423	1.3272	Vasoconstrictor
FBN1	Fibrillin 1	281154	10	61654541..61919167	1.0283	Extracellular matrix glycoprotein
KCNMA1	Potassium calcium-activated channel subfamily M alpha 1	282573	28	32610164..33387551	1.5703	Large conductance ion channel
NOX4	NADPH oxidase 4	378474	29	6120515..6303004	2.2042	Catalytic subunit the NADPH oxidase complex; Acts as an oxygen sensor
P2RY6	Pyrimidinergic receptor P2Y6	539703	15	52754742..52790314	-1.1681	G-protein coupled receptor; Mediates inflammatory responses
PLA2G4A	Phospholipase A2 group IVA	525072	16	67907024..68081280	1.1096	Catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid
RCAN1	Regulator of calcineurin 1	539640	1	882073..1002231	1.7262	Calcium/calmodulin-dependent phosphatase
RGS4	Regulator of G protein signaling 4	617437	3	6290862..6297919	1.8992	Regulator/inhibitor of G-protein signaling
THBS4	Thrombospondin 4	541281	10	11006321..11060136	2.5556	Adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions

[1]Chromosome

[2]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[3]Average Log_2 fold change estimated from differential expression analysis of total counts from RNA-sequencing.

Primer design

Specific primer pairs for reference and candidate genes were designed and selected for *Bos taurus* cattle utilizing the National Center from Biotechnology Information Primer Blast tool (https://www.ncbi.nlm.nih.gov). Reference gene primers included: 18S rRNA, ACTB, B2M, GAPDH, HMBS, HPRT1, RPLP0, and RPS9 (Table 5-4). Candidate genes included: ASIC2, EDN1, FBN1, KCNMA1, NOX4, P2RY6, PLA2G4A, RCAN1, RGS4, and THBS4 (Table 5-5). All primers were designed with the following parameters: a minimum amplicon size of 70 bp, a maximum amplicon size of 300 bp, and the primer must span an exon-exon junction. The most suitable forward and reverse primer pair for each reference and candidate gene was selected based upon the amplicon length, the span of 2 or more exons, guanine-cytosine (G-C) content less than 60% (for melting and annealing stability), a melting temperature 58 to 60°C, with no more than 3°C difference between the pair, and the lowest self-complementarity values. Primer pairs were synthesized by Integrated DNA Technologies, Inc. Primer pair oligos (25 nmole) were reconstituted with nuclease free water and aliquoted to 10 μM in 100 μL prior to use.

Table 5-4. Primer sequences of reference or endogenous control genes used for mRNA expression analysis.

Gene[1]	Accession Number	Amplicon size	Primer	Sequence (5'→3')	Tm[2] (°C)	GC%[3]
18S rRNA	-	-	Forward	GTAACCCGTTGAACCCCATT	55.30	50.00
			Reverse	CCATCCAATCGGTAGTAGCG	55.10	55.00
ACTB	NM_173979.3	282	Forward	AGAGCTACGAGCTTCCTGAC	58.90	55.00
			Reverse	GCGCGATGATCTTGATCTTCATT	59.81	43.48
B2M	NM_173893.3	195	Forward	AAGTGGGATCGAGACCTGTA	57.47	50.00
			Reverse	ACATGGACATGTAGCACCCA	59.01	50.00
GAPDH	NM_001034034.2	259	Forward	AGGTCGGAGTGAACGGATTC	59.47	55.00
			Reverse	CCAGCATCACCCCACTTGAT	60.03	55.00
HMBS	NM_001046207.1	283	Forward	CCCGACACCGGAGGACATT	61.66	63.16
			Reverse	ACAGGCTCTTCTCTCCAATCTTAG	59.59	45.83
HPRT1	NM_001034035.2	131	Forward	AGCTACTGTAACGACCAGTCA	58.49	47.62
			Reverse	AGCAAAGTCTGCATTGTCTTCC	59.44	45.45
RPLP0	NM_001012682.1	276	Forward	TCGTGTGAGTGACATCGTCTT	59.39	47.62
			Reverse	CGGGTTGTTTTCCAGATGCC	59.76	55.00
RPS9	NM_001101152.2	239	Forward	CGTTGGCTTAGGCGCAGA	60.43	61.11
			Reverse	GGGTCTTTCTCATCCAGCGT	59.75	55.00

[1]18S rRNA = Ribosomal RNA 18 subunit; ACTB = Actin beta; B2M = Beta-2-Microglobulin; GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase; HMBS = Hydroxymethylbilane Synthase; HPRT1 = Hypoxanthine Phosphoribosyltransferase 1; RPLP0 = Ribosomal Protein Lateral Stalk Subunit P0; RPS9 = Ribosomal Protein S9

[2]Melting temperature

[3]Percentage of nitrogenous bases that were either guanine (G) or cytosine (C)

Table 5-5. Primer sequences of candidate genes used for mRNA expression analysis.

Gene[1]	Accession Number	Amplicon size	Primer	Sequence (5'→3')	Tm[2] (°C)	GC%[3]
ASIC2	NM_001076484.1	224	Forward	GCGAGACAGAGGAAACGACA	60.04	55.00
			Reverse	ACAGGCGGTGATGCTGTAAA	59.96	50.00
EDN1	NM_181010.2	289	Forward	CCAGAGCACGTTGTTCCGTA	60.32	55.00
			Reverse	TGGCCTCCAACCTTCTTGTTT	60.06	47.62
FBN1	XM_015473179.2	300	Forward	GACAGAGTGCCGAGACATTGA	60.07	52.38
			Reverse	TTCATGCAGATCCCAGGTGT	59.01	50.00
KCNMA1	NM_174680.2	115	Forward	CTAACCTGGAGCTGGAAGCCT	61.52	57.14
			Reverse	GCATCTGCTGACTCTATCTTGACT	60.20	45.83
NOX4	NM_001304775.1	279	Forward	GAGATGCTGGGGCTAGGATTG	60.27	57.14
			Reverse	TTCGACAAAATCCTCGCGGT	60.32	50.00
P2RY6	NM_001192295.1	239	Forward	ACAGGAGGCTGTATGACCATTG	60.09	50.00
			Reverse	CTCGGTAGACGCAGTTGGTG	60.73	60.00
PLA2G4A	XM_024976260.1	261	Forward	GGGACGACAACGTTTCCCAT	60.60	55.00
			Reverse	CACAGGCACATCACGTGTAGA	60.34	52.38
RCAN1	NM_001034679.1	237	Forward	GATGCCAGACTCCAGCTACA	59.17	55.00
			Reverse	ATACTTTTCCCCTGGCCCTA	57.70	50.00
RGS4	NM_001046600.2	210	Forward	AGTGGTGATTTGTCAGAGGGTG	60.22	50.00
			Reverse	ATCTTTTTGGCCTTGGGACT	57.30	45.00
THBS4	NM_001034728.1	245	Forward	AGAGGAAGGCACACGACTCT	60.54	55.00
			Reverse	TTCGGCTATGGTGTTTCGCA	60.32	50.00

[1]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *P2RY6* = Pyrimidinergic Receptor P2Y6; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator Of Calcineurin 1; *RGS4* = Regulator Of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[2] Melting temperature

[3] Percentage of nitrogenous bases that were either guanine (G) or cytosine (C)

Synthesis of cDNA

Reagents for synthesis of cDNA were provided in the ImProm-II Reverse Transcription System kit (Promega; PRA3802). To prepare the cDNA, 2 µL total RNA was mixed with 2 µL of nuclease free water and 1 µL of primer mix per template. The primer mix comprised equal parts of random hexamer (50 ng/µL) and oligo$(dT)_{20}$ (50 µM) primers. The reaction mix was incubated at 70°C for 5 min in a thermocycler and immediately chilled on ice (4°C) for a minimum of 5 min. The reverse transcription (RT) master mix was prepared for a 1x reaction as follows: 4 µL ImProm-II 5x buffer, 2.4 µL $MgCl_2$ (25 mM), 5 µL dNTP, 1 µL RNase Out (40 U/µL), 1 µL R.T, and 1.6 µL nuclease free water to adjust the volume to 15 µL. The RT master mix was added to 5 µL prepared cDNA for a total volume of 20 µL for each template. The reaction was then incubated in the thermocycler at 25°C for 5 min, 42°C for 1 hour, 70°C for 15 min, and was then cooled and held at 4°C until further processing. All cDNA samples were evaluated through a melt curve analysis to ensure a single product was created.

Primer efficiency

Standard dilutions were utilized to estimate and optimize the efficiency of each primer pair. Pooled cDNA (n = 22) was utilized for the efficiency testing. Samples were randomly selected through a random number generator for the pooled cDNA, where tissues (n = 11) were represented twice, and every animal (n = 20) was represented at least once. The pooled cDNA was 1:10 diluted, resulting in 6 dilutions: 1x, 0.1x, 0.01x, 0.001x, 0.0001x, and 0.00001x. Standard curves were estimated with a 1x PCR master mix of: 10 µL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems; A25778), 0.6 µL primer, 7.4 µL nuclease free water, and 2 µL pooled cDNA template. Results were visualized and analyzed with QuantStudio™ Design and Analysis Software (version 5?). For samples with sub-optimal efficiencies, both 1:5 and 1:2 serial dilutions were conducted.

Qbase^{+} software was utilized to estimate and rank the stability of the reference genes tested through the geNorm program (Vandesompele et al., 2002). The most stable reference was utilized to estimate gene expression. Appendix D provides additional primer efficiency results.

Quantitative reverse transcription PCR

A gene maximization strategy was implemented with reactions on both candidate and reference genes. Both reference and candidate genes were repeated across 384-well plates (so-called inter-run calibrator samples) in order to detect and remove inter-run variation. Hypertensive and normotensive groups represented biological replicates in each of the tissues. Technical replicates were represented through triplicate of every sample and gene on each plate. Non-template controls were utilized to monitor contamination and primer-dimer formation that could produce false positive results. Appendix E provides an example of the 384-well plate arrangement for all samples (n = 20) and genes (n = 12) on a single tissue (i.e., Right ventricle bottom).

The PCR master mix included a 1x reaction mix of 10 µL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems; A25778), 5.6 µL nuclease free water, and 2 µL pooled cDNA template. Primers were diluted from 10 µM to 2.5 µM concentrations to increase volume added to the plate. Primer was the rate-limiting factor, therefore 2.4 µL of each primer was added to bottom of a 96-well plate followed by 17.6 µL of PCR master mix. Triplicate samples were plated in a 384-well plate at 6 µL each (APPENDIX E). Gene expression was analyzed with a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Cycling conditions were as follows: 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 sec for elongation and 60°C for 1 min for annealing. The melt curve analysis was then performed with heating to 95°C for 15 sec, 60°C for 1 min, and final 95°C for 15 sec. Estimation of fluorescence was complete at the end of each of the 40 cycles and additionally at the end of the melt curve

analysis steps. Real-time PCR monitors amplification of the target in real-time via a target-specific fluorescent signal emitted during amplification. QuantStudio™ Design and Analysis Software (version 5) and Expression Suite software was used to define the crossing threshold (C_T) for each amplification reaction. Automatic thresholds were set for candidate gene *RCAN1* and reference gene *RPS9*. All other candidate and reference genes were adjusted to eliminate background noise amplification. Thresholds ranged from 0.045 to 0.104. Results from all tissues and samples were exported to Microsoft Excel for further analysis.

Data analysis

There are two widely accepted methods for estimating expression differences from RT-qPCR results. The Livak method may be used to calculate relative changes in gene expression, with the assumption that candidate and reference gene efficiencies were the same (Livak and Schmittgen, 2001). The Pfaffl method is also used to calculate relative gene expression while correcting for differences in primer efficiencies (Pfaffl, 2001). It is important to differentiate among the two methodologies as they have different assumptions, which have an effect on results. Both methods have been implemented to understand how the results of these analyses differ. However, the methodology and results for the delta delta C_T method (Livak and Schmittgen) are provided in Appendix F.

The Pfaffl method (efficiency correction)

To calculate the relative gene expression using the Pfaffl method, candidate and reference gene efficiencies were accounted for within the analyses. Appendix D provides additional information on primer efficiencies of each gene based up serial dilution curves described previously (Tables A-1 and A-3). As with the Livak method, estimated C_T values were first averaged across PCR technical replicates (triplicates). First, establish a calibrator. All C_T values

for the normotensive steer group in a given gene and within a single tissue were utilized as the calibrator and established by estimating their average C_T. This is completed for both the reference and candidate genes.

The delta C_T values were then calculated as

$$\Delta C_T = C_{T_{calibrator}} - C_T \quad (1)$$

where C_T was the crossing threshold value for the either the reference or the candidate gene and $C_{T_{calibrator}}$ was the average crossing threshold value for calibrator, or all C_T values for the normotensive steer group. The ΔC_T values were calculated for both the reference and candidate genes with their respective calibrators. There were 9 or 10 animals represented in the normotensive group for all tissues, therefore the average expression for the control group should not be exactly 1.0, as classically estimated. Calculations were repeated with any additional reference genes in separate analyses.

To calculate the relative gene expression, primer efficiency percentages were converted using the equation

$$Converted\ primer\ efficiency = \left(\frac{primer\ efficiency\ \%}{100}\right) + 1 \quad (2)$$

where an efficiency of 100% is represented in with value of 2.0. Relative gene expression, estimated as a ratio, was then calculated through the equation

$$Gene\ expression\ ratio = \frac{efficiency_{target}^{\Delta C_{T_{target}}}}{efficiency_{reference}^{\Delta C_{T_{reference}}}} \quad (3)$$

where $efficiency_{target}$ was the converted efficiency for the respective candidate gene from equation 2, $\Delta C_{T_{target}}$ was the estimate ΔC_T for the candidate gene from equation 1, $efficiency_{reference}$ was the converted efficiency for the reference gene, and $\Delta C_{T_{reference}}$ was the estimate ΔC_T for the reference gene.

Mann-Whitney Test (Wilcoxon Rank Sum Test)

Analyses were between unpaired or independent subjects. A Levene's test was implemented to test for heterogeneity of variance between the two populations (normotensive versus hypertensive). The analyses revealed that the two populations had unequal variance. A Shapiro-Wilks test was utilized to test for the normality of the C_T values for each candidate gene in each tissue. The analyses revealed that some of the populations violated normality. A Mann-Whitney (Wilcoxon Rank Sum) non-parametric test was subsequently utilized to test the hypothesis

$$H_0: \mu_1 = \mu_2 \tag{4}$$

$$H_A: \mu_1 \neq \mu_2$$

where μ_1 was the average relative gene expression for the first population (normotensive) and μ_2 was the average relative gene expression for the second population (hypertensive). The average of the relative gene expression was calculated in equations 3 for the Pfaffl method. Estimations with the Livak method were presented in Appendix F. Mann-Whitney U-test defines the statistic *U* by the following formula

$$U_1 = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1 \tag{5}$$

$$U_2 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

where R_1 denoted the sum of the ranks for the first group, R_2 denoted the sum of the ranks for the second group, n_1 and n_2 were sample sizes for the first and second group, respectively, and where $U_1 + U_2 = n_1 * n_2$. The Mann-Whitney U-tests were repeated for each gene (n = 9) and across each tissue (n =11). Significance was adjusted for multiple comparisons testing to the number of genes within a tissue, as comparisons were not conducted between tissues. Therefore, a standard significance of 0.05 was divided by 9 to yield a threshold significance of 0.0055.

Outlier tests

A Grubbs' test was used to detect potential outliers in the gene expression results. The test assumes normality of the population; however, as discussed previously the data were non-normally distributed. Detection of outliers was dependent upon significance of the test ($P < 0.05$).

RESULTS AND DISCUSSION

RNA quality

Moderator band produced lower concentrations of RNA compared to all other tissues (Table 5-3). The low concentrations may be attributed to numerous factors. The moderator band is a fibrous muscle, which can influence RNA purity and integrity. The required amount of tissue for sufficient RNA yields was 50 to 100 mg (Appendix C). Collection methodology generated small yields of the muscle (< 50 mg), where the quantity of tissue available was completely utilized for some samples (i.e., steer #2385 and #2392). These low sample yields will directly influence RNA concentrations, and downstream expression results as well.

In addition, collection, preservation, and storage of tissues will have an effect on RNA quality. Right and left ventricle middle samples, as well as moderator band samples were degraded in appearance (i.e., brown in color, shriveled). The exact cause is unknown. It was speculated that samples were improperly preserved, either through the snap freezing process in liquid nitrogen, in their long-term storage over liquid nitrogen vapor, or in transit. These factors were considered throughout the research process and in evaluation of research results.

Relative gene expression

Minimal differences were observed between the two methods (Livak vs. Pfaffl) for estimating gene expression (Table A-4; APPENXID F). The Livak method estimates relative gene expression with two assumptions: 1) amplification efficiency between primer sets does not differ

more than 5%, and 2) candidate and reference gene efficiencies amplify near 100%, or there is optimal doubling of the gene DNA during each PCR cycle. As displayed in Appendix D Tables A-1 and A-3, this is not an accurate assumption for our data, which could result in erroneous quantification of transcript amounts (Livak and Schmittgen, 2001). The Livak method is typically utilized in qPCR analyses involving a large number of genes, as quantifying efficiency of each candidate gene may be challenging. Given the small number of genes utilized in this study, the most appropriate method to estimate relative expression was the Pfaffl method. Table 5-7 displays the average gene expression for the hypertensive and normotensive groups across all candidate genes and all tissues utilizing the Pfaffl estimation method with efficiency correction. In addition, results are graphically represented in Appendix G.

Candidate gene validation and differential expression

Table 5-6 displays the validation of genes between the RNA-seq and qPCR fold change results. Fold change was not calculated exactly the same between the two methodologies, as the dynamic range or scale of expression differed, therefore we were unable to compare the quantitative measures of fold change directly. We in-turn opted to compare the directional (either more or less abundant) change of gene expression between hypertensive and normotensive steers. All candidate genes except *ASIC2*, were estimated to be more abundant in the hypertensive steers as compared to the control normotensive steers. Several other studies have estimated a correlation between RNA-seq and qPCR expression results (Asmann et al., 2009; Griffith et al., 2010; Wu et al., 2013; Shi and He, 2014).

Table 5-6. Comparison of fold change (FC) directionality from RNA-seq and qPCR analyses for right ventricle middle tissues.

Gene[1]	RNA-seq FC[2]	qPCR FC[2]
ASIC2	less	less
EDN1	more	more
FBN1	more	more
KCNMA1	more	more
NOX4	more	more
PLA2G4A	more	more
RCAN1	more	more
RGS4	more	more
THBS4	more	more

[1]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator Of Calcineurin 1; *RGS4* = Regulator Of G Protein Signaling 4; *THBS4* = Thrombospondin 4
[2]Estimated through comparison of hypertensive to normotensive; less = less abundant expression in the hypertensive steers when compared to the normotensive steers; more = more abundant expression in the hypertensive steers when compared to the normotensive steers

Differences between hypertensive and normotensive groups were observed in several tissues; specifically, right papillary muscle, and right ventricle bottom, middle and top (Table 5-7). An objective of the study was to validate results from RNA-seq for the candidate genes, in which all of the genes were differentially expressed in the right ventricle RNA-seq data, and some of which were also differentially expressed in left ventricle. Therefore, it was not surprising to have those genes validated in the qPCR model for the right ventricle.

The right ventricle is a highly influential muscle regulating heart function. Pulmonary hypertension develops through remodeling of the vasculature of the heart and lung and an inability of the animal to overcome the necessary force to eject the blood through the pulmonary artery, leading to hypertrophy of the right ventricle (Pugliese et al., 2015; Krafsur et al., 2019). The muscles of the right ventricle of the heart enlarge to compensate, and as the impedance increases,

the heart could eventually fail. Heart failure is the resulting action that can take place in cattle with PH, if the heart succumbs to these pathophysiological changes (Voelkel et al., 2006).

In general, the results appear to demonstrate that the majority of the candidate genes regulating calcium availability and utilization are upregulated in the hypertensive steers (Figure 4-5; Table 5-6). This would imply that modifying the regulation of these genes (i.e., downregulation as opposed to upregulation) could be an option in altering susceptibility of cattle to PH. Important to note however is the polygenic nature of bovine PH, in which the candidate genes are likely just a subset of the number of genes controlling PH susceptibility.

Table 5-7. Summary of relative gene expression (Mean ± Standard Error) and Wilcoxon rank sum test[1] (p-value) for muscle tissues (n = 11) and calcium-related candidate genes[2] (n = 9) between hypertensive and normotensive[3] groups.

		ASIC2		*EDN1*		*FBN1*		*KCNMA1*	
Tissue	Group[3]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]
Apex	HT	0.77 ± 0.27	0.243	3.29 ± 1.34	0.133	1.39 ± 0.16	1.000	1.95 ± 0.51	0.549
	NT	1.15 ± 0.24		1.18 ± 0.24		1.37 ± 0.14		1.60 ± 0.39	
Brisket	HT	1.34 ± 0.27	1.000	1.28 ± 0.63	0.905	0.96 ± 0.20	0.315	0.97 ± 0.26	0.356
	NT	1.37 ± 0.31		1.46 ± 0.45		1.23 ± 0.19		1.12 ± 0.13	
Longissimus dorsi	HT	1.32 ± 0.18	0.447	1.02 ± 0.15	0.604	1.13 ± 0.29	0.447	0.97 ± 0.10	0.315
	NT	1.11 ± 0.15		1.05 ± 0.11		1.31 ± 0.25		1.23 ± 0.17	
Left ventricle, bottom	HT	1.82 ± 0.71	0.684	2.87 ± 1.25	0.075	1.25 ± 0.19	0.684	1.60 ± 0.68	0.631
	NT	1.91 ± 0.94		1.06 ± 0.11		1.08 ± 0.13		1.11 ± 0.19	
Left ventricle, middle	HT	1.29 ± 0.42	0.661	1.33 ± 0.12	0.095	0.6 ± 0.14	0.780	0.82 ± 0.16	0.604
	NT	1.41 ± 0.40		1.07 ± 0.14		0.59 ± 0.13		0.64 ± 0.13	
Left ventricle, top	HT	1.01 ± 0.38	0.190	2.00 ± 0.72	0.063	0.82 ± 0.06	0.105	0.93 ± 0.21	0.353
	NT	1.18 ± 0.22		1.08 ± 0.14		1.03 ± 0.08		1.12 ± 0.17	
Moderator band	HT	0.51 ± 0.13	0.010	1.09 ± 0.18	0.661	1.34 ± 0.27	0.604	1.86 ± 0.41	0.113
	NT	1.22 ± 0.20		1.38 ± 0.46		1.06 ± 0.24		1.20 ± 0.53	
Right papillary muscle	HT	0.55 ± 0.15	0.043	1.94 ± 0.58	0.218	1.65 ± 0.18	0.023	4.20 ± 1.13	0.063
	NT	1.30 ± 0.29		1.10 ± 0.15		1.05 ± 0.11		1.43 ± 0.39	
Right ventricle, bottom	HT	0.35 ± 0.21	0.009	1.82 ± 0.22	0.007	1.23 ± 0.11	0.247	2.14 ± 0.61	0.353
	NT	1.60 ± 0.43		1.23 ± 0.35		1.13 ± 0.20		3.71 ± 2.65	
Right ventricle, middle	HT	0.19 ± 0.11	***< 0.001***	2.13 ± 0.78	0.222	1.32 ± 0.13	0.387	2.66 ± 1.46	1.000
	NT	1.32 ± 0.34		1.19 ± 0.27		1.08 ± 0.15		1.41 ± 0.42	
Right ventricle, top	HT	0.13 ± 0.06	***< 0.001***	2.86 ± 1.01	***0.003***	1.27 ± 0.19	0.631	2.22 ± 0.39	0.015
	NT	1.20 ± 0.24		1.03 ± 0.08		1.02 ± 0.07		1.08 ± 0.14	

[1]Non-parametric analysis; H_0: Population means are equal; H_A: Population means are not equal

[2]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator Of Calcineurin 1; *RGS4* = Regulator Of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[3]Physiological group based upon pulmonary arterial pressures (PAP); See Figure 3-2.

[4]Signifance adjusted for multiple testing; Significance set at $P < 0.005555$; Bold and italicized p-values met significance threshold

Table 5-7. Continued…

		NOX4		*PLA2G4A*		*RCAN1*		*RGS4*		*THBS4*	
Tissue	Group[3]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]
Apex	HT	1.81 ± 0.45	0.315	1.89 ± 0.25	0.028	2.53 ± 0.59	0.010	1.67 ± 0.36	0.113	1.23 ± 0.30	0.842
	NT	1.21 ± 0.22		1.14 ± 0.19		1.20 ± 0.31		1.05 ± 0.10		1.15 ± 0.21	
Brisket	HT	1.11 ± 0.21	0.720	0.79 ± 0.14	0.133	1.05 ± 0.17	0.720	2.06 ± 1.24	0.661	1.89 ± 0.68	0.356
	NT	1.04 ± 0.09		1.09 ± 0.15		1.68 ± 0.78		1.26 ± 0.26		1.12 ± 0.19	
Longissimus dorsi	HT	1.56 ± 0.27	0.243	1.32 ± 0.18	0.315	1.42 ± 0.27	0.447	1.18 ± 0.22	0.780	1.20 ± 0.14	0.549
	NT	1.14 ± 0.18		1.02 ± 0.07		1.14 ± 0.19		1.11 ± 0.16		1.03 ± 0.08	
Left ventricle, bottom	HT	1.64 ± 0.59	0.631	1.57 ± 0.32	0.280	1.98 ± 0.7	0.190	1.32 ± 0.34	0.912	1.44 ± 0.31	0.393
	NT	1.12 ± 0.16		1.05 ± 0.10		1.10 ± 0.17		1.08 ± 0.13		1.06 ± 0.13	
Left ventricle, middle	HT	0.89 ± 0.13	0.447	1.04 ± 0.14	1.000	1.16 ± 0.19	0.447	0.87 ± 0.10	0.497	0.86 ± 0.13	0.315
	NT	1.17 ± 0.20		1.13 ± 0.19		1.17 ± 0.28		1.14 ± 0.21		1.07 ± 0.13	
Left ventricle, top	HT	0.87 ± 0.16	0.190	0.99 ± 0.08	0.579	1.91 ± 0.56	0.353	0.61 ± 0.09	0.043	0.87 ± 0.10	0.218
	NT	1.08 ± 0.14		1.03 ± 0.08		1.07 ± 0.13		1.16 ± 0.21		1.06 ± 0.12	
Moderator band	HT	3.07 ± 0.91	0.133	1.63 ± 0.42	0.447	2.08 ± 0.46	0.133	1.19 ± 0.21	0.243	3.29 ± 0.93	0.043
	NT	1.27 ± 0.27		1.25 ± 0.36		1.20 ± 0.23		2.47 ± 1.51		1.37 ± 0.41	
Right papillary muscle	HT	5.51 ± 1.52	***< 0.001***	3.04 ± 0.74	***0.002***	3.61 ± 0.79	***0.001***	3.16 ± 0.71	0.019	3.34 ± 0.57	***0.005***
	NT	1.09 ± 0.15		1.08 ± 0.14		1.12 ± 0.20		1.08 ± 0.14		1.17 ± 0.20	
Right ventricle, bottom	HT	3.02 ± 0.62	***0.002***	1.85 ± 0.38	0.035	6.47 ± 1.98	***0.001***	1.75 ± 0.52	0.190	3.44 ± 0.89	***0.002***
	NT	1.19 ± 0.24		1.40 ± 0.51		1.23 ± 0.29		2.5 ± 1.74		1.20 ± 0.28	
Right ventricle, middle	HT	3.76 ± 1.10	***0.004***	2.63 ± 0.54	***0.001***	3.29 ± 0.87	0.008	3.21 ± 0.82	0.040	5.13 ± 1.35	***0.001***
	NT	1.12 ± 0.19		1.08 ± 0.15		1.14 ± 0.20		1.18 ± 0.23		1.25 ± 0.38	
Right ventricle, top	HT	2.90 ± 0.72	***0.002***	1.70 ± 0.22	***0.002***	3.22 ± 0.53	***0.004***	2.31 ± 0.60	0.029	3.54 ± 0.96	***< 0.001***
	NT	1.05 ± 0.12		1.03 ± 0.08		1.07 ± 0.14		1.04 ± 0.09		1.06 ± 0.13	

[1]Non-parametric analysis; H_0: Population means are equal; H_A: Population means are not equal

[2]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator Of Calcineurin 1; *RGS4* = Regulator Of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[3]Physiological group based upon pulmonary arterial pressures (PAP); See Figure 3-2.

[4]Signifance adjusted for multiple testing; Significance set at $P < 0.005555$; Bolded p-values met significance threshold

Right papillary muscle

Differences between hypertensive and normotensive groups were observed in the right papillary muscle tissues (Table 5-7). The contraction of the papillary muscle is important for the effectiveness of the atrioventricular valves, separating the atria and ventricles (Semafuko and Bowie, 1975). This provides opportunity to relate our results to those of previous research examining expression differences in mitral and tricuspid valve function. There are limited reports describing gene expression differences within right papillary muscle. However, an increase in protein synthesis during hemodynamic overload within the papillary muscle resulted in marked hypertrophy of the muscle (Peterson et al., 1972; Cooper et al., 1985; Komuro and Yazaki, 1993). Additionally, in pressure-overloaded papillary muscles in cats, a linear relationship was observed between increased stiffness and decreased contractile performance (Natarajan et al., 1979). Crawford et al. (2010) provided results to support the idea that right papillary muscles may lead to premature ventricular contractions and ventricular tachycardia, of which can have an effect on cardiac function. The right papillary muscle appears to be influenced by PH status in Angus cattle as per the validation of candidate genes examined.

The NADPH oxidase 4 (*NOX4*) gene encodes a protein that acts as an oxygen sensor and catalyzes the reduction of molecular oxygen to various reactive oxygen species (Bedard and Krause, 2007). Expression of both *NOX2* and *NOX4* isoforms are was identified in cardiomyoctyes (Heymes et al., 2003). Overexpression of *NOX4* in vascular smooth muscle cells is commonly associated with progression of cardiovascular disease (Ellmark et al., 2005; Sturrock et al., 2007; Manea et al., 2010). Increased expression of *NOX4* was discovered in the pulmonary vasculature and lungs of hypoxia-exposed mice and humans with PH (Mittal et al., 2007). *NOX4* was upregulated by hypoxia in adventitial fibroblasts from patients with idiopathic PH (Li et al., 2008).

Expression of NOX4 was upregulated in right papillary muscle of hypertensive steers as compared to normotensive steers. Limited research is available on expression of NOX4 in the right papillary muscle.

Phospholipase A2 Group IVA (*PLA2G4A*) is an enzyme activated by calcium concentration and(or) phosphorylation, promotes binding to the membrane, and is a key contributor to inflammatory processes (Leslie, 1997; Gilroy et al., 2004; Diouf et al., 2006; Linkous and Yazlovitskaya, 2010). This gene is regulated by several mechanisms, and its role is dependent upon the species or pathological state being examined (Ait-Mamar et al., 2005). Research by Zheng et al. (2009) estimated an upregulation of *PLA2G4A* in mice with mitral valve regurgitation. Mitral valve regurgitation is a condition in which the mitral valve of the heart fails to close tightly, forcing blood to flow backward. Contrary, Mahmut et al. (2014) estimated that *PLA2G4A* was significantly down-regulated in stenotic or narrowing valves. There is potential that valve changes may be associated with the proper function of the papillary muscle. Expression of *PLA2G4A* was significantly upregulated in hypertensive steers has compared to normotensive steers. Additional research is necessary to delineate the function of *PLA2G4A* in the right papillary muscle.

Regulator of calcineurin 1 (*RCAN1*; aka calcipressin-1) is a protein activated by increases in cytoplasmic calcium, is upregulated by the NFAT signaling pathway, and stimulates genes regulating cardiac remodeling (Cavasin et al., 2014; Grabner et al., 2015). *RCAN1* functions to inhibit calcineurin-dependent transcriptional responses by binding to the catalytic domain of calcineurin. Reduced expression of NFATc1, a gene targeting *RCAN1* expression and inhibited by *RCAN1* expression through a negative feedback loop, was associated with pulmonary valve homeostasis (Johnson et al., 2003; Chang et al., 2004). This echoed in earlier research by de La

Pompa et al. (1998) and Ranger et al. (1998) in which NFATc had a critical role in early development of cardiac valves. Downregulation of NFAT downregulates *RCAN1* expression, resulting in decreased valve homeostasis, potentially reflected in ineffective contraction of the papillary muscle. In our study, expression of *RCAN1* was upregulated in hypertensive steers as compared to normotensive steers in the right papillary muscle. Previous research does not provide exact evidence to support nor refute these reports. Pulmonary hypertension in our steers may be reflected in a hyper-responsive right papillary muscle to the stress stimuli and upstream regulators of *RCAN1* expression.

Thrombospondin 4 (*THBS4*; aka *TSP-4*) belongs to a family of adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions, where secretion of the protein is suppressed by calcium (Lynch et al., 2012; Duquette et al., 2014). *THBS4* expression decreased in pressure unloaded left ventricle papillary muscle and compared to normal muscle, leading to tissue growth and remodeling (Haggart, 2010; Haggartet al., 2013). Our results indicated an upregulation of THBS4 in hypertensive steers as compared to normotensive steers. These results appear to reiterate the results presented above from other research efforts.

Right ventricle

Acid-sensing ion channels (*ASIC*s) belongs to the degenerin/epithelial Na^+ channel family and serves many functions. There are four genes in this complex (*ASIC1-ASIC4*), which encode six distinct subunits (Sherwood et al., 2012). Under acidosis, overexpression *ASIC2* can activate the calcineurin and NFAT signaling pathways (Zhou et al., 2017). Activation of the calcineurin and NFAT signaling pathways can increase the hypertrophic response of the tissue leading to hypertension, hypertrophy, and remodeling. The *ASIC2* gene was downregulated in nodose ganglia of hypertensive rates (Abboud and Benson, 2015). Nodose ganglia are responsible in part for

sensations in the heart. Gronda et al. (2014) and Lu et al. (2009) studied the development of increased blood pressure in association with *ASIC2*-null mice, through the impairment of arterial baroreceptor signaling. Likewise, increased expression of *ASIC2* inhibited remodeling and decrease blood pressure via vascular smooth muscle cell migration (de Campos Grifoni et al., 2008). To our knowledge, the *ASIC2* gene has not been studied in right ventricle tissue relative to PH or HF. Gene expression differences were estimated in both right ventricle middle and top tissues, where expression was less abundant in the hypertensive steers as compared to the normotensive steers (Table 5-7). With the exception of tissue, the results were consistent with reports of other research. This provides new knowledge of our understanding of the influence of *ASIC2* gene in right ventricle tissue.

Endothelin 1 (*EDN1*) is a peptide hormone that is a potent vasoconstrictor, is involved in a diverse number of biological actions, and its receptors are therapeutic targets in the treatment of PH (Stow et al., 2011). Endothelin-1 is a positive stimulus on heart rate, and indirect positive stimulus on contraction of cardiac muscle (Ogino et al., 1995; Strewler, 2000). It functions as a downstream regulatory of intracellular calcium availability through transcriptional regulation of inositol 1,4,5-trisphosphate (IP3) and nuclear factor of activated T-cells (NFAT) pathways (Rinne and Blatter, 2010). Multiple studies have established associations between increased *EDN1* expression and left ventricular hypertrophy and risk of developing hypertension (Arai et al., 1995; Tiret et al., 1999; Castro et al., 2007). Research by Elton et al. (1992) estimated a tendency for the expression of *EDN1* to increase in the right ventricle with increasing time of hypoxia exposure. Gene expression differences were observed in right ventricle top tissues in the current study. Expression fold change yielded more abundant expression in the hypertensive steers as compared to the normotensive steers. These reports corroborate the results of previous research.

Basic functions of *NOX4* were described previously. In mice, *NOX4* expression contributes to age-dependent changes in contractility of ventricular myocytes through altered calcium utilization (Rueckschloss et al., 2010). Multiple studies have investigated the mechanisms *NOX4*, providing evidence that *NOX4* can be targeted to reduce the effect of hypoxia-induced pulmonary vascular remodeling and right ventricular dysfunction (Green et al., 2012; Frazziano et al., 2013; Li et al., 2014). In addition, *NOX4* interacts with other biomarkers regulating right ventricular remodeling, where expression of *NOX4* was up-regulated in the right ventricle of patients with PH (He et al., 2017). Differences were observed in all right ventricle tissues, top, middle, and bottom in the current study (Table 5-7). Expression was more abundant in the hypertensive steers as compared to the normotensive steers. These results support those of other research studies.

Description and function of *PLA2G4A* was described previously. In ventricular cardiomyoctyes, there appears to be an association between *PLA2G4A* expression, beta-adrenergic receptors (β_1-AR, β_2-AR), and arachidonic acid in the reduction of cardiac contractility (Madamanchi, 2007; Ait-Mamar et al., 2005). Mice deficient in *PLA2G4A* had increased right and left ventricle hypertrophy (Haq et al., 2003). This could have implications to the hypertrophy observed in the right ventricle of cattle suffering from PH. Expression was significantly different in right ventricle middle and bottom tissues, in which hypertensive steers had more abundant expression then the normotensive steers. These results appeared to be contradictory to the reports of other research studies, but concordant with the RNA-seq results. One explanation of the differing results may be the tissue examined. The studies described previously, in addition to others, utilized a both left and right ventricle tissues or a mix of ventricular muscle cells to estimate expression, whereas in the current study we estimated differences strictly in right ventricle middle and bottom tissue. Another potential reason for the different expression results is the species

examined. Most research has previously been conducted in rodent models, whereas the current study was conducted in bovine. Additional research is necessary to delineate if functional differences exist for the candidate genes across species.

Increased expression of *RCAN1* (function described previously) was associated with decreased RV dysfunction, hypertrophy, and lower risk of heart failure in RV tissues from rats and mice with PH (Vega et al., 2003; Cavasin et al., 2014; Wang et al., 2016). Pharmacological drugs have been identified to inhibit the activation of the NFAT pathway by *RCAN1*, which provides opportunity for a potential therapeutic target to reduce or mitigate the extent of hypertrophy and RV pressure overload in cattle (Molkentin et al., 1998; Kapur et al., 2014). Results of the current study were concordant with the reports of previous research outlined previously, as more abundant expression was estimated in hypertensive steers as compared to normotensive in right ventricle bottom and top tissues.

Deficiency of the glycoprotein *THBS4* increased heart fibrosis in mice with PH, and appears to play an important role in myocardial structure, function, and remodeling (Frolova et al., 2012). Thrombospondin 4 expression rises in response to vascular endoplasmic reticulum stress (i.e., hypertension) in the mesenteric arteries of rats (Mustonen et al., 2008; Palao et al., 2015; Sure and Katakam, 2016). Expression of *THBS4* in left ventricle tissues was highly correlated with connective tissue growth factor, which induces pulmonary vascular remodeling and PH (Gabrielsen et al., 2007; Chen et al., 2011). Expression fold change yielded significantly more abundant expression in the hypertensive steers as compared to normotensive steers in all right ventricle tissues (top, middle, bottom). These results directly reflect the findings of research described previously for the THBS4 candidate gene.

Outlier identification

A Grubb's test was implemented to identify outliers from the gene expression results for each gene and within each tissue. Table 5-8 displays the ID numbers of the steer identified as an outlier for its gene expression. Non-significant outliers were excluded. It is important to mention is the necessity to identify those animals that are more susceptible to developing PH. Therefore, expression identified as a significant outlier could be an indicator of that susceptibility. Of particular interest was the outliers identified for the candidate gene *NOX4*. All of the tissues identified hypertensive steers with outlying gene expression for *NOX4*, with 5 out of the 9 outliers being steer #2108. Of the 57 identified outliers, 14 were steer #2108, and 3 were steer #2162, both of which were confirmed or symptomatic for HF.

Table 5-8. Steer IDs representing statistically significant outliers identified with a Grubb's outlier identification test from the estimates of gene expression within each gene (n = 9) for each tissue (n = 11).

	Candidate Gene[1,2]								
Tissue	*ASIC2*	*EDN1*	*FBN1*	*KCNMA1*	*NOX4*	*PLA2G4A*	*RCAN1*	*RGS4*	*THBS4*
Apex	2299	**2108**	*ns*	2113	**2108**	*ns*	*ns*	2024	*ns*
Brisket	*ns*	2151	*ns*	*ns*	2342	*ns*	2156	2342	2342
Longissimus dorsi	*ns*	*ns*	*ns*	*ns*	2046	2045	*ns*	*ns*	*ns*
Left ventricle, bottom	2107	**2108**	2046	2046	2046	2046	**2108**	*ns*	2342
Left ventricle, middle	*ns*	*ns*	2113	*ns*	*ns*	*ns*	2156	2352	*ns*
Left ventricle, top	2045	**2108**	*ns*	*ns*	*ns*	*ns*	**2108**	2107	*ns*
Moderator band	*ns*	2385	2352	2300	**2108**	*ns*	*ns*	2385	*ns*
Right papillary muscle	*ns*	**2108**	*ns*	*ns*	**2108**	**2162**	**2108**	*ns*	*ns*
Right ventricle, bottom	2299	*ns*	2410	2410	**2108**	2410	**2108**	2410	2342
Right ventricle, middle	*ns*	2151	2410	2222	2222	**2162**	**2162**	2222	2151
Right ventricle, top	*ns*	**2108**	2222	*ns*	**2108**	2342	*ns*	2342	*ns*

[1]ns = no significant outlier

[2]Red IDs denote hypertensive steers; those bolded were confirmed or symptomatic for heart failure; black IDs denote normotensive steers

Typically, outliers are excluded from data to avoid problems with statistical analyses and significantly different estimated than the real parameters of the entire population (Osborne and Overbay, 2004; Barnett and Lewis, 1974). However, the exclusion of these data would result in loss of valuable information for this particular trait. We were limited in sample size, so the exclusion of such data would inadvertently decrease our statistical power to detect differences between animals, with additional effects on accuracy and error variance (Osborne and Overbay, 2004). Grubb's method performs well with and is generally accepted for small sample sizes. Exclusion (or acceptance) procedures of outliers are not only dependent upon a statistical basis, but also the evaluation of other data criteria and standards (i.e., normality, sample size, study design; Barbato et al., 2011). Quality control measures were put in place through the study to limit the occurrence of erroneous errors in gene expression. These measures included: 1) a gene maximization plate arrangement to limit inter-plate variation, 2) inclusion of endogenous control or reference genes across all plates, 3) analyzing samples in triplicate, 4) elimination of erroneous sample amplification, and 5) normalization of all expression with the reference gene. We therefore believe the inclusion of such data is important to the overall goal of the study.

CONCLUSION

This study demonstrates validated expression differences of calcium-related 6 candidate genes (*ASIC2*, *EDN1*, *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*) between hypertensive and normotensive Angus steers. Right papillary and ventricle muscles appear to be influential tissues in PH as differences in PAP groups was estimated. Our data suggested that genes regulating the availability and utilization of calcium are associated PH status in Angus cattle. The validation of the candidate genes provides opportunity to implement selection strategies, for example through the identification of causal variant, for reduced susceptibility to PH and heart failure.

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CHAPTER 6

SNP DETECTION: VARIANTS IN CALCIUM-RELATED GENES IN ASSOCIATION WITH PULMONARY HYPERTENSION IN ANGUS CATTLE FED AT HIGH ALTITUDE

SUMMARY

Elucidating the underlying genetic mechanisms regulating pulmonary hypertension in cattle is of importance to high altitude beef production systems. Cattle can be categorized by their pulmonary arterial pressure measures (PAP) as hypertensive (HT) or normotensive (NT) for pulmonary hypertension. Genes regulating the availability and downstream utilization of intracellular calcium may be influential to cardiopulmonary maladaptation in animals with hypertension. The utilization of transcriptomics concurrently with sequence polymorphism detection provides opportunities to understand gene regulation and functional polymorphisms within a gene. The objectives of this study were to: 1) detect single nucleotide polymorphisms (SNP) in the transcriptome of 6 tissues (aorta, middle left ventricle, *longissimus dorsi* muscle, lung, pulmonary artery, and middle right ventricle), and 2) identify functional consequences of those variants within validated candidate genes (*ASIC2*, *EDN1*, *NOX4*, *PLA2G4A*, *RCAN1*, *THBS4*). We hypothesized that a minimal number of exonic SNP will be identified in the 6 candidate genes associated with regulating calcium availability and utilization in RNA-Seq data from tissue samples of Angus steers. Transcriptome (RNA-Sequencing) data were available on the 6 tissues from steers (n = 14) raised at high altitude (2,150 m). Variant detection and annotation analyses were conducted with the ARS-UCD1.2 bovine reference in CLC Genomics Workbench software (version 11.0.1). Ensembl Variant Effect Predictor was utilized to determine functional consequences of detected SNP within candidate genes. In total, 104,949 SNP were previously annotated within the 6 candidate genes. Of the 1,445 exonic SNP, 375 synonymous and 1,087 non-

synonymous SNP were identified in the Angus steers used in this study. The pooled Angus sample analysis revealed 68 SNP in the 6 candidate genes, of which 38 SNP were unique in the HT group and 8 were unique in the NT group. In the latter, a novel missense variant in *EDN1* was identified which will alter the amino acid from an alanine to serine. In the HT group, a novel nonsense mutation in *EDN1* was identified leading to a premature stopped transcript, as well as a missense mutation (rs109862098) in *NOX4* altered the amino acid from a serine to threonine. Ten of the 68 identified SNP are utilized on commercially available high-density SNP chips. Analysis of transcriptome data identified SNP within genes regulating calcium availability and utilization. These analyses enhanced our understanding of sequence polymorphisms that may be involved in regulating pulmonary hypertension in Angus cattle raised at high altitude.

INTRODUCTION

Pulmonary hypertension (PH), represented by pulmonary arterial pressures (PAP), pose problems for the beef industry (Holt and Callan, 2007). These problems arise as some cattle are unable to acclimate to altitude and physiological changes of the cardiopulmonary system (Neary et al., 2015; Pugliese et al., 2015; Ryan et al., 2015). Calcium is a key mediator of muscle cell function, specifically in cardiac contraction and relaxation mechanisms (Hasenfuss and Pieske, 2002; Stanfield, 2011). Previous studies have identified associations between sequence polymorphisms and PH in cattle (Zeng, 2016; Cockrum et al., 2019). However, minimal research has been conducted to understand the specific variants within genes regulating calcium availability and utilization. There is a lack in knowledge of the potential influence these variants may have on PH in Angus steers.

Increased utilization and implementation of '-omics' technologies has provided increased opportunity in livestock research to investigate difficult to measure traits such as disease

susceptibility. Detection of single nucleotide polymorphisms and functional consequences of these variants with transcriptome (RNA-Seq) data provide insight into translational influence on gene function. The objectives of this study were to: 1) detect single nucleotide polymorphisms (SNP) in the transcriptome of 6 tissues, and 2) identify functional consequences of those variants associated with validated candidate genes from qPCR analyses. We hypothesized that a minimal number of exonic SNP will be identified in the 6 candidate genes associated with regulating calcium availability and utilization in RNA-Seq data from tissue samples of Angus steers fed at high altitude. Results of the current study provide an informative perspective on the utilization of RNA-Seq and functional influence of variants calcium-regulated candidate genes.

MATERIALS AND METHODS

Animal Care and Use Committee approval was not obtained because data were acquired from an existing sample database (Protocol # 13-4111).

Tissues/Samples

Chapter 3 of this dissertation outlines the animal population and tissue samples available and utilized in this study. In short, cardiac muscle (n = 9) and muscle control tissues (n = 2) from yearling Angus steers (n = 20) were used.

A second set of data, also from the Colorado State University Beef Improvement Center, was utilized to determine the association between identified SNP on commercially available chips and PAP in a secondary population. The study received approval from the Colorado State University Animal Care and Use Committee prior to the sampling or handling of any animals (Protocol # 13-4136A). Angus steers (n = 65) were approximately 6 months of age when DNA was collected for genotyping. Genotypic data included a panel of 777,962 SNP (BovineHD BeadChip, Illumina, San Diego, CA) by GeneSeek (Neogen, Lincoln, NE). A subset of identified

SNP in the other data was extracted from the SNP panel for analyses. Additional data information can be found in the dissertation of Joseph Neary (2014).

RNA isolation and sequencing

For the collected tissue samples, a Trizol reagent (Invitrogen) protocol was utilized for RNA isolation. Total RNA quality was evaluated using the RNA Integrity Number (RIN) value with Experion BioRad and was converted to double stranded cDNA. Adapters and indices were added to each sample. Sequence libraries were prepared with Illumina TruSeq stranded mRNA Sample Preparation kit. Sequencing was performed HiSeq 2000 sequencer analyzer (Illumina, San Diego, CA), and generated approximately 30 million single read sequences (100 bp) for each sample (Cánovas et al., 2014; Cánovas et al., 2016). Sequence analyses were conducted with CLC Genomics Workbench software (version 11.0.1; CLC Bio, Aarhus, Denmark) with the ARS-UCD1.2 bovine reference and ARS-UCD1.2.95 annotation. Quality control measures were implemented, resulting in 68 samples for RNA-Seq. Graphical information on poor quality samples is provided in Appendix A.

Variant detection and annotation

Detection analyses were performed by pooling all samples. As described by Piskol et al. (2013), pooling of sequence reads during assembly increases the number of available reads to increase coverage (i.e., alignment to the reference genome); therefore, increasing the power of variant detection. This concept was echoed in research results of Lehne et al. (2011) and Dias et al. (2017). A Fixed Ploidy Variant Detection tool in CLC Genomics Workbench software (version 11.0.1) was implemented as the ploidy of bovine (diploid; 30 chromosomes) was known or fixed. The fixed ploidy variant caller was used to detect germline variants, discarding variants assumed to be a result of sequencing errors or mapping artifacts (Qiagen Bioinformatics). Parameters for

the pooled analyss were set as follows: ploidy: 2, variant probability: 90%, minimum coverage (reads): 20, minimum count (reads): 2, minimum variant frequency (%): 5, minimm central quality: 20, minimum neighborhood quality: 15, and relative read direction filter (%): 1.

Variants subject to detection were within validated candidate genes: Acid sensing ion channel subunit 2 (*ASIC2*), Endothelin 1 (*EDN1*), NADPH oxidase 4 (*NOX4*), Phospholipase A2 group IVA (*PLA2G4A*), Regulator of calcineurin 1 (*RCAN1*), and Thrombospondin 4 (*THBS4*; Table 6-3). Target genes were estimated as differentially expressed with RNA-Seq in ventricle tissues (Chapter 4) and subsequently validated with qPCR methodology (Chapter 5). Annotating amino acid changes and predicting the splice site effect of the variants were also determined within the CLC Genomics Workbench software.

Table 6-1. List of validated candidate genes from RNA-Seq and qPCR gene expression studies.

Gene	Name	Gene ID	Chr[1]	Location[2]	Exons[3]	Function
ASIC2	Acid sensing ion channel subunit 2	617930	19	16022575..17228963	11	Membrane ion channel; Activator of the calcineurin/NFAT signaling pathways
EDN1	Endothelin 1	281137	23	44156440..44163423	5	Vasoconstrictor
NOX4	NADPH oxidase 4	378474	29	6120515..6303004	18	Catalytic subunit the NADPH oxidase complex; Acts as an oxygen sensor
PLA2G4A	Phospholipase A2 group IVA	525072	16	67907024..68081280	19	Catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid
RCAN1	Regulator of calcineurin 1	539640	1	882073..1002231	5	Calcium/calmodulin-dependent phosphatase
THBS4	Thrombospondin 4	541281	10	11006321..11060136	22	Adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions

[1]*Bos taurus* chromosome

[2]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[3]Number of exons in the gene

Prediction of variant effects

The Variant Effect Predictor (VEP) tool, available on the Ensembl database (release 95), was utilized to determine the effect of the identified variants, in addition to identifying novel variants (McLaren et al., 2016). Important to note was that more than one functional consequence can be assigned to a variant. Therefore, SNP overlapping or concordant between the pooled analyses were evaluated to reduced false positive variant identification. A Fisher exact test was implemented to identify variants more common in case samples as compared to control samples. In our analyses, HT steers were utilized as cases, and NT steers were the controls.

SNP-chip inclusion

Detected SNP from the candidate genes were identified within commercially available SNP-chips. The goal of this effort was to determine the current utilization of these SNP within the beef industry.

Genotype differences by PAP and tissue specificity

Kruskal-Wallis analyses, comparing the mean ranks of the groups (i.e., three genotypes of a SNP), were completed to determine if differences in PAP of the 10 HT and 10 NT steers existed between genotypes of identified SNP. These were completed between a subset of identified SNP identified in both the HT and NT groups, those identified solely in the HT group, and solely in the NT group. Likewise, tissue specificity was assessed for all identified SNP.

Significant differences between genotypes were estimated for 5 of the 68 identified SNP (Figures 6-3 to 6-7). Utilizing R statistical software, the genotype-to-phenotype associations of these 5 SNP were conducted with a linear regression model of: PAP = SNP Genotype. The SNP genotypes were fit as a continuous, linear covariate for the number of B alleles (i.e., A/A = 0, A/B = 1, B/B = 2). An analysis of variance (ANOVA) test was utilized to determine significant sources

of variation of mPAP and the percent variation in PAP phenotypes explained by genotype. Heterogeneity of variance was also tested, where a skewed, bimodal distribution was observed (APPENDIX H). Violations of assumptions of normality were investigated using a Shapiro-Wilk normality test and quantile-quantile plots (APPENDIX H). A Box-Cox analysis was used to determine the most appropriate transformation for the data, yielding a power value (λ; lambda) of -0.060 for PAP (Box and Cox, 1964). The ANOVA was then repeated to determine significant sources of variation with the log transformed data.

The above analyses were repeated on supplementary genomic data available on 65 steers from the Colorado State University Beef Improvement Center (APPENDIX I). Genotypes of interest were those the 4 identified on the Illumina BovineHD BeadChip (Table 6-6), as this was accessible data for our use. Genotype was an insignificant predictor of PAP for all of the SNP of interest (P = 0.40 to 0.97). The Box-Cox analysis yielded a power value of -2.97 for PAP (Box and Cox, 1964). The ANOVA was then repeated to determine significant sources of variation with the transformed data, indicating genotype was still an insignificant predictor of PAP (P = 0.54 to 0.87). Kruskal-Wallis analyses yielded non-significant PAP differences (P = 0.46 to 0.56) between genotypes for all of the 4 SNP on the commercially available chips. Appendix I provides additional results for this subset of data.

RESULTS AND DISCUSSION

Variant detection

In the Ensembl SNP database (release 95), 104,949 SNP were previously annotated within the 6 candidate genes. Of the 1,445 exonic SNP, 375 synonymous and 1,087 non-synonymous SNP were identified. The variant detection analyses of the pooled RNA sequences revealed 68 SNP in the 6 candidate genes. Figure 6-2 displays the comparison of identified SNP between

hypertensive and normotensive steer groups. Of the 68 SNP, 38 were uniquely detected in the HT group and 8 were detected in the NT group. Thirty of the 68 SNP (44.1%) were intronic. Non-coding variants have the potential to affect phenotype of the individual through alternative splicing, as detected SNP using RNA-Sequencing reads are expected to be exonic because of intronic splicing from pre-cursor messenger RNA (pre-mRNA; Wang and Cooper, 2007). A limitation to the use of RNA-Seq data for SNP discovery is limited knowledge of SNP outside of coding regions. Whole genome sequencing can identify SNP in other regions, such as the promoter or enhancer regions, which could have a significant effect on regulation and expression of the genes of interest.

Previous RNA-Seq analyses attributed the identification of intronic SNP to accuracy of the sequence and annotation of the bovine reference. Discordances were identified between previous bovine genome assemblies (UMD 3.1 and Btau 4.6) as compared to the newest bovine assembly (ARS-UCD1.2), where the older assemblies were only approximately 60 to 80% accurate in annotating genes and their locations (Zhou et al., 2015; Utsunomiya et al., 2016). The newest bovine assembly was utilized for sequence and annotation; however, the results allude to the potential for improvement of annotated genes and their locations. An additional explanation for the large number of intronic SNP may be the efficacy of mRNA extraction. Intron-containing pre-mRNA may be captured in the isolation of mRNA through certain methods of purifying mRNA molecules, resulting in intronic SNP emerging within our variant detection methods (Piskol et al., 2013; Yousefi et al., 2018).

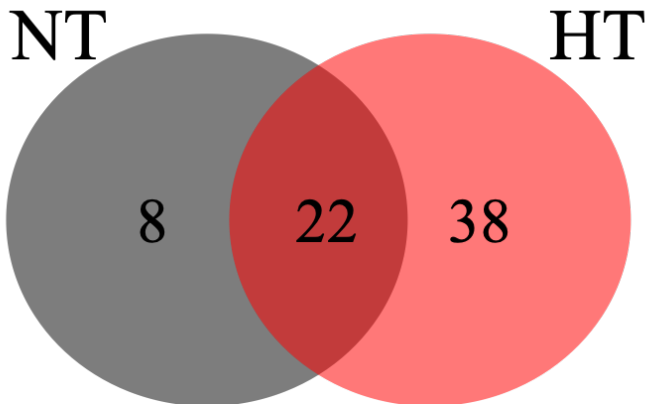

Figure 6-1. Comparison of the single nucleotide polymorphisms discovered using transcriptome (RNA-Seq) data from Angus steers using a pooled assembly approach across 6 tissues (right and left ventricle, pulmonary artery, aorta, lung, and *longissimus dorsi*) between hypertensive (HT) and normotensive (NT) steer groups.

Functional consequences

Twenty-two SNP were concordant between the two groups, where 19 SNP were previously identified variants, and 3 SNP were novel variants. Table 6-4 provides additional information on the functional consequences of the concordant SNP. The majority of the concordant SNP were synonymous (72.7%), in which the result of the nucleotide change was the same amino acid. This would imply that the variant was not a causative mutation to differentiating PH susceptibility.

An important finding in the concordant SNP was that none of the SNP had non-synonymous functional consequences. Non-synonymous SNP have the potential to influence the phenotype of interest through a change in the amino acid and protein structure (Koufariotis et al., 2014; Iso-Touru et al., 2016). The results allude to the influence of SNP, not related to differences attributed by breed or by PH physiological status, on this subset of steers. Filtering criteria was implemented to remove SNP that were the same as the reference allele (i.e., from Dominette, Line 1 inbred Hereford), making these Angus-specific SNP, and concordant SNP between both hypertensive and normotensive Angus steers.

A possible explanation for the emergence of concordant SNP in both HT and NT steers was the lack of breed diversity of the samples. As discussed in Chapter 3 on the study animal population, all samples came from Angus steers of the CSU-BIC, and in addition from the same birth year. Statistical modeling and analyses can be utilized to account for the relational ties in the data, however this was not implemented in the variant detection methods. As outlined by Bulmer (1971) and Gomez-Raya and Burnside (1990), genetic variation and genetic change is highly dependent upon the correlation between pairs of loci, where more pairs of loci limits change, in which less herd diversity will limit possible genetic change in a trait(s) of interest.

Table 6-2. Description of single nucleotide polymorphisms (SNP) in calcium-related candidate genes in association with both hypertensive and normotensive[1] Angus cattle.

	Gene		SNP				
Chr[2]	Name[3]	Location[4]	ID[4]	Position[5]	Mutation	Allele	Functional Consequence
1	*RCAN1*[6]	882081..1002223	rs110943703	1001634	T/C	C	3' Untranslated
1	*RCAN1*[6]	882081..1002223	rs109816298	1001841	C/T	T	3' Untranslated
10	*THBS4*	11005591..11060139	rs43615529	11032374	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs137156180	11032404	A/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs43616140	11036010	A/G	G	Synonymous
10	*THBS4*	11005591..11060139	rs135439748	11036019	C/A	A	Synonymous
10	*THBS4*	11005591..11060139	rs109358835	11037910	G/A	A	Synonymous
10	*THBS4*	11005591..11060139	rs109466013	11052646	A/G	G	Synonymous
10	*THBS4*	11005591..11060139	rs109630324	11052664	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs136947208	11052679	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs135163910	11052682	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs137811570	11052691	C/T	T	Synonymous
10	*THBS4*	11005591..11060139	rs110094046	11052739	C/G	G	Synonymous
10	*THBS4*	11005591..11060139	rs135687688	11052751	C/T	T	Synonymous
10	*THBS4*	11005591..11060139	rs132840500	11052760	G/A	A	Synonymous
10	*THBS4*	11005591..11060139	rs110860537	11053625	C/T	T	Splice Region; Synonymous
10	*THBS4*	11005591..11060139	rs43615501	11060032	C/T	T	3' Untranslated
16	*PLA2G4A*	67906979..68081283	rs110861016	67907075	C/A	A	5' Untranslated
16	*PLA2G4A*[6]	67906979..68081283	rs109652349	68046471	G/T	T	Synonymous
23	*EDN1*	44156426..44163955	-	44157399	C/T	A	3' Untranslated
23	*EDN1*	44156426..44163955	-	44160663	T/C	G	Intronic
23	*EDN1*	44156426..44163955	-	44161460	G/A	T	Synonymous

[1]NT = normotensive, 39.9 ± 5.6 mmHg; HT = hypertensive, 76.0 ± 21.9 mmHg; SNP identified in both groups

[2]*Bos taurus* Chromosome number

[3]*RCAN1* = Regulator of Calcineurin 1, *THBS4* = Thrombospondin 4, *PLA2G4A* = Phospholipase A2 Group IVA, *EDN1* = Endothelin 1

[4]Reference SNP cluster identification assigned by National Center for Biotechnology Information (NCBI); Missing ID refer to novel SNP

[5]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[6]Multiple transcripts associated with gene

Table 6-5 displays the 8 unique SNP identified in the pooled normotensive Angus steer samples. Variants were classified only in candidate genes *EDN1*, *PLA2G4A*, and *RCAN1*. Two of the 8 SNP were existing variants in dbSNP of cattle, while the remaining six SNP were novel variants. Of particular importance, was the novel missense variant located within candidate gene *EDN1*, which altered the amino acid from an alanine to serine. The Sorting Tolerant From Intolerant (SIFT) score (0.15) predicted the amino acid substitution effect to be tolerable in its effect on protein function (Kumar et al., 2009). This scoring technique provided through the Ensembl database was developed based upon the evolutionary conservation of amino acids within protein families, in which highly conserved regions are more intolerant to amino acid changes, and less conserved regions more tolerable to the changes (Kumar et al., 2009). Endothelin-1 (*EDN1*) secretes a potent vasoconstrictor peptide, and expression leads to an increase in intracellular calcium and activation of protein kinase C (Humbert et al., 2004). Additionally, *EDN1* gene receptors are therapeutic targets in the treatment of pulmonary arterial hypertension (Langleben et al., 1999; Montani et al., 2007). The identification of this variant in *EDN1* suggests a potential association of genetic variability of PH in Angus cattle and should be examined further.

Table 6-3. Description of single nucleotide polymorphisms (SNP) in calcium-related candidate genes in association with normotensive[1] Angus cattle.

	Gene		SNP				Functional
Chr[2]	Name[3]	Location[4]	ID[5]	Position[6]	Mutation	Allele	Consequence
1	*RCAN1*[7]	882081..1002223	rs110356008	997718	C/G	G	Intronic
1	*RCAN1*[7]	882081..1002223	-	997749	G/A	T	Intronic
16	*PLA2G4A*[7]	67906979..68081283	rs473113169	68026048	G/A	A	Synonymous
23	*EDN1*	44156426..44163955	-	44157526	G/A	T	3' UTR
23	*EDN1*	44156426..44163955	-	44160822	G/A	T	Intronic
23	*EDN1*	44156426..44163955	-	44161432	G/T	A	Missense[8]
23	*EDN1*	44156426..44163955	-	44163308	A/G	C	5' UTR
23	*EDN1*	44156426..44163955	-	44163357	C/T	A	5' UTR

[1]NT = normotensive, 39.9 ± 5.6 mmHg

[2]*Bos taurus* Chromosome number

[3]*RCAN1* = Regulator of Calcineurin 1, *PLA2G4A* = Phospholipase A2 Group IVA, *EDN1* = Endothelin 1

[4]Megabases upstream..downstream

[5]Reference SNP cluster identification assigned by National Center for Biotechnology Information (NCBI); Missing ID refer to novel SNP

[6]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[7]Multiple transcripts associated with gene

[8]Amino acid change from Alanine to a Serine

Table 6-6 presents the 38 unique SNP detected in the pooled HT Angus steer samples. Almost all of the candidate genes, with the exception of *PLA2G4A*, contained SNP. Twenty (73.7%) SNP were previously identified variants and 18 (47.4%) were novel. Sixteen novel variants were identified in the *EDN1* candidate gene, and 2 in *ASIC2*. It is thought that the identification of this novel SNP may be population dependent. Studies on the functional role of these novel variants are warranted to understand the mechanism underlying the development of PH in cattle. Additionally, an independent population must to utilized to validate novel SNP. Steps can be implemented to identify, validate, and commercialize novel SNP (Barendse, 2005; Van Eenennaam et al., 2014).

Of particular importance, was a novel stop gain or nonsense mutation in *EDN1*, and a missense mutation in *NOX4*. The nonsense mutation in *EDN1* introduced a single base change in the codon sequence leading to a premature stop and shortened transcript. Nonsense variants have high impact on the resulting gene transcript. As previously discussed, *EDN1* has known associations with PH. Zero nonsense (i.e., stop gain or stop lost) variants have been previously identified in the *EDN1* gene of *Bos taurus* cattle. If validated, the finding has the potential to be of great importance to the understanding the genetic mechanisms of PH susceptibility.

The missense mutation (rs109862098) identified in *NOX4* altered that amino acid from a serine to threonine. The SIFT score (0.19) predicted the amino acid substitution effect to be tolerable in its effect on protein function. Nicotinamide adenine dinucleotide phosphate oxidase 4 (*NOX4*) is highly expressed in cardiovascular tissues and has been associated with PH and cardiac failure (Chen et al., 2012). Overexpression of *NOX4* in vascular smooth muscle cells is commonly associated with progression of cardiovascular disease (Ellmark et al., 2005; Sturrock et al., 2007; Manea et al., 2010). Additionally, expression of *NOX4* was increased in lungs and adventitial

fibroblasts of mice and humans with PH (Mittal et al., 2007; Li et al., 2008). Evidence has been provided from thirteen difference sources identifying this variant in *Bos taurus* cattle (Ensembl, release 95).

Table 6-4. Description of single nucleotide polymorphisms (SNP) in calcium-related candidate genes in association with hypertensive[1] Angus cattle.

	Gene		SNP				Functional
Chr[2]	Name[3]	Location[4]	ID[4]	Position[5]	Mutation	Allele	Consequence
1	*RCAN1*[6]	882081..1002223	rs136779353	997375	G/T	T	Intronic
1	*RCAN1*[6]	882081..1002223	rs133085792	997387	C/T	T	Intronic
1	*RCAN1*[6]	882081..1002223	rs110964656	998301	C/T	T	Intronic
1	*RCAN1*[6]	882081..1002223	rs385707409	999581	C/T	T	Intronic
10	*THBS4*	11005591..11060139	rs379030450	11046640	C/T	T	Synonymous
10	*THBS4*	11005591..11060139	rs209570150	11046709	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs209956810	11047169	T/C	C	Synonymous
10	*THBS4*	11005591..11060139	rs209206437	11058108	C/T	T	Intronic
10	*THBS4*	11005591..11060139	rs43615493	11058202	G/A	A	Intronic
10	*THBS4*	11005591..11060139	rs133100564	11058248	G/T	T	Intronic
10	*THBS4*	11005591..11060139	rs133459953	11058484	C/A	A	Intronic
10	*THBS4*	11005591..11060139	rs43615495	11058937	T/C	C	Intronic
10	*THBS4*	11005591..11060139	rs43615496	11059073	A/G	G	Intronic
19	*ASIC2*	16022746..17228096	-	16513323	C/T	T	Intronic
19	*ASIC2*	16022746..17228096	-	16513330	T/C	C	Intronic
23	*EDN1*	44156426..44163955	-	44157052	G/A	T	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157199	C/T	A	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157327	G/A	T	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157334	T/C	G	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157445	A/G	C	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157461	T/C	G	3' Untranslated Region
23	*EDN1*	44156426..44163955	-	44157571	G/A	T	Synonymous
23	*EDN1*	44156426..44163955	-	44158325	A/G	C	Intronic
23	*EDN1*	44156426..44163955	-	44159580	C/T	A	Stop Gain
23	*EDN1*	44156426..44163955	-	44160106	T/A	T	Intronic
23	*EDN1*	44156426..44163955	-	44160245	G/A	T	Intronic
23	*EDN1*	44156426..44163955	-	44160497	A/G	C	Intronic
23	*EDN1*	44156426..44163955	-	44160605	A/G	C	Intronic

23	*EDN1*	44156426..44163955	-	44160647	G/A	T	Intronic
23	*EDN1*	44156426..44163955	-	44161179	G/C	G	Intronic
23	*EDN1*	44156426..44163955	-	44161845	A/G	C	Intronic
29	*NOX4*	6120515..6303004	rs42399155	6196214	T/C	C	Synonymous
29	*NOX4*	6120515..6303004	rs42400583	6275818	G/A	A	Synonymous
29	*NOX4*	6120515..6303004	rs134637460	6283155	G/A	A	Synonymous
29	*NOX4*	6120515..6303004	rs109862098	6300890	T/A	A	Missense
29	*NOX4*	6120515..6303004	rs42401521	6302346	A/T	T	3' Untranslated Region
29	*NOX4*	6120515..6303004	rs42401520	6302379	G/A	A	3' Untranslated Region
29	*NOX4*	6120515..6303004	rs457170496	6302890	G/A	A	3' Untranslated Region

[1]HT = hypertensive, 76.0 ± 21.9 mmHg

[2]*Bos taurus* Chromosome number

[3]*RCAN1* = Regulator of Calcineurin 1, *THBS4* = Thrombospondin 4, *ASIC2* = Acid Sensing Ion Channel Subunit 2, *EDN1* = Endothelin 1, *NOX4* = NADPH Oxidase 4

[4]Reference SNP cluster identification assigned by National Center for Biotechnology Information (NCBI); Missing ID refer to novel SNP

[5]Position according to Bos taurus ARS-UCD1.2 (Ensembl genome database); Megabases upstream..downstream

[6]Multiple transcripts associated with gene

Availability of commercial SNP-chips

Ten of the 68 SNP were identified on commercially available SNP chips (Table 6-7). Candidate genes represented were *NOX4*, *PLA2G4A*, *RCAN1*, and *THBS4*. The SNP in *NOX4* (rs42399155, rs42401520, rs134637460) had functional consequences of synonymous, 3' UTR, and synonymous, respectively. The 3-prime untranslated region (3' UTR) often contains regulatory regions that post-transcriptionally influence gene expression (Conne et al., 2000). Three-prime mutations have the potential to affect the termination codon, polyadenylation signal, and secondary structure in various disease states (Chatterjee and Pal, 2009). These three SNP were located on the Affymetrix Axiom Bovine chip, which contains 648,875 SNP probes. Each of these three SNP were identified solely in the HT samples.

Two SNP were identified in *RCAN1*. An intronic SNP (rs136779353), identified in only the HT samples, was utilized on the Illumina BovineHD BeadChip (777,962 SNP), and would in theory provide little information to transcript or protein level expression. However, as previously discussed, detected SNP using RNA-sequencing reads are expected to be exonic. Therefore, there is the potential that this SNP may be more informative to PH susceptibility from a transcript or protein level as the introns and exons in these gene may be incorrectly annotated. The second SNP (rs110943703) was a 3' untranslated region mutation identified in both hypertensive and normotensive steer samples. This SNP was utilized on 3 commercially available chips: Illumina BovineHD BeadChip, GeneSeek Genomic Profiler HD (76,879 SNP), and the GeneSeek Genomic Profiler HDv2 (139,480 SNP). Additional genomic data from 65 Angus steers was available for use from the Illumina BovineHD BeadChip, in which the 2 *RCAN1* SNP were included. Analyses were completed to determine if these SNP were associated with PAP utilizing the additional data (APPENDIX I).

From those analyses, 3 SNP within *THBS4* were identified to be within a variety of commercially available SNP chips. A synonymous SNP (rs209956810) identified in HT samples was present on the Affymetrix HD chip. The intronic SNP (rs43615495), also identified in the HT samples, was used on the Illumina BovineHD chip. Important to note is that the majority of SNP on most large commercially available chips are not exonic and of functional significance. The Affymetrix HD chip was designed from a database of 3 million validated SNP, with the expectation of covering genetically diverse populations and receiving reliable results. The SNP on these chips were chosen to be uniformly distributed across the entire genome, with an average gap size between SNP of 3.43 kilobases, providing excellent SNP density for detection in cattle. (Khatkar et al., 2007). Many SNP therefore fall within intronic or intergenic regions. This is a limiting factor in the usefulness of SNP information to associations with phenotypes. Sequencing and SNP detection strategies provide insight into the transcript (functional) level properties of genes and their association to phenotypes of interest. The third SNP identified in both the hypertensive and normotensive PAP samples, resided in the 3' untranslated region of the *THBS4* gene (rs43615501). The Illumina BovineHD and the GeneSeek Genomic Profiler HDv2 chips both contained this SNP. Analyses were completed to determine if these SNP were associated with PAP utilizing the additional genomic data on 65 Angus steers (APPENDIX I).

Two SNP located within the *PLA2G4A* gene were identified in both the hypertensive and normotensive PAP samples on the Affymetrix HD chip. The first (rs110861016) was a 5' untranslated region mutation, and the second (rs109652349) was a synonymous SNP. Five-prime untranslated regions (5' UTR) of messenger RNA influence translation through its length, thermal stability (G-C allele content), location of secondary structures, existence of multiple open reading

frames and binding sites for proteins that can repress or promote translation. These changes have the potential to cause disease under altered conditions (Chatterjee and Pal, 2009).

The non-synonymous SNP identified in *EDN1* and *NOX4* candidate genes were not on commercially available chips. Genomic (SNP) data is commonly used for genome-wide association studies. However, specific gene studies would warrant a more direct approach to associated variants to phenotypes. As demonstrated by Crawford et al., (2018), single SNP association analyses with a large sample size have the potential to provide information and validation to findings other research. It would be of benefit to increase the sample size and re-evaluate the initial findings of the current research.

Table 6-5. List of single nucleotide polymorphisms (SNP) represented on commercially available SNP chips.

Gene[1]	SNP ID[2]	SNP Name	Bovine Chip Name[3]
RCAN1	rs136779353	BovineHD0100000104	Illumina HD
RCAN1	rs110943703	BovineHD0100000106	Geneseek GPP HD; Geneseek GGP HD v.2; Illumina HD
THBS4	rs209956810	AX-18655848	Affymetrix HD
THBS4	rs43615495	BovineHD1000003711	Illumina HD
THBS4	rs43615501	BovineHD1000003713	Geneseek GGP HD v.2; Illumina HD
PLA2G4A	rs110861016	AX-21139196	Affymetrix HD
PLA2G4A	rs109652349	AX-21139735	Affymetrix HD
NOX4	rs42399155	AX-24696746	Affymetrix HD
NOX4	rs134637460	AX-24697120	Affymetrix HD
NOX4	rs42401520	AX-24697199	Affymetrix HD

[1]*NOX4* = NADPH Oxidase 4, *RCAN1* = Regulator of Calcineurin 1, *THBS4* = Thrombospondin 4
[2]Reference SNP cluster identification assigned by National Center for Biotechnology Information (NCBI)
[3]Illumina BovineHD BeadChip (777,962 SNP); GeneSeek Genomic Profiler HD (76,879 SNP); GeneSeek Genomic Profiler HDv2 (139,480 SNP); Affymetrix Axiom Bovine (648,875 SNP probes)

Genotype differences in PAP

Of the 22 identified SNP in both the HT (n = 10) and NT (n = 10) groups, the genotypes of 3 SNP (rs110943703, rs109358835, rs43615501) had differences in their mean PAP. Figures 6-3, 5-4, and 5-5 plot the distribution of PAP for each genotype of the respective SNP. In identifying concordant SNP in the HT and NT samples, this provides opportunity to determine if the mutations are diagnostic (i.e., to differentiate samples into PH groups based on their genotype).

An important aspect to discuss in both Figures 6-4 and 6-5 is the single PAP measurement representing the mean for the first genotypes. This genotype was represented in multiple tissue samples (i.e., right and left ventricle) but from the same animal (i.e., ID #2352). Therefore, the PAP value was the same. Research by Ba et al., (2018) stated that there was a reduction of diagnostic power of SNP markers if samples were unrepresented in the data. Therefore, the ability to utilize the two SNP in THBS4 (rs109358835, rs43615501) as diagnostic SNP was diminished as not all samples were represented. The data additionally suggested these were no tissue specific (TS) SNP, as the SNP were present in multiple tissues. This will be discussed further in a following section.

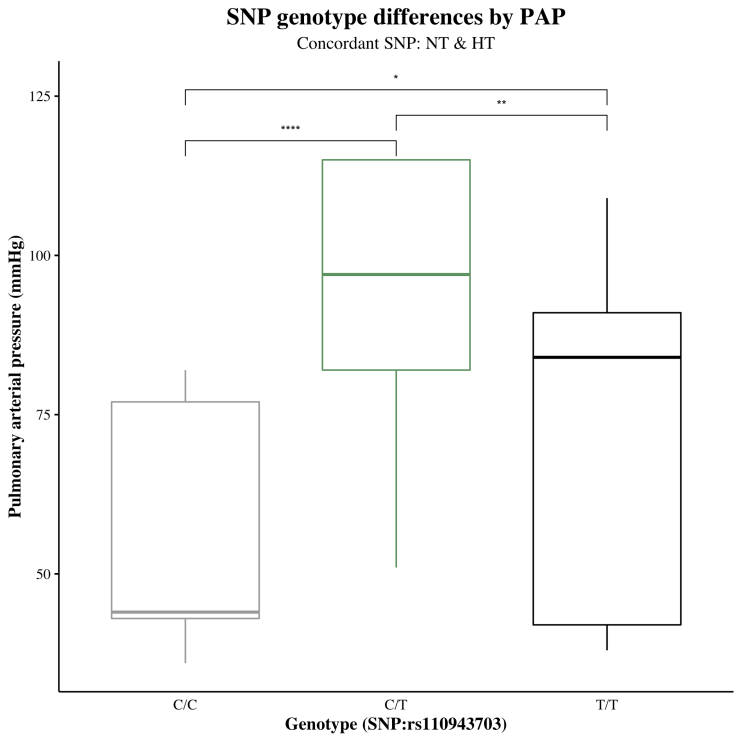

Figure 6-2. Mean pulmonary arterial pressure (PAP) differences by genotype for concordant SNP (rs110943703) in both hypertensive (HT) and normotensive (NT) steer samples. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.000$; *ns* Non-significant.

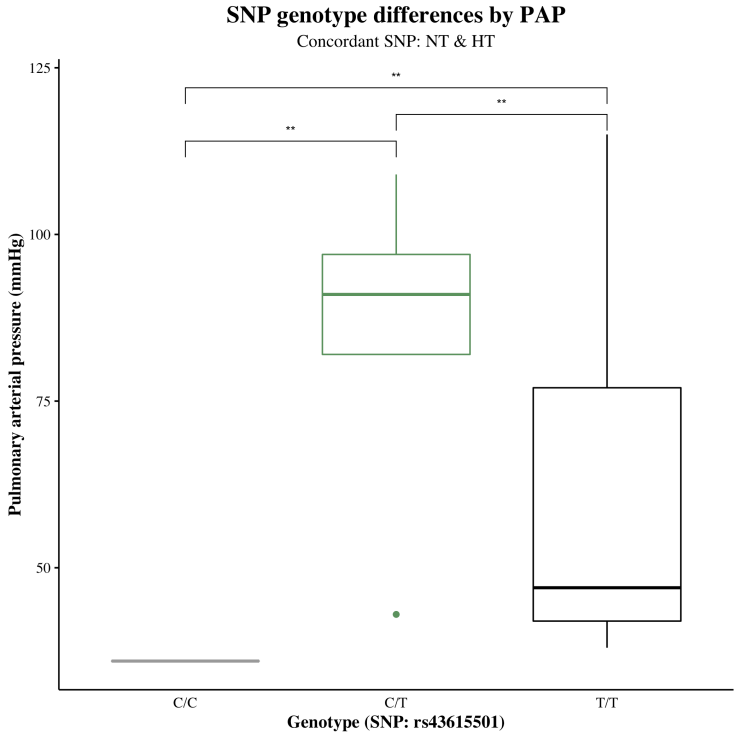

Figure 6-3. Mean pulmonary arterial pressure (PAP) differences by genotype for concordant SNP (rs43615501) in both hypertensive (HT) and normotensive (NT) steer samples. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.000$; *ns* Non-significant.

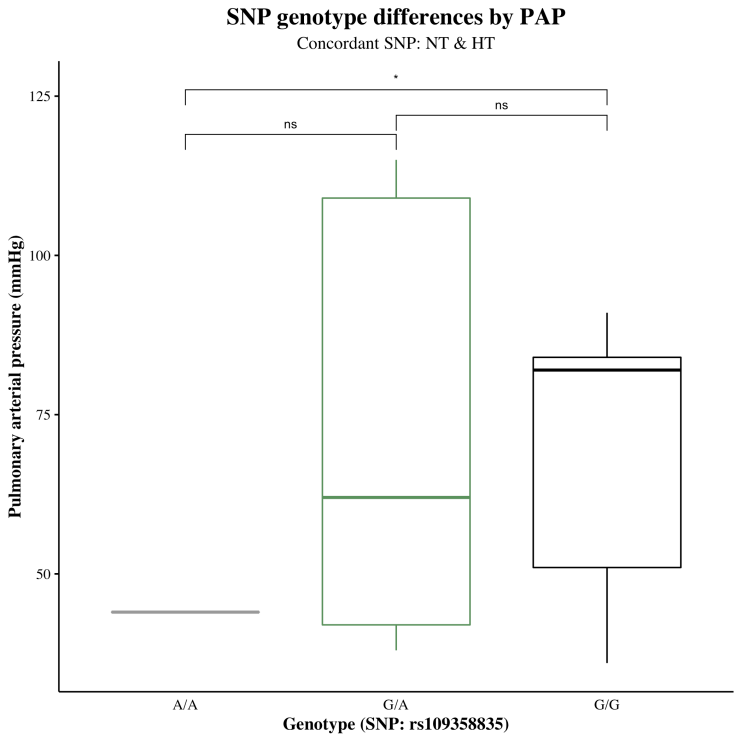

Figure 6-4. Mean pulmonary arterial pressure (PAP) differences by genotype for concordant SNP (rs109358835) in both hypertensive (HT) and normotensive (NT) steer samples. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.000$; *ns* Non-significant.

Associations were also studied with SNP identified in HT only and NT only samples. Although SNP were only identified as a variant in one group or the other, genotypes for all samples were collected. This provided opportunity to determine if the SNP were additive, in which increased PAP was associated with an increase (or decrease) in the number of a particular allele (Lewis, 2002). The additive model was previously used to understand genetic variation and gene expression in disease traits (Powell et al., 2013).

Differences in PAP were estimated in genotypes for a single SNP (rs209956810) identified in HT samples in the *THBS4* gene ($P < 0.001$). Figure 6-6 plotted the distribution of PAP by genotype for the SNP. Important to note is there were no samples identified with the C/C genotype for this SNP. For all but one of the identified SNP (rs110943703), variants were unable to be called. In the fixed ploidy variant detection method, the required variant probability parameter was

90%, meaning variants with a probability of 90% or greater would be called. The stringency of the parameter reduced the number of variants called and was likely why there were missing genotypes. Therefore, comparison of PAP could only be completed between the T/C and T/T genotypes of this SNP. These results suggested a trend of high PAP associated with the C allele of the SNP. However, additional data and study are necessary to establish if the SNP has an additive affect.

Of the other 37 identified SNP in the HT samples, no significant differences were estimated in PAP across genotypes of the 14 steers. Twenty-seven of the 38 SNP had a significant number of missing genotypes (> 55) out of the 68 available samples. As suggested, this was likely due to the high variant probability parameter, which hindered our ability to compare PAP differences across genotypes as sample size was reduced.

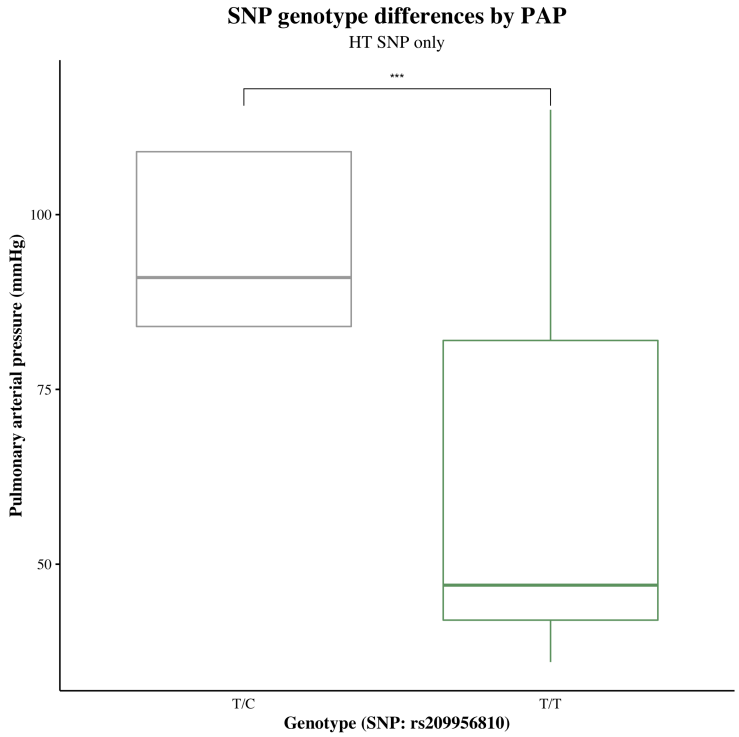

Figure 6-5. Mean pulmonary arterial pressure (PAP) differences by genotype for SNP (rs209956810) identified in hypertensive (HT) steer samples. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.000$; *ns* Non-significant

Of the 8 identified SNP in the NT samples, differences in PAP between genotypes was estimated in a single SNP (rs473113169; $P < 0.05$). Figure 6-7 plotted the distribution of PAP by genotype for the SNP. Similar to the results discussed previously, no samples had the A/A genotype for this SNP, and therefore PAP could only be compared between the G/A and G/G genotypes. These results suggested a trend of low PAP associated with the A allele of the SNP. However, additional data and study are necessary to establish if the SNP has an additive effect. Of the 8 identified SNP in the NT group, missing genotypes (> 50) for 6 SNP in the 68 samples limited our ability to estimate if associations existed with PAP in thee Angus steers.

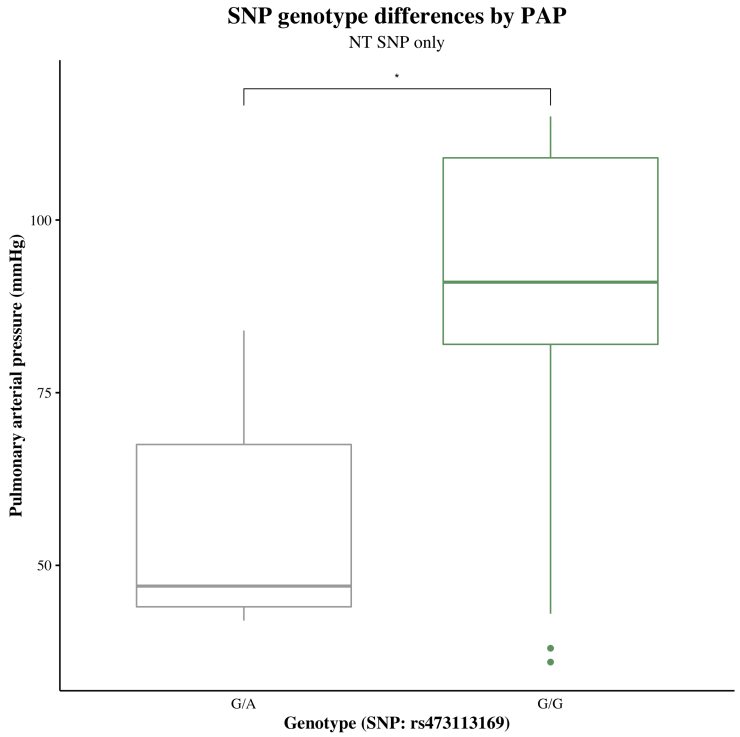

Figure 6-6. Mean pulmonary arterial pressure (PAP) differences by genotype for SNP (rs473113169) identified in normotensive (NT) steer samples. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.000$; *ns* Non-significant

Tissue-specific SNP

Venn diagrams were used to visualize tissue-specificity of the identified SNP. Figure 6-8 represents the concordant SNP from both HT and NT samples. Of the 22 concordant SNP, 2 SNP were detected in all 6 tissues (rs110943703, rs109816298). This observation would suggest that these particular SNP were not tissue specific. On the contrary, a single novel SNP the in *EDN1* candidate gene was identified only in lung tissue. This would suggest that regulation of this mutation in *EDN1* was dependent upon expression within the lung. Research examining tissue-specificity has provided insights and opportunity to expand our understanding of regulation of genes, but also understanding the phenotypic consequences of these genes (GTEx Consortium, 2015; Barbeira et al., 2018).

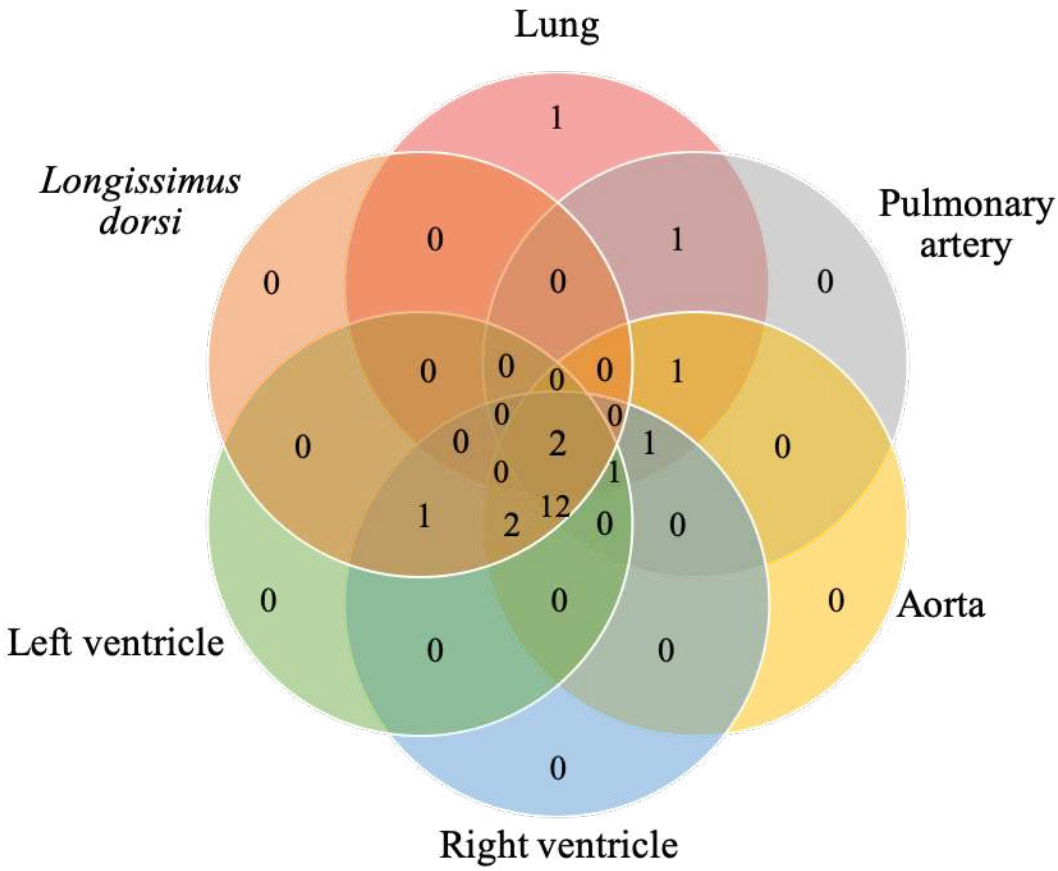

Figure 6-7. Venn diagram of the number of SNP identified in both the hypertensive and normotensive steer samples for each of the 6 tissues (aorta, left ventricle, longissimus dorsi, lung, pulmonary artery, right ventricle).

CONCLUSIONS

Cardiopulmonary transcriptome data identified 68 SNP in the 6 candidate genes. Of the 68 SNP, 38 were uniquely detected in the HT group and 8 were detected in the NT group. In total, 22 concordant SNP between Angus steers with PH and those without. The variants were located in genes associated with regulating calcium availability and utilization. Non-synonymous SNP were identified in candidate genes: *EDN1* and *NOX4*. The comparative analysis (NT vs. HT) enhanced our understanding of the sequence polymorphisms regulating pulmonary hypertension in Angus steers raised at high altitude. Additional data and analyses are necessary to determine causal SNP to enhance selection opportunities.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

The research of this dissertation transcends multiple levels of technology utilization to understand genetic regulation of pulmonary hypertension in Angus beef cattle. The use of transcriptome data to estimate differential gene expression provided insight into the association of calcium-related genes distinguishing normotensive and hypertensive steers and allowed to the identification of candidate genes for validation. Quantitative polymerase chain reaction methodologies resulted in the validation of a subset of those candidate genes in a larger cohort of Angus steers and across additional tissues. Single nucleotide polymorphism detection methods gave understanding to genetic variants differentiating normotensive and hypertensive steers. The methodology of this research can be utilized as a framework for future studies on how to conduct hypothesis-based research, where a focused question is targeted. The results of this research elucidate the importance of genes regulating the availability and utilization of calcium can be of significance to cardiac function and pulmonary hypertension susceptibility in beef cattle.

Although this research effort provides valuable insight, additional opportunities exist to enhance our understanding of the association of calcium-regulated genes to pulmonary hypertension in beef cattle. Three areas of additional study may include: 1) validation of results is subsequent populations, 2) longitudinal differences in expression, and 3) selection of candidate genes with genomic coverage. It is pertinent to validate the results of the current study in a larger population of beef cattle. The current study focused on yearling Angus steers from a single herd. However, all breeds and ages of cattle are affected by pulmonary hypertension, therefore a validation of the identified candidate genes would prove importance. Secondly, a longitudinal study would provide valuable information on how expression of either the candidate genes in the

current study, or additional genes of interest change over time. This would help to deteremine if a change in disease state or progression can be identified through expression changes. Longitudinal studies are difficult to conduct and are limited by the resources (i.e., time, finances, animals/samples) available. Lastly, an additional opportunity exists to select candidate genes that are known to have multiple SNP located on commercially available SNP chips. This could increase the likelihood of identifying variants in candidate genes associated with your phenotype and that can ultimately be used in selection strategies to delineate susceptibility to bovine pulmonary hypertension and heart failure.

APPENDIX A

POOR QUALITY RNA-SEQUENCING READS

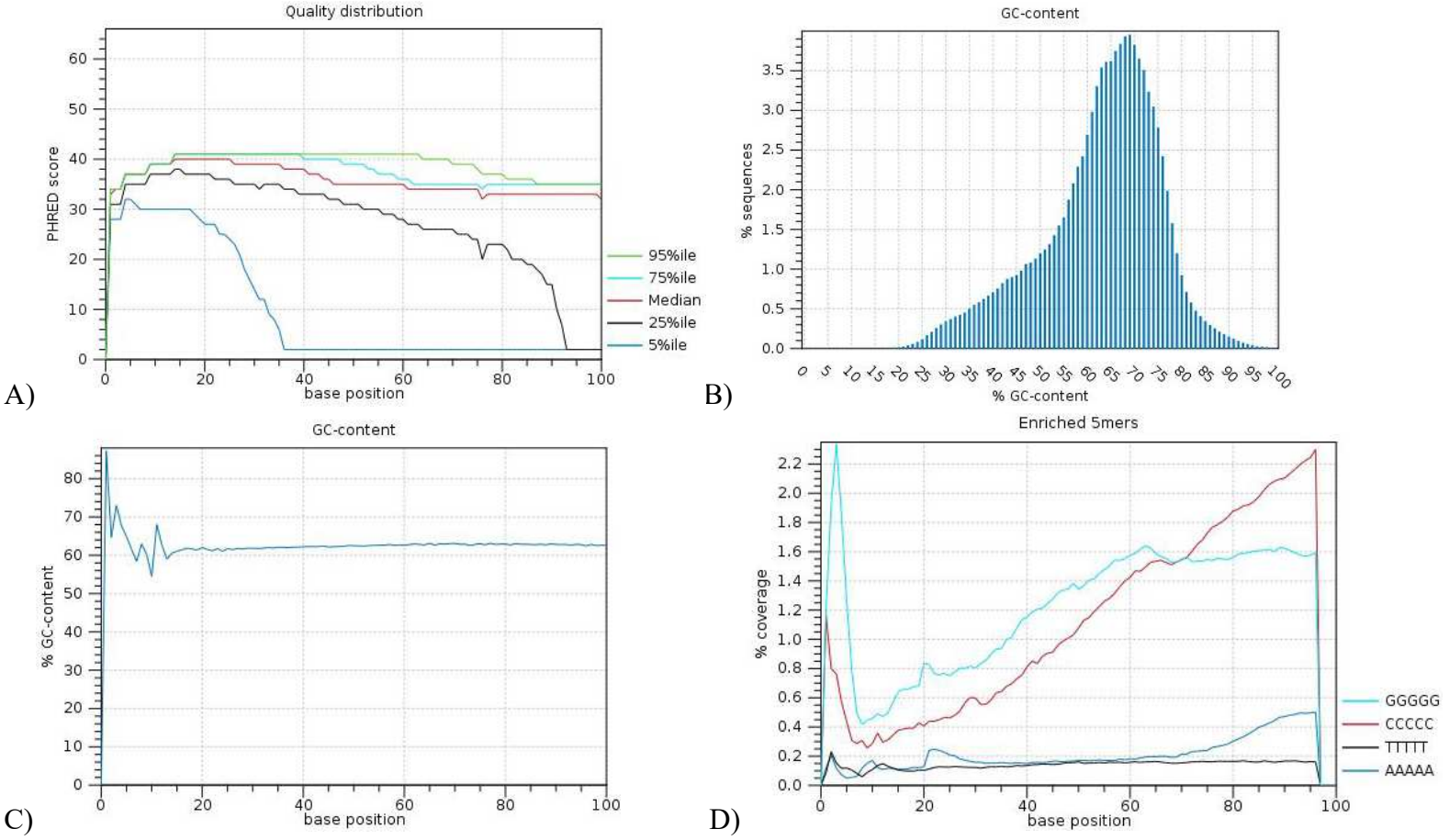
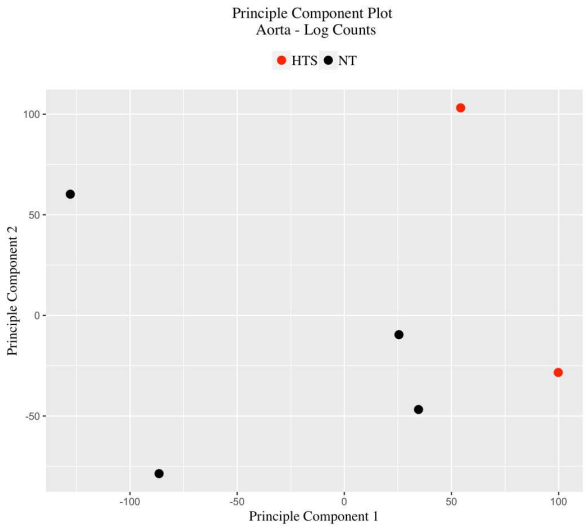


Figure A-1. Graphical output of quality control analyses on poor RNA-Seq reads of 2 samples completed in CLC Genomics Workbench software (version 11.0.1). A) Distribution of the read quality; B) Distribution of G-C content; C) Percent of G-C content; D) Plot of enriched 5-mers (stings of bases).

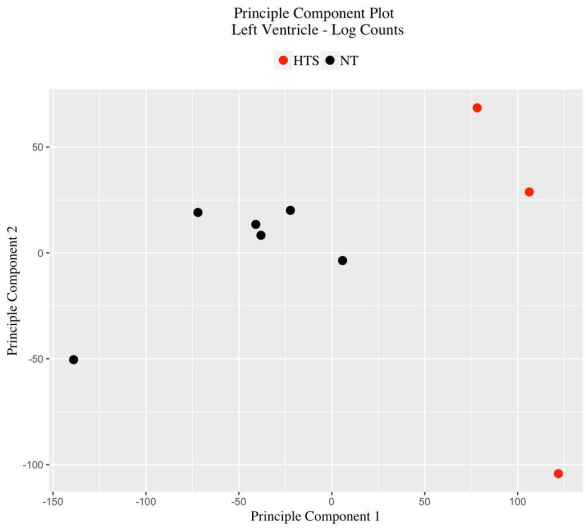
APPENDIX B

PRINCIPLE COMPONENT PLOTS OF LOG TOTAL COUNTS OF EXPRESSION BY TISSUE

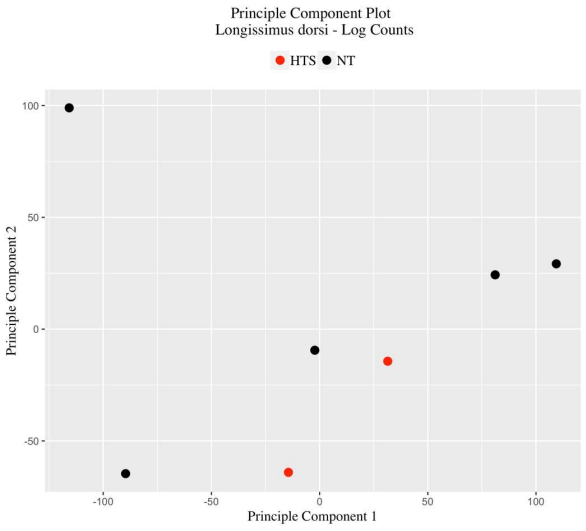
A)



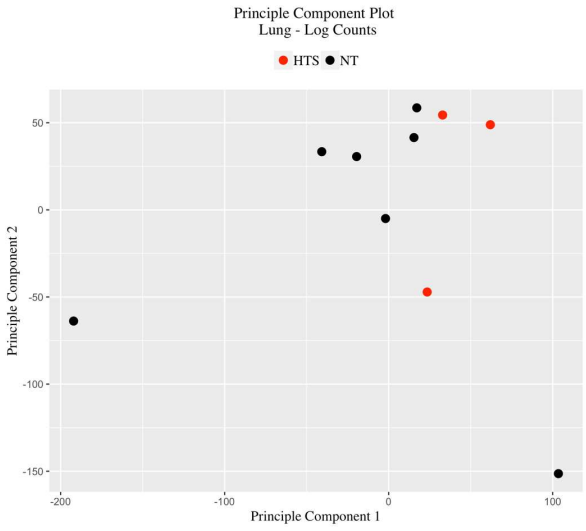
B)

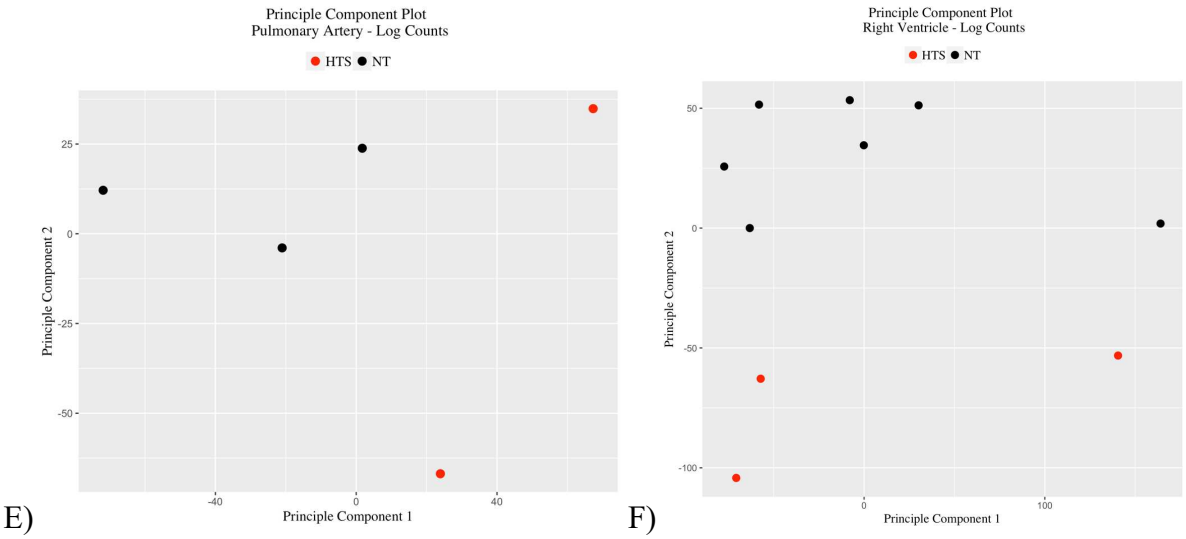


C)



D)





E) F)

Figure A-2. Principle component plots of transcriptome results (log total counts) of normotensive (NT) and hypertensive symptomatic (HTS) steer tissues. A) Aorta B) Left ventricle C) *Longissimus dorsi* D) Lung E) Pulmonary artery F) Right ventricle.

APPENDIX C

RNA ISOLATION PROTOCOL (TRI REAGENT)

-Homogenization

1. Homogenize tissue samples in TRI Reagent (1mL/50-100 mg tissue)
    - 3 times in Bead Bug for 30 seconds
    - 1 minute in ice in-between

-Phase Separation

2. Store the homogenate for 5 minutes at room temperature
3. Transfer homogenate to new 2mL tube
4. Add 200 μL chloroform per 1mL TRI Reagent
5. Vortex 15 seconds
6. Store at room temperature for 10 minutes
7. Centrifuge at 12,000 g for 15 minutes at 4^{o}C

-RNA Precipitation

8. Transfer top aqueous phase to fresh 2mL tube, save the other phases
9. Add 500 μL of isopropanol and vortex
10. Store samples at room temperature for 10 minutes
    - Put in -20^{o}C if a break is needed
11. Centrifuge at 12,000 g for 8 minutes at 4-25^{o}C

-RNA Wash

12. Remove supernatant, keep the pellet
13. Add 1mL of 75% ethanol and vortex
14. Centrifuge at 7,500 g for 5 minutes at 4-25^{o}C
15. Remove supernatant, keep the pellet
16. Add 1mL of 75% ethanol and vortex
17. Centrifuge at 7,500 g for 5 minutes at 4-25^{o}C

-RNA Solubilization

18. Remove supernatant
    - Spin down in mini-spinner to get all residual ethanol removed
19. Air dry pellet for 3-5 minutes
20. Add at least 30 μL nuclease-free water
21. Dissolve pellet
22. Incubate at 55-60^{o}C for 15 minutes (flick or vortex/spin down 2-3 times at this step)

-Check concentration/purity with NanoDrop prior to DNAse treatment

-DNAse Treatment (Ambion)

23. Add 2 μL 10X DNAse I Buffer
24. Add 1 μL rDNAse I and vortex/spin down
25. Incubate for 20 minutes at 37^{o}C
26. Add 2 μL of DNAse Inactivation Reagent
27. Be sure everything is re-suspended and mixed by vortexing
28. Incubate for 2 minutes at room temperature
29. Centrifuge at 10,000 g for 1.5 minutes
30. Transfer clear liquid to a new 2mL tube

-Check concentration/purity with NanoDrop again

APPENDIX D

PRIMER EFFICIENCY AMPLIFICATION AND STANDARD CURVES

Reference genes

Eight common endogenous control genes were utilized to establish the most stable to utilize for the gene expression analysis. All genes have been previously utilized in gene expression studies involving bovine (Robinson et al., 2007; Lisowski et al., 2008; Pérezet al., 2008). Slope of the standard curve, R^2, and amplification efficiency are provided in Table A-1. In the case where serial dilutions were repeated (i.e., 10x, 5x, 2x), the table displays the best values of those repeated attempts. Ideally, standard curves for every gene would include a slope of -3.32, an R^2 of 1.0, and an efficiency of 100%. Reference genes *HMBS*, *HPRT1,* and *RPLP0* had notable high amplification efficiencies. This, in concordance with their amplification and standard curves, signified that these control genes were lowly expressed in our tissues of interest (Figures A-8 to A-10). We therefore eliminated them as endogenous control options for gene expression. Similarly, the amplification efficiency of *GAPDH* was lower than the desired efficiency of 90 to 110% and was eliminated as an endogenous control option. One reason for the poor amplification of these genes could be attributed to their design. As explained, all primer sets were designed based upon a set of criteria. There was the potential to optimize by re-designing the primers; however, the objective was to establish the single most stable reference to utilize, and re-design was unnecessary.

Table A-1. Estimates from primer efficiency testing of reference genes (endogenous controls).

Gene[1]	Slope of inhibition curve	R^2	PCR amplification efficiency (%)
18S rRNA	-3.424	0.974	95.894
ACTB	-3.119	0.976	109.221
B2M	-3.363	0.975	98.302
GAPDH	-4.034	0.971	76.972
HMBS[2]	-2.178	0.969	187.789[3]
HPRT1[2]	-1.826	0.951	252.797[3]
RPLP0[2]	-1.909	0.998	234.047[3]
RPS9	-3.322	0.967	99.980

[1] 18S rRNA = Ribosomal RNA 18 subunit; *ACTB* = Actin Beta; B2M = Beta-2-Microglobulin; *GAPDH* = Glyceraldehyde-3-Phosphate Dehydrogenase; *HMBS* = Hydroxymethylbilane Synthase; *HPRT1* = Hypoxanthine Phosphoribosyltransferase 1; *RPLP0* = Ribosomal Protein Lateral Stalk Subunit P0; *RPS9* = Ribosomal Protein S9
[2] Gene primer was lowly expressed in all serial dilutions (10x, 5x, 2x), and was therefore not used in further analyses.
[3] Gene primer had poor efficiency for all serial dilutions (10x, 5x, 2x). Values reflect the best efficiency (serial dilution 5x).

The 18s rRNA gene primer had good overall efficiency values. However, upon examining the amplification data for the gene, it amplified too quickly in comparison to the other reference genes, as well as the candidate genes. This is problematic as an important aspect in choosing the best reference is that the product amplifies near the same time (cycle number) as the candidate genes. Therefore, the 18s rRNA reference was eliminated as well. The remaining reference genes (*ACTB*, *B2M*, and *RPS9*) were utilized in qPCR and tested for stability across all tissues.

To estimate gene expression differences in the candidate genes, a single endogenous control is typically chosen. The geNorm software by Qbase$^+$ provided stability (M) and coefficient of variation (CV) estimates for the three tested reference genes. Table A-2 and Figure A-3 display these values, where the gene with the lowest M and CV values was most stable. The reference

ACTB was therefore established to be the most stable reference and was subsequently used to estimate expression differences in the candidate genes.

Table A-2. Estimates of the stability (M) and coefficient of variation (CV) of three reference genes using Qbase^{+} software.

Gene[1]	M	CV
ACTB	0.230	0.062
B2M	0.307	0.121
RPS9	0.297	0.149

[1]*ACTB* = Actin beta; *B2M* = Beta-2-Microglobulin; *RPS9* = Ribosomal Protein S9

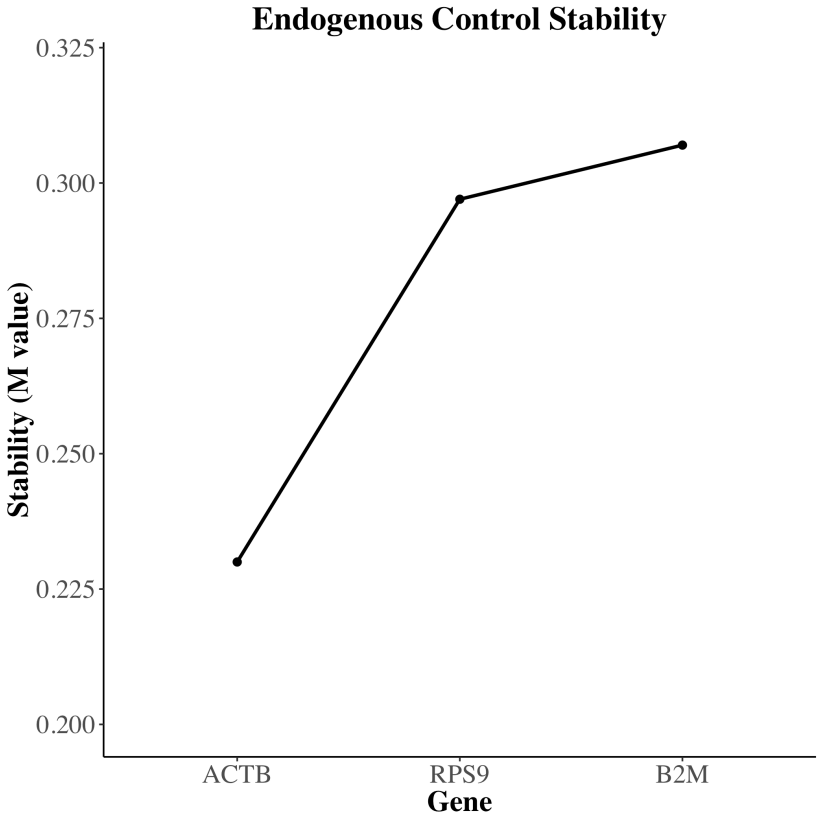


Figure A-3. Graph of calculated stability values (M) associated with reference genes ACTB, B2M, and RPS9. Good reference genes have an M < 0.5. Most stable reference with the lowest M value.

Candidate genes

Amplification and efficiency of all but one candidate genes were sufficient (Table A-3). We were unable to calculate efficiency above the acceptable values for the candidate gene *P2RY6*. The amplification and standard curves present additional concerns regarding expression and efficiency. The gene was lowly expressed across the pool of tissues, amplifying at later PCR cycles then many of the other candidate genes. Additionally, it was difficult to differentiate expression in serial dilations. These issues may be attributed to low quality RNA (cDNA) for the reaction or poor primer design (ThermoFisher Scientific, 2015). As mentioned, a gene maximization plate arrangement strategy was implemented, where the goal was to maximize the number of genes that would fit on a single plate of all samples. We were therefore limited to a total of 12 genes per plate, including the three reference genes. This plate arrangement was implemented to remove inter-run variation (Appendix E). For these reasons, we chose to eliminate the use of the P2RY6 candidate gene from subsequent qPCR analyses.

In addition to the P2YR6 gene, candidate gene KCNMA1 also had low expression across the pool of tissues, amplifying at later PCR cycles then many of the other genes. This gene was previously identified in a genome wide association study as associated with PH (results unpublished; D. Brown). Although expression was low, we chose to include the gene to validate not only the RNA-seq expression, but also the results of previous association analyses.

Table A-3. Estimates from primer efficiency testing of candidate genes used for mRNA expression analysis.

Gene[1]	Slope of inhibition curve	R^2	PCR amplification efficiency (%)
ASIC2	-3.141	0.988	108.157
EDN1	-3.262	0.984	102.585
FBN1	-3.24	0.962	103.550
KCNMA1	-3.292	0.990	101.256
NOX4	3.134	0.986	108.498
P2RY6[2]	-2.543	0.990	147.307 [3]
PLA2G4A	-3.348	0.979	98.903
RCAN1	-3.427	0.970	95.810
RGS4	-3.203	0.982	105.205
THBS4	-3.389	0.975	97.271

[1] *ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *P2RY6* = Pyrimidinergic Receptor P2Y6; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1*= Regulator Of Calcineurin 1; *RGS4* = Regulator Of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[2] Gene primer was lowly expressed in all serial dilutions (10x, 5x, 2x), and was therefore not used in further analyses.

[3] Gene primer had poor efficiency for all serial dilutions (10x, 5x, 2x). Values reflect the best efficiency (serial dilution 5x).

Below are the amplification and standard curve graphs for each of the reference and candidate genes. Tables A-1 and A-3 provide a synopsis of the information presented in the graphs. The amplification and standard curves for reference genes *GAPDH*, *HMBS*, *HPRT1*, and *RPLP0* (Figures A-7 to A-10). Efficiencies for each of these reference genes were outside acceptable range (i.e., < 90 or > 110%). In the case where serial dilutions were repeated (i.e., 10x, 5x, 2x), the table displays the best values of those repeated attempts. The amplification and standard curves of the candidate genes are also provided (Figures A-12 to A-21).

Of particular interest was the amplification and standard curves of genes *KCNMA1* and *P2RY6* (Figures A-15 & A-17). Although efficiency and other curve characteristics were acceptable, the gene product had very low expression and therefore amplified much later in the

qPCR cycle process. This gene was of particular interest as it not only fit within the hypothesis of this study but was previously associated with PH in beef cattle (results unpublished; D. Brown). We chose to utilize this gene in our expression analyses in hopes to delineate if expression could be validated. For the candidate gene P2RY6, all characteristics of the curve (i.e., slope, R^2, and efficiency) were outside the acceptable ranges. It was additionally difficult to differentiate the serial dilutions, as shown in the amplification graph. One reason for the poor amplification could be the design of the primer. It is possible that the primer was not specific enough to capture the gene product of interest. For these reasons, P2RY6 was not utilized to estimate gene expression.

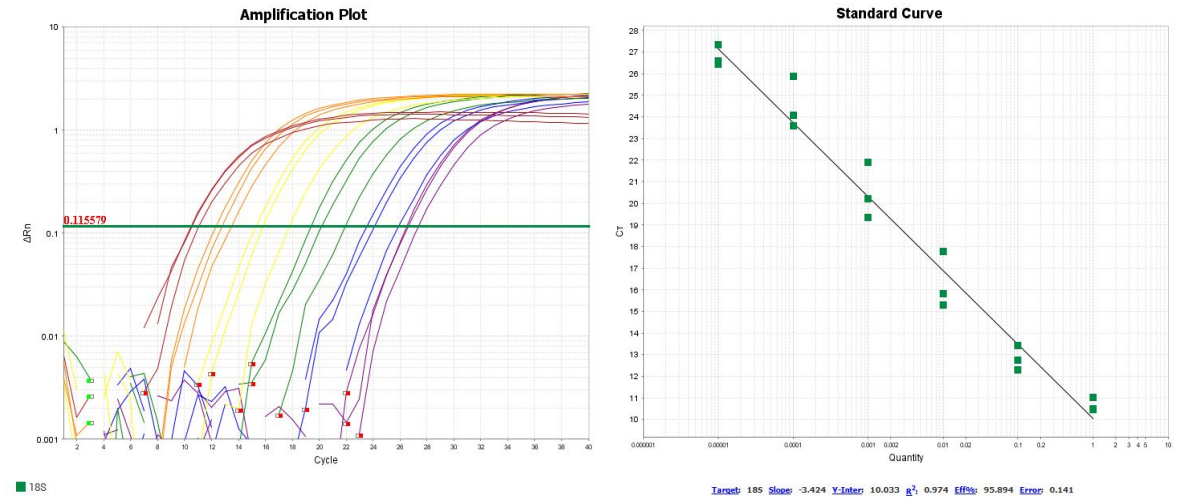


Figure A-4. Reference: *18S rRNA*

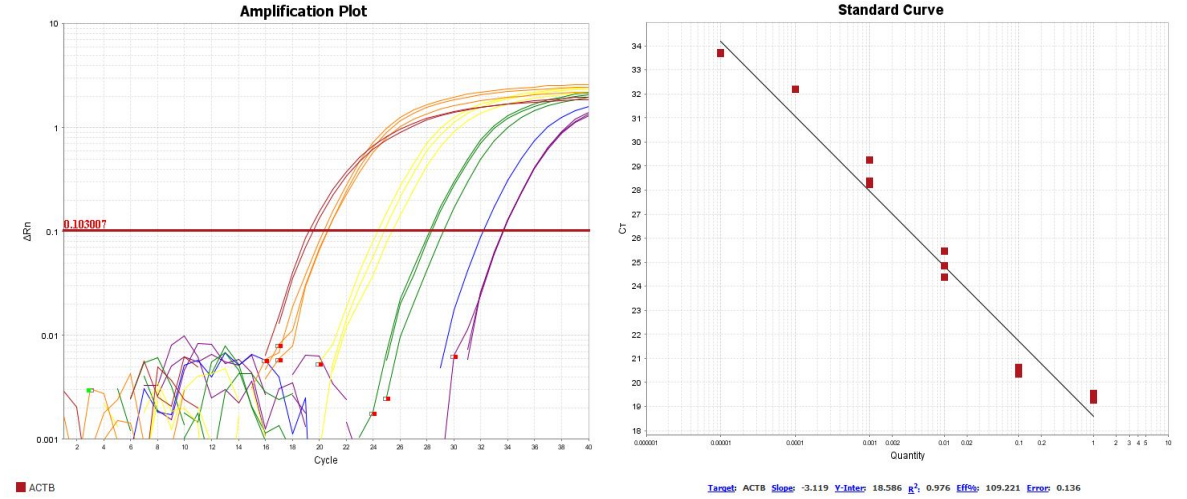


Figure A-5. Reference: *ACTB*

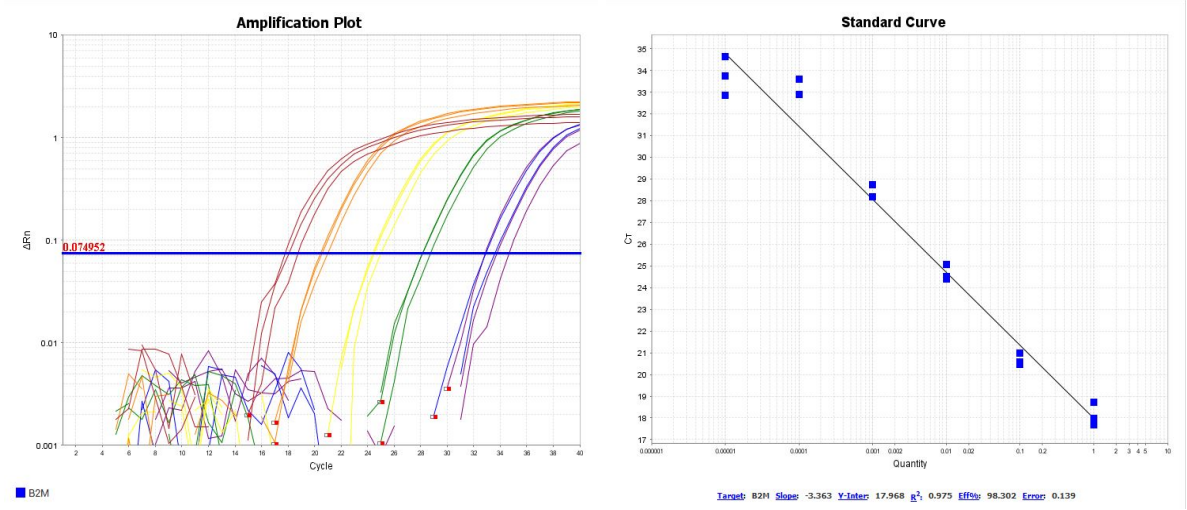


Figure A-6. Reference: *B2M*

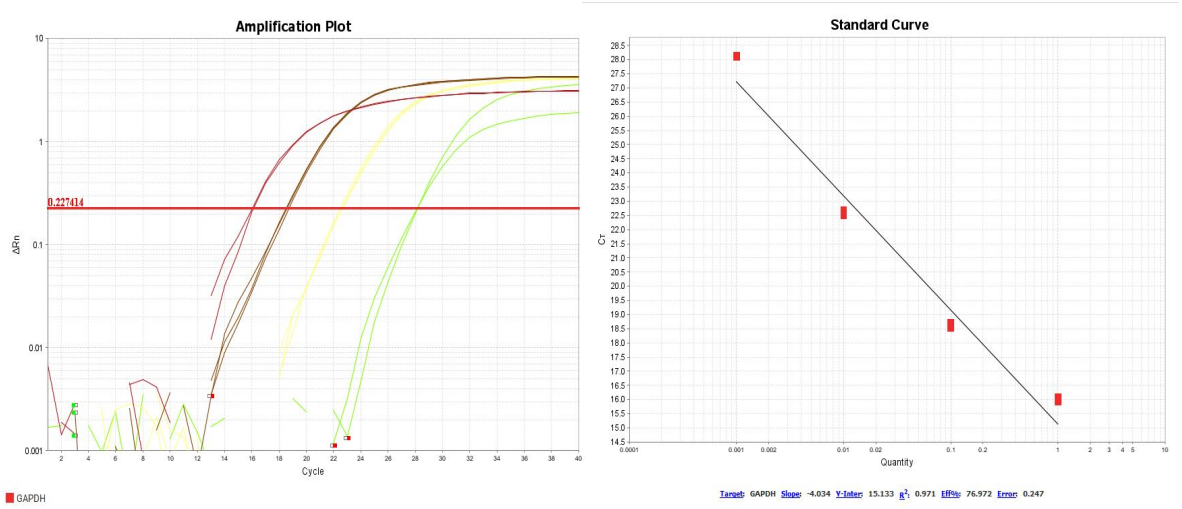


Figure A-7. Reference: *GAPDH*

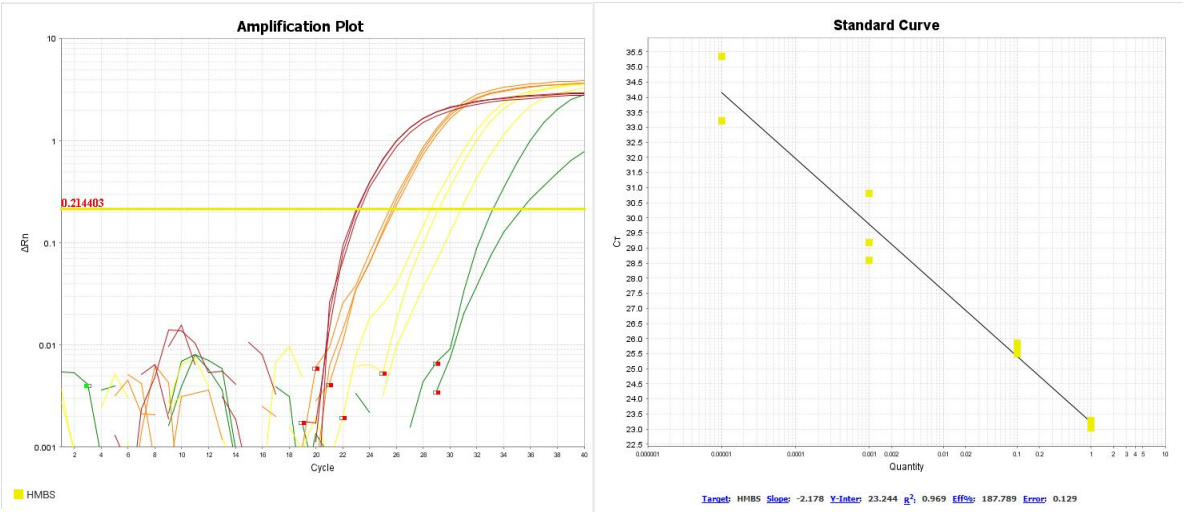


Figure A-8. Reference: *HMBS*

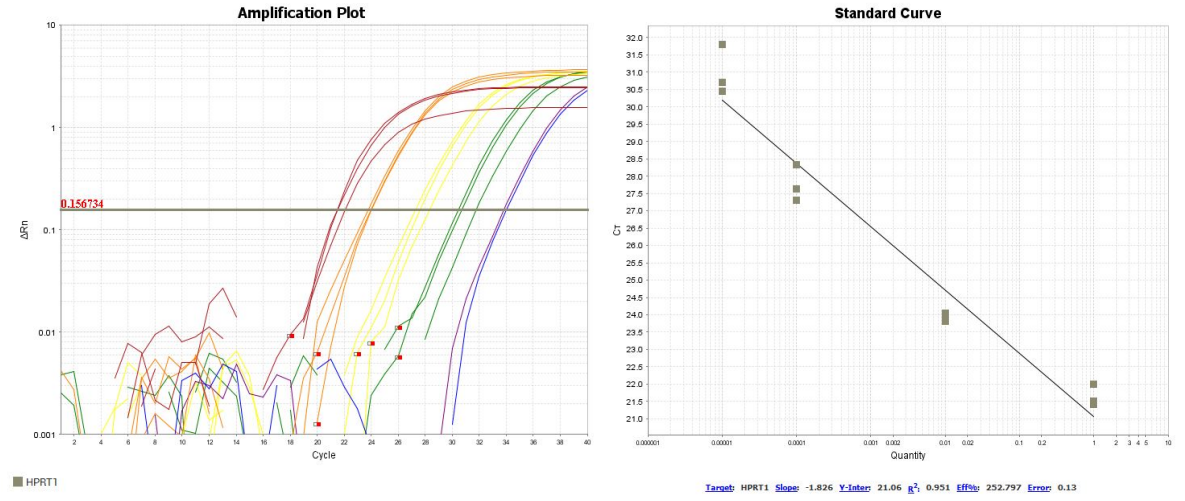


Figure A-9. Reference: *HPRT1*

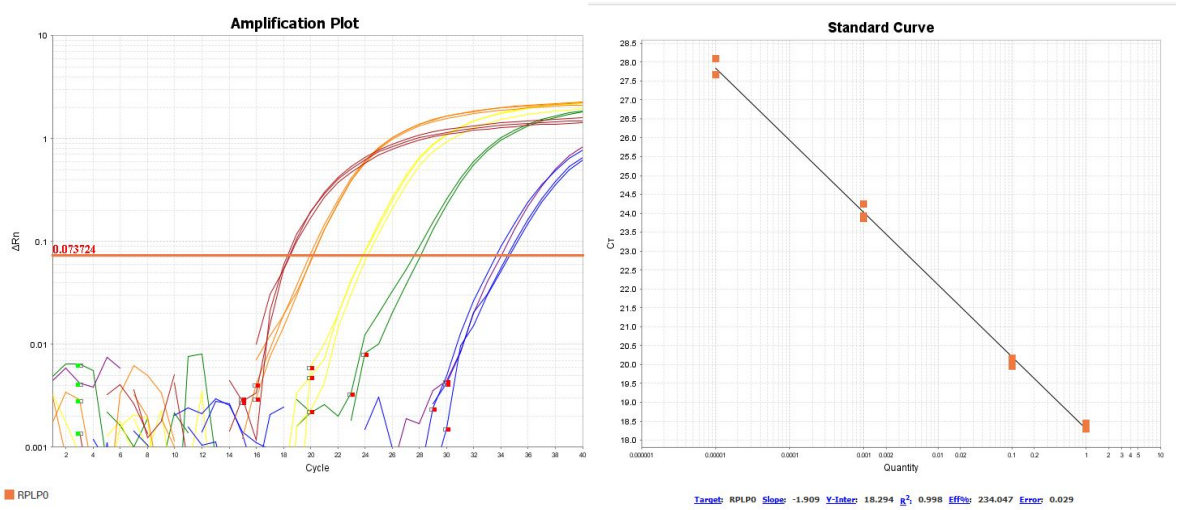


Figure A-10. Reference: *RPLP0*

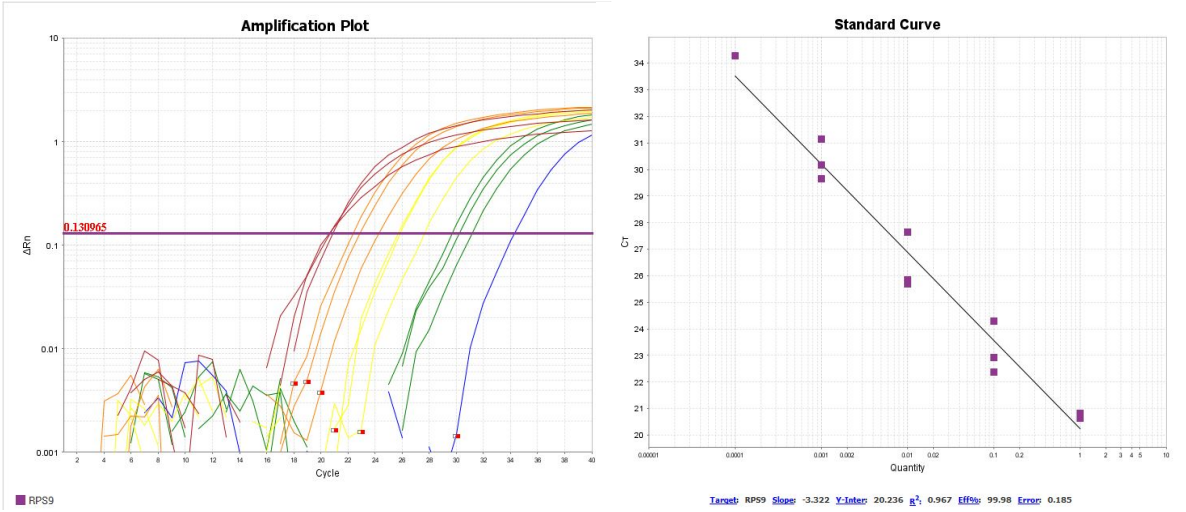


Figure A-11. Reference: *RPS9*

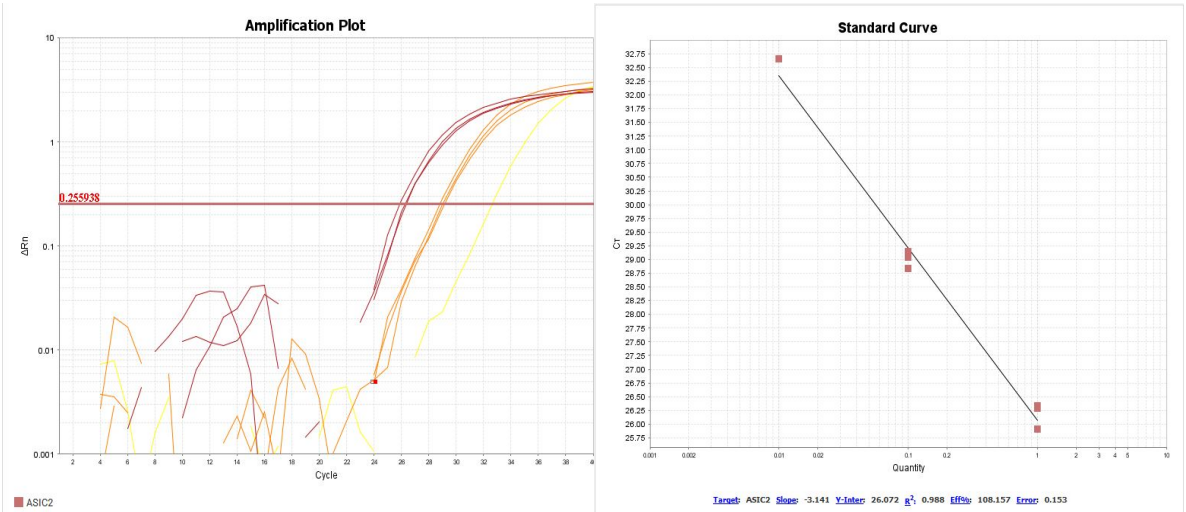


Figure A-12. Candidate: *ASIC2*

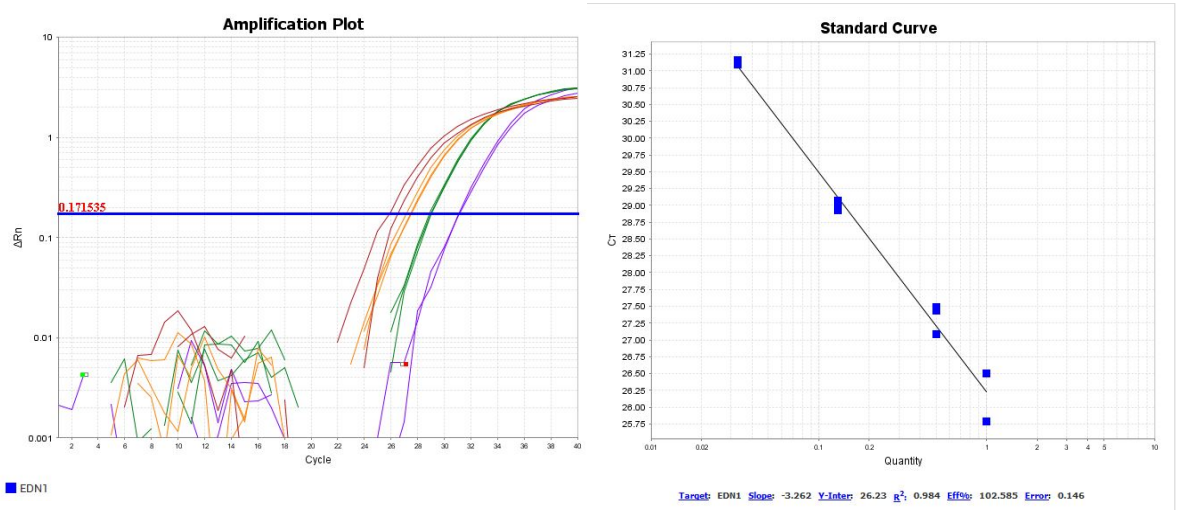


Figure A-13. Candidate: *EDN1*

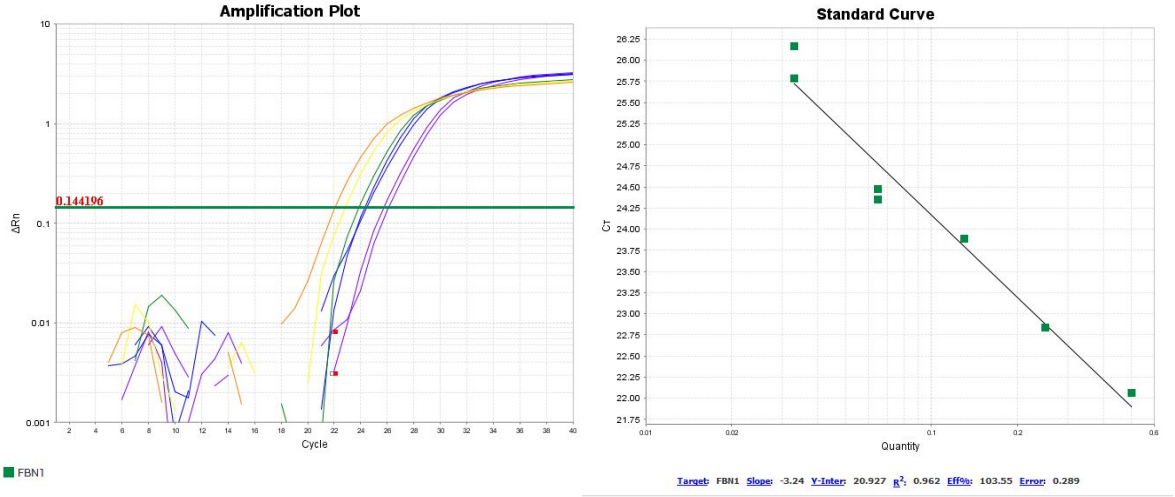


Figure A-14. Candidate: *FBN1*

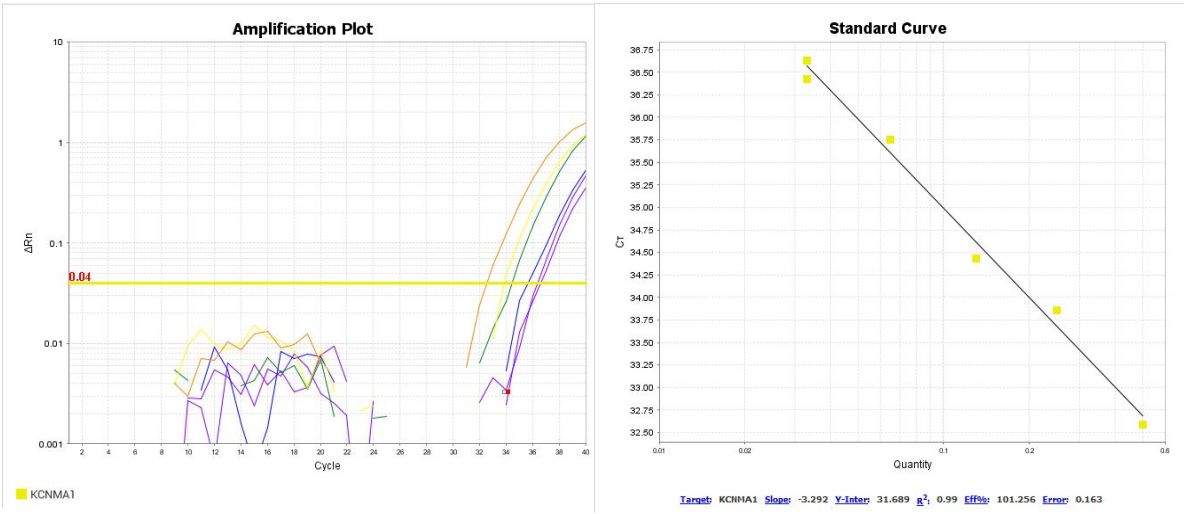


Figure A-15. Candidate: *KCNMA1*

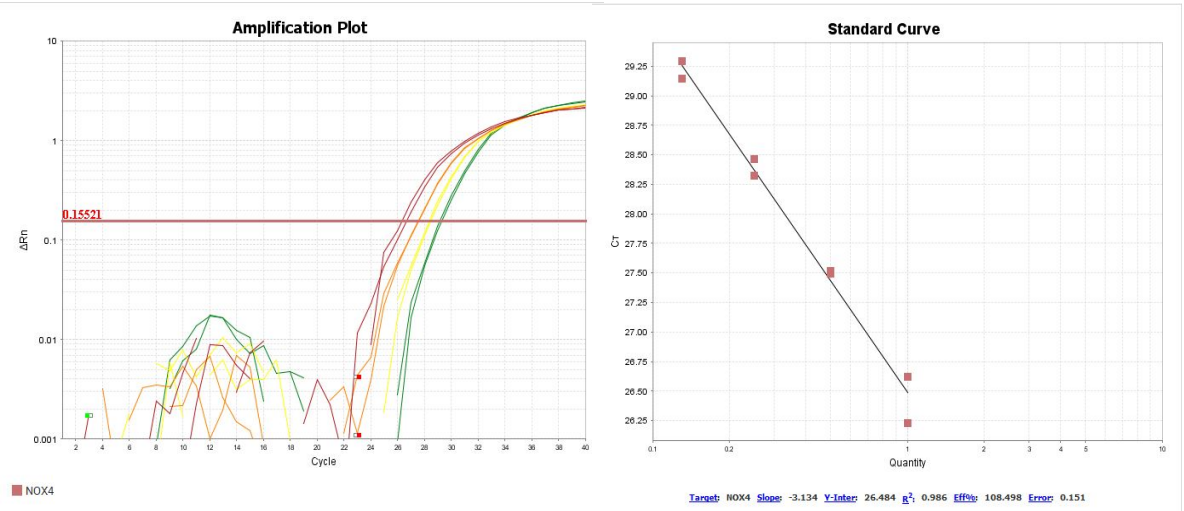


Figure A-16. Candidate: *NOX4*

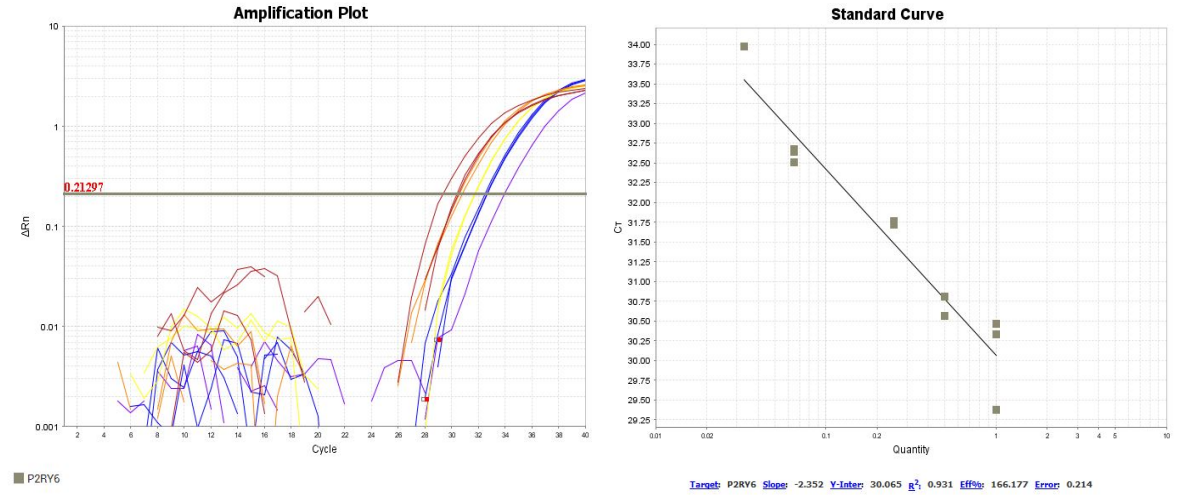


Figure A-17. Candidate: *P2RY6*

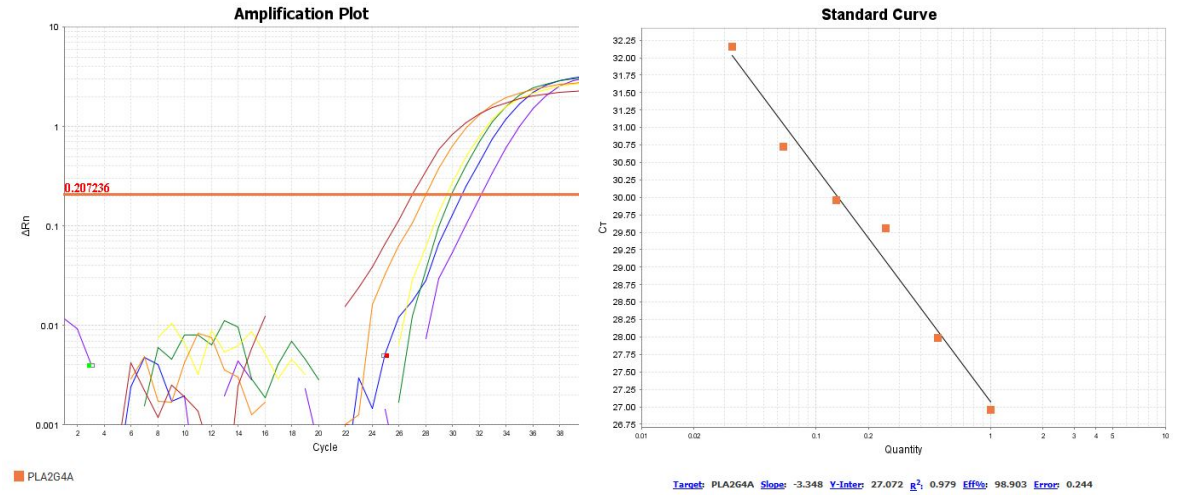


Figure A-18. Candidate: *PLA2G4A*

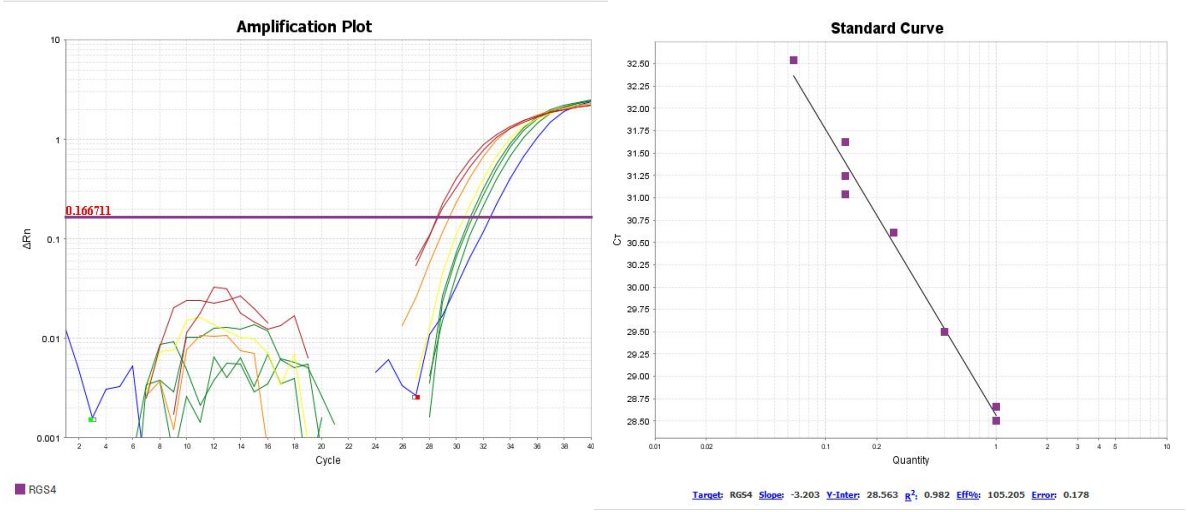


Figure A-19. Candidate: *RGS4*

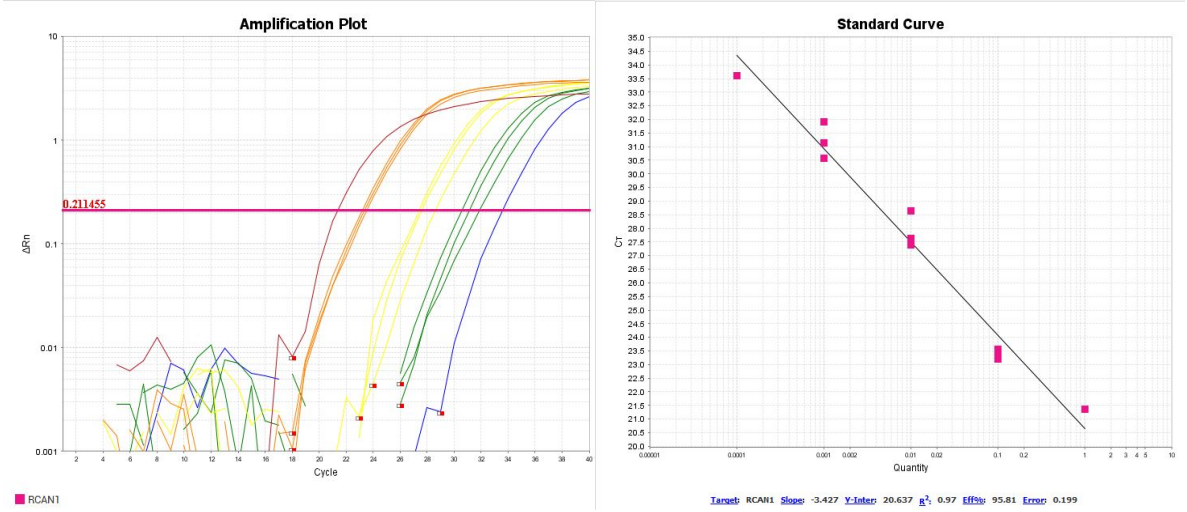


Figure A-20. Candidate: *RCAN1*

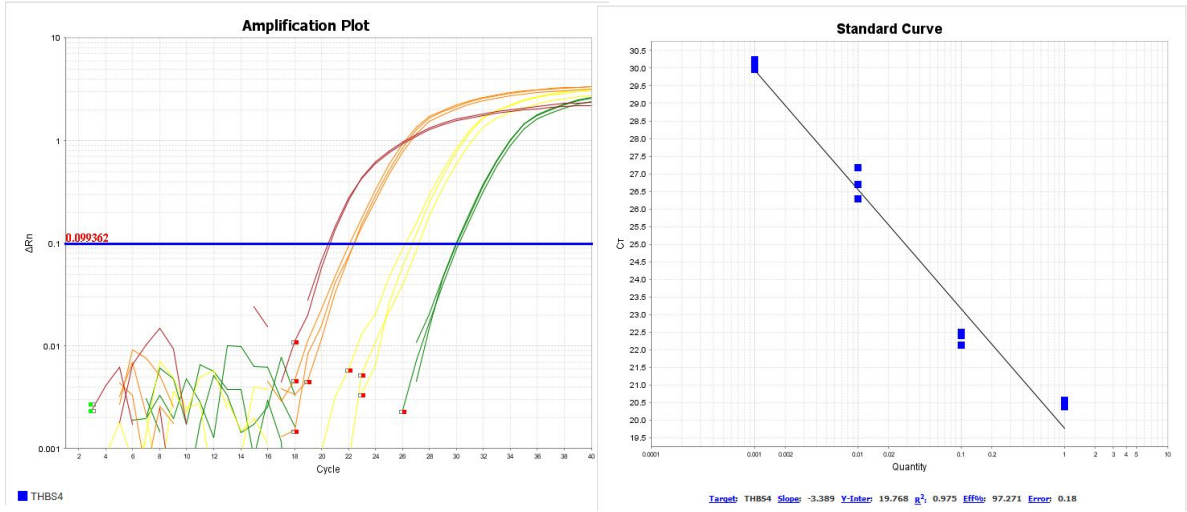


Figure A-21. Candidate: *THBS4*

APPENDIX E

384-WELL PLATE ARRANGEMENT FOR QPCR

Tissue: Right Ventricle Bottom

384-Well Plate #1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	ASIC2_1	ASIC2_1	ASIC2_1	ASIC2_2	ASIC2_2	ASIC2_2	ASIC2_3	ASIC2_3	ASIC2_3	ASIC2_4	ASIC2_4	ASIC2_4	ASIC2_5	ASIC2_5	ASIC2_5	ASIC2_6	ASIC2_6	ASIC2_6	ASIC2_7	ASIC2_7	ASIC2_7	ASIC2_8	ASIC2_8	ASIC2_8
B	B2M_1	B2M_1	B2M_1	B2M_2	B2M_2	B2M_2	B2M_3	B2M_3	B2M_3	B2M_4	B2M_4	B2M_4	B2M_5	B2M_5	B2M_5	B2M_6	B2M_6	B2M_6	B2M_7	B2M_7	B2M_7	B2M_8	B2M_8	B2M_8
C	EDN1_1	EDN1_1	EDN1_1	EDN1_2	EDN1_2	EDN1_2	EDN1_3	EDN1_3	EDN1_3	EDN1_4	EDN1_4	EDN1_4	EDN1_5	EDN1_5	EDN1_5	EDN1_6	EDN1_6	EDN1_6	EDN1_7	EDN1_7	EDN1_7	EDN1_8	EDN1_8	EDN1_8
D	PLA2G4A_1	PLA2G4A_1	PLA2G4A_1	PLA2G4A_2	PLA2G4A_2	PLA2G4A_2	PLA2G4A_3	PLA2G4A_3	PLA2G4A_3	PLA2G4A_4	PLA2G4A_4	PLA2G4A_4	PLA2G4A_5	PLA2G4A_5	PLA2G4A_5	PLA2G4A_6	PLA2G4A_6	PLA2G4A_6	PLA2G4A_7	PLA2G4A_7	PLA2G4A_7	PLA2G4A_8	PLA2G4A_8	PLA2G4A_8
E	FBN1_1	FBN1_1	FBN1_1	FBN1_2	FBN1_2	FBN1_2	FBN1_3	FBN1_3	FBN1_3	FBN1_4	FBN1_4	FBN1_4	FBN1_5	FBN1_5	FBN1_5	FBN1_6	FBN1_6	FBN1_6	FBN1_7	FBN1_7	FBN1_7	FBN1_8	FBN1_8	FBN1_8
F	RCAN1_1	RCAN1_1	RCAN1_1	RCAN1_2	RCAN1_2	RCAN1_2	RCAN1_3	RCAN1_3	RCAN1_3	RCAN1_4	RCAN1_4	RCAN1_4	RCAN1_5	RCAN1_5	RCAN1_5	RCAN1_6	RCAN1_6	RCAN1_6	RCAN1_7	RCAN1_7	RCAN1_7	RCAN1_8	RCAN1_8	RCAN1_8
G	ACTB_1	ACTB_1	ACTB_1	ACTB_2	ACTB_2	ACTB_2	ACTB_3	ACTB_3	ACTB_3	ACTB_4	ACTB_4	ACTB_4	ACTB_5	ACTB_5	ACTB_5	ACTB_6	ACTB_6	ACTB_6	ACTB_7	ACTB_7	ACTB_7	ACTB_8	ACTB_8	ACTB_8
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I	KCNMA1_1	KCNMA1_1	KCNMA1_1	KCNMA1_2	KCNMA1_2	KCNMA1_2	KCNMA1_3	KCNMA1_3	KCNMA1_3	KCNMA1_4	KCNMA1_4	KCNMA1_4	KCNMA1_5	KCNMA1_5	KCNMA1_5	KCNMA1_6	KCNMA1_6	KCNMA1_6	KCNMA1_7	KCNMA1_7	KCNMA1_7	KCNMA1_8	KCNMA1_8	KCNMA1_8
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M	ASIC2_9	ASIC2_9	ASIC2_9	KCNMA1_9	KCNMA1_9	KCNMA1_9	RGS4_9	RGS4_9	RGS4_9	ASIC2_10	ASIC2_10	ASIC2_10	KCNMA1_10	KCNMA1_10	KCNMA1_10	RGS4_10	RGS4_10	RGS4_10	NTC					
N	FBN1_9	FBN1_9	FBN1_9	PLA2G4A_9	PLA2G4A_9	PLA2G4A_9	RPS9_9	RPS9_9	RPS9_9	FBN1_10	FBN1_10	FBN1_10	PLA2G4A_10	PLA2G4A_10	PLA2G4A_10	RPS9_10	RPS9_10	RPS9_10	NTC					
O	EDN1_9	EDN1_9	EDN1_9	NOX4_9	NOX4_9	NOX4_9	THBS4_9	THBS4_9	THBS4_9	EDN1_10	EDN1_10	EDN1_10	NOX4_10	NOX4_10	NOX4_10	THBS4_10	THBS4_10	THBS4_10	NTC					
P	ACTB_9	ACTB_9	ACTB_9	RCAN1_9	RCAN1_9	RCAN1_9	B2M_9	B2M_9	B2M_9	ACTB_10	ACTB_10	ACTB_10	RCAN1_10	RCAN1_10	RCAN1_10	B2M_10	B2M_10	B2M_10	NTC					

384-Well Plate #2

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B	B2M_11	B2M_11	B2M_11	B2M_12	B2M_12	B2M_12	B2M_13	B2M_13	B2M_13	B2M_14	B2M_14	B2M_14	B2M_15	B2M_15	B2M_15	B2M_16	B2M_16	B2M_16	B2M_17	B2M_17	B2M_17	B2M_18	B2M_18	B2M_18
C	EDN1_11	EDN1_11	EDN1_11	EDN1_12	EDN1_12	EDN1_12	EDN1_13	EDN1_13	EDN1_13	EDN1_14	EDN1_14	EDN1_14	EDN1_15	EDN1_15	EDN1_15	EDN1_16	EDN1_16	EDN1_16	EDN1_17	EDN1_17	EDN1_17	EDN1_18	EDN1_18	EDN1_18
D	PLA2G4A_11	PLA2G4A_11	PLA2G4A_11	PLA2G4A_12	PLA2G4A_12	PLA2G4A_12	PLA2G4A_13	PLA2G4A_13	PLA2G4A_13	PLA2G4A_14	PLA2G4A_14	PLA2G4A_14	PLA2G4A_15	PLA2G4A_15	PLA2G4A_15	PLA2G4A_16	PLA2G4A_16	PLA2G4A_16	PLA2G4A_17	PLA2G4A_17	PLA2G4A_17	PLA2G4A_18	PLA2G4A_18	PLA2G4A_18
E	FBN1_11	FBN1_11	FBN1_11	FBN1_12	FBN1_12	FBN1_12	FBN1_13	FBN1_13	FBN1_13	FBN1_14	FBN1_14	FBN1_14	FBN1_15	FBN1_15	FBN1_15	FBN1_16	FBN1_16	FBN1_16	FBN1_17	FBN1_17	FBN1_17	FBN1_18	FBN1_18	FBN1_18
F	RCAN1_11	RCAN1_11	RCAN1_11	RCAN1_12	RCAN1_12	RCAN1_12	RCAN1_13	RCAN1_13	RCAN1_13	RCAN1_14	RCAN1_14	RCAN1_14	RCAN1_15	RCAN1_15	RCAN1_15	RCAN1_16	RCAN1_16	RCAN1_16	RCAN1_17	RCAN1_17	RCAN1_17	RCAN1_18	RCAN1_18	RCAN1_18
G	ACTB_11	ACTB_11	ACTB_11	ACTB_12	ACTB_12	ACTB_12	ACTB_13	ACTB_13	ACTB_13	ACTB_14	ACTB_14	ACTB_14	ACTB_15	ACTB_15	ACTB_15	ACTB_16	ACTB_16	ACTB_16	ACTB_17	ACTB_17	ACTB_17	ACTB_18	ACTB_18	ACTB_18
H	RGS4_11	RGS4_11	RGS4_11	RGS4_12	RGS4_12	RGS4_12	RGS4_13	RGS4_13	RGS4_13	RGS4_14	RGS4_14	RGS4_14	RGS4_15	RGS4_15	RGS4_15	RGS4_16	RGS4_16	RGS4_16	RGS4_17	RGS4_17	RGS4_17	RGS4_18	RGS4_18	RGS4_18
I	KCNMA1_11	KCNMA1_11	KCNMA1_11	KCNMA1_12	KCNMA1_12	KCNMA1_12	KCNMA1_13	KCNMA1_13	KCNMA1_13	KCNMA1_14	KCNMA1_14	KCNMA1_14	KCNMA1_15	KCNMA1_15	KCNMA1_15	KCNMA1_16	KCNMA1_16	KCNMA1_16	KCNMA1_17	KCNMA1_17	KCNMA1_17	KCNMA1_18	KCNMA1_18	KCNMA1_18
J	THBS4_11	THBS4_11	THBS4_11	THBS4_12	THBS4_12	THBS4_12	THBS4_13	THBS4_13	THBS4_13	THBS4_14	THBS4_14	THBS4_14	THBS4_15	THBS4_15	THBS4_15	THBS4_16	THBS4_16	THBS4_16	THBS4_17	THBS4_17	THBS4_17	THBS4_18	THBS4_18	THBS4_18
K	NOX4_11	NOX4_11	NOX4_11	NOX4_12	NOX4_12	NOX4_12	NOX4_13	NOX4_13	NOX4_13	NOX4_14	NOX4_14	NOX4_14	NOX4_15	NOX4_15	NOX4_15	NOX4_16	NOX4_16	NOX4_16	NOX4_17	NOX4_17	NOX4_17	NOX4_18	NOX4_18	NOX4_18
L	RPS9_11	RPS9_11	RPS9_11	RPS9_12	RPS9_12	RPS9_12	RPS9_13	RPS9_13	RPS9_13	RPS9_14	RPS9_14	RPS9_14	RPS9_15	RPS9_15	RPS9_15	RPS9_16	RPS9_16	RPS9_16	RPS9_17	RPS9_17	RPS9_17	RPS9_18	RPS9_18	RPS9_18
M	ASIC2_19	ASIC2_19	ASIC2_19	KCNMA1_19	KCNMA1_19	KCNMA1_19	RGS4_19	RGS4_19	RGS4_19	ASIC2_20	ASIC2_20	ASIC2_20	KCNMA1_20	KCNMA1_20	KCNMA1_20	RGS4_20	RGS4_20	RGS4_20	NTC					
N	FBN1_19	FBN1_19	FBN1_19	PLA2G4A_19	PLA2G4A_19	PLA2G4A_19	RPS9_19	RPS9_19	RPS9_19	FBN1_20	FBN1_20	FBN1_20	PLA2G4A_20	PLA2G4A_20	PLA2G4A_20	RPS9_20	RPS9_20	RPS9_20	NTC					
O	EDN1_19	EDN1_19	EDN1_19	NOX4_19	NOX4_19	NOX4_19	THBS4_19	THBS4_19	THBS4_19	EDN1_20	EDN1_20	EDN1_20	NOX4_20	NOX4_20	NOX4_20	THBS4_20	THBS4_20	THBS4_20	NTC					
P	ACTB_19	ACTB_19	ACTB_19	RCAN1_19	RCAN1_19	RCAN1_19	B2M_19	B2M_19	B2M_19	ACTB_20	ACTB_20	ACTB_20	RCAN1_20	RCAN1_20	RCAN1_20	B2M_20	B2M_20	B2M_20	NTC					

Figure A-22. Example (i.e., right ventricle bottom) 384-well plate for the qPCR reactions including candidate (n = 9) and reference (n = 3) genes. Hypertensive and normotensive steers were randomized on each 384-well plate in alternating order (represented by numbers 1-20 next to each gene). Each 384-well plate contained 10 steers.

APPENDIX F

LIVAK METHOD ($2^{-\Delta\Delta C_T}$)

The derived $2^{-\Delta\Delta C_T}$ estimated amplification based upon the number of target molecules in the reaction. However, expression in this experiment was estimated based upon the reference gene and is therefore calculated slightly different. For a valid $\Delta\Delta C_T$ calculation, the main assumption was that candidate and reference efficiencies must be approximately equal. As shown in Tables 5-6 and 5-8, there is small variation in these efficiencies, which could represent a violation to the assumption. The estimated C_T values were first averaged across PCR technical replicates (triplicates). The delta C_T values were calculated as

$$\Delta C_T = C_{T_{target}} - C_{T_{reference}} \quad (1)$$

where $C_{T_{target}}$ was the crossing threshold value for the candidate gene and $C_{T_{reference}}$ was the crossing threshold value for the reference gene. Only a single reference gene was utilized, and therefore the calculations were repeated with any additional reference genes. Delta delta C_T values were estimated through the equation

$$\Delta\Delta C_T = \Delta C_{T_{target}} - Calibrator \quad (2)$$

where the calibrator represented the average ΔC_T of the control samples. The control samples in this experiment were all normotensive animals ($\Delta C_{T_{NT}}$). Therefore, the amount of candidate gene, normalized to a reference and relative to a calibrator, is given by

$$amount\ of\ target = 2^{-\Delta\Delta C_T}. \quad (3)$$

Table A-4. Livak Method ($\Delta\Delta C_T$) - Summary of relative gene expression (Mean ± Standard Error) and Wilcoxon rank sum test[1] (p-value) for all tissues (n = 11) and candidate genes[2] (n = 9) between hypertensive and normotensive[3] groups. P-values in red represent differences (> 0.1) from Pfaffl method results (Table 5-7).

		ASIC2		*EDN1*		*FBN1*		*KCNMA1*	
Tissue	Group[3]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]	μ ± SE	p-value[4]
Apex	HT	0.76 ± 0.26	0.243	3.12 ± 1.23	0.113	1.35 ± 0.17	0.497	2.12 ± 0.69	0.278
	NT	1.13 ± 0.22		1.17 ± 0.23		1.14 ± 0.19		1.34 ± 0.38	
Brisket	HT	1.31 ± 0.25	0.968	1.24 ± 0.59	0.968	0.77 ± 0.13	0.211	0.86 ± 0.20	0.447
	NT	1.33 ± 0.29		1.42 ± 0.42		1.06 ± 0.12		1.10 ± 0.17	
Longissimus dorsi	HT	1.30 ± 0.17	0.447	1.01 ± 0.14	0.661	1.01 ± 0.19	0.400	0.98 ± 0.16	0.356
	NT	1.10 ± 0.14		1.05 ± 0.11		1.04 ± 0.10		1.08 ± 0.14	
Left ventricle, bottom	HT	1.70 ± 0.64	0.684	2.74 ± 1.16	0.052	1.23 ± 0.17	0.684	1.53 ± 0.62	0.684
	NT	1.78 ± 0.82		1.05 ± 0.11		1.08 ± 0.13		1.11 ± 0.18	
Left ventricle, middle	HT	1.25 ± 0.38	0.661	1.33 ± 0.12	0.079	0.89 ± 0.12	0.400	1.36 ± 0.28	1.000
	NT	1.36 ± 0.36		1.06 ± 0.14		1.11 ± 0.22		1.45 ± 0.36	
Left ventricle, top	HT	0.98 ± 0.35	0.190	1.98 ± 0.70	0.052	0.82 ± 0.06	0.075	0.93 ± 0.20	0.353
	NT	1.16 ± 0.21		1.07 ± 0.13		1.02 ± 0.07		1.12 ± 0.17	
Moderator band	HT	0.52 ± 0.13	0.010	1.10 ± 0.17	0.604	1.20 ± 0.16	0.315	2.06 ± 0.51	0.780
	NT	1.20 ± 0.19		1.31 ± 0.40		1.05 ± 0.12		1.97 ± 0.67	
Right papillary muscle	HT	0.56 ± 0.15	0.043	1.86 ± 0.54	0.218	1.60 ± 0.17	0.015	4.01 ± 1.06	0.052
	NT	1.27 ± 0.27		1.09 ± 0.14		1.05 ± 0.10		1.41 ± 0.38	
Right ventricle, bottom	HT	0.35 ± 0.20	0.009	1.78 ± 0.22	0.007	1.21 ± 0.11	0.280	2.07 ± 0.58	0.393
	NT	1.53 ± 0.39		1.21 ± 0.33		1.11 ± 0.19		3.49 ± 2.44	
Right ventricle, middle	HT	0.17 ± 0.10	***< 0.001***	1.49 ± 0.30	0.222	1.17 ± 0.19	0.387	2.17 ± 1.05	1.000
	NT	1.39 ± 0.35		1.14 ± 0.21		1.15 ± 0.21		1.42 ± 0.36	
Right ventricle, top	HT	0.14 ± 0.06	***< 0.001***	2.78 ± 0.99	***0.003***	1.24 ± 0.18	0.684	2.16 ± 0.37	0.015
	NT	1.18 ± 0.22		1.03 ± 0.08		1.02 ± 0.07		1.08 ± 0.14	

[1]Non-parametric analysis; H_0: Population means are equal; H_A: Population means are not equal

[2]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator of Calcineurin 1; *RGS4* = Regulator of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[3]Physiological group based upon pulmonary arterial pressures (PAP); HT = hypertensive, 98 ± 15 mmHg; NT = normotensive, 48 ± 20 mmHg.

[4]Signifance adjusted for multiple testing; Significance set at P < 0.005555; Bold and italicized p-values met significance threshold

Table A-4. Continued…

Tissue	Group[2]	*NOX4* μ ± SE	*NOX4* p-value[3]	*PLA2G4A* μ ± SE	*PLA2G4A* p-value[3]	*RCAN1* μ ± SE	*RCAN1* p-value[3]	*RGS4* μ ± SE	*RGS4* p-value[3]	*THBS4* μ ± SE	*THBS4* p-value[3]
Apex	HT	1.72 ± 0.40	0.315	1.86 ± 0.24	0.028	2.54 ± 0.59	0.013	1.62 ± 0.33	0.133	1.22 ± 0.30	0.968
	NT	1.18 ± 0.21		1.12 ± 0.18		1.19 ± 0.29		1.05 ± 0.10		1.15 ± 0.20	
Brisket	HT	1.10 ± 0.20	0.720	0.79 ± 0.13	0.113	1.05 ± 0.16	0.661	1.92 ± 1.10	0.661	1.88 ± 0.66	0.315
	NT	1.03 ± 0.09		1.09 ± 0.14		1.65 ± 0.75		1.24 ± 0.25		1.12 ± 0.18	
Longissimus dorsi	HT	1.51 ± 0.25	0.243	1.30 ± 0.18	0.315	1.42 ± 0.28	0.400	1.17 ± 0.21	0.780	1.20 ± 0.15	0.447
	NT	1.12 ± 0.16		1.02 ± 0.07		1.16 ± 0.19		1.10 ± 0.16		1.03 ± 0.08	
Left ventricle, bottom	HT	1.55 ± 0.53	0.631	1.53 ± 0.29	0.280	1.99 ± 0.71	0.218	1.28 ± 0.31	0.912	1.43 ± 0.31	0.315
	NT	1.10 ± 0.15		1.05 ± 0.10		1.10 ± 0.16		1.07 ± 0.13		1.06 ± 0.13	
Left ventricle, middle	HT	0.89 ± 0.12	0.447	1.05 ± 0.13	0.968	1.19 ± 0.20	0.315	0.88 ± 0.09	0.497	0.87 ± 0.13	0.356
	NT	1.15 ± 0.19		1.12 ± 0.18		1.18 ± 0.30		1.13 ± 0.20		1.06 ± 0.13	
Left ventricle, top	HT	0.87 ± 0.15	0.190	0.99 ± 0.08	0.631	1.99 ± 0.62	0.353	0.62 ± 0.09	0.035	0.87 ± 0.10	0.190
	NT	1.07 ± 0.13		1.03 ± 0.08		1.07 ± 0.13		1.14 ± 0.20		1.06 ± 0.13	
Moderator band	HT	2.84 ± 0.79	0.133	1.70 ± 0.46	0.447	2.22 ± 0.51	0.095	1.19 ± 0.20	0.278	3.55 ± 1.04	0.028
	NT	1.24 ± 0.25		1.19 ± 0.29		1.19 ± 0.24		2.22 ± 1.28		1.37 ± 0.41	
Right papillary muscle	HT	4.92 ± 1.26	***< 0.001***	2.96 ± 0.72	***0.002***	3.59 ± 0.78	***0.002***	2.96 ± 0.64	0.023	3.33 ± 0.58	0.007
	NT	1.08 ± 0.14		1.08 ± 0.13		1.11 ± 0.19		1.07 ± 0.14		1.17 ± 0.20	
Right ventricle, bottom	HT	2.81 ± 0.54	***0.002***	1.82 ± 0.37	0.052	6.87 ± 2.23	***0.001***	1.68 ± 0.48	0.165	3.44 ± 0.90	***0.002***
	NT	1.16 ± 0.22		1.37 ± 0.48		1.25 ± 0.30		2.29 ± 1.52		1.18 ± 0.26	
Right ventricle, middle	HT	2.78 ± 0.65	***0.004***	2.28 ± 0.53	***0.001***	2.72 ± 0.74	0.006	2.60 ± 0.60	0.050	3.88 ± 0.73	***0.001***
	NT	1.19 ± 0.24		1.09 ± 0.15		1.18 ± 0.23		1.18 ± 0.23		1.27 ± 0.36	
Right ventricle, top	HT	2.70 ± 0.62	***0.002***	1.65 ± 0.20	***0.003***	3.26 ± 0.55	***0.004***	2.20 ± 0.55	0.029	3.52 ± 0.95	***< 0.001***
	NT	1.04 ± 0.11		1.03 ± 0.08		1.06 ± 0.13		1.04 ± 0.09		1.06 ± 0.13	

[1]Non-parametric analysis; H_0: Population means are equal; H_A: Population means are not equal

[2]*ASIC2* = Acid Sensing Ion Channel Subunit 2; *EDN1* = Endothelin 1; *FBN1* = Fibrillin 1; *KCNMA1* = Potassium Calcium-Activated Channel Subfamily M Alpha 1; *NOX4* = NADPH Oxidase 4; *PLA2G4A* = Phospholipase A2 Group IVA; *RCAN1* = Regulator of Calcineurin 1; *RGS4* = Regulator of G Protein Signaling 4; *THBS4* = Thrombospondin 4

[3]Physiological group based upon pulmonary arterial pressures (PAP); HT = hypertensive, 98 ± 15 mmHg; NT = normotensive, 48 ± 20 mmHg.

[4]Signifance adjusted for multiple testing; Significance set at P < 0.005555; Bolded p-values met significance threshold

APPENDIX G

GRAPHICAL REPRESENTATION OF GENE EXPRESSION DIFFERENCES

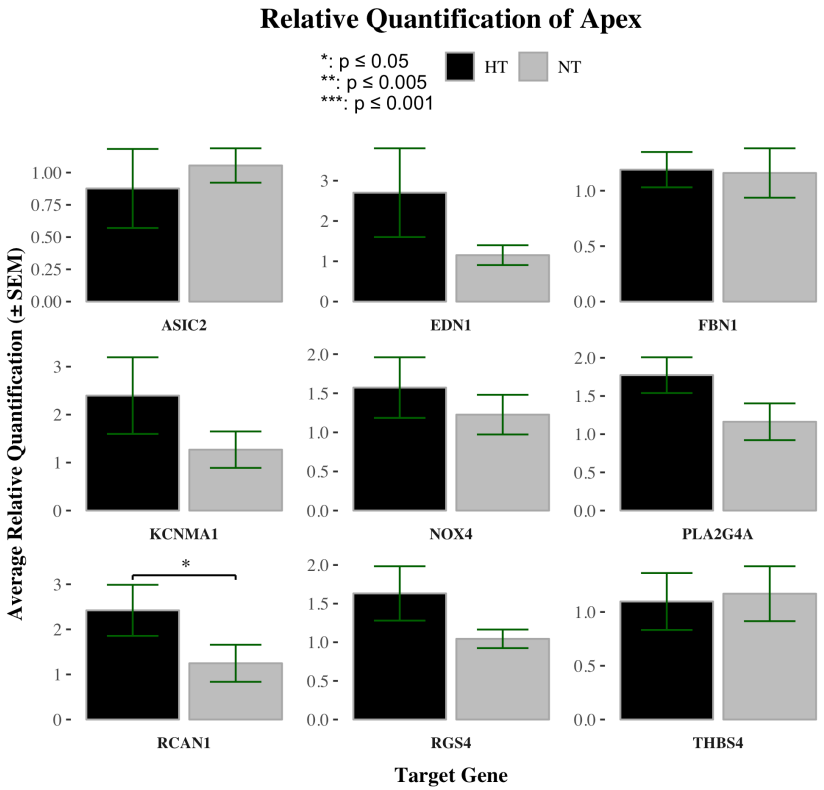


Figure A-23

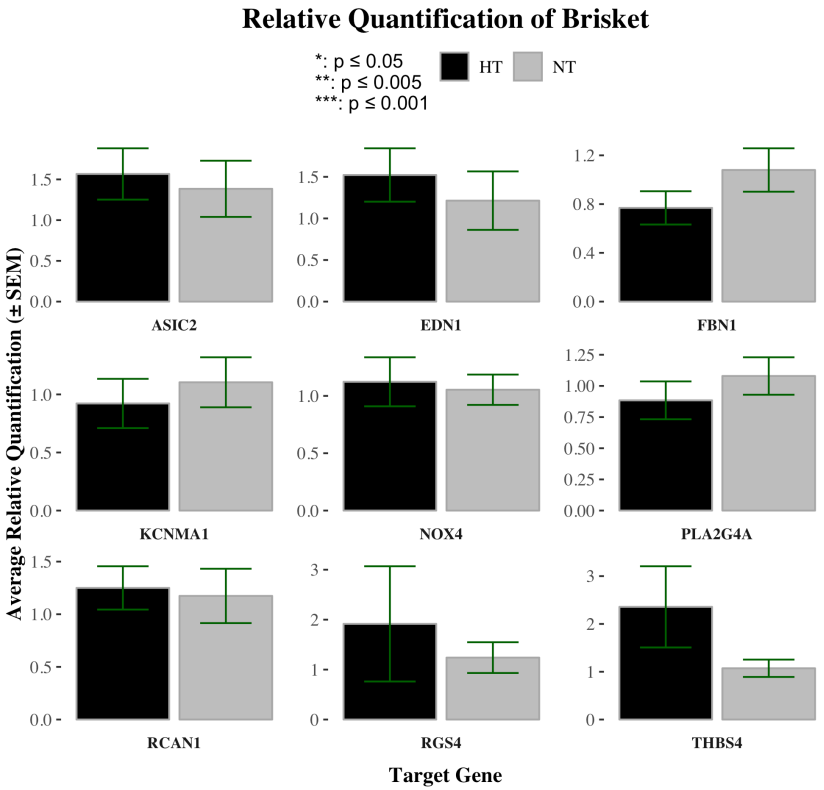


Figure A-24

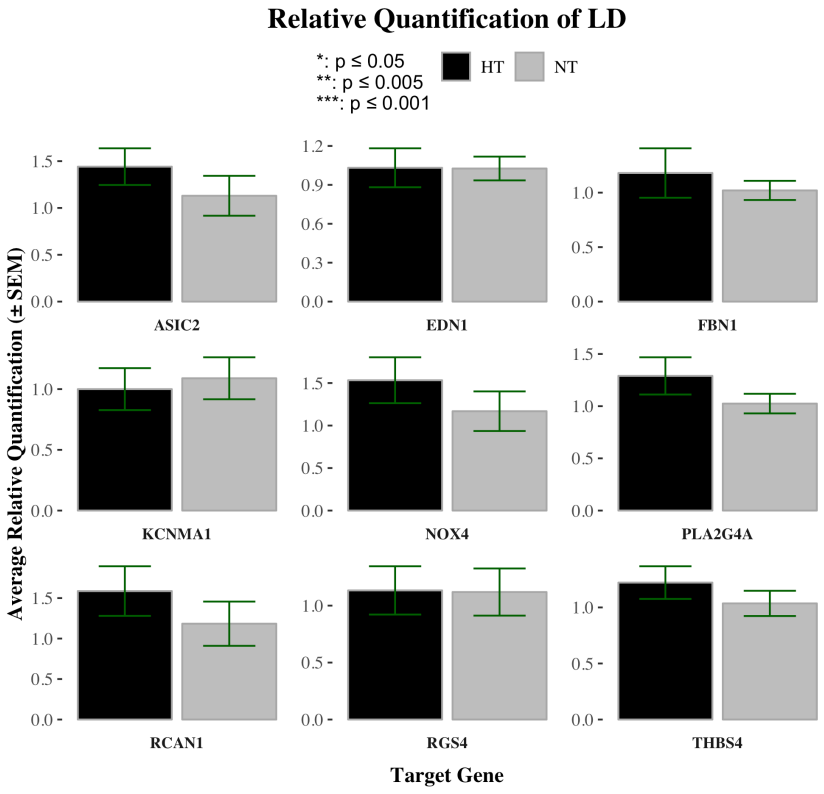


Figure A-25

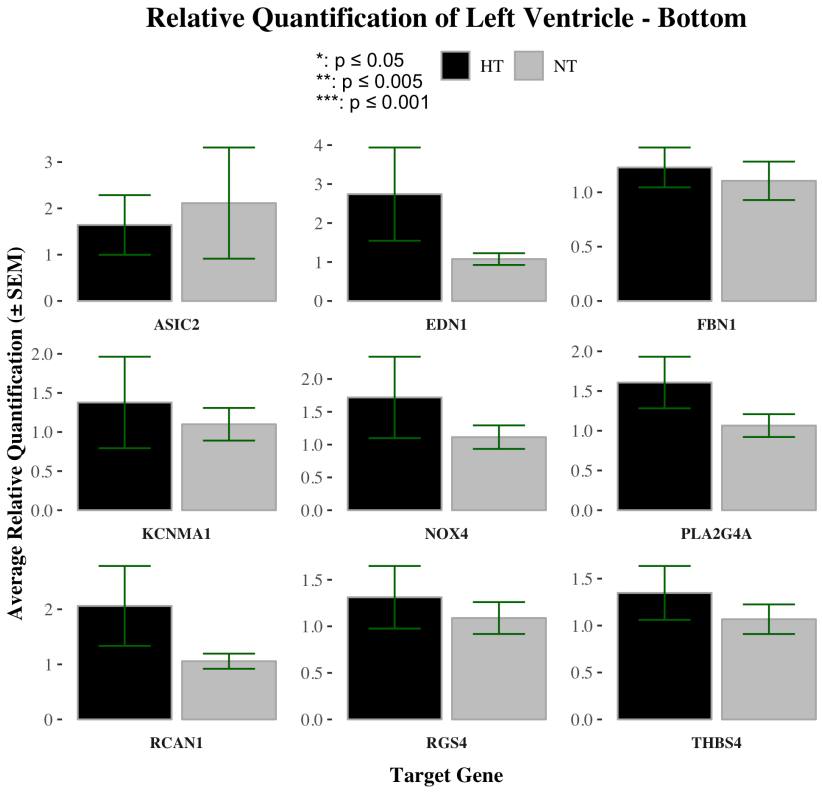


Figure A-26

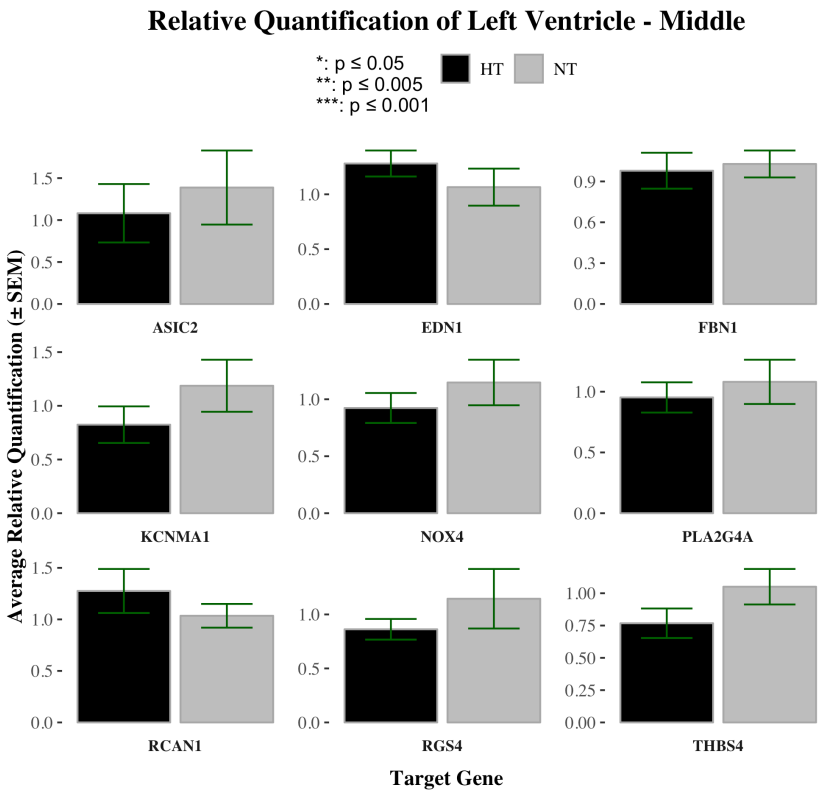


Figure A-27

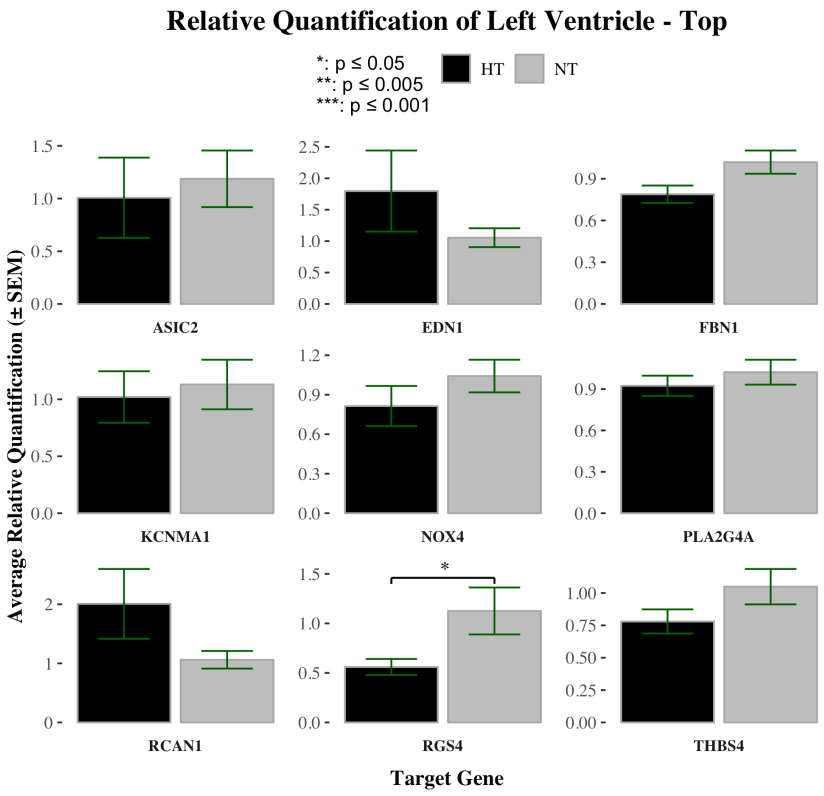


Figure A-28

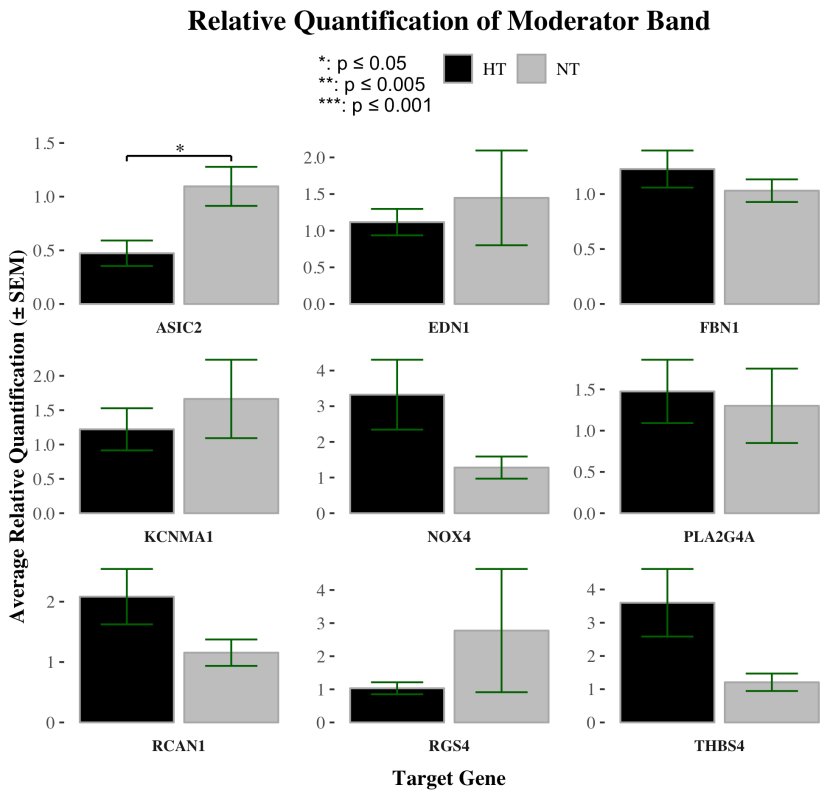


Figure A-29

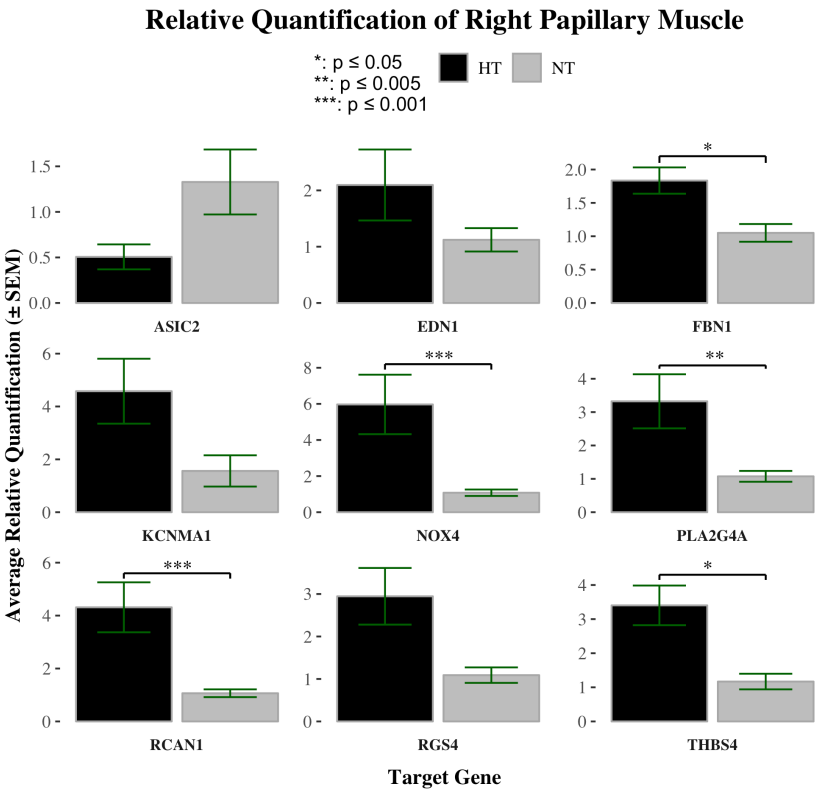


Figure A-30

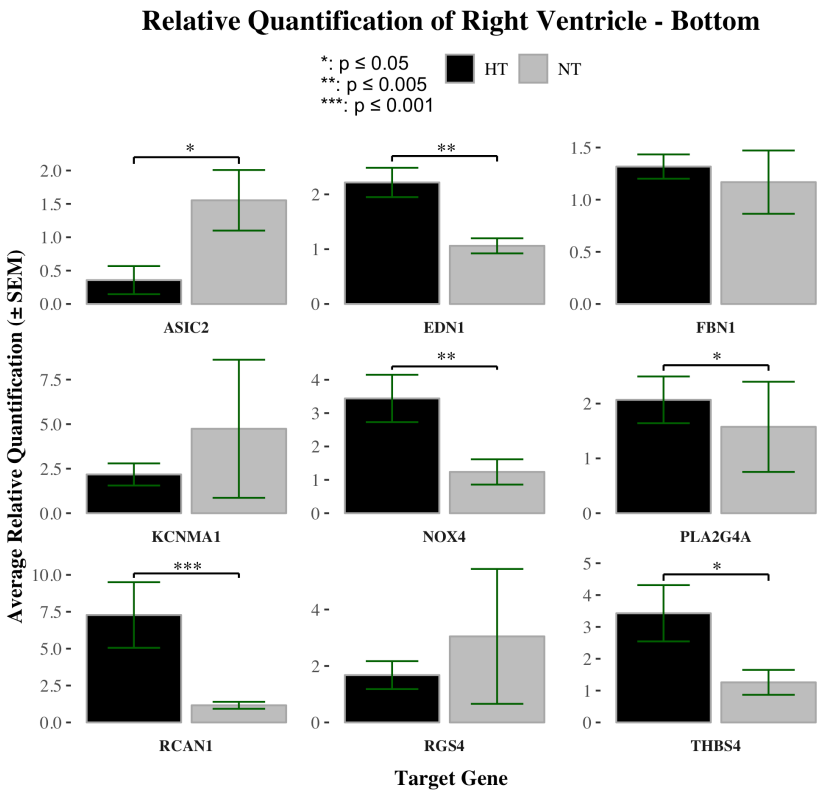


Figure A-31

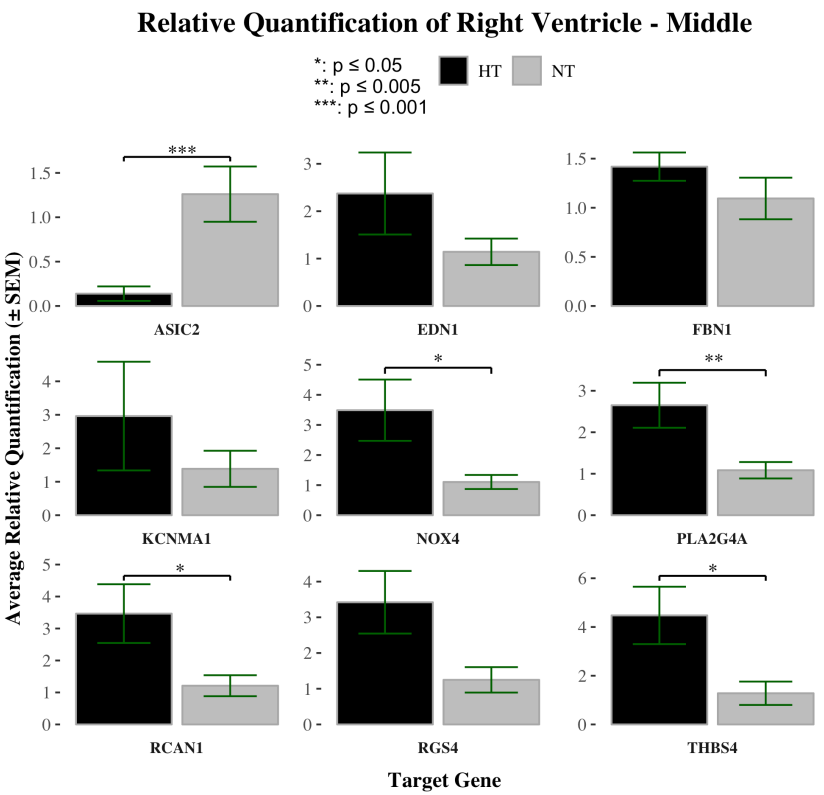


Figure A-32

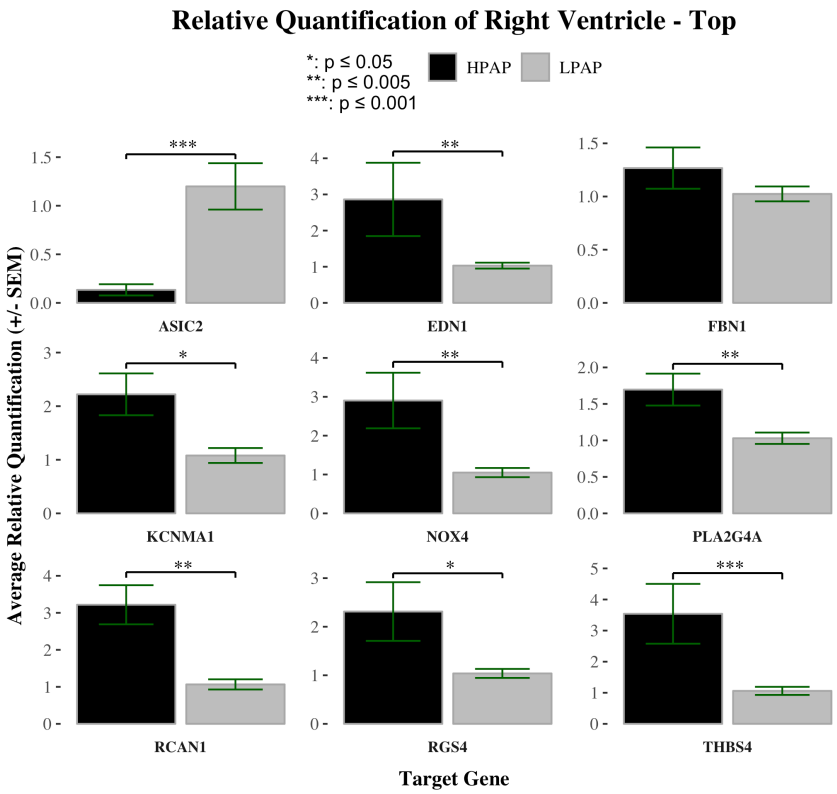
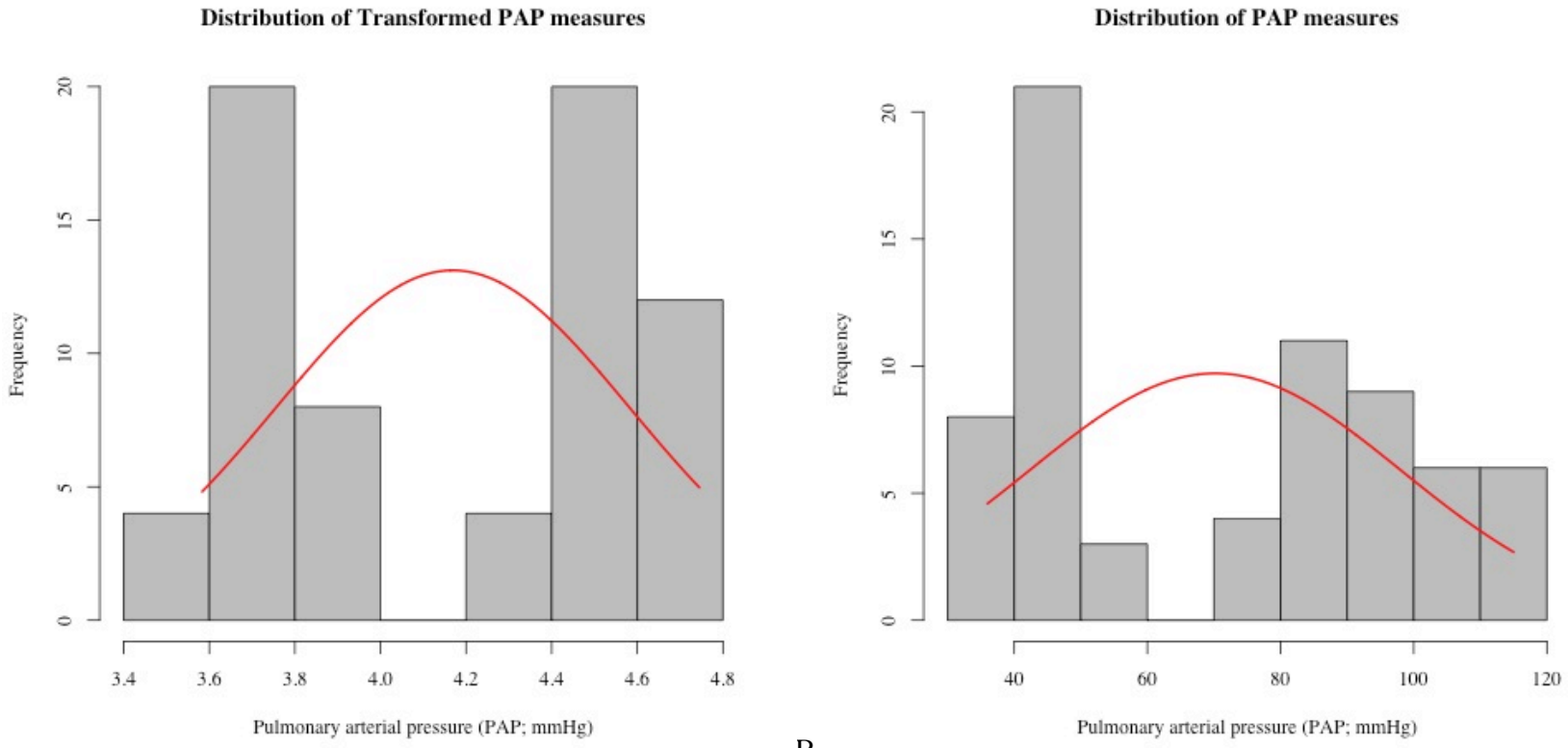


Figure A-33

Figures A-23 through A-33. Histograms of the mean relative quantification (i.e., gene expression) for each gene and within each tissue are shown below. These were differentiated by PH status of hypertensive (HT) or normotensive (NT). This is the average expression for the PH groups and a standard error of the mean. Differences ($P < 0.0055$) were represented above the histogram pairs. Significant differences were estimated in right papillary muscle and all of the right ventricle tissues for a selected number of candidate genes.

APPENDIX H

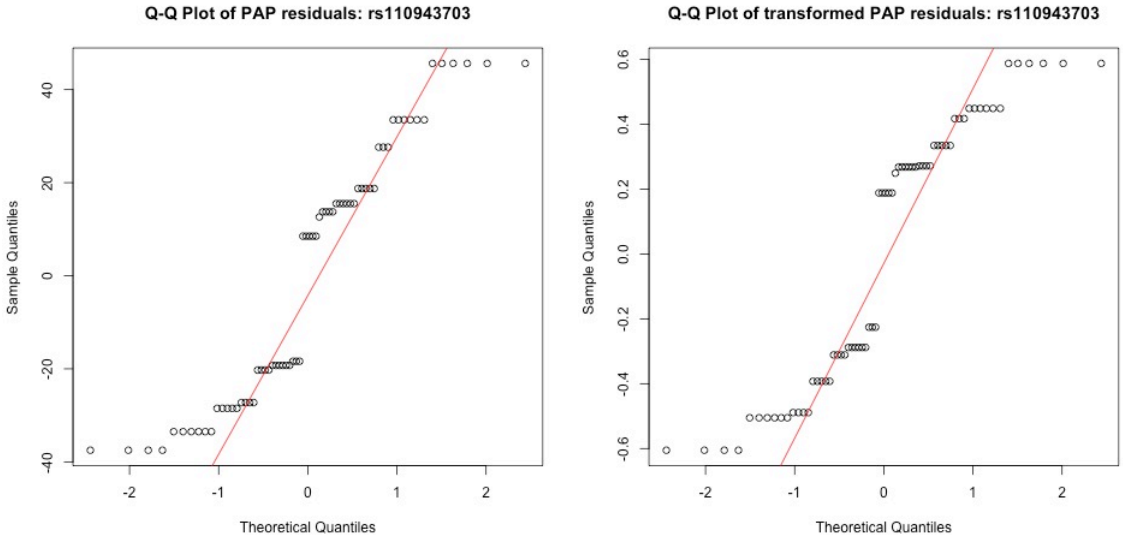
TRANSCRIPTOME DATA: HETEROGENEITY OF VARIANCE OF UNTRANSFORMED AND TRANSFORMED PAP



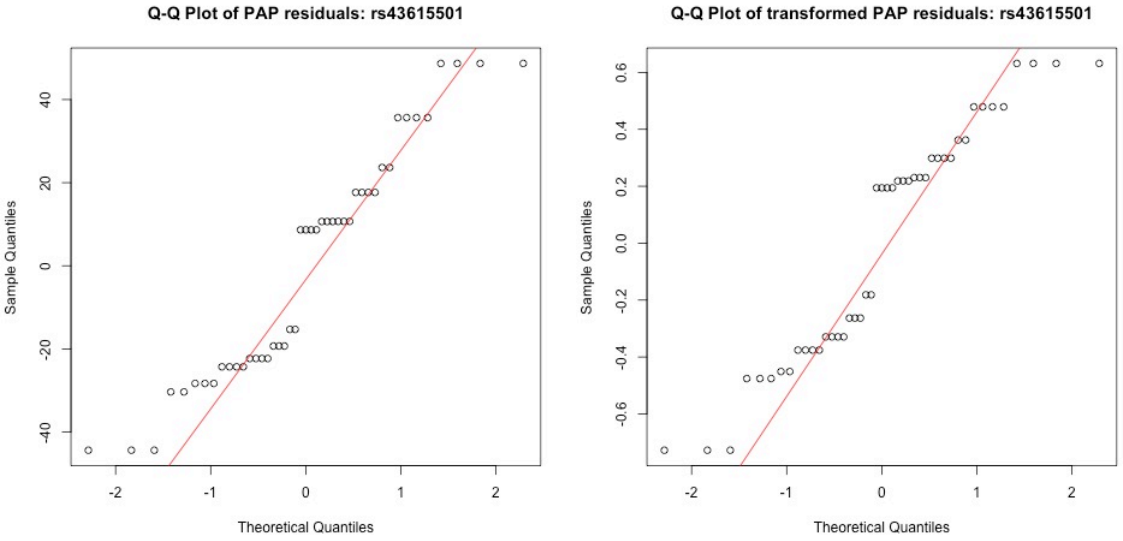
A. B.

Figure A-34. Transcriptome data from calf-fed yearling Angus steers (n = 14). Distribution of pulmonary arterial pressure (PAP) measures untransformed (A) and transformed (log; B). Both estimated as non-normally distributed based up Box-Cox transformation method and Shapiro-Wilks normality test ($P < 0.001$).

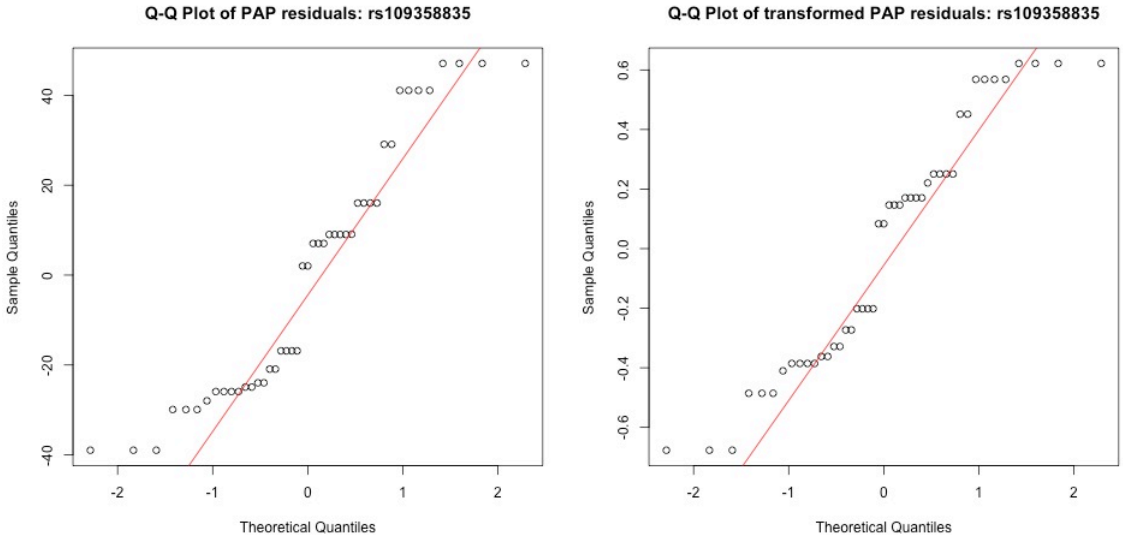
A.


Q-Q Plot of PAP residuals: rs110943703
Sample Quantiles
Theoretical Quantiles
Q-Q Plot of transformed PAP residuals: rs110943703
Sample Quantiles
Theoretical Quantiles

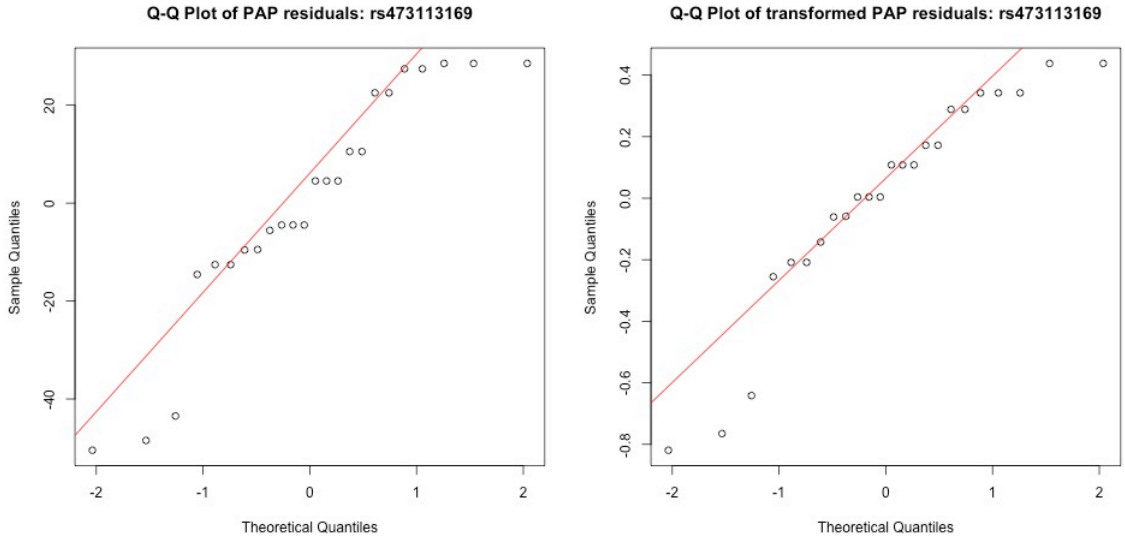
B.

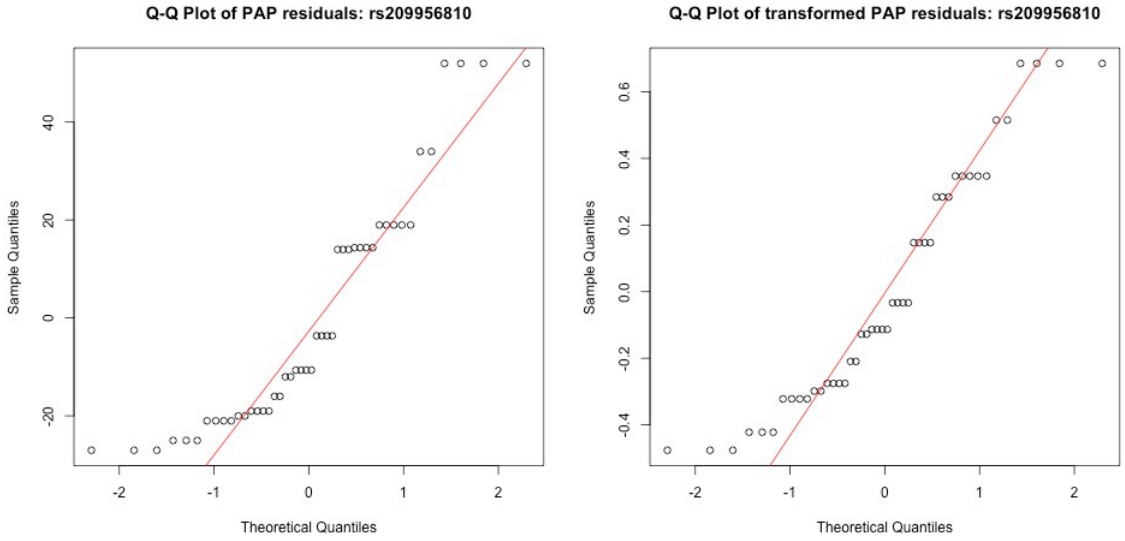

Q-Q Plot of PAP residuals: rs43615501
Sample Quantiles
Theoretical Quantiles
Q-Q Plot of transformed PAP residuals: rs43615501
Sample Quantiles
Theoretical Quantiles

C.


Q-Q Plot of PAP residuals: rs109358835
Sample Quantiles
Theoretical Quantiles
Q-Q Plot of transformed PAP residuals: rs109358835
Sample Quantiles
Theoretical Quantiles

D.


Q-Q Plot of PAP residuals: rs473113169
Sample Quantiles
Theoretical Quantiles
Q-Q Plot of transformed PAP residuals: rs473113169
Sample Quantiles
Theoretical Quantiles

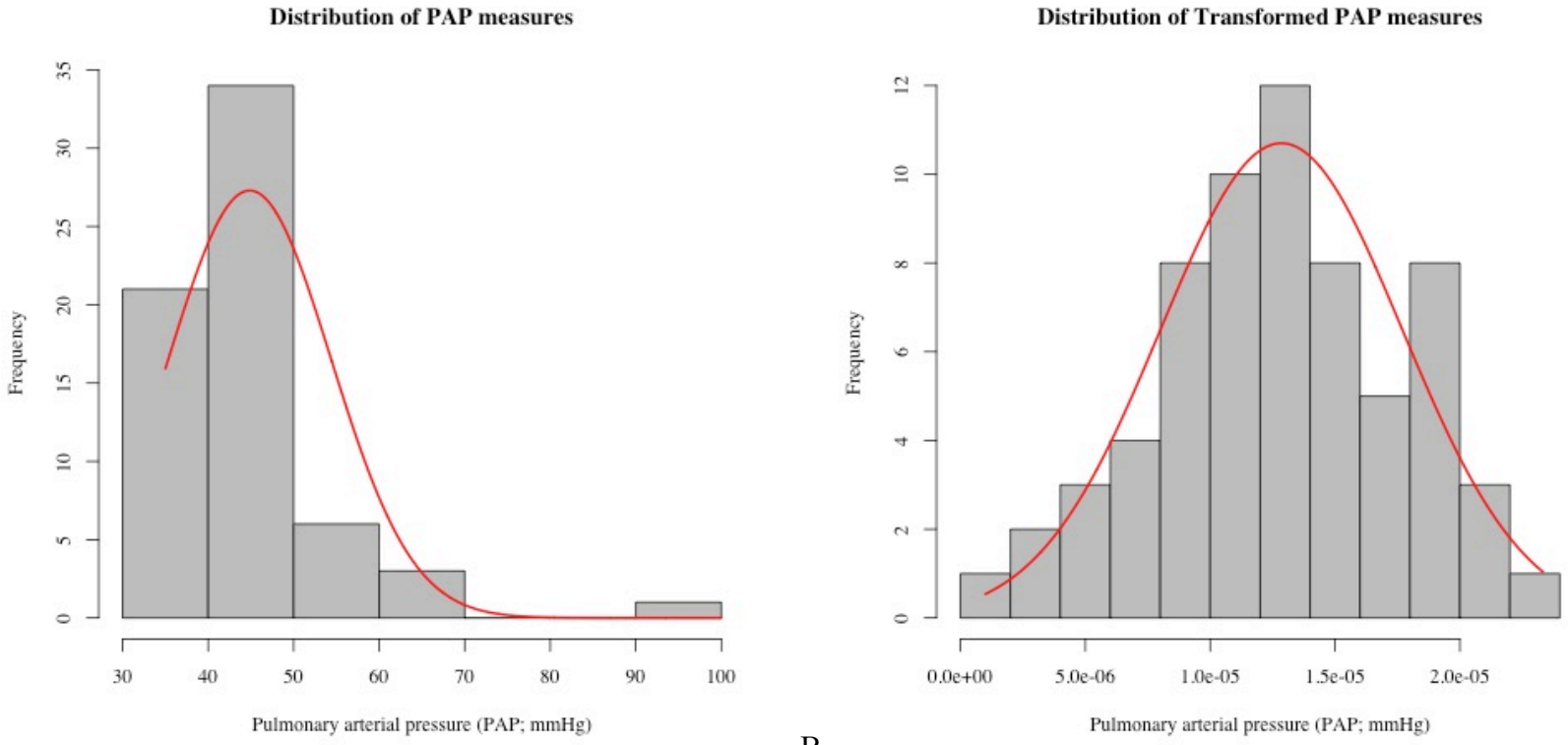


E.

Figure A-35. Quantile-quantile plots of the residuals of untransformed pulmonary arterial pressure (PAP) measures and log transformed PAP for each of the 5 identified SNP. A. rs110943703; B. rs43615501; C. rs109358835; D. rs473113169; E. rs209956810. Transformation estimated by Box-Cox method.

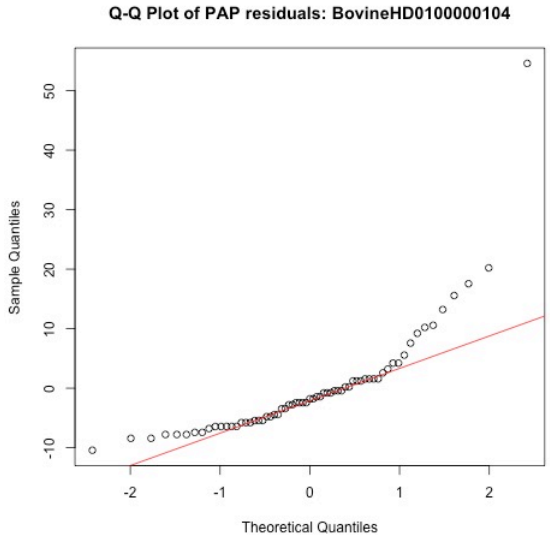
APPENDIX I

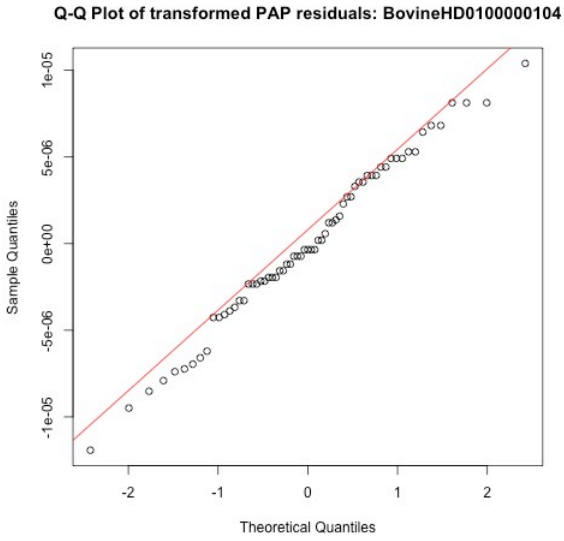
GENOME DATA: HETEROGENEITY OF VARIANCE OF UNTRANSFORMED AND TRANSFORMED PAP



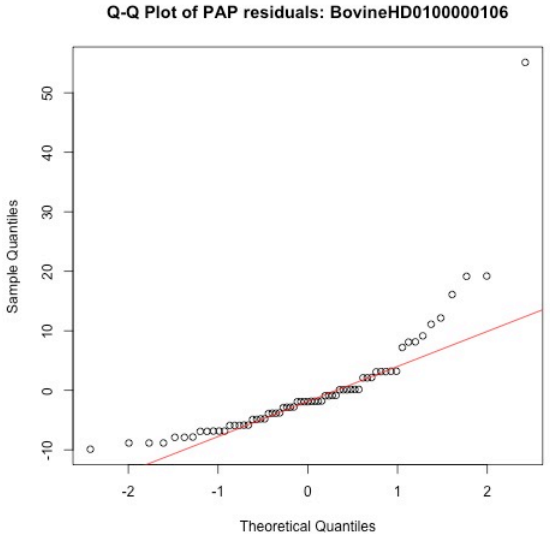
A. B.

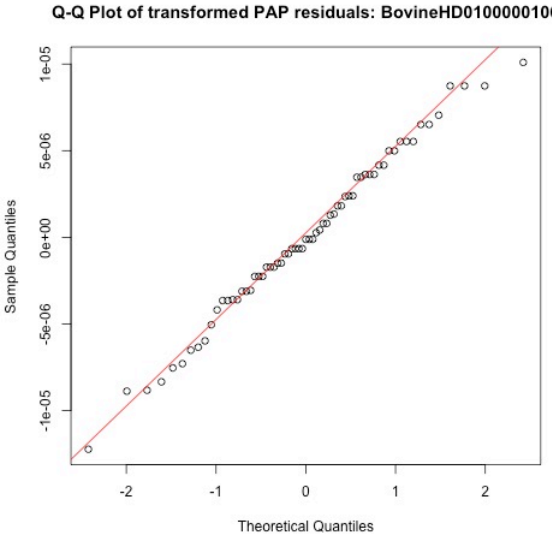
Figure A-36. Genome (SNP) data from 6-month Angus steers (n = 65). Distribution of pulmonary arterial pressure (PAP) measures (A) untransformed and (B) transformed. Transformation estimated used Box-Cox method. Untransformed PAP estimated as non-normal ($P < 0.001$) and transformed PAP as normally distributed ($P = 0.77$) using the Shapiro-Wilks normality test.


Q-Q Plot of PAP residuals: BovineHD0100000104
Sample Quantiles
Theoretical Quantiles
-10
0
10
20
30
40
50
-2
-1
0
1
2

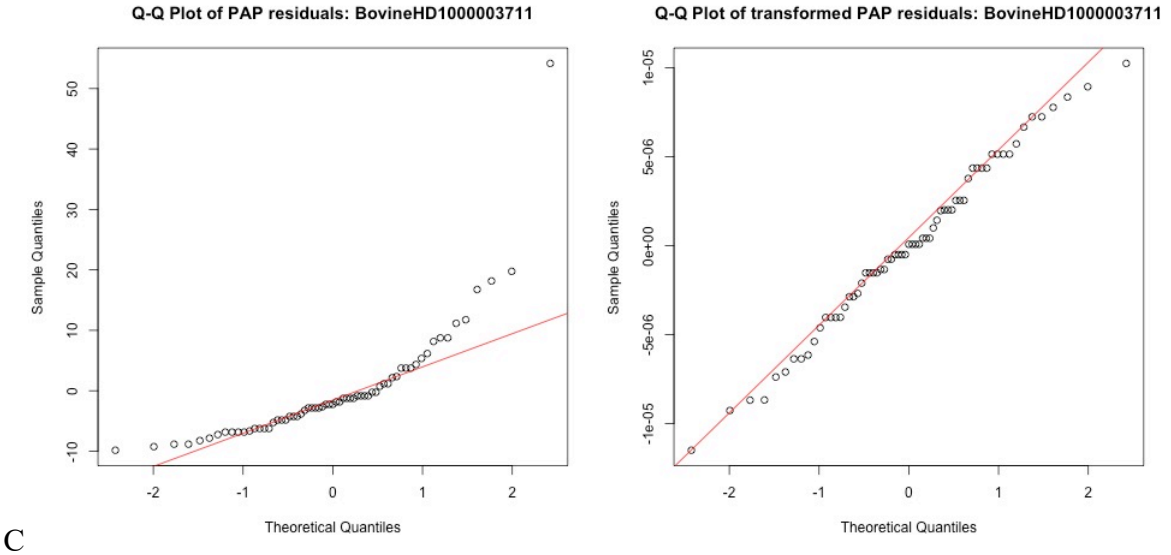

Q-Q Plot of transformed PAP residuals: BovineHD0100000104
Sample Quantiles
Theoretical Quantiles
-1e-05
-5e-06
0e+00
5e-06
1e-05
-2
-1
0
1
2

A

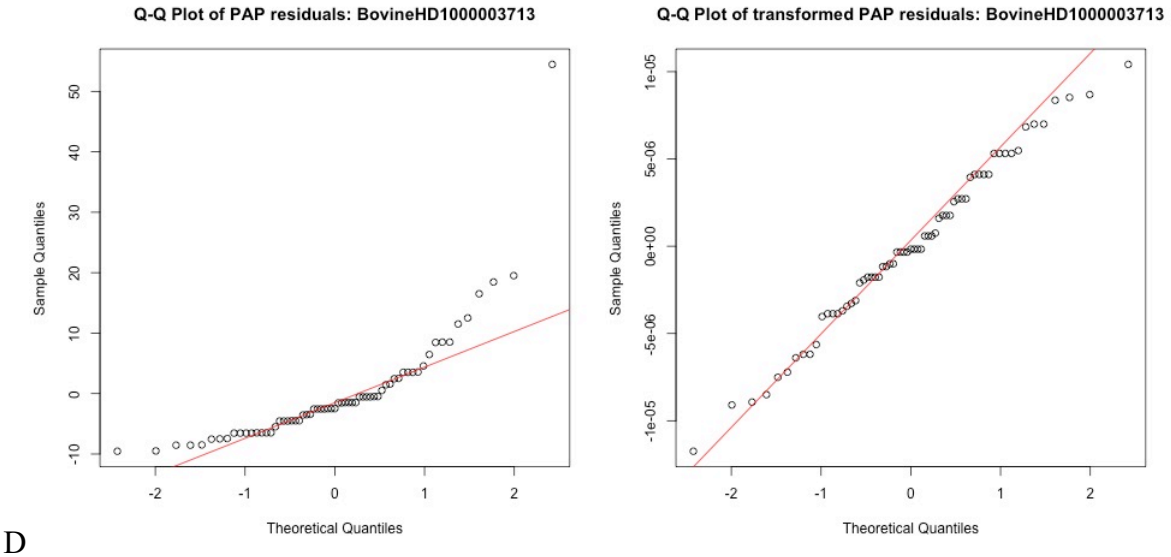

Q-Q Plot of PAP residuals: BovineHD0100000106
Sample Quantiles
Theoretical Quantiles
-10
0
10
20
30
40
50
-2
-1
0
1
2


Q-Q Plot of transformed PAP residuals: BovineHD0100000106
Sample Quantiles
Theoretical Quantiles
-1e-05
-5e-06
0e+00
5e-06
1e-05
-2
-1
0
1
2

B



C



D

Figure A-37. Quantile-quantile plots of the residuals of untransformed pulmonary arterial pressure (PAP) measures and log transformed PAP for each of the 4 SNP located on the Illumina BovineHD BeadChip (777,962 SNP). A. BovineHD0100000104; B. BovineHD0100000106; C. BovineHD1000003711; D. BovineHD1000003713. Transformation estimated by Box-Cox method.