GENE EXPRESSION CHANGES IN MUSCLE AND LIVER TISSUE OF GROWING BEEF CALVES WITH BOVINE RESPIRATORY DISEASE

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ABBREVIATIONS AND ACRONYMS

AA - arachidonic acid
ACACA - acetyl-CoA carboxylase
ACC - acetyl-CoA carboxylase
ADG - average daily gain
ADIPOR - adiponectin receptor
AMPK - adenosine monophosphate kinase
APP - acute phase protein
APR - acute phase response
BACE1 - β-site amyloid precursor protein cleaving enzyme 1
BAD - BCL2-associated agonist of cell death
BAFFR - B-cell activating factor of the TNF family receptor
BCAA - branched chain amino acid
BRD - bovine respiratory disease
BVDV - bovine viral diarrhea virus
CD36/FAT - fatty acid translocase
ChREBP - carbohydrate response element binding protein
CPT - carnitine palmitoyl transferase
CTMP - carboxy-terminal modulator protein
D6D - Δ6-desaturase
DHA - docosahexaenoic acid
EPA - eicosopentaenoic acid
FADS2 - fatty acid desaturase 2
FASN - fatty acid synthase
FATP - fatty acid transporter protein
FBXO32 - atrogin-1
GH - growth hormone
GLUL - glutamine synthetase
HCW - hot carcass weight
H-FABP - heart-fatty acid binding protein
IFN - interferon
IGF - insulin-like growth factor
IGFBP - insulin-like growth factor binding protein
IKK - inhibitor of NF-κB

IL - interleukin
IL-1R - interleukin-1 receptor
LPS - lipopolysaccharide
LTβR - lymphotoxin-β receptor
MAFbx - muscle atrophy F-box
MEKK3 - mitogen-activated protein kinase kinase 3
MuRF-1 - muscle RING finger 1
MUSTN1 - musculoskeletal embryonic nuclear protein 1
NF-κB - nuclear factor κB
NIK - NF-κB inducing kinase
p38-MAPK - p38 mitogen-activated protein kinase
PI-BVDV - persistently infected with bovine viral diarrhea virus
PKB - protein kinase B
PPAR - peroxisome proliferator-activated receptor
PUFA - polyunsaturated fatty acid
rbTNF - recombinant bovine tumor necrosis factor
RT-PCR - reverse transcription polymerase chain reaction
S100A8 - S100 calcium binding protein A8
SAA - serum amyloid A
SCD - stearoyl-CoA desaturase
SEPN1 - selenoprotein 1
SREBP - sterol regulatory binding protein
ST6Gal I - α2,6-sialyltransferase
THEM4 - thioesterase superfamily member 4
TLR4 - Toll-like receptor 4
TNFR - tumor necrosis factor receptor
TNF-α - tumor necrosis factor-α

CHAPTER I

INTRODUCTION

Beef production in the modern-day United States is concentrated in the central part of the country. The most common management scheme is to wean calves from brood cows and take them immediately to local livestock auctions were they are commingled with unfamiliar, aggressive, and possibly diseased animals. Following this initial pooling of animals, they may then be sent to several other locations and interact with an assortment of cattle before being hauled hundreds to thousands of miles to Great Plains cattle feeders. Because of acute stress to animals from this transition, many will develop bovine respiratory disease (**BRD**).

Bovine respiratory disease is a multifactorial disease that is considered to be the most prevalent ailment of cattle in this country. A recent review of the occurrence of BRD in feedlot cattle at the Meat Animal Research Center from 1987 to 2001 revealed that 17.0% of the calves were affected annually (Snowder et al., 2006). This respiratory disease complex has been shown to affect 90% of unvaccinated calves and 20% of vaccinated calves that are placed in a feedlot environment resulting in up to 6.4% mortality in untreated animals (Nyamusika et al., 1994). In a survey of feedlot cattle, Edwards (1996) found that 67-82% of moribund animals had some form of respiratory disease. Prevalence of respiratory disease overshadows other illnesses in feedlot cattle in that digestive and miscellaneous disorders contribute only 3-7% and 14-28% of morbidity in feedlot cattle, respectively (Edwards, 1996). Furthermore, 44.1% of all deaths of feedlot cattle are attributed to BRD. Such high mortality translates to 0.128% of feedlot cattle monthly (Smith, 1998).

While it is difficult to place an exact figure to economic losses attributable to BRD, when one considers that there were 27.6 million cattle on feed in the U.S. in 2007, the estimated losses are staggering (USDA, 2007). Nyamusika and coworkers (1994) estimated that mild and moderate cases of BRD cost feeders \$2.25·per head daily from veterinary expenses, while severe cases cost feeders $\$5.70 \cdot hd^{-1} \cdot d^{-1}$. This group further postulated that BRD led to \$8.12 to \$29.19/hd for veterinary expenses for vaccinated and unvaccinated animals receiving treatment, respectively. These estimates agree with other statistics from the 1992-1995 Texas A&M Ranch to Rail program, which state that veterinary costs in feedlot cattle range from \$20.76-37.90 per animal treated (Smith, 1998). Additionally, this survey estimates losses from purchase weight to be between \$0.19-0.35/kg. Treatment of cattle has been shown to decrease carcass quality, and economic losses associated with treatment of moribund cattle can lead to approximately \$31 decrease in carcass value. When cattle are treated two or more times during finishing, there is an approximate decrease in the profit margin of \$208/animal (Roeber et al., 2001). With the mortality losses, treatment costs, and decreased carcass quality, it is understandable that economic losses associated with BRD exceed \$3 billion annually (Griffin, 1997). Current market prices, which are near record highs, may make losses from BRD much higher than these estimates (Lawrence and Ellis, 2010).

Animal health has been shown to have a direct impact on carcass characteristics of feedlot cattle. Cattle treated during the finishing period have decreased capacity for gaining weight compared to healthy cattle. Gardner and coworkers (1999) found that cattle treated two or more times for BRD had average daily gains (**ADG**) that were 10% lower that cattle treated only once. However, a more recent study has shown that during

the finishing phase, ADG and feed efficiency increased linearly with increased number of times treated (Holland et al., 2010). Equivocal data regarding the impact of lung lesions on cattle performance exist. When cattle were stratified by the presence of lung lesions at slaughter, Gardner and associates found that cattle having lung lesions had 11% lower ADG than cattle not presenting lesions (1999). Interestingly, those researchers found that cattle with active lymph nodes at slaughter had 18% lower ADG than cattle with inactive lymph nodes. On the other hand, Holland and coworkers found no differences in the overall presence of pulmonary lesions at harvest (2010). These groups seem to have conflicting results on the effects of BRD on carcass characteristics. While some groups have observed lighter hot carcass weights (**HCW**) and decreased dressing percentages in treated calves (Gardener et al., 1999; Roeber et al., 2001), more recent data suggests that such differences do not exist in calves fed to a common physiologic endpoint with the exception of calves with chronic illness (Holland et al., 2010). The caveat is that in the aforementioned study, calves treated 3 times or those with chronic infection were on feed 19 and 26 days longer, respectively, when compared to those treated on 2 or fewer occasions. If one calculates the HCW/total DMI, one finds that calves treated 3 times or chronically ill calves have roughly 90% of the efficiency of the others to convert feed to a saleable product.

Morbidity in feedlot cattle has been shown to have an effect on fat deposition and protein accretion. Cattle treated for BRD have less external and internal fat with concomitantly lower yield grades scores and decreased *longissimus dorsi* muscle area (Gardener et al., 1999; Roeber et al., 2001). Cattle treated for BRD had lower marbling scores when compared to healthy cattle and as a consequence carcasses from these cattle

were more likely to receive USDA quality grades of Select and Standard at the expense of Choice and Prime grades (Gardener et al., 1999; Roeber et al., 2001; Busby et al., 2008; Holland et al., 2010).

RESEARCH OBJECTIVES

Based on these previous findings, we hypothesize that calves treated for BRD, particularly those treated 3 or more times, are less efficient at utilizing metabolizable nutrients for tissue accretion thus making them less profitable than healthy calves. The first objective of the current research was to evaluate gene expression changes during the acute phase response in *longissimus dorsi* muscle and hepatic tissues when calves are experimentally infected with two common BRD pathogens, namely *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) and bovine viral diarrhea virus (**BVDV**). This was accomplished by exposing experimental beef steers to a calf persistently infected with bovine viral diarrhea viral for 72 hours and subsequently receiving intratracheal inoculation of *M. haemolytica*. *Longissimus dorsi* and hepatic tissues were evaluated by microarray and RT-PCR analysis to identify genes of interest.

A second objective was to evaluate the differences in expression of these selected genes during chronic disease. This was achieved by taking biopsy samples of *longissimus dorsi* muscle and liver at the time of treatment of calves identified as having clinical signs of BRD that were treated once, twice, or thrice. Gene expression was determined by RT-PCR analysis.

CHAPTER II

REVIEW OF LITERATURE

As previously discussed, BRD is the most costly animal illness facing the beef industry in the United States. Not only are costs for treatment incurred, but significant profit losses result from lighter and lower quality beef carcasses. Skeletal muscle tissues are the major amino acid reserves in vertebrates. During disease conditions, these muscle tissues are called upon to release amino acids making them available for immune responses, particularly for acute phase protein production by the liver. Therefore, for immune purposes it is necessary for this amino acid mobilization to occur but at the expense of the beef product. If it were possible to uncouple the catabolism that occurs in skeletal muscle from the anabolism of the liver, there may exist the opportunity to supply the liver with amino acids from another source. Research has demonstrated that inflammation can lead to nuclear factor-κB (**NF-κB**) mediated muscle cachexia by promoting protein catabolism, suppressing protein synthesis, and inhibiting myoblast differentiation, which leads to decreased muscle mass (Hong et al. 2008; Peterson and Guttridge, 2008). However, muscle-specific gene targets of NF-κB signaling, which would allow for such homeostatic remodeling to occur have not been identified.

Research and field studies support the idea that bovine respiratory disease can become a chronic condition. We have demonstrated that calves chronically ill with BRD have poor performance and decrease carcass qualities (Holland et al., 2010). Researchers have reported that 37% of steers appearing clinically healthy during the feeding phase had lung lesions at slaughter (Gardner et al., 1999). Furthermore, 9% of these animals had active bronchial lymph nodes at slaughter (Gardner et al., 1999). This would suggest that bovine respiratory disease causes chronic subclinical inflammation.

MUSCLE CACHEXIA

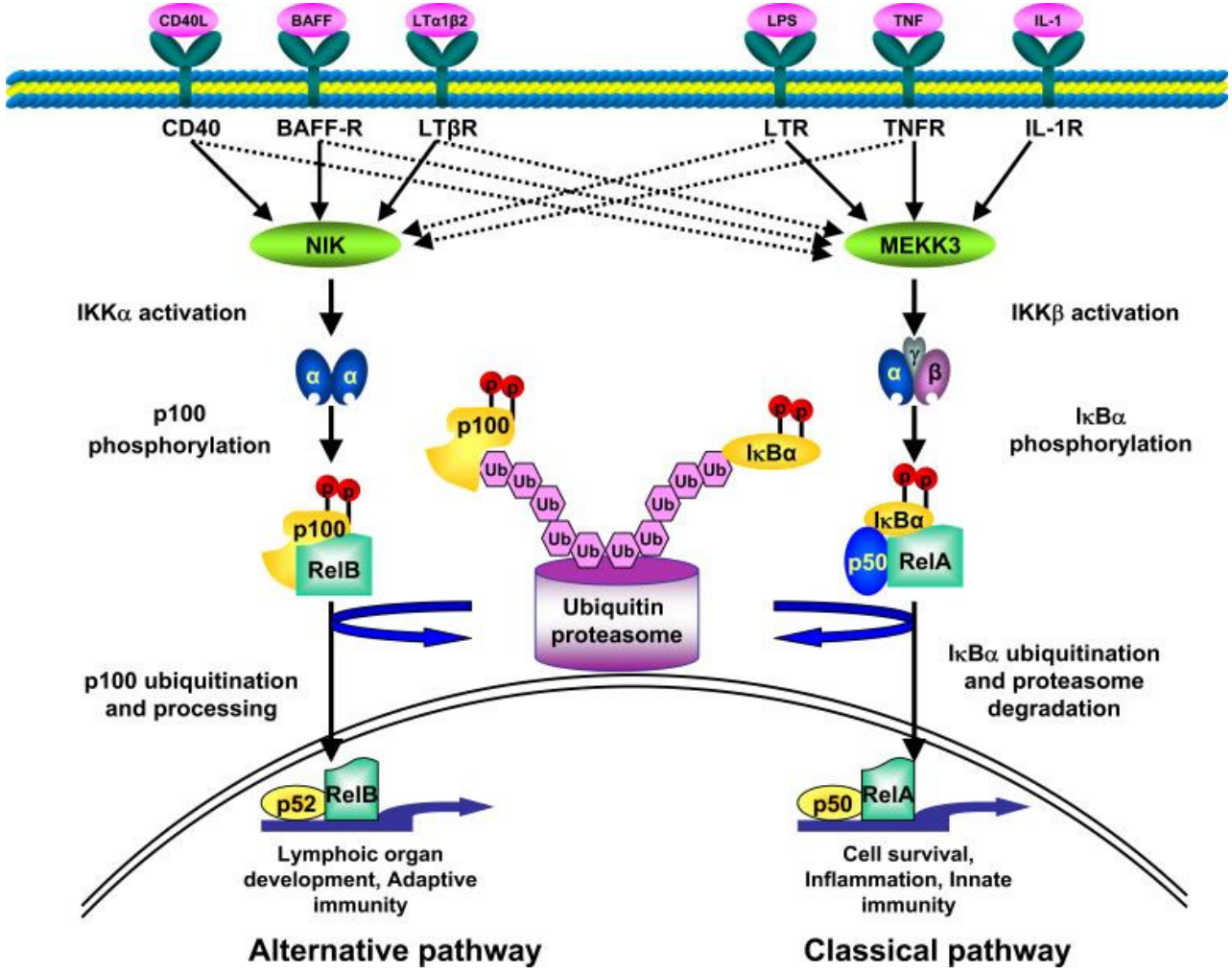
In humans, chronic diseases such as chronic obstructive pulmonary disorder, Duchenne muscular dystrophy, sepsis, cancer, acquired immunodeficiency syndrome, and chronic renal disease produce severe weight loss and cachexia (Hong et al. 2008; Peterson and Guttridge, 2008). Researchers have shown *in vivo* that inflammation results in increased protein degradation in skeletal muscle (Voison et al., 1996; Cai et al. 2004; Acharyya et al., 2007). Furthermore, *in vitro* experiments have shown that protein synthesis is attenuated during atrophy (Eley and Tisdale, 2007).

The protein disequilibrium observed in cachexia has been shown to be mediated by NF-κB (Cai et al., 2004; Bakkar et al., 2005; Acharyya et al., 2007). Nuclear factor-κB signaling has been identified in diverse cell types and activates several signaling pathways; the most well characterized being inflammation and cell survival. In brief, NF-κB activation occurs through two convergent pathways (Fig. 2.1). The classical pathway indicates that multiple extracellular stimuli, such as tumor necrosis factor-α (**TNF-α**), interleukin-1 (**IL-1**), and lipopolysaccharide (**LPS**), interact with their cognate receptors [**TNFR**, **IL-1R**, and Toll-like receptor-4 (**TLR4**)] leading to downstream signal transduction through mitogen-activated protein kinase kinase 3 (**MEKK3**) and phosphorylates the β-subunit of the inhibitor of NF-κB complex kinase (**IKK**). Activated IKK phosphorylates inhibitor of NF-κB (**IκB**) bound to NF-κB freeing it to be degraded through the ubiquitin-proteasome pathway. Upon its release, NF-κB crosses into the nucleus where it acts as a transcription factor for multiple genes (Li et al., 2008; Peterson and Guttridge, 2008). The alternative pathway is mediated through extracellular stimuli interacting with their receptors (e.g. **CD40**, a costimulatory receptor on antigen

presenting cells; B-cell activating factor of the TNF family receptor, **BAFFR**; or lymphotoxin-β receptor, **LTβR**) to promote signaling through NF-κB-inducing kinase (**NIK**) which acts on IKK-α leading to phosphorylation of p100 (a subunit of IκB) causing it to be degraded through the ubiquitin-proteasome pathway resulting in transnucleation of NF-κB (Li et al., 2008; Peterson and Guttridge, 2008).

Nuclear factor-κB signaling has been shown to increase muscle catabolism by inducing proteins in the ubiquitin-proteasome pathway, which is the principle route of protein degradation in the cell. Researchers have found that *in vivo* and *in vitro* exposure to TNF-α resulted in increased ubiquitin mRNA and protein degradation in rat skeletal muscle (Garcia-Martinez et al., 1995; Llovera et al., 1997). Others have shown that atrogin-1 (a.k.a. muscle atrophy F-box, **MAFbx**) and muscle RING finger 1 (**MuRF-1**) is a muscle specific ubiquitin-ligase found in atrophying skeletal muscles (Bodine et al. 2001; Gomes et al., 2001). Inflammatory ligands such as LPS, dexamethasone, and TNF-α have been shown to upregulate expression of these atrogenes with long-term effects; however, inhibition of these pathways does not mitigate their expression (Frost et al., 2007). Indeed, the anabolic hormone, insulin-like growth factor-1 (**IGF-1**), could suppress atrogin-1, but not MuRF-1 expression (Frost et al., 2007). Proteolysis inducing factor (**PIF**) increases the 'chymotrypsin-like' activity of the proteasome in myotubes (Whitehouse and Tisdale, 2003). Another ubiquitin-ligase, Nedd4, has been shown to be upregulated in atrophying muscle and is specific for suspended muscle (Koncarevic et al., 2007). Several ubiquitin ligases can be induced during skeletal muscle atrophy and are specific for the type of stimuli received.

Figure 2.1. Nuclear factor-κB signaling pathways (created by Li et al., 2008).



In chronic inflammation, serum levels of pro-inflammatory cytokines (i.e. TNF-α and IL-6) increase and are associated with decreased muscle mass (Tracey et al. 1988; Visser et al., 2002). In rats injected with TNF-α, anorexia soon appeared, but whole-body protein reserves were depleted significantly more than in pair-fed control rats (Tracey et al., 1988). Endotoxin and raised levels of TNF-α and IL-1β lead to decreased levels of IGF-I and growth hormone (**GH**), both of which are involved in myogenesis (Fan et al., 1995; Fan et al., 1996; Frost et al., 2003). Furthermore, these cytokines decrease IGF-I mediated protein synthesis by utilizing ceramide as a second messenger to downregulate myogenin and MyoD expression, two myogenic proteins (Strle et al., 2004). It has been demonstrated that NF-κB signaling, mediated by PIF and angiotensin II, can affect translation efficiency by inhibiting binding of methionyl-tRNA to the 43S ribosome (Eley and Tisdale, 2007). To complicate the situation, research suggests that protein degradation and decreased protein synthesis are responsible for the increased muscle protein turnover during acute sepsis; however, compensatory protein synthesis occurs during chronic sepsis with a concomitant increase in proteolysis (Voison et al., 1996).

Lastly, pro-inflammatory cytokines, namely TNF-α and IL-1β, can inhibit differentiation and decrease protein content of myoblasts through NF-κB transactivation (Langen et al., 2001). What is of interest is that NF-κB signaling may be necessary for myoblast proliferation. Typically, myoblasts will differentiate into myotubes by fusing with existing myofibers, but ablation of NF-κB results in decreased myoblast proliferation and differentiation by inducing cyclin D expression and promoting cell cycle progression from G1 to S phase (Guttridge et al., 1999). Specifically, NF-κB may exert its inhibitory effects during the late stages of differentiation. YinYang1 (**YY1**) is a

negative regulator of myogenesis and researchers have discovered it is induced by NF-κB activation resulting in decreased levels of myofibrillar genes encoding troponins, myosin heavy and light chains, and α-actin, thereby preventing maturation into myotubes (Wang et al., 2007). Activation of NF-κB has been shown to destabilize MyoD transcripts through the inducible nitric oxide synthase-nitric oxide pathway (Sitcheran et al., 2003; Di Marco et al., 2005). Under these circumstances, repair of differentiated myofibers would be suppressed by NF-κB activation. This was demonstrated by Mourkioti and coworkers (2006), who found that inhibiting IKK2 activity improved morphology and size of myofibers. Overall, NF-κB activation is necessary for myoblast proliferation, but its activity prevents myoblast differentiation and may impair muscle regeneration following injury.

AMINO ACIDS AND PROTEOLYSIS

Protein homeostasis is essential to an animal's health, growth, and maintenance of life. Proteins provide the basic elements that provide structure (e.g. collagen), movement (e.g. actin and myosin), metabolism (e.g. enzymes), respiration (e.g. hemoglobin), and immunity (e.g. immunoglobulins). It has long been known that twenty amino acid species are the building blocks of proteins, and these can be stratified into those that are an indispensable (essential) part of the diet and those that are dispensable (nonessential), which can be made in the body from other precursors given the appropriate nutritional environment and stage of development (McNurlan and Garlick, 2000).

Since animals have no storage capacity of amino acids, the free amino acid pool is essentially the crux of protein homeostasis with inputs coming from the diet (particularly

the indispensable amino acids), *de novo* synthesis, and proteolysis of existing proteins. Amino acids exit this pool by incorporation into proteins, catabolism to urea and carbon dioxide, or as precursors of signaling molecules (Chaveroux et al., 2010; McNurlan and Garlick, 2000). Net protein balance, the difference between protein synthesis and its degradation, has been shown to fluctuate relative to consumption of a meal. During the absorptive period, plasma amino acid concentrations will rise (especially the branched chain amino acids) accompanied by a net uptake of amino acids into skeletal muscle (Aoki et al., 1976; Huntington and Prior, 1985). Postruminal supply of amino acids to steers fed a protein restricted diet resulted in increased muscle proteogenesis and proteolysis, with the net result of increase muscle protein accretion (Reecy et al., 1996). Additionally, this research group observed postruminal supplementation of amino acids promoted cell cycle progression and proliferation and protein synthesis in myogenic cells *in vitro*.

In contrast, a negative protein balance can result during the post-absorptive period or in response to deleterious situations such as malnutrition, including total amino acid deficit, imbalanced amino acid profile, or disease. Prolonged protein deficiency in children leads to decreased plasma amino acid, especially the essential amino acids, in particular, the branched chain amino acids (Arroyave et al., 1962; Baertl et al., 1974). Shortage of amino acids leads to growth arrest attributable to changes in the somatomedins (GH, IGF-1, and IGF-2). Animals with severe and moderate malnutrition had increased circulating concentrations of GH while concomitantly decreasing plasma IGF-1 levels (Breier et al., 1986; Renaville et al., 2002). The stimulatory effects of IGF-1 can be modulated by expression of IGF binding proteins (**IGFBP**). Young bulls subjected

to prolonged undernutrition exhibited lower concentrations of circulating levels of IGF-1 corresponding with increased IGFBP-2 levels. Realimentation of this group of animals reversed this condition with IGF-1 levels increasing and IGFBP-2 decreasing (Renaville et al., 2000). The same trends are apparent in humans with decreases in circulating amino acids being responsible for stimulation of IGFBP-1 production and growth arrest (Straus et al. 1994). The increase in IGFBP-1 results from the increased expression of GH (Chaveroux et al., 2010). Therefore, the growth restriction seen in young malnourished animals results from increased GH, which, in turn, increases levels of IGFBP, thereby reducing levels of IGF-1 and decreasing its somatotrophic activity.

Pathological situations such as sepsis, cancer, and injury result in decreases in body mass and alterations in metabolism leading to negative nitrogen balance. While there is an observable increase in protein synthesis, there exists a concomitant increase in proteolysis that outpaces the synthesis. (Wykes et al., 1996; Jahoor et al., 1999; Orellana et al., 2004; Mercier et al., 2002; Orellana et al., 2002; Langen et al., 2006; Frick et al., 2008; Balage et al., 2010)

MUSCLE GENES

Glutamine Synthetase

While there are several gene targets whose expression is characteristically changed during muscle atrophy, our interest lies in those involved in proteolysis and transducing signals from growth stimulatory mediators. In this review, we present a few candidate genes of interest in an effort to understand their possible role in decreasing muscle mass in calves treated for BRD.

The cachexia associated with activation of the NF-κB signaling pathway could explain the decreased muscle mass in calves diagnosed with BRD. Increased protein degradation leads to accumulation of intracellular ammonia that must be eliminated. Glutamine synthetase (also known as glutamate-ammonia ligase; **GLUL**) is an ATP-dependent enzyme that ligates ammonia to glutamate forming glutamine. Glutamine is the most abundant free amino acid, but 95% of this amino acid is stored intracellularly, with skeletal muscle being its major repository (Stipanik and Watford, 2000). Additionally, inflammation depletes muscle glutamine levels by as much as 50% (Calder and Yaqoob, 1999). Chakrabarti (1998) discovered that TNF-α stimulated GLUL in a dose-dependent manner with maximal stimulation observed at 1,000 units/ml TNF-α added to murine skeletal muscle cell lines. That same experiment observed that the same concentration of TNF-α increased GLUL in a time-dependent manner. Following 24 hours of TNF-α stimulation, GLUL activity rose 67% and reached maximum activity by 48 hours with a 3-4 fold increase. This increase in activity corresponded with increased GLUL transcription which peaked with a 90% increase in mRNA at twelve hours and only slightly decreased transcripts at 24 hours following TNF- α.

Glutamine may play a pivotal role in skeletal muscle myopathy observed in cattle infected with common BRD pathogens. *In vitro* and *ex vivo* research demonstrate that glutamine supplementation can increase the phagocytic capabilities of human macrophages and neutrophils (Parry-Billings et al., 1990; Furukawa et al., 2000). In clinical cases, parenteral supplementation of glutamine can increase intramuscular glutamine concentrations and nitrogen balance in post-operative patients (Stehle et al., 1989). However, Wells and coworkers (1999) demonstrated that production of pro-

inflammatory cytokines in murine macrophages, namely TNF-α, IL-1β, and IL-6, was enhanced by glutamine supplementation *in vivo* during inflammation in the paws of rats, suggesting that increased glutamine exacerbated inflammation; however, those rats receiving additional glutamine demonstrated decreased swelling in the affected appendage (Nascimento et al., 2005). A survival rodent model demonstrated that glutamine supplementation significantly increased the chances of survival (Wilmore and Shabert, 1998).

Adiponectin Receptor 1

Adiponectin is one of a class of protein hormones secreted by adipocytes called adipokines (Freubis et al., 2001; and Yamauchi et al., 2001). Adiponectin is the most abundant adipokine in plasma of humans and rodents, and its concentrations decline in models of obesity and type 2 diabetes (Kadowaki and Yamauchi, 2005; Dyck et al., 2006). Moreover, administration of this adipokine can alleviate hyperglycemia and hyperinsulinemia in mouse models of insulin resistance (Freubis et al., 2001; Yamauchi, 2001). Adiponectin has been shown to increase removal of triglycerides and free fatty acids in mice fed a high fat diet (Freubis et al., 2001). This removal was facilitated by an increase cellular uptake potentiated by increased gene expression of fatty acid transporters (i.e., CD36; Freubis et al., 2001; Yamauchi et al., 2005). In addition, this hormone decreased triglyceride content in skeletal muscle by increasing gene expression of acyl-CoA oxidase and uncoupling protein 2 and as a result increasing fatty acid oxidation by as much as 19% (Freubis et al., 2001; Yamauchi et al., 2001).

The effects of adiponectin are mediated by two integral membrane receptors, adiponectin receptor-1 and -2 (**ADIPOR1** and **ADIPOR2**); the former being most abundantly expressed in skeletal muscle, and the latter being most prolific in liver (Yamauchi et al., 2003). Yamauchi's group observed that transfection of C2C12 murine myocytes with ADIPOR1 increased peroxisome proliferator-activated receptor-α (**PPAR-α**) activation, which partially explains the increased fatty acid oxidation that they observed. Similarly, ADIPOR1 expression increased the phosphorylation of adenosine monophosphate kinase (**AMPK**), acetyl-CoA carboxylase (**ACC**), and p38 mitogen-activated protein kinase (**p38-MAPK**) which in turn accounts for the increase in glucose uptake and fatty acid oxidation (Yamauchi et al., 2003). The phosphorylation of ACC would lead to decreases in fatty acid biosynthesis (Tong, 2005).

In human *in vivo* studies, there is no consensus on the effects of glucocorticoids on adiponectin levels. Intravenous administration of hydrocortisone resulted in decreased plasma concentration of adiponectin in healthy subjects (Fallo et al., 2004). Fallo et al. (2004) further observed an inverse relationship between urinary cortisol levels and adiponectin levels in plasma of patients diagnosed with Cushing's syndrome. Conversely, a more recent study indicated that oral administration of dexamethasone in both healthy and diabetic patients resulted in small increases in plasma adiponectin (Jang et al., 2008). In contrast, *in vitro* research indicates that dexamethasone addition to human adipocytes decreased adiponectin release by up to 16% (Degawa-Yamauchi et al., 2005). Additional research with 3T3-L1 murine adipocytes has similar findings with a 50% decrease in adiponectin mRNA following dexamethasone treatment (Fasshauer et al., 2002). Rodent models are equally unclear. In obese and non-obese rats, chronic intraperitoneal injection

of hydrocortisone decreased serum adiponectin levels and mRNA in white adipose tissue (Shi et al., 2010). Another group observed that adrenalectomy increased adiponectin mRNA and serum concentrations in obese mice compared with the sham controls; however, in wild-type mice, there was a significant drop in these parameters in the adrenalectomized group relative to the sham-operated group (Makimura et al., 2002). Less information is available regarding the effects of glucocorticoids on ADIPOR1 expression. Jang and coworkers (2008), found that ADIPOR1 mRNA expression in human skeletal muscle was unaffected by dexamethasone treatment. Glucocorticoid-mediated suppression of adiponectin may explain the decrease in ADIPOR1. Results from a study of lean human subjects found that myotubes stimulated with globular adiponectin increased expression of ADIPOR1, but myotubes from obese, diabetic, or weight loss subjects were refractory to such stimulation (McAinch et al., 2006). Depressed levels of adiponectin induced by glucocorticoid may explain the trend for ADIPOR1 suppression in the calves in both treatment groups at the 24H time point.

Inflammatory cytokines, such as TNF-α, can reduce adiponectin release by murine adipocytes *in vitro* (Degawa-Yamauchi et al., 2005). In human subjects, TNF-α neutralization resulted in increased total adiponectin levels in serum by more than 10% above baseline (Lo et al., 2007). Anorexia is common among morbid animals, which may alter adiponectin effects. Investigators have reported increases in ADIPOR1 transcripts in skeletal muscle of fasted mice which were restored to original levels upon re-feeding (Tsuchida et al., 2004).

It may be that effects from the stressful handling conditions and decreases in feed intake exacerbate the deleterious effects of BRD. If levels of adiponectin and its receptors

are altered during BRD infections, this may explain the reduction in muscle accretion often seen in calves treated for BRD. In models of diabetic mice, insulin resistance is associated with skeletal muscle atrophy. This muscle wasting is observed with low plasma concentrations of adiponectin, which is further implicated in increased levels of E3 ubiquitin ligases (i.e. atrogin-1 and muscle RING finger-1) involved in the ubiquitin-proteasome pathway leading to increased protein degradation (Zhou et al., 2007; Wang et al., 2006).

Thioesterase Superfamily Member 4

Little is known about thioesterase superfamily member 4 (**THEM4**) protein, and we were unable to find information regarding the effects of stress on its expression. THEM4, also known as carboxyl-terminal modulator protein (**CTMP**), was first identified in 2001 as an inhibitor of protein kinase B (**PKB/Akt**) and was thought to be localized to the plasma membrane in various *in vitro* cell cultures (Maira et al., 2001). The kinase activity of PKB/Akt was stimulated by insulin and insulin-like growth factor-1, but this was abolished by expression of THEM4. Furthermore, this group found that THEM4 lessened phosphorylation of glycogen synthase kinase-3β, a downstream target of PKB/Akt activity. In contrast, Ono's group, using similar *in vitro* modeling, indicated that overexpression of THEM4 increase phosphorylation of PKB/Akt in both basal and insulin stimulated states (Ono et al., 2007). They observed that THEM4 increased phosphorylation of glycogen synthase kinase-3β and demonstrably increased glycogen synthesis in 3T3-L1 adipocytes. Additional findings in that report suggest that THEM4 overexpression may increase cell viability following UV irradiation.

More recent reports indicate that THEM4 is a nuclear encoded protein found in the inner membrane and intermembrane space of mitochondria that is released into the cytosol following apoptotic signaling (Parcellier et al., 2009; Piao et al., 2009). Overexpression of THEM4 resulted in increased incidence of cell death and was integral in increasing cleavage of pro-apoptotic enzymes (i.e. caspase-3 and poly ADP ribose polymerase). This increased induction of THEM4 delayed phosphorylation of PKB/Akt. In addition, mutants of THEM4 that are sequestered in the mitochondria do not affect phosphorylation of PKB. THEM4 further demonstrated the ability to sequester heat shock protein 70 promoting apoptosis (Piao et al., 2009). Therefore, the majority of the evidence suggests that THEM4 is an inhibitor of PKB/Akt that functions to sensitize cells to apoptotic stimuli. It is further noteworthy that THEM4/Akt heterodimer is an effective thioesterase with particular affinity for medium to long chain fatty acids (Zhao et al., 2009). Yet with the experimentation performed with this enzyme, relatively little is known about its induction and regulation; its effects and responses in muscle tissues have not been reported.

ACUTE PHASE RESPONSE

Following breach of host defenses by bacterial and/or viral pathogens, trauma, stress, surgery, or cancer, the innate immune system initiates a rapid and potentially systemic acute inflammatory reaction. Such a response is termed the acute phase response (**APR**) and usually occurs within the first 48 hours of infection or injury. This response is initiated by the production of proinflammatory cytokines (e.g. TNF-α, IL-1, and IL-6) by phagocytic immune tissues and leads to fever, anorexia, muscle catabolism, coagulation, increased glucocorticoid hormones, changes in liver protein synthesis, and

leukocytosis (Jeschke and Herndon, 2004; Gruys et al., 2005; Cray et al., 2009; Eckersall and Bell, 2010). While such a response is invaluable to the health of the animal, it can have severe growth consequences. If the systemic immune response cannot be regulated and becomes chronic, it can be detrimental to the animal by increasing morbidity and mortality, organ failure, and risk of sepsis (De Maio et al., 1998; Selzman et al. 1998).

Hepatic Protein Metabolism

The parenchymal cells of the liver are responsible for the production of the acute phase proteins (**APP**). These can be classified into two groups; the positive APPs are those that are stimulated and the negative APPs are those that are inhibited during this response (Gruys et al., 2005; Cray et al., 2009). The positive APPs are so named because they typically increase more than 25% from their baseline values during the APR (Eckersall and Bell, 2010). These APPs are induced by pro-inflammatory cytokines and can be further categorized into Type I and II depending upon the stimulus. Type I APPs are stimulated by the IL-1 like cytokines (i.e. TNF-α, IL-1α, and IL-1β) and include haptoglobin and α_1-acid glycoprotein. Type II APPs such as α_2-microglobulin, fibrinogen, and serum amyloid A respond to IL-6 like cytokines (i.e. IL-6 and IL-11; Jeschke et al., 2004). While there is a plethora of acute phase proteins identified in other species, ruminants, particularly cattle, are unique in that haptoglobin and serum amyloid A are the major APPs observed to increase during infections (Cray et al., 2009; Eckersall and Bell, 2010).

Haptoglobin (Hp) has been extensively studied, and its major function is somewhat bacteriostatic in that it sequesters iron from free hemoglobin in the blood

during inflammation preventing its utilization by pathogens (Peterson et al., 2004). Changes in Hp levels have been seen in several bacterial and viral infections, including *M. haemolytica* and *Pasteurella multocida*, BVDV and bovine respiratory synctial virus all of which are common BRD pathogens (Peterson et al., 2004; Eckersall and Bell, 2010). Normal serum Hp concentration in healthy cattle is less than 20 mg/L, but this can increase 100 fold or more and is a useful biomarker for clinical infection (Horadagoda et al., 1999, Carter et al., 2002; Nikunen et al., 2007, Aich et al., 2009, Burciaga-Robles et al., 2010). Previous research from feedlot and dairy calves showed significant increases in Hp, serum amyloid A (**SAA**), fibrinogen, α1-glycoprotein, and lipopolysaccharide binding protein associated with infection of common BRD pathogens (Horadagoda et al., 1999, Carter et al., 2002; Nikunen et al., 2007). In feedlot conditions, Hp concentration was increased in calves diagnosed with BRD and the ratio of Hp to serum amyloid A was correlated with the numbers antimicrobial treatments administered to morbid calves (Carter et al., 2002). In a similar experimental challenge study, calves were inoculated with BVDV and 5 days later infected with *M. haemolytica* (Gånheim et al., 2003). Haptoglobin level increased in all groups compared with controls. In calves inoculated with BVDV and *M. haemolytica*, Hp concentrations increased above baseline (0.13 g/l) 24 hours following joint infection with concentrations 8.5- to 16.9-fold higher observed at 3 to 6 days later and finally returning to baseline 10 to 13 days post-infection with both pathogens (Gånheim et al., 2003). Research from our laboratory has shown that experimental exposure of beef calves to *M. haemolytica* resulted in a 10-fold increase in serum haptoglobin concentrations 18 hours following bacterial infection, reached maximal levels at 40 hours and remained elevated for 4 days (Burciaga-Robles et al.,

2010). Similarly, Aich and coworkers (2009) experimentally induced BRD in calves with bovine herpesvirus-1 (BHV-1) and *M. haemolytica* four days post viral infection, and using 2D-gel electrophoresis, they observed several subunits of haptoglobin protein and apolipoprotein A1 were significantly increased in all infected calves (independent of subsequent survival) at day 4 following viral infection compared to d 0.

Serum amyloid A is a second definitive positive APP in cattle and is shown to be increased during acute inflammation and BRD (Horadagoda et al., 1999, Nikunen et al., 2007). In an experimental model of chronic infusion of lipopolysaccharide (LPS) in heifers, Werling and associates found that SAA concentration in serum was significantly increased at 30 minutes post infusion and reach a plateau at 4.5 hours with an approximate 5-fold increase that was sustained through 6 hours post infusion (1996). Similarly, others observed that SAA rose steadily following 2 hours of intratracheal inoculation with *P. haemolytica* (Horadagoda et al., 1994). As previously mentioned in the Gånheim experiment, SAA levels increased in all groups compared with controls. In calves inoculated with BVDV and *M. haemolytica*, SAA concentration changes were biphasic with the first phase 1 to 3 days post-infection of both pathogens with peak levels increased approximately 5-fold above baseline (25.6 g/l).The second phase occurred 5 to 7 days post infection and was similar in peak concentrations, but was more variable. Lastly, SAA values returned to normal levels 10 days post infection with both pathogens (Gånheim et al., 2003).

The picture that emerges from these studies is clear that significant changes in protein production occur in the liver, which surely modifies amino acid requirements for that tissue. In fact, previous research from our laboratory has demonstrated that during

experimental *M. haemolytica* challenges there is a net uptake of essential and non-essential amino acids by the liver, resulting in net removal of total amino acids from portal blood that is greater than unchallenged control animals (Burciaga-Robles, 2007). Further, our group observed significantly increased net hepatic removal of aromatic, branched chain, and gluconeogenic amino acids (unpublished data). Waggoner and associates experimentally challenged beef steers with endotoxin and plasma concentrations of isoleucine and leucine significantly decreased, especially following 4 hours post-infusion with LPS when compared to their unchallenged counterparts (Waggoner et al., 2009). Similarly, clearance kinetics in human subjects intravenously perfused with amino acids indicated greater removal of several amino acids in patients with microbial sepsis when compared to healthy controls, presumably as a result of hepatic extraction (Druml et al., 2001). Notably, branched chain and gluconeogenic amino acid clearance was 97% greater in septic patients than in control subjects (Druml et al., 2001). Low amounts of branched-chain amino acid transferase in the liver make metabolism of branched chain amino acids (**BCAA**) to their ketoacids negligible since perfused liver does not readily metabolize BCAA (Smith and Elia, 1983). Little is known of hepatic metabolism of BCAA during acute inflammation; however, it may be that these amino acids are incorporated into nascent proteins or involved in signaling protein translation (Kimball and Jefferson, 2006). Interestingly, Reeds and co-workers compared the amino acid content of major APPs (C-reactive protein, fibrinogen, α_1-glycoprotein, α_1-antitrypsin, SAA, and Hp) and muscle protein and found a significant disparity between the two (1994). They calculated the demand for the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) would lead to more than twice the amount of

amino acids in skeletal muscle to be mobilized to accommodate APP synthesis. This need for specific amino acids indicates the detriment that the APR can have on muscle in beef cattle.

Hepatic Energy Metabolism

The hypermetabolic state that occurs during acute phase of illness results in alterations in carbohydrate and lipid metabolism (Chioléro et al., 1997). Lipopolysaccharide depletes carbohydrate stores in animals. In cattle infused with LPS or recombinant bovine TNF-α (**rbTNF**), there was an initial hyperglycemic state as early as 30 min. post-infusion with hypoglycemia occurring at 3 hours and sustaining for 6 to 10 hours (Werling et al., 1996; Kushibiki et al., 2000). Additionally, Montgomery and coworkers (2009) found that heifers treated for apparent BRD had lower glucose levels compared with those that were untreated. In rodent models of endotoxin challenge, researchers have demonstrated LPS leads to hypoglycemia and hepatic glycogen depletion in fasted rodents (McCallum and Berry, 1973; Filkins and Cornell, 1974). Furthermore, using radioactively labeled substrates (alanine, pyruvate, and glucose), these rodent models established that LPS decreased gluconeogenesis, glycogenesis, and glyconeogenesis *in vivo*. Similarly *in vitro* enzyme activity and kinetic experiments indicate endotoxemia decreased activity and substrate flux of phosphoenolpyruvate carboxykinase (**PEPCK)**, the rate limiting enzyme in gluconeogenesis; glycogen synthase, the primary enzyme in glycogenesis, and pyruvate kinase (PK), an important enzyme in glyconeogenesis (McCallum and Berry, 1973; Jones and Titheradge, 1993).

Reduction in these enzyme activities is potentiated by the pro-inflammatory cytokines. Tumor necrosis factor-α is a transcriptional regulator of PEPCK gene expression in endotoxemic rodents and is capable of repressing glucocorticoid- and glucagon/cAMP-mediated induction of this gene (Hill and McCallum, 1991; Hill and McCallum, 1992; Waltner-Law et al., 2000). In transition dairy cattle administered daily low doses of TNF-α for seven days, glucose-6-phosphatase taken from liver tissues decreased 50%. Additionally, PEPCK expression tended to be suppressed by this inflammatory cytokine, supporting the fact that gluconeogenesis suppression in cattle is similar to that in other species (Bradford et al., 2009). However, these results are equivocal given that perfused liver slices taken from dairy cattle in mid-lactation tended to exhibit increased conversion of radio-labeled propionate to glucose when LPS was administered *in vivo.* This finding led the authors to conclude that those cattle were in an increased gluconeogenic state (Waldron et al., 2003). Surprisingly, these results did not correspond with results for other gluconeogenic substrates (lactate and alanine) or with plasma glucose levels observed in the animals.

Other cytokines involved in the APR have been implicated in these changes in carbohydrate metabolism. Interleukin-6 mitigated the mRNA transcription and abundance, and enzyme activities increase of PEPCK and glucokinase, the initial committed enzyme of glycolysis, by glucagon and insulin, respectively, in cultured rat hepatocytes (Christ et al., 1994; Christ et al., 1997). In a similar study, IL-1β and TNF-α both virtually abrogated the glucagon induction of PEPCK transcription and activity, which resulted in decreased glucose formation from radioactive lactate (Christ and Nath, 1996). These effects on PEPCK appear to be potentiated by posttranscriptional

degradation of mRNA transcripts and decreased translation of these genes (Christ et al., 1994; Christ and Nath, 1996).

Furthermore, inflammatory cytokines are known to alter glycogen metabolism. In rat hepatocytes stimulated with insulin, IL-6 and IL-1β lessened the increase of glycogen deposition (Lee et al., 1993; Kanemaki et al., 1998). Further, in these studies, these two cytokines were able to inhibit the activity of glycogen synthase and to stimulate glycogen phosphorylase activity, thereby attenuating the effects of insulin on these two enzymes (Lee et al., 1993; Kanemaki et al., 1998). Kanemaki and coworkers (1998) demonstrated that IL-6 was very effective, as it decreased glycogen increase by 30% within 1 hour and nearly abolished its increase within 4 hours of cytokine treatment, whereas IL-1β showed no significant effects until 4 hours.

Research has established that inflammatory conditions such as sepsis can increase whole body resting energy expenditure (Chioléro et al., 1997). Furthermore, the ability of pro-inflammatory cytokines to alter transcription, translation, and activity of metabolic enzymes involved in glycolysis, gluconeogenesis, glyconeogenesis, glycogenesis, and glycogenolysis. The overall picture is that *de novo* glucose production and utilization is inhibited; similarly, glycogen synthesis is inhibited while its degradation is promoted. This shift in carbohydrate mobilization supports the theory that hepatic tissues diminish carbohydrate utilization to meet the energy demands of peripheral tissues. With markedly increased levels of proinflammatory cytokines in cattle with BRD during the acute phase response (Burciaga-Robles, 2010), one can presume this would greatly alter expression of metabolic genes in cattle. However, there is no known literature that addresses the effects of bovine respiratory disease on specific genes in carbohydrate metabolism. Additional

research would be of interest given the important differences in carbohydrate metabolism of ruminants compared with monogastric species.

Increased lipid utilization is a common characteristic in pyogenic bacterial infections, particularly those that induce sepsis. These infections increase fatty acid oxidation, particularly by peripheral tissues, and decrease glucose usage (Stoner, 1987). In lactating cows, LPS administration lead to an increase in non-esterified fatty acids (NEFA) within 2 hours of administration with return to basal levels an hour later (Waldron et al., 2003). Steiger et al. (1999) used a more prolonged infusion of LPS (100 min.) to dairy heifers and observed a biphasic response of free fatty acids (FFA) and glycerol in plasma. There was an initial increase in occurring within 1 hour of infusion, a decrease to levels similar to control heifers by 3 hours, and another peak around 4.5 to 5 hours post-infusion. Free fatty acids, however, elevated for the duration of the observations (22 hours). Recombinant bovine TNF-α given to dairy heifers caused similar results. There was an elevation in NEFA as early as 45 min. and persistence for up to 24 hours following dosing (Kushibiki et al., 2000; Kushibiki et al., 2002). Triglyceride (TG) levels of heifers were lowered 2.5 hours post-infusion with LPS and remained low for 10 hours, but surprisingly increased to above control levels at 22 hours (Steiger et al., 1999). This response is mediated in part by TNF-α. In studies with Holstein heifers, administering rbTNF led to an early increase in TG concentration at 30 to 45 min. following dosing that was followed by diminished levels at 2.5 hours through 8 hours after treatment with the cytokine (Kushibiki et al., 2000; Kushibiki et al., 2002). LPS administration lessened plasma concentration of β-hydroxybutyrate (BHB) within 2 hours of infusion (Werling, et al., 1996; Steiger et al., 1999; Waldron et al., 2003). There

is a variable longer term response in BHB levels with LPS infusion. Following the initial decrease, BHB remained suppressed in lactating cows (Waldron et al., 2003). In studies with beef heifers, BHB levels were decreased and remained low for 6 hours following infusion (Werling et al., 1996). In contrast, dairy heifers with a similar infusion time, exhibited an initial decrease similar to the other studies, but BHB levels increased by 6 hours post-infusion to match those in healthy control heifers (Steiger et al., 1999). On the contrary, in dairy cattle with clinical mastitis, there were no significant changes in NEFA or ketone levels, but there were chronic decreases in cholesterol (Huszenicza et al., 1998). Even with these variable results, it is evident that endotoxin can disrupt fatty acid and intermediary metabolism.

In healthy animals, the liver is actively involved in packaging FFA by producing very-low-density lipoproteins (VLDL; Jones, 1982). In rodents treated with endotoxin or TNF, there was an increase in triglycerides and cholesterol in serum following administration (Feingold and Grunfeld, 1986; Feingold et al., 1992). Further that laboratory group observed increased fatty acid and cholesterol content in the liver with TNF-α treatment and *in vitro* analysis of liver explants revealed increased incorporation of acetate into fatty acids without concomitant changes in acetate oxidation to carbon dioxide (Feingold and Grunfeld, 1986). Interleukin-1 also affects lipid metabolic aberrations. Injection of IL-1 into chow-fed and sucrose-fed rats resulted in increased serum levels of tritiated TG while decreasing glycerol concentrations, indicating that the cytokine stimulated TG secretion by liver while suppressing lipolysis (Feingold et al., 1990). In addition, they observed that IL-1 injected rats had increased hepatic fatty acid and cholesterol content when compared to pair-fed controls (Feingold et al., 1990).

Therefore, IL-1 increased fatty acid synthesis and decreased fatty acid catabolism in liver, presumably supplying the energy substrate to peripheral tissues. Closely related to this is secretion of VLDL. Studies with septic and endotoxemic rats and liver explants indicated increased levels and secretion of apolipoprotein B (apoB) protein, which represents an increase in VLDL and probable role in the hypertriglyceridemia commonly observed with sepsis (Tripp et al., 1993; Aspichueta et al., 2005; Bartolomé et al., 2008). Similarly, *in vitro* stimulation of HepG2 and mouse primary liver cells by IL-1β and IL-6, but not TNF-α, resulted in increased mRNA and protein levels of apoB (Yokoyama et al., 1988; Bartolomé et al., 2008).

Many of the changes in intermediary metabolism brought about by the APR and several cytokines, including TNF-α, IL-1, and IL-6, are due to changes in gene expression in hepatic tissues that alter fatty acid, cholesterol, and sphingolipid metabolism as well as the lipoproteins involved in their transport (Khovidhunkit et al., 2004). This review will focus on the changes in hepatic gene expression with regard to fatty acid metabolism. Past research focused on the activity of hepatic enzymes in response to TNF-α and showed that the activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), the rate limiting enzymes of fatty acid synthesis, were increased 58% and 35%, respectively, in rats 16 hours after treatment (Grunfeld et al., 1988). Further, studies demonstrate that such a response was due to a coordinated increase in citrate concentrations, an allosteric modulator of ACC, and decrease in acyl-CoA, an allosteric inhibitor of the same (Grunfeld et al., 1988; Grunfeld et al., 1990). A more recent study shows that *in vivo* stimulation with LPS in rodents, and TNF-α, and IL-1, but not IL-6 stimulation in Hep3B cell lines suppresses transcription of ACC, FAS,

and PK (Feingold et al., 2011). These changes in gene expression were a result in part by the reduction of mRNA and protein levels for the transcription factor, carbohydrate response element binding protein (ChREBP). Other transcription factors in the liver are reduced in response to LPS and other APR cytokines and hormones. Namely, the peroxisome proliferator-activated receptors (PPAR)-α and γ are suppressed resulting in decreased levels of their target genes, FATP (fatty acid transporter protein), CD36/FAT (fatty acid translocase), H-FABP (heart-fatty acid binding protein), and carnitine palmitoyl transferase (CPT)-Iα resulting in decreased fatty acid transport into and within the liver cell as well as decreased fatty acid oxidation (Khovidhunkit et al., 2004). Therefore, during the APR, hepatic tissues increase lipogenesis somewhat independently of transcription as a result of allosteric modulators and possibly the stability of key fatty acid synthesis enzymes and decrease lipolysis and fatty acid oxidation. This results in hypertriglyceridemia and increased VLDL levels that carry the fatty acids to peripheral tissues for utilization.

In summary, the increased demand for amino acids for production of APP by the liver are supplied by muscle protein catabolism and amino acid uptake by the liver, whereas the nucleotides required for the synthesis of the positive APPs are at the expense of the transcription and mRNA degradation of the negative APPs (see Christ et al., 1994). Furthermore, the liver likely provides energy substrates to the peripheral tissues in exchange for the amino acid substrates needed for protein synthesis.

CHAPTER III

Transcriptome analysis of muscle tissue from calves infected with bovine viral diarrhea virus and *Mannheimia haemolytica*

ABSTRACT

Bovine respiratory disease (BRD) has been shown to negatively impact carcass characteristics by leading to lighter carcasses and poorer carcass quality. To understand the effects of BRD on muscle gene expression, 16 crossbred beef steers (280.8 $\pm$ 32.5 kg) were divided into 2 treatment groups: the control group (CON) was not exposed to a calf persistently infected with bovine viral diarrhea virus type 1b (PI-BVDV) and received an intratracheal dose of isotonic saline; an infected group (INF) was exposed to a PI-BVDV calf for 72 h followed by intratracheal inoculation with *Mannheimiahaemolytica* serotype A. Muscle biopsies were taken from the *longissimus dorsi* prior to exposure to both pathogens (PRE) and 24 hours (24H) following inoculation. Total RNA was extracted and bovine oligonucleotide microarray hybridization performed on the INF samples (n = 6). Significance was set at fold change greater than |2.0| and $P < 0.05$. Microarray results were validated using RT-PCR. Bioinformatic pathway analysis was conducted using Ingenuity Pathway Analysis software. We observed 28 genes were induced and 104 genes were suppressed following 24H of dual infection. Pathway analysis revealed a network of genes centered on nuclear factor-κB. PCR results demonstrated that glutamine synthetase was significantly induced at the 24H sampling (+2.57 fold, $P < 0.01$), but did not differ between treatments ($P > 0.10$). Adiponectin receptor 1 expression tended to be suppressed at the 24H sampling (-2.15; $P = 0.08$), and there was a significant treatment x time interaction ($P < 0.05$). Direct comparison of the CON and INF groups at 24H sampling indicated INF was significantly higher in expressing ADIPOR1 (+3.89; $P = 0.03$). In the INF group thioesterase family member 4 was suppressed more than 3 fold at the 24H sampling compared to the PRE, although results were not significant ($P =$

0.12). These results indicate that there are no substantive changes that occur in muscle tissues of beef calves at 24H post-infection with BVDV and *M. haemolytica*. Further research is needed to understand such physiological changes related to muscle atrophy in cattle and to better understand tissue nutrient requirements during morbidity.

Keywords: beef cattle, bovine respiratory disease, gene expression, skeletal muscle

INTRODUCTION

Bovine respiratory disease (**BRD**) is considered to be the most prevalent and economically impacting disease facing the U.S. beef cattle industry. Estimates suggest that BRD affects approximately 20 percent of all feedlot calves in the U.S. and could create actual and potential profit losses in excess of $3 billion (Edwards, 1996; Griffin, 1997; Snowder et al. 2006). The disease complex is multifactorial in its etiology. Exposed to stressors such as transportation, dietary and climatic changes, handling, and commingling with unfamiliar, aggressive, and/or sick animals can lead to immunosuppression in calves sufficient to allow primary infection from viral agents like parainfluenza virus 3, infectious bovine rhinotracheitis virus, bovine respiratory syncytial virus, and bovine viral diarrhea virus (Lillie, 1974, Fulton et al. 2000). These primary viral infections further compromise the calves' immune systems permitting secondary bacterial infections to occur, primarily with *Pasteurella* spp. like *Mannheimia haemolytica* and *Pasteurella multocida* (Lillie, 1974).

Animal health has been shown to have a direct impact on carcass characteristics of feedlot cattle. Gardner and coworkers (1999) showed that cattle treated two or more times for BRD had lower average daily gains (**ADG**) than cattle treated only once. This

decrease in weight gain was further exacerbated in cattle with evidence of lung lesions and/or active lymph nodes at the time of harvest. These decreases in ADG may translate into lighter hot carcass weights and decreased dressing percentages (Gardner et al., 1999; Roeber et al., 2001). This evidence suggests that BRD has long-term effects on muscle composition in growing calves. Therefore the objective of this study was to examine the gene expression profile of calves acutely infected with common BRD pathogens, namely bovine viral diarrhea virus and *M. haemolytica*.

MATERIALS AND METHODS

Cattle Management

All procedures performed with the animals were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Protocol # AG075). Sixteen crossbred *Bos taurus* steers (280.8 $\pm$ 32.5 kg) were procured from the university's research herd, housed at the Nutrition and Physiology Unit, Stillwater, OK, and fed a standard growing diet according to the nutrient requirements set forth by the National Research Council (NRC, 1996). Steers selected for pathogen challenge (**INF**) were transported 5.1 km to the Willard Sparks Beef Research Center, Stillwater, OK where they were commingled together in a 6 x 10.8-m pen with 2 steers confirmed to be persistently infected with bovine viral diarrhea virus type 1b (**PI-BVDV**) using genotyping and immunohistochemistry (Fulton et al., 2006). Following a 72 h exposure to the PI-BVDV cattle, these steers were returned to the Nutrition and Physiology Unit. Following a 12 h rest period, steers were intratracheally challenged at 0800 h with *M. haemolytica* serotype 1 by inoculation with 10 mL of a solution containing 6 x 10^9 cfu of

bacteria similar to the method described by Dowling et al. (2002). In short, following restraint, a broncheoalveolar lavage tube (Bivona Medical Technology, Gary, IN) was sterilized with chlorhexidine solution, rinsed with saline, inserted into the nostril, directed into the trachea within 2 to 3 cm of the bifurcation allowing challenge material to be delivered to both lungs. *M. haemolytica* serotype 1 bacteria was grown before the challenge and reconstituted as described by Mosier et al. (1998). The control group of steers (**CON**) was not transported nor exposed to a calf persistently infected with bovine viral diarrhea virus type 1b (BVDV) and received an intratracheal dose of 10 mL of PBS solution (pH 7.4; Sigma Aldrich, St. Louis, MO). Subsequently, all steers were housed in individual stanchions and remained at the Nutrition and Physiology Unit.

Biopsy Procedure

Muscle biopsies from the *longissimus dorsi* were taken at two time points: prior to exposure to both pathogens (**PRE**) and 24 hours (**24H**) following joint inoculation (Fig. 3.1). Infection status was determined by serology (Confer et al.,1995). All CON calves showed no titers for either pathogen, while all INF calves in the experiment seroconverted for both pathogens (data not shown). Muscle biopsy procedures were performed similarly to those described by Meijer et al. (1995). Calves were restrained in stanchions and a small area (5 x 5 cm) was clipped approximately 10 cm caudal to the last rib and approximately 8 cm lateral to the vertebrae. At 24H, the biopsy site was approximately 10 cm distal to the PRE sampling site in the same muscle. To provide aseptic conditions, the area was cleaned with iodine soap and rinsed with sterile water. Local anesthetic (lidocaine 2%, 3 to 15 mL) was administered subcutaneously in the designated area (without making contact with the muscle itself). The area was cleaned a

second time with iodine solution and ethanol (75%). A 1-2 cm stab incision was made through the skin and a sterile Bergstrom biopsy trochar (7 mm in diameter) was inserted into the *longissimus dorsi* muscle and 100 to 200 mg of tissue was removed and immediately placed in liquid nitrogen (Dunn et al., 2003; Pampusch et al., 2003). Samples were stored at -80^{o}C until lab analysis was performed.

RNA Isolation

Muscle tissue samples were placed in 2 mL TRIzol Reagent^{TM} (Invitrogen; Carlsbad, CA) and centrifuged at 12,000 x g for 15 min. at 4^{o}C. Supernatant was collected and phenol-chloroform extraction was performed to isolate total RNA according to the manufacturer's protocol with minor modifications. Briefly, samples were allowed to stand at room temperature (23^{o}C) for 5 min. Chloroform was added to the samples at 0.2 mL per 1 mL of TRIzol reagent used and samples were allowed to stand for 10 min. at room temperature. Samples were then centrifuged at 12,000 × g for 15 min. at 4^{o}C. The aqueous phase was collected and RNA precipitated by adding an equal volume of absolute isopropanol. Samples were kept overnight at -20^{o}C followed by a second centrifugation as previously mentioned. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 12,000 × g for 2 min. The ethanol supernatant was removed and samples were allowed to dry at room temperature. RNA was suspended using diethylpyrocarbonate treated water (42^{o}C) and concentration determined by spectrophotometry (NanoDrop ND-1000; Thermo Scientific; Wilmington, DE). Agarose gel (1%) electrophoresis was used to determine RNA integrity.

Microarray Analysis

Microarray analysis was performed on the PRE and 24H INF muscle samples. Aminoallyl-amplified RNA (**aRNA**) was synthesized according to the protocol of TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101 (EPICENTRE; Madison, WI). First-strand cDNA was synthesized using 500 ng mRNA and a synthetic oligo(dT) primer containing a promoter sequence at the 5' end for a phage T7 RNA polymerase and reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen; Carlsbad, CA). From the RNA:cDNA hybrids, the RNA was digested into fragments for priming of the second strand synthesis using RNase H. Second strand synthesis was performed using the DNA polymerase provided by the manufacturer creating a double stranded cDNA complex. This double stranded cDNA was transcribed *in vitro* and 5-(3-aminoallyl)-UTP was incorporated into the nascent strands. Aminoallyl-aRNA was purified using RNeasy MinElute Cleanup columns (Qiagen Inc.; Valencia, CA) and subsequently labeled using fluorescent dyes: PRE and 24H samples were labeled with Alexa Fluor® 555 and 647, respectively (Invitrogen; Carlsbad, CA). Dye-coupled aminoallyl-aRNA was purified using the aforementioned purification column. Labeled aminoallyl-aRNA was hybridized to bovine genome oligonucleotide (70mer) microarray slides (n = 8; Bovine Microarray Consortium; Columbia, MO) and incubated at 42^{o}C for 16 to 18 hours. Slides were scanned using the ScanArray Express (PerkinElmer; Waltham, MA).

Reverse Transcription Polymerase Chain Reaction

Microarray results were validated by reverse-transcription polymerase chain reaction (RT-PCR). Reverse transcription was performed with 900 ng RNA using QuantiTect reverse transcription kit according to the manufacturer's protocol (Qiagen Inc.; Valencia, CA). Concentration of cDNA was assessed by spectrophotometry (NanoDrop ND-1000; Thermo Scientific; Wilmington, DE). Quantitative PCR was conducted with 100 ng cDNA and using the PerfeCTa® SYBR® Green FastMix® Kit (Quanta Biosciences Inc.; Gaithersburg, MD) according to the following reaction protocol: cycle 1: (1X) 95ºC for 2 min. 30s; cycle 2: (40X), step 1: 95ºC for 10s, step 2: primer-specific temperatures (Table 3.1) for 30s, and step 3: 72ºC for 30s; cycle 3: (81X) 55 to 95º C for 30s. The reaction was performed and fluorescent intensity measured using the BioRad MyiQ™ Real Time PCR Detection System (Bio-Rad Laboratories, Inc.; Hercules, CA). Optimal melt temperatures for all primers were determined using a temperature gradient qPCR. Amplification efficiency was assessed by performing qPCR with a standard curve of serial dilutions of cDNA (Table 3.1). Primer sequences are presented in Table 3.1.

Data Analysis

Microarray. Microarray images were preprocessed for spot recognition, spot correlation with gene array list, and GenePix Results (GPR) files were generated using GenePix Pro 5.0 software (Molecular Devices, Sunnyvale, CA). Signal filtering, background noise correction, global intensity Loess normalization, and statistical analysis of GPR files (n = 6) were conducted by GenePix Auto Processor 3.2 software (Weng and

Ayoubi, 2005; http://darwin.biochem.okstate.edu/gpap). Data were analyzed using log2 transformation of signal intensities calculated as: M = log2(24H / PRE). Fold change was calculated as follows: for M values ≥ 0, fold change = 2 ^ (M); for M values < 0, fold change = (-1) * 2 ^ (M). Differential gene expression was considered to be statistically significant if a minimum of 2 spots were represented, the fold change ≥ |2|, and $P < 0.05$. Gene ontology was assigned to each gene according to its biological process or molecular function using DAVID Bioinformatics Resources 2008 (Dennis et al., 2003; Huang et al., 2009; http://david.abcc.ncifcrf.gov/) or KEGG (Ogata et al., 1999; http://www.genome.jp/kegg/). A subset of the genes was selected using increased stringency ($P < 0.01$) for pathway analysis using Ingenuity Pathway Analysis Software (Ingenuity Systems, Inc.; Redwood City, CA).

RT-PCR. The experiment was conducted in a completely randomized design with infection, time of biopsy, and their interaction used at the treatment effects and individual animal within infection x time represented the random effects. Data from RT-PCR was analyzed as the difference (ΔCt) between the threshold cycle (Ct) for *18S* and that for gene of interest. ΔCt values were analyzed using the MIXED procedure (SAS v. 9.2, SAS Institute Inc.; Cary, NC). Fold change was determined according to the following formula: for ΔCt values < 0, fold change = 2 ^ [(-1) (Least squares mean 24H – Least squares mean PRE)]; for ΔCt values ≥ 0, fold change = (-1) * 2 ^ [(Least squares mean 24H – Least squares mean PRE)]. Error bars represent the standard error of the mean of the ΔCt values. Gene expression was considered significantly different at $P < 0.05$.

RESULTS

Microarray Analysis

Microarray analysis of the entire bovine genome identified 132 differentially expressed genes when 24H was compared to PRE in the INF calves ($P < 0.05$). Among the genes (28) that were induced in muscle tissues following 24 h of joint infection of BVDV and *M. haemolytica*, the most affected categories were the cell cycle (14%), transcription regulation (11%), amino acid metabolism (7%), inflammation (7%), and ion (7%) and protein binding (7%; Fig. 3.2). In the category of suppressed genes (104) with known functions, transcription regulation (6%), oxidation/reduction (5%), transport (5%), ion binding (4%), protein binding (4%) and ubiquitin-mediated proteolysis (4%) were the biological processes most altered (Fig. 3.3). Pathway analysis indicated that the network containing the most differentially expressed genes (16) was one involved in cellular assembly and organization, skeletal and muscular system development and function, and cell death. This network of genes was focused around nuclear factor-κB (Fig. 3.4).

Microarray Validation

Quantitative PCR was normalized to *18S* rRNA gene expression. All the results presented heretofore will represent the 24H sampling time as compared to the PRE. Twelve genes were assayed by RT-PCR to compare results to validate the findings of the microarray. Comparison of microarray results with those observed using RT-PCR indicates differences in values between the two methods, but of the individual genes evaluated, seven shared the same directional change as the findings from the microarray analysis. Results indicated there was a tendency for an increase in glutamine synthetase

expression (*GLUL*, + 3.53 fold, $P < 0.01$) and a tendency for thioesterase superfamily member 4 to be suppressed in the 24H group relative to the PRE group (*THEM4*, -3.45 fold, $P = 0.12$). Other genes examined revealed no differences in expression between the two biopsy sampling times (Table 3.2).

Comparison of Infected and Control Groups

A subset of genes was analyzed to compare the INF calves with the CON calves (Table 3.3). Of note, adiponectin receptor 1 (*ADIPOR1*) showed no effects due to joint infection with *M. haemolytica* and BVDV ($P > 0.15$); however, there was a tendency for the expression of this gene to be suppressed at the 24H time point compared to the PRE (-2.15; $P = 0.08$). Additionally, there was an infection x time interaction for this gene such that the CON and INF at 24H were down-regulated compared to the PRE time point of the CON group (-5.26, $P < 0.01$; -1.35, $P = 0.05$; respectively). Glutamine synthetase (*GLUL*) was unaffected by the treatment conditions ($P > 0.15$), but was significantly induced at the 24H sample collection time (+2.57; $P < 0.01$). There was no treatment x time interaction for *GLUL* expression ($P > 0.15$). Similarly, thioesterase superfamily member 4 (*THEM4*) expression was unaffected by treatment or the treatment x time interaction ($P > 0.15$), but its expression was numerically decreased at 24H (-1.38; $P = 0.15$). We further compared gene expression of the CON versus INF groups at the 24H time point and observed that *ADIPOR1* was significantly increased in the INF group (+3.89; $P < 0.05$) while *GLUL* and *THEM4* expression was similar between the two groups (+1.57, -1.25; $P > 0.15$).

DISCUSSION

Research has shown that BRD in feedlot cattle has deleterious effects on carcass merit as evidenced by decreased yield grades concomitant with lighter hot carcass weights indicating an attenuation of muscle accretion (Garcia et al., 2009; Holland et al., 2010). Bovine respiratory disease has the potential to become a chronic condition in calves. Researchers have reported that 37 to 66% of calves that appeared clinically healthy during the feeding phase had lung lesions at harvest (Gardner et al., 1999; Holland et al., 2010). Gardner et al. (1999) reported that 9% of these healthy animals presented active bronchial lymph nodes at the time of harvest. Because BRD can have long term effects in clinically healthy calves, we set out to investigate the changes in gene expression of muscle tissues in growing calves during the acute stage of illness to begin to elucidate the negative carcass effects seen at harvest in calves treated for BRD.

Microarray

Results from this experiment show similarities between results from the microarray analysis and RT-PCR. However, 5 of 12 genes had differing directional changes. Both procedures have their own limitations. Microarray analysis can be influenced by dye imbalances either in efficiency of incorporation and spot intensity or location on the slide (Smyth and Speed, 2003; Morey et al., 2006). Results from microarray analysis can be a product of random and/or cross hybridization (Morey et al., 2006). Real-time PCR can amplify non-specific products such as misprimed genes or primer dimers (Morey et al, 2006). In general, results from microarray and RT-PCR are highly correlated with 67 to 73% of genes evaluated exhibiting similar directional

changes in the two techniques (Dallas et al., 2005; Morey et al., 2006). Although the percentage of genes that shared similar up- or down-regulation patterns between the microarray analysis and RT-PCR (58%) in the current experiment was lower than previous experiments, the small pool size of genes evaluated may have allowed for more variability. To achieve similar results, only one additional gene would need to share similarity of directional expression. There is variability in the degree of gene expression change in validating microarray data with that of PCR. Some research has shown that expression of at least 1.3-fold in either direction show correlation between microarray and RT-PCR (Wurmbach et al., 2003; Moyer et al., 2006). Others have indicated that gene expression levels less than 1.5-fold are less reliable (Dallas et al., 2005). Of the genes in the current experiment that exhibited dissimilar directional changes, three had fold changes as determined by RT-PCR that were less than 1.5-fold which may explain the discrepancy. These RT-PCR results do not invalidate those from the microarray; however, it should be emphasized that RT-PCR is much more sensitive in quantifying mRNA expression when compared to microarray analysis.

The result of pathway analysis from the current experiment indicates that a network of genes centering on NF-κB signaling are induced in muscle tissue of growing calves acutely infected with BVDV and *M. haemolytica*. While we did not specifically assay typical genes in NF-κB signaling, the stratification of affected genes by their biological process and/or molecular function indicates many of the functions are congruent with common effects of such signaling, namely cell cycle, transcriptional regulation, inflammation, and proteolysis. Nuclear factor-κB signaling has been identified in diverse cell types and activates several signaling pathways; the most well

characterized being inflammation and cell survival. The NF-κB pathway is activated by multiple extracellular stimuli, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and lipopolysaccharide (LPS). Nuclear factor-κB signal transduction has been shown to increase muscle catabolism by inducing proteins in the ubiquitin-proteasome pathway, which is the principle route of protein degradation in the cell. Researchers have observed that *in vivo* and *in vitro* exposure to TNF-α resulted in increased ubiquitin mRNA and protein degradation in rat skeletal muscle (Garcia-Martinez et al., 1995; Llovera et al., 1997). Inflammatory ligands such as LPS, dexamethasone, and TNF-α have been shown to upregulate expression of the genes involved in the ubiquitin-proteasome systems with long-term effects (Frost et al., 2007). It could be hypothesized that chronic exposure to NF-κB stimulatory mediators leads to the attenuation of muscle accretion seen in beef cattle identified with BRD.

Glutamine Synthetase

In the microarray, glutamine synthetase expression was increased in steers challenged with *M. haemolytica* and bovine viral diarrhea virus at the 24H time point compared to the pre-challenge samples. Glutamine synthetase is an ATP-dependent enzyme that ligates ammonia to glutamate forming glutamine. Glutamine is the most abundant free amino acid, but 95% of this amino acid is stored intracellularly, with skeletal muscle being its major repository (Stipanik and Watford, 2000). Inflammation has been shown to deplete muscle glutamine levels by as much as 50% (Calder and Yaqoob, 1999). In addition, pro-inflammatory cytokines have shown the ability to increase GLUL transcripts and activity 24 h following treatment (Chakrabarti, 1998). In the present experiment, the increased expression of glutamine synthetase (GLUL) was

not influenced by infection. Instead time of sampling had the greatest effect. In the current experiment, GLUL transcripts were increased more than 2.5-fold at 24H compared with PRE which was consistent with induction of the gene in rodent models by glucocorticoids suggesting that stress may have played a role in the present experiment possibly explaining the differences seen between these time points. It is a further possibility that there was residual inflammation in the muscle caused by the previous sampling, thereby accounting for the increase in GLUL transcripts in the CON group at 24H compared to PRE.

It has been well established that glucocorticoids and other stress hormones can lead to muscle protein catabolism (Menconi et al., 2007). This is due in part to increases in activity of the ubiquitin-proteasome pathway where endogenous proteins are degraded (Frost et al., 2007; Menconi et al., 2007). In efforts to deal with the increased ammonia from degradation, GLUL is induced to ligate the ammonia to glutamate producing glutamine. Dexamethasone administration has demonstrated the ability to increase GLUL activity 2-fold by 24H post-injection but can increase mRNA levels by approximately 5-fold which is greater than the degree of increase obtained in the current study (Max et al., 1988; Abcouwer et al., 1995). Since muscle catabolism by glucocorticoids and inflammation affect similar pathways involved in degradation, it is possible that the stress created by prolonged restraint may be masking the effects that inflammation would have on GLUL expression in the present study. Experiments under field conditions may be necessary to clarify the effects of BRD on GLUL.

Adiponectin Receptor 1

Results from this experiment indicate that there was no significant effect of ADIPOR1 expression associated with infection of two common BRD pathogens. Interestingly, there was a sharp decline in ADIPOR1 expression occurring in the CON group at 24H compared to the same group at PRE. This decrease in ADIPOR1 transcripts may be a result of stress induced by the management of the steers during the study. This suppression is difficult to interpret because of equivocal data in the literature. In human *in vivo* studies, there is no consensus on the effects of glucocorticoids on adiponectin and its associated receptor. Intravenous administration of hydrocortisone resulted in decreased plasma concentration of adiponectin in human subjects (Fallo et al., 2004), while others observed dexamethasone slightly increased adiponectin levels (Jang et al., 2008). Jang et al. (2008) also observed increased levels of glucose and insulin following dexamethasone administration to fasting human subjects. Rodent models are equally unclear. In obese and non-obese rats, chronic intraperitoneal injection of hydrocortisone decreased serum adiponectin levels and mRNA in white adipose tissue (Shi et al., 2010). Another group observed that adrenalectomy increased adiponectin mRNA and serum concentrations in obese mice compared with the sham controls; however in wild-type mice there was a significant drop in these parameters in the adrenalectomized group relative to the sham operated group (Makimura et al., 2002). Less information is available regarding the effects of glucocorticoids on ADIPOR1 expression. Glucocorticoid-mediated suppression of adiponectin may explain the decrease in ADIPOR1. Results from a study of lean human subjects showed that myotubes stimulated with globular adiponectin increased expression of ADIPOR1 (McAinch et al., 2006). On the other

hand, Jang and coworkers (2008) observed that ADIPOR1 mRNA expression in human skeletal muscle was unaffected by dexamethasone treatment. Depressed levels of adiponectin induced by stress may explain the trend for ADIPOR1 suppression in the calves in both treatment groups at the 24H time point.

Surprisingly, when the CON and INF groups were compared at only the 24H time point, there was an increase in ADIPOR1 transcripts in the INF group. This may be a result of the dramatic decrease of ADIPOR1 expression in the CON. The INF calves in this study exhibited significantly higher rectal temperatures and clinical signs as well as decreased feed intake compared with the CON group (data not shown). With the higher feed intake of CON steers, insulin levels may have been higher than those of the infected group. Research suggests that insulin can affect ADIPOR1 expression in a species specific manner. A study of fasted mice demonstrated that ADIPOR1 levels can increase 2-fold following a 48 h fast which is similar to those observed in comparison of CON and INF at 24 h. Administration of insulin to C2C12 myocytes *in vitro* leads to decreased mRNA for the receptor (Staiger et al., 2004; Tsuchida et al., 2004). However, these results are controversial because similar studies in human subjects indicate that ADIPOR1 is positively correlated with insulin levels in the blood (Debard et al., 2004; Staiger et al., 2004). Furthermore, infusion of insulin increased ADIPOR1 levels 2-fold in human skeletal muscle (Debard et al., 2004). Changes in feed intake and possible changes in insulin may explain the changes in ADIPOR1 expression seen in these steers. It may also be that genetic variation in the ADIPOR1 gene affected expression values. One steer in the study had Ct and ΔCt values that were just outside of 2 standard deviations, but the values for the 18S reference gene were in line with the others which justified including

the steer's data in the analysis. Adiponectin receptors have shown to be highly polymorphic in human populations and were related to insulin resistance (Crimmins and Martin, 2007). This genetic variation may explain the extreme response by the aforementioned steer and seems a more likely explanation for the low levels of ADIPOR1 transcripts in the CON group at 24H.

Thioesterase Superfamily Member 4

Evidence from our findings indicates that THEM4 tends to be slightly down-regulated at the 24H time point in comparison to the PRE time point. Additionally, when the PRE and 24H were compared with only the infected animals, there was greater than 3 fold suppression of the gene. Expression of this gene was not influenced by treatment with the pathogens. Little is known about this protein and we were unable to find information regarding the effects of stress on its expression. THEM4, also known as carboxyl-terminal modulator protein (CTMP), was first identified in 2001 as an inhibitor of protein kinase B (PKB/Akt) and was thought to be localized to the plasma membrane in various *in vitro* cell cultures (Maira et al., 2001). Researchers have noted that THEM4 may be involved in cell survival (Ono et al., 2007). Following apoptotic signaling this protein is released into the cytosol, and overexpression of THEM4/CTMP resulted in greater incidence of cell death and was integral in increasing cleavage of pro-apoptotic enzymes (i.e., caspase-3 and poly ADP ribose polymerase) (Parcellier et al., 2009; Piao et al., 2009). Another group demonstrated that THEM4/CTMP is capable of sequestering heat shock protein 70 and promoting apoptosis (Piao et al., 2009). Therefore, the evidence suggests that THEM4/CTMP functions to sensitive cells to apoptotic stimuli. This makes is appear that suppression of the gene may in effect be an attempt to attenuate

muscle tissue atrophy, yet with the experimentation performed regarding this enzyme relatively little is known about its induction and regulation; its effects and responses in muscle tissues have not been reported.

Results from the expression changes in the genes previously discussed indicate muscle tissue breakdown when steers are stressed, but was not related to BRD at the timepoint samples were collected in the present experiment. The suppression of ADIPOR1 is indicative of decreased circulating adiponectin and hence decreased insulin sensitivity. Such a change leads to muscle atrophy and increased expression of components of the ubiquitin-proteasome pathway to mobilize amino acids. These results are congruent with the increased transcripts of GLUL, which would be needed to process the increased ammonia levels resultant from such proteolysis. Similarly, increased GLUL expression may occur in order to supply glutamine for increased nucleotide synthesis for transcription of vital survival genes (Stipanik and Watford, 2000). Furthermore, it is plausible to suggest that the induction of GLUL is coordinated to meet the demands of the immune cells during times of inflammation. It is well established that glutamine utilization is increased in proliferating lymphocytes and that such clonal expansion is dependent upon glutamine concentrations (Ardawi and Newsholme, 1983; Kew et al., 1999). In addition, the tissue macrophages would be induced to stimulate phagocytosis of cellular debris that inevitably would occur during muscle atrophy. Macrophages have high protein turnover and hence high transcriptional activity. It has been demonstrated that macrophages have high glutaminase relative to other immune cells indicating high glutamine demands for nucleotide synthesis for mRNA production (Newsholme et al., 1986).

What is of equal interest is the association of all 3 genes evaluated, GLUL, ADIPOR1 and THEM4, centers on the Akt/protein kinase B (PKB) signaling pathway. This kinase transduces signals from several growth factors including insulin and IGF (Whiteman et al., 2002; Sen et al., 2003). Akt/PKB signaling has been shown to promote cell survival by phosphorylating pro-apoptotic proteins, Bad and FOXO transcription factors (Sen et al., 2003). Furthermore, this signaling pathway has been shown to promote cell cycle progression (Sen et al., 2003). Akt/PKB plays an important role in muscle tissues in response to insulin by inducing GLUT4 translocation to vesicles thereby increasing glucose transport, as well as phosphorylating phosphofructokinase and glycogen synthase kinase and subsequently increasing glycolysis and glycogen synthesis (Whiteman et al., 2002; Sen et al., 2003). Lastly, activation of Akt/PKB modulates protein synthesis by promoting translation through activation of the mTOR pathway (Whiteman et al., 2002).

This experiment was intended to identify molecular targets in muscle tissue of growing beef calves that are exposed to common respiratory pathogens. The possible stress from handling the cattle in our model may have masked some of the effects of BRD infection. However, the identification of these molecular targets that may be involved in Akt signaling, a pleiotropic pathway affecting energy metabolism, cell growth, and survival warrants further investigation. Increased understanding of these transcriptional events may allow manipulation of these molecular targets which could decrease or ameliorate muscle losses associated with bovine respiratory disease.

Table 3.1. Primer sequences, melt temperatures, amplification efficiencies, and standard curve r^2 values

Gene	Forward Sequence	Reverse Sequence	T_m, ºC*	Efficiency	r^2
18S	TTCGAACGTCTGCCCTATCAA	GATGTGGTAGCCGTTTCTCAGG	55.0	92.4%	0.998
ADIPOR1	CCTCCCTCACCCCTGTCCTGA	AGGCCCGAGAAAGATGGGACCAG	61.4	100.3%	0.922
CTSH	CCAGGATGGTGACTGCAAG	CCTCCTCATCGTTCAGTGTG	61.4	108.3%	0.905
CWF19L2	TCAGAGGGATGAATGGATGA	TCAAGTGCCTGGCTTTTCTC	55.9	111.7%	0.916
GLUL	ACATTGAGGAGGCCATTGAG	TTGATGTTGGAGGTTTCGTG	57.5	100.9%	0.975
HNMT1	GCAATTCCACGGAGCACCAGTGT	CCTGCACCACCGCCAATGCTT	61.4	143.5%	0.924
MAP3K1	AGCAAGCTGAGGGAACTGCAA	AGACCACCATGCCCACCACA	61.4	108.5%	0.979
RBBP6	ATAAGTCGAACTGAACCAGTGATGGG	AGCAACGTGTAGAAAAAGTGTGAGA	61.4	118.5%	0.920
RFXANK	ATCTGAGGAAAGGCGACAAC	TCAAGCAAGAAGCGGACAG	61.4	100.1%	0.976
SMAD7	GGAGTCCTTTCTCTCTCAAAGCAC	ACGTTGACACTGCTCTTAGCTCAAT	61.4	136.3%	0.962
SPTBN1	CAAACGGGAGAAGGACAAAG	TGTGTTGGTTCTGAGCTTGC	57.1	110.0%	0.972
THEM4	GGAAATGCCAGAAAACTGAAGAAAGG	TCCAAGACAACAGAGGTGAAGGGT	63.3	100.9%	0.886
TPM4	GTGGCAGCTCTCAATCGAC	TCACCTTCATCCCTCTCTCAC	60.3	91.3%	0.963

* T_m represents the melting temperature used for primer annealing in qPCR reactions.

Table 3.2. Comparison of microarray and RT-PCR results for selected genes in calves following 24 hours of joint infection with *Mannheimia haemolytica* and bovine viral diarrhea virus

Gene Symbol	Gene Name	Fold Change		
		Microarray	RT-PCR	Pr > 1*
ADIPOR1	Adiponectin receptor 1	-6.31	1.14	0.84
CTSH	Cathepsin H	2.09	2.08	0.35
CWF19L2	CWF19-like 2, cell cycle control (*S. pombe*)	-1.66	-1.11	0.85
GLUL	Glutamine synthetase	2.91	3.53	< 0.01
HNMT	Histamine N-methyltransferase	-6.54	-2.39	0.58
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	2.04	-1.22	0.68
RBBP6	Retinoblastoma binding protein 6	-2.04	-1.71	0.23
RFXANK	Regulatory factor X-associated ankyrin-containing protein	-2.38	2.03	0.38
SMAD7	SMAD family member 7	-2.20	-2.26	0.21
SPTBN1	Spectrin, beta, non-erythrocytic 1	5.11	-1.44	0.60
THEM4	Thioesterase superfamily member 4	5.17	-3.45	0.12
TPM4	Tropomyosin 4	2.84	1.82	0.45

*****Represents analysis of gene expression changes between PRE and 24H sampling times obtained from RT-PCR.

Figure 3.1. Timeline of muscle biopsy and pathogen exposure and inoculation events.

P.I. = persistently infected.

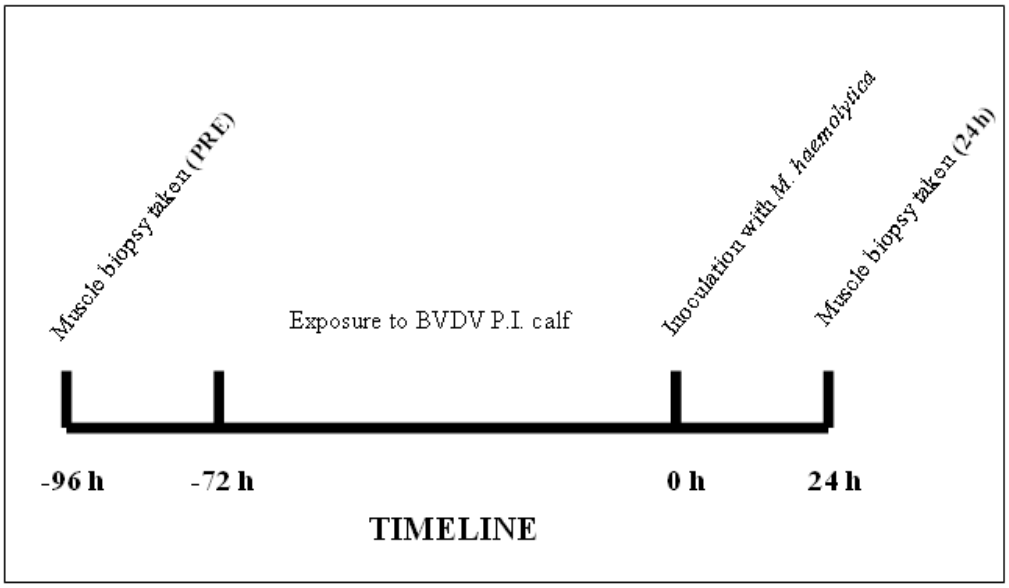


Figure 3.2. Categories of genes upregulated in muscle tissue following 24 hours of joint infection with BVDV and *M. haemolytica*.

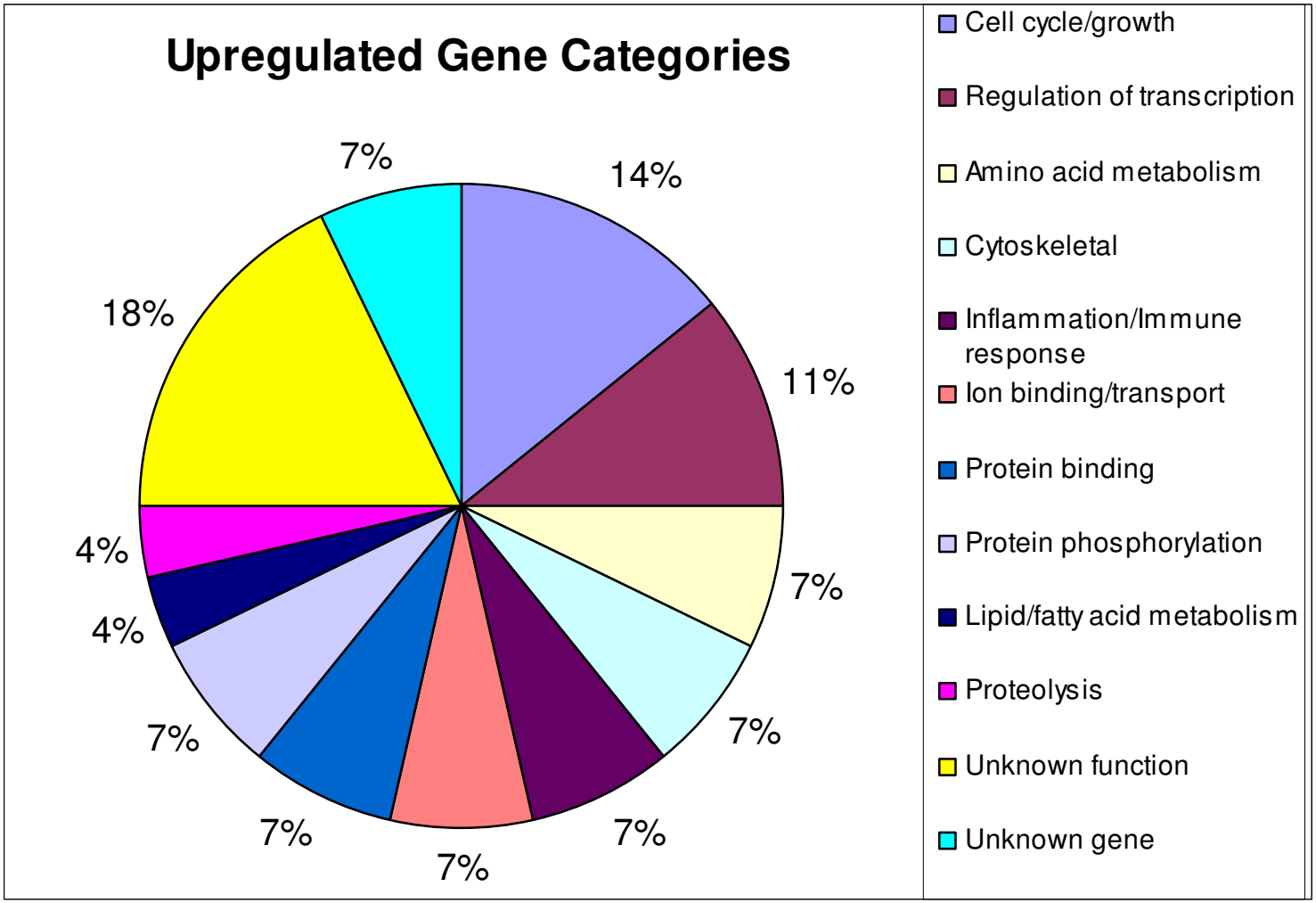


Figure 3.3. Categories of genes downregulated in muscle tissue following 24 hours of joint infection with BVDV and *M. haemolytica*.

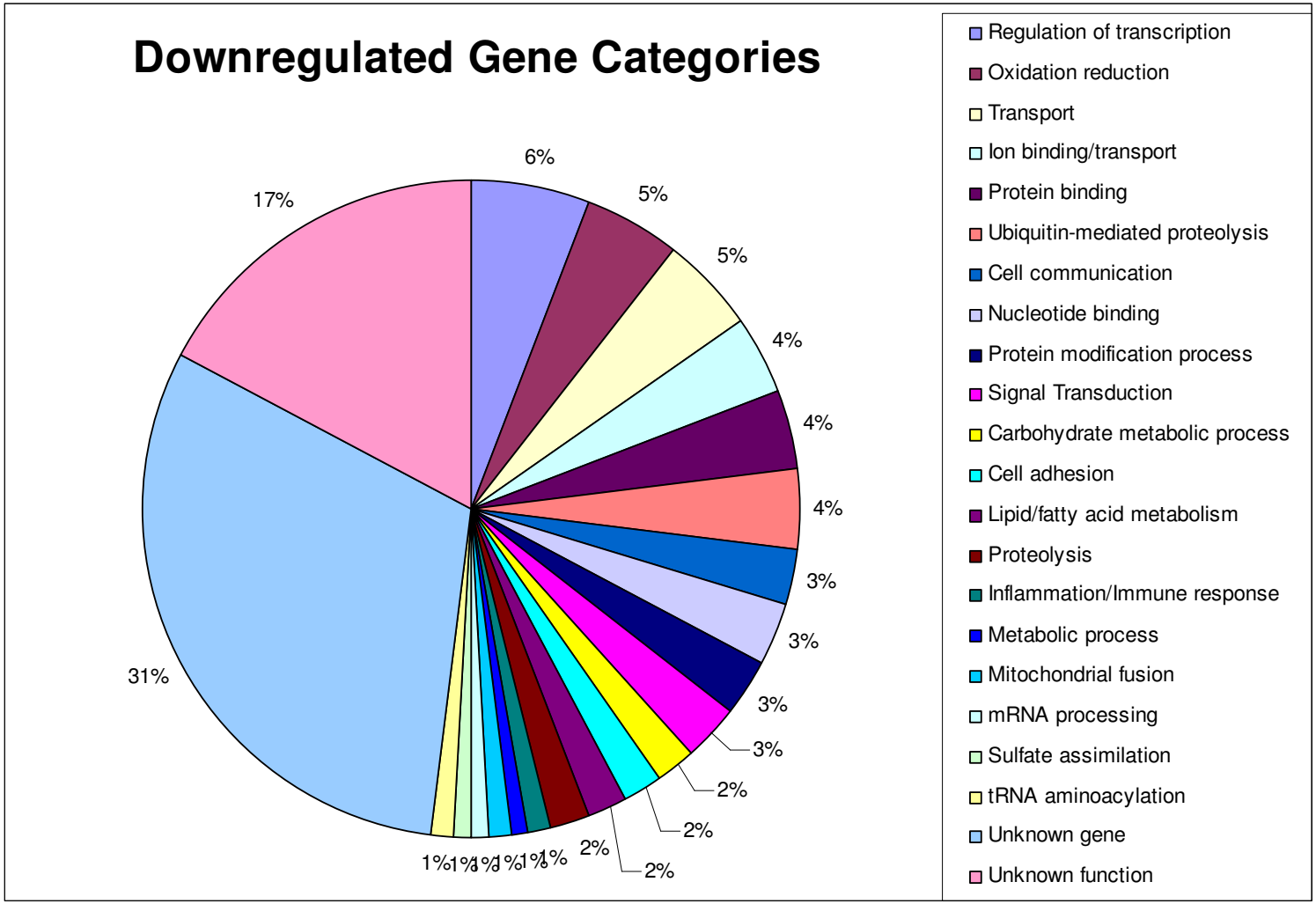


Figure 3.4. Network of related genes identified by Ingenuity Pathway Analysis in microarray experiments conducted with muscle tissue of steers following 24 hours of joint infection with *Mannheimia haemolytica* and bovine viral diarrhea virus compared with calves unexposed to pathogens. Red indicates upregulation of gene expression, green indicates downregulation of gene expression, and white indicates related genes.

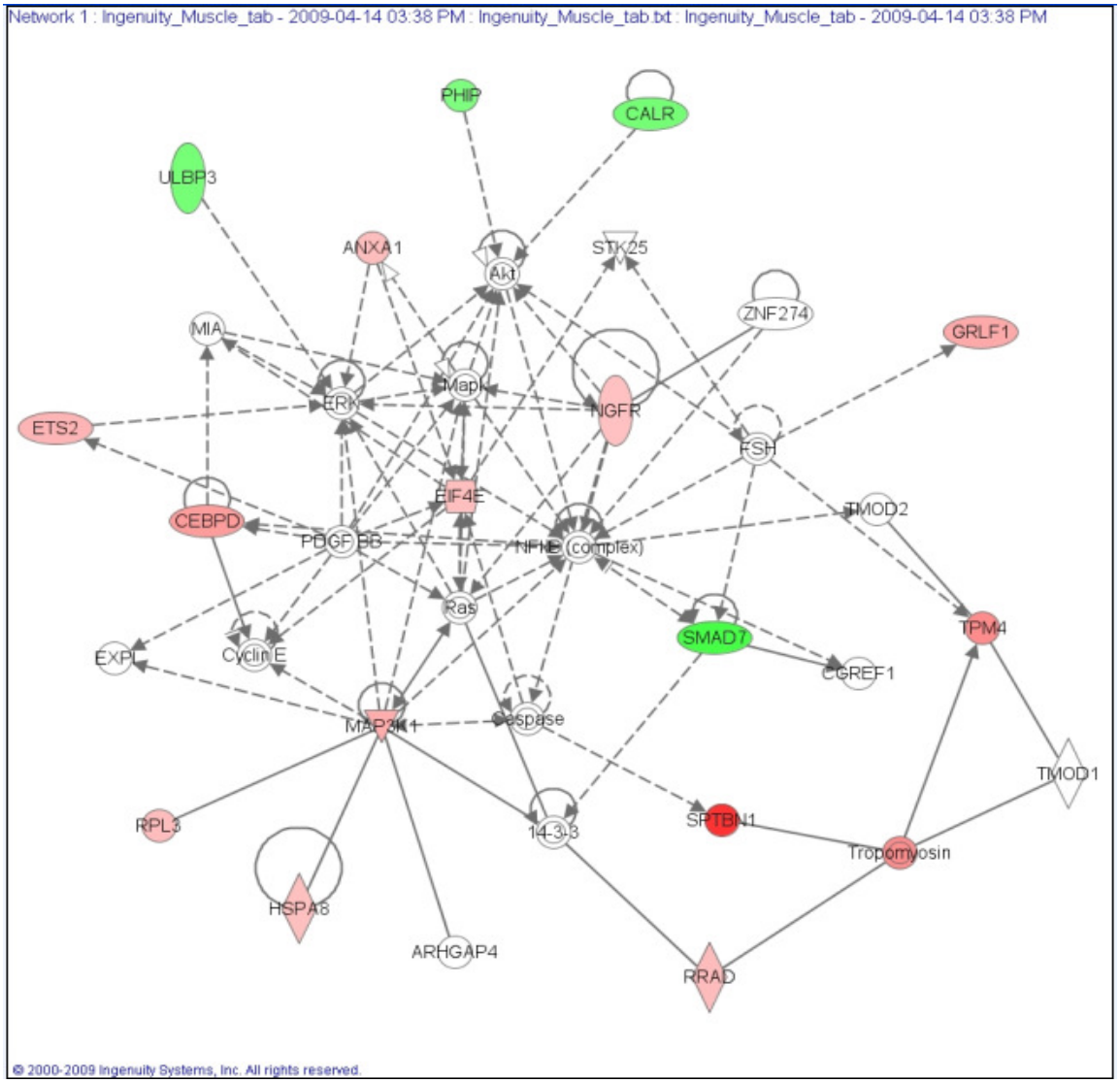
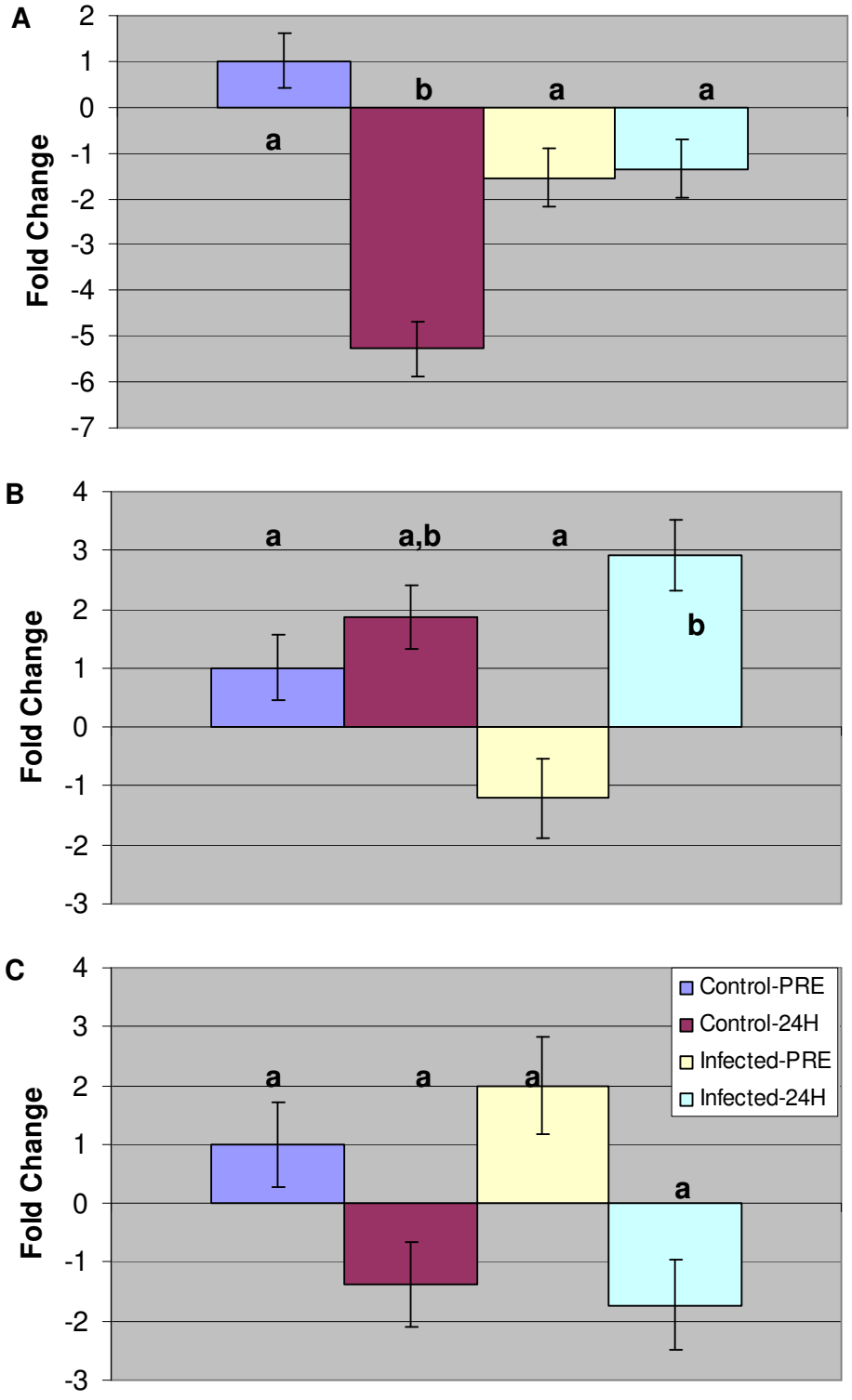


Figure 3.5. Effects of *M. haemolytica* and BVDV infection on gene expression compared with controls. [a,b] Groups with different letters differ ($P < 0.05$). A = *ADIPOR1*; B = *GLUL*; C = *THEM4*.



CHAPTER IV

Transcriptome analysis of liver tissue from calves infected with bovine viral diarrhea virus and *Mannheimia haemolytica*

ABSTRACT

Bovine respiratory disease (BRD) is the most prevalent ailment of feedlot cattle and negatively impacts animal growth and performance and subsequently leads to less desirable carcass characteristics. To understand the effects of BRD on liver gene expression, 16 crossbred beef steers (280.8 $\pm$ 32.5 kg) were divided into 2 treatment groups: the control group (CON) was not exposed to a calf persistently infected with bovine viral diarrhea virus type 1b (PI-BVDV) and received an intratracheal dose of isotonic saline; an infected group (INF) was exposed to a PI-BVDV calf for 72 h followed by intratracheal inoculation with *Mannheimia haemolytica* serotype A. Liver biopsies were taken from the right side between the 11^{th} and 12^{th} ribs prior to exposure to both pathogens (PRE), 12, (12H) and 24 hours (24H) following bacterial inoculation. Total RNA was extracted and bovine oligonucleotide microarray hybridization performed on PRE and 24H sampling times (n = 6). Significance for the microarray was set at fold change greater than |2| and $P < 0.001$. Quantitative RT-PCR was performed to both validate microarray results and to examine gene expression at the indicated time points with significance set at $P < 0.05$. Bioinformatic pathway analysis was conducted using Ingenuity Pathway Analysis. Microarray analysis found 44 genes were induced and 45 genes were suppressed following 24H of dual infection. Pathway analysis revealed that lipid metabolism, molecular transport, cellular assembly and organization, carbohydrate metabolism, and small molecule biochemistry were the molecular and cellular functions most affected in the current experiment. PCR results showed that beta-site APP-cleaving enzyme 1 (BACE1) was increased 2.3-fold in INF compared to CON ($P < 0.05$). S100 calcium binding protein A8 (S100A8) transcripts were increased 28.9-fold in INF versus

CON ($P < 0.001$), were also increased more than 21-fold in 12H and 24H above PRE ($P < 0.01$), and exhibited an infection × time interaction with INF-12H and INF-24H showing mRNA increases of 44- and 42-fold above CON-PRE, respectively ($P < 0.01$). Fatty acid desaturase 2 (FADS2) was suppressed 73.2-fold in INF relative to CON ($P = 0.05$) and tended to be down-regulated 29.5- and 95.1-fold at 12H and 24H versus PRE ($P = 0.09$). Gene expression changes in liver are indicative of hepatic inflammation which may suggest calves with BRD are susceptible to the development of fatty liver.

Keywords: beef cattle, bovine respiratory disease, gene expression, liver

INTRODUCTION

Bovine respiratory disease (**BRD**) remains the most prevalent and economically important disease facing the beef industry in the U.S. Older estimates suggest that this ailment affects 1 in 5 feedlot calves and potentially causes profit losses in excess of $3 billion (Edwards, 1996; Griffin, 1997; Snowder et al., 2006). The disease can develop as a result of variety of stressors resulting in immune compromise in the calf permitting viral infections such as bovine viral diarrhea virus which precede secondary bacterial infections by such species as *Mannheimia haemolytica* and/or *Pasteurella multocida* (Lillie, 1974; Fulton et al., 2000).

Morbidity can have deleterious effects on calf performance and carcass characteristics (Gardner et al., 1999; Holland et al., 2010). Following bacterial and/or viral infections, the innate immune system initiates an acute systemic inflammatory response leading to metabolic changes in the liver (Cray et al., 2009; Eckersall and Bell, 2010). Research from feedlot and dairy calves showed significant increases in hepatic

synthesis of acute phase proteins during infection with common BRD pathogens (Horadagoda et al., 1999, Nikunen et al., 2007; Burciaga-Robles et al., 2010). Other rodent studies indicate decreased gluconeogenesis, glycogenesis, and glyconeogenesis in the liver during acute inflammation (McCallum and Berry, 1973; Jones and Titheradge, 1993). The acute phase response alters intermediary metabolism through changes in expression of genes that alter fatty acid, cholesterol, and sphingolipid metabolism as well as the lipoproteins involved in their transport (Khovidhunkit et al., 2004). However, no research is available to address changes in liver metabolism during BRD in growing beef calves. Therefore, the objective of this study was to examine the gene expression profile of liver tissue in calves during acute infection with common BRD pathogens, namely *M. haemolytica* and bovine viral diarrhea virus.

MATERIALS AND METHODS

Cattle Management

All procedures performed with the animals were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Protocol # AG075). Sixteen crossbred *Bos taurus* steers (280.8 $\pm$ 32.5 kg) were procured from the university's research herd , housed at the Nutrition and Physiology Unit, Stillwater, OK, and fed a standard growing diet according to the nutrient requirements set forth by the National Research Council (NRC, 1996). Steers selected for pathogen challenge (INF) were transported 3.2 miles to the Willard Sparks Beef Research Center, Stillwater, OK, where they were commingled together in a 6 x 10.8-m pen with a 2 steers confirmed to be persistently infected with bovine viral diarrhea virus type 1b (**PI-BVDV**) using

genotyping and immunohistochemistry (Fulton et al., 2006). Following a 72 h exposure to the PI-BVDV cattle, these steers were returned to the Nutrition and Physiology Unit. Following a 12 h rest period, steers were intratracheally challenged at 0800 h with *M. haemolytica* serotype 1 by inoculation with 10 mL of a solution containing 6 x 10^9 cfu of bacteria similar to the method described by Dowling et al. (2002). In short, following restraint, a broncheoalveolar lavage tube (Bivona Medical Technology, Gary, IN) was sterilized with chlorhexidine solution, rinsed with saline, inserted into the nostril and directed into the trachea within 2 to 3 cm of the bifurcation allowing challenge material to be delivered to both lungs. *M. haemolytica* serotype 1 bacteria was grown before the challenge and reconstituted as described by Mosier et al. (1998). The control group of steers (CON) was not transported nor exposed to a calf persistently infected with bovine viral diarrhea virus type 1b (BVDV) and received an intratracheal dose of 10 mL of PBS solution (pH 7.4; Sigma Aldrich, St. Louis, MO). Infection status was determined by serology (Confer et al.,1995). All CON calves showed no titers for either pathogen, while all INF calves in the experiment seroconverted for both pathogens (data not shown). Subsequently, all steers were housed in individual stanchions and remained at the Nutrition and Physiology Unit.

Biopsy Procedure

Liver biopsy sampling was performed similar to that described by Swanson et al., 2000. In brief, calves were restrained while standing. An area on the right cranial abdominal cavity was clipped in such a way as to surround the intercostal space of the 11^{th} and 12^{th} ribs. The entire surgical area was aseptically prepared by scrubbing the designated area thrice with iodine soap, ethanol (75%) solution, and rinsed with sterile

water; scrubbing was performed in a circular motion beginning at the center of the focal area and moving outward. Following the third wash, the area was cleansed with iodine solution using the same scrubbing motion. Next, a local anesthetic (4 to 10 ml of lidocaine 2%) was injected subcutaneously and in the intercostal muscle to desensitize the surgical area. Following a 3 to 5 minute pause, a stab incision (1 to 2 cm) using a scalpel blade was made through the skin. Subsequently, the biopsy needle [12-gauge × 10 cm with a penetration depth of 22 mm; Bard® Monopty® Biopsy Instrument (C. R. Bard, Inc., Covington, GA)] was advanced through the intercostal muscle on the caudal side of the 11^{th} rib, through the peritoneum and diaphragm, and into the liver. Further, this biopsy instrument had mechanical action such that only liver tissue was taken and avoiding contamination from other tissue types. Upon collection of the sample, the instrument was retracted and the specimen was placed in a sterile vial, immediately placed in liquid nitrogen (-196º C), and stored at -80º C until analysis was performed.

RNA Isolation

Liver tissue samples were placed in 1 ml TRIzol Reagent™ (Invitrogen; Carlsbad, CA) and homogenized for 15-30 s on ice (4^{o} C). Supernatant was collected and phenol-chloroform extraction was performed to isolate total RNA according to the manufacturer's protocol with minor modifications. Briefly, samples were allowed to stand at room temperature (23^{o} C) for 5 min. to promote protein dissociation. Chloroform was added to the samples at 0.2 ml per 1 ml of TRIzol reagent used and samples were allowed to stand for 10 min. at room temperature. Samples were then centrifuged at 12,000 x g for 15 min. at 4^{o}C. The aqueous phase was collected and RNA precipitated by adding an equal volume of absolute isopropanol. Samples were kept overnight at -20 ^{o} C

followed by a second centrifugation as previously mentioned. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 12,000 x g for 2 min. The ethanol supernatant was removed and samples were allowed to dry at room temperature. RNA was suspended using diethylpyrocarbonate treated water (42^{o} C) and concentration determined by spectrophotometry (NanoDrop ND-1000; Thermo Scientific; Wilmington, DE). Agarose gel (1%) electrophoresis was used to determine RNA integrity. Samples were treated with DNase (DNase I, Amplification Grade; Invitrogen Corp., Carlsbad, CA) to eliminate contamination from genomic DNA according to the manufacturer's protocol.

Microarray Analysis

Aminoallyl-aRNA (**aRNA**) was synthesized according to the protocol of TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101 (EPICENTRE; Madison, WI). First-strand cDNA was synthesized using 500ng mRNA and a synthetic oligo(dT) primer containing a promoter sequence at the 5' end for a phage T7 RNA polymerase and reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen; Carlsbad, CA). From the RNA:cDNA hybrids the RNA was digested into fragments for priming of the second strand synthesis using RNase H. Second strand synthesis was performed using the DNA polymerase provided by the manufacturer creating a double stranded cDNA complex. This double stranded cDNA was transcribed *in vitro* and 5-(3-aminoallyl)-UTP was incorporated into the nascent strands. Aminoallyl-aRNA was purified using RNeasy MinElute Cleanup columns (Qiagen Inc.; Valencia, CA). Aminoallyl-aRNA was labeled using fluorescent dyes: PRE and 24H samples were labeled with Alexa Fluor® 555 and 647, respectively (Invitrogen; Carlsbad, CA). Dye-

couple aminoallyl-aRNA was purified using the aforementioned purification column. Labeled aminoallyl-aRNA was hybridized to bovine genome oligonucleotide (70mer) microarray slides (n = 8; Bovine Microarray Consortium; Columbia, MO) and incubated at 42^{o}C for 16 to 18 hours. Slides were scanned using the ScanArray Express (PerkinElmer; Waltham, MA).

Reverse Transcription Polymerase Chain Reaction

Microarray results were validated by reverse-transcription polymerase chain reaction (RT-PCR). Reverse transcription was performed with 1800 ng RNA using QuantiTect reverse transcription kit (Qiagen Inc.; Valencia, CA) according to the manufacturer's protocol. Quantitative PCR was conducted with 100 ng cDNA using the PerfeCTa® SYBR® Green FastMix® Kit (Quanta Biosciences Inc.; Gaithersburg, MD) according to the following reaction protocol: initial denaturation: 95ºC for 2 min. 30s; denaturation: 95ºC for 10s, annealing at 58ºC for 30s, and extension at 72ºC for 30s (forty cycles). The reaction was performed and fluorescent intensity measured using the BioRad MyiQ™ Real Time PCR Detection System (Bio-Rad Laboratories, Inc.; Hercules, CA). Amplification efficiency was assessed by performing qPCR with a standard curve of serial dilutions of cDNA (Table 4.1). Primer sequences are presented in Table 4.1.

Data Analysis

Microarray. Microarray images were preprocessed for spot recognition and spot correlation with gene array list and GenePix Results (GPR) files were generated using GenePix Pro 5.0 software (Molecular Devices, Sunnyvale, CA). Signal filtering,

background noise correction, global intensity Loess normalization, and statistical analysis of GPR files (n = 6) were conducted by GenePix Auto Processor (GPAP) 3.2 software (Weng and Ayoubi, 2005; http://darwin.biochem.okstate.edu/gpap). Data was analyzed with log2 transformation using signal intensities calculated as log2(24H / PRE) (M value). Fold change was calculated as follows: for M values ≥ 0, fold change = 2 ^ (M value); for M values < 0, fold change = (-1) * 2 ^ (M value). Differential gene expression was considered to be statistically significant if a minimum of 3 spots were represented, the fold change $\geq |2.0|$, and $P < 0.001$. Gene ontology was assigned to each gene according to its biological process or molecular function using DAVID Bioinformatics Resources 2008 (Huang et al., 2009; Dennis et al., 2003; http://david.abcc.ncifcrf.gov/) or KEGG (Ogata et al., 1999; http://www.genome.jp/kegg/). Genes were allocated to pathways using Ingenuity Pathway Analysis Software (Ingenuity Systems, Inc.; Redwood City, CA).

RT-PCR. The experiment was conducted in a completely randomized design with infection (TRT), sampling time (TIME), and their interaction as main effects with animal(TRT × TIME) used as the residual error. Data from RT-PCR was analyzed as the difference (ΔCt) between the threshold cycle (Ct) for *18S* and that for gene of interest. Fold change values were analyzed using the MIXED procedure (SAS v. 9.2, SAS Institute Inc.; Cary, NC). Fold change was determined using relative quantification, otherwise known as the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene expression was considered significantly different at $P < 0.05$.

RESULTS

Microarray Analysis

Microarray analysis of the entire bovine genome identified 89 differentially expressed genes when 24H was compared to PRE in the INF calves ($P < 0.001$). Among the genes (44) that were induced in muscle tissues following 24 h of joint infection of BVDV and *M. haemolytica*, the most affected categories were protein metabolism and modification (16%), immunity and defense (9%), signal transduction (9%), DNA processing (7%), intracellular protein transport (7%) and mRNA transcription and processing (7%; Fig. 4.1). In the category of suppressed genes (45) with known functions, protein metabolism and modification (21%); lipid, fatty acid, and steroid metabolism (14%); amino acid metabolism (7%); signal transduction (7%); DNA processing (7%); and immunity and defense (7%) were the biological processes most altered (Fig. 4.2). Pathway analysis indicated that the network containing the most differentially expressed genes (18) was one associated with cancer, reproductive system disease, and the cell cycle. Other identified networks were as follows: small molecule biochemistry, amino acid metabolism and molecular transport; lipid metabolism, molecular transport, and small molecule biochemistry; and lastly, cell-to-cell signaling and interaction, cardiovascular system development and function, and cellular movement. The top five molecular and cellular functions were lipid metabolism, molecular transport, small molecule biochemistry, cellular assembly and organization, and finally, carbohydrate metabolism.

Microarray Validation

Quantitative PCR was normalized to *18S* rRNA gene expression. All the results presented heretofore will represent the 24H sampling time as compared to the PRE in the INF steers only. Ten genes were assayed by RT-PCR to compare results with the findings of the microarray. Comparison of microarray results with those observed using RT-PCR indicates differences in values between the two methods, but of the individual genes evaluated, six shared the same directional change as the findings from the microarray analysis. Quantitative PCR results indicated there was a an increase in S100 calcium binding protein A8 expression (***S100A8***, + 42.13 fold, $P < 0.01$) and a tendency for suppression of fatty acid desaturase 2 (***FADS2***, -184.27 fold, $P = 0.10$) and selenoprotein N 1 (***SEPN1***, -2.34 fold, $P = 0.06$) in the 24H group relative to the PRE group. Other genes examined revealed no differences in expression between the two biopsy sampling times (Table 4.2).

Comparison of Infected and Control Groups

A subset of genes was analyzed to compare the INF calves with the CON calves (Table 4.3). Of note, β-site APP-cleaving enzyme 1 (***BACE1***) showed a 2.3-fold increase of mRNA transcripts in INF compared with CON ($P = 0.03$); however, there were no effects of TIME or the TRT × TIME interaction on its expression ($P > 0.10$). S100 calcium binding protein A8 was significantly up-regulated in INF steers compared to CON (28.9-fold; $P = 0.0001$). Additionally, there was a significant increase due to TIME ($P = 0.008$) with expression being increased at 12H (21.3-fold) and remaining elevated through 24H (21.4-fold) compared to PRE. We observed a significant TRT × TIME

interaction ($P = 0.007$) for S100A8 expression with INF-12H and INF-24H having 44- and 42-fold increased transcripts in relation to CON-PRE while the other groups were similar. Fatty acid desaturase 2 was suppressed in the INF group versus the CON group (-73.2-fold; $P = 0.05$). There was a tendency for TIME to affect *FADS2* expression ($P = 0.10$); 12H and 24H decreased expression by 32.8- and 91.9-fold relative to PRE. There was no significant TRT × TIME interaction on expression of *FADS2* ($P = 0.15$).

DISCUSSION

Informative research has been conducted to delineate the negative effects of BRD on the performance and carcass merit of feedlot calves (Gardner et al., 1999; Holland et al., 2010). In a companion paper, we recently showed that experimental challenge of growing beef calves with BRD pathogens resulted in transcriptional changes in muscle that indicated muscle breakdown suggesting an acute systemic inflammatory response (unpublished data). An important purpose of muscle protein catabolism is to supply amino acids for production of acute phase proteins that are induced during infection with common BRD pathogens in growing cattle (Horadagoda et al., 1999, Nikunen et al., 2007; Burciaga-Robles et al., 2010). Further it has been established that an acute systemic inflammatory response can alter hepatic metabolism (Cray et al., 2009; Eckersall and Bell, 2010). Because the literature was silent regarding gene expression changes in liver tissues of growing beef calves with BRD, our goal was characterize the hepatic response to acute infection with two common BRD pathogens, BVDV type 1b and *M. haemolytica*.

Microarray Analysis

We observed similar numbers of genes that were up-regulated (44) and down-regulated (45). Among these there were similar percentages of genes in each group that were altered in protein metabolism, immunity, signal transduction, and DNA processing. This is expected given that the acute infection promotes expression of the positive acute proteins at the expense of the negative acute phase proteins (Gruys et al., 2005; Cray et al., 2009; Eckersall and Bell, 2010). The categories of molecular/biological function that were most divergent were those for amino acid and fatty acid metabolism. This was corroborated by pathway analysis that categorized 16 genes (both induced and suppressed) into a network of amino acid metabolism and 12 genes (both induced and suppressed) into a network of lipid metabolism.

Protein production occurring in the liver modifies amino acid requirements for that tissue. In fact, previous research from our laboratory has demonstrated that during experimental *M. haemolytica* challenges there is a net uptake of essential and non-essential amino acid by the liver, resulting in net removal of total amino acids that is greater than unchallenged control animals (Burciaga-Robles, 2007). Similarly, clearance kinetics in human subjects intravenously perfused with amino acids indicated greater removal of several amino acids in patients with microbial sepsis when compared to healthy controls, presumably as a result of hepatic extraction (Druml et al., 2001). However, catabolism of these extracted amino acids is lowered suggesting that they are directed toward incorporation into nascent proteins and/or used in signaling for protein translation (Smith and Elia, 1983).

The suppression of fatty acid metabolism is more difficult to explain. A recent study shows that the acute phase response suppresses transcription of enzymes involved in fatty acid synthesis (acetyl-CoA carboxylase, fatty acid synthase, and pyruvate kinase; Feingold et al., 2011). These changes in gene expression were a result in part by the reduction of mRNA and protein levels for the transcription factor, carbohydrate response element binding protein (**ChREBP**). Other transcription factors in the liver have shown to be reduced in response to LPS and other APR cytokines and hormones. Namely, the peroxisome proliferator-activated receptors (**PPAR**)-α and γ are suppressed resulting in decreased levels of their target genes, FATP (fatty acid transporter protein), CD36/FAT (fatty acid translocase), H-FABP (heart-fatty acid binding protein), and carnitine palmitoyl transferase (CPT)-Iα resulting in decreased fatty acid transport into and within the liver cell as well as decrease fatty acid oxidation (Khovidhunkit et al., 2004).

Microarray Validation

Results from this experiment show some discrepancies between results from the microarray analysis and quantitative RT-PCR. These results showed that 70% of the genes evaluated by qPCR were in agreement with the microarray. Generally, results from these two assays are highly correlated with 67 to 73% or genes evaluated exhibiting similar directional changes (Dallas et al., 2005; Morey et al., 2006). These RT-PCR results validate those from the microarray and it must be emphasized that RT-PCR is much more sensitive in quantifying mRNA expression when compared to microarray analysis as seen in the large divergence in expression values of *FADS2*, *MUSTN1*, and *S100A8*.

Comparison of Infected and Control Groups

To follow up on the results from the microarray validation, we compared the expression of the genes in the CON and INF groups and the three sampling times to make sure that the expression changes seen were a result of the model or the pathogen infection. Quantitative PCR uncovered three genes whose expression differed between these groups; *BACE1*, *FADS2*, and *S100A8*. In this experiment, β-site APP cleaving enzyme 1 (**BACE1**) was slightly, but significantly up-regulated as a result of the infection with the BRD pathogens. This enzyme is an aspartic acid protease and is the rate-limiting catalyst involved in the sequential cleavage of amyloid precursor protein (**APP**) into amyloid-β peptide which commonly associated with the pathogenesis of Alzheimer's disease (Cole and Vassar, 2007; Heneka et al., 2010). β-site APP cleaving enzyme 1 (also known as Asp2) is thought to be ubiquitously expressed with greatest expression in the brain and pancreas, but only slightly in peripheral tissues including liver (Vassar et al., 1999; Yan et al., 1999; Lange-Dohna et al. 2003). The preponderance of experiments has been conducted in the pathophysiology of neural tissues with a paucity of information regarding its role in the normal physiology and much less so in the liver (Cole and Vassar, 2007).

Research involving neurons, astrocytes, and glial cells demonstrated that *BACE1* transcription can be affected by several inflammatory signals as mediated by a variety of transcription factors. One such factor is nuclear factor (**NF**)-κB. An i*n vitro* experiment was conducted to evaluate the role of NF-κB signaling in neurons and astrocytes and researchers observed that TNF-α stimulated *BACE1* promoter activity and transcription through NF-κB subunits binding in the promoter region (Bourne et al., 2007; Chen et al.,

2011). Other research in *in vivo* and *in vitro* experiments with mouse astrocytes demonstrated interferon (**IFN**)-γ stimulated *BACE1* mRNA transcription which further translated into increased BACE1 protein and increased levels of its cleavage product, amyloid-β which was mediated by JAK2 and ERK1/2 signaling whereby phosphorylated STAT1 bound *BACE1* promoter stimulating its expression (Hong et al., 2003; Cho et al., 2007; Yamamoto et al., 2007). Interestingly, IFN-β stimulation increased BACE1 protein expression in a time-dependent manner, where BACE1 expression increased to plateau levels at 12H and only slightly increased until 24H (Yamamoto et al., 2007). This corresponds with the *BACE1* transcription on our study, where its expression peaked at 12H and remained elevated at 24H.

The BACE1 protein has been shown to cleave other protein products involved in the immune response such α2,6-sialyltransferase (**ST6Gal I**), which was identified as upregulated by our microarray analysis (Cole and Vassar, 2007). This substrate is important in that it is highly expressed in the liver and indeed the majority of its secretion is by the liver (Kitagawa and Paulson, 1994). Furthermore, ST6Gal I secretion is promoted during the acute phase response by the activity of IL-6 (Kaplan et al., 1983; Dalziel et al., 1999). In a series of experiments, one research group demonstrated that BACE1 may play a role in the acute phase response. First, in an *in vitro* transfection study where *BACE1* gene was over-expressed, there was a significant increase in secretion of ST6Gal I (Kitazume et al., 2001). Later, an *in vivo* study showed knockout mice for *BACE1* had reduced plasma concentrations of ST6Gal I compared to wild-type. Conversely, over-expression of *BACE1* lead to increased ST6Gal I levels in plasma (Kitazume et al, 2005). In addition, that research group found that there was increased

ST6Gal I activity in plasma of rats known for hepatic pathology. This activity was propagated by increased *BACE1* mRNA while *ST6GAI* transcription remained stable (Kitazume et al., 2005). In their most recent experiment, those researchers found increased levels of ST6GalI in plasma were correlated to increased levels of the haptoglobin and α2-macroglobulin in response to hepatic injury (Kitazume et al., 2009). Therefore, in summary, one can hypothesize that the *BACE1* mRNA increase in our experiment is a result of pro-inflammatory signaling involved in the acute phase response to stimulate ST6Gal I levels in plasma. However, with limited research of this gene in peripheral tissues, additional research would be needed to clarify its role in the hepatic response to inflammation and other potential cleavage products.

A second marker of hepatic inflammation was evidenced by the significant increase (40-fold) in *S100A8*. This protein was originally discovered in granulocytic cells and was considered to have antimicrobial activities (Dale et al., 1983; Steinbakk et al., 1990). S100 calcium binding protein A8 along with its homolog, S100A9, have been shown to account for 30-45% of the cytosolic proteins in granulocytes (Dale et al., 1983). Abundant research has demonstrated the S100A8/S100A9 heterodimeric complex is secreted in inflammatory lesions and may function as a chemoattractant for infiltrating leukocytes, particularly neutrophils, in response to pro-inflammatory cytokines (Xu and Geczy, 2000; Nacken et al., 2003; Gebhardt et al., 2006). In fact, microscopy studies have evidenced significant neutrophil infiltration in the sinusoids and parenchyma of the liver during endotoxic shock and promotion of hepatocellular injury (Jaeschke et al., 1991; Chosay et al., 1997). A more recent study using immunohistochemistry demonstrated these infiltrating neutrophils to be highly expressive of S100A8/S100A9

(Koike et al., 2011). Additionally, high levels of S100A8 have been shown to increase death rates in endotoxic mice (Vogl et al., 2007) and are inversely related to survival of septic human patients (Payen et al., 2008). It is worth mentioning that S100A8/S100A9 heterodimer is capable of binding the polyunsaturated fatty acids, α-linolenic, γ-linolenic, and arachidonic acid (AA), with the latter having a higher affinity for the heterodimer than the former two (Kerkhoff et al., 1999). Further research by that group indicated that S100A8/S100A9 functions as an extracellular transporter of AA effectively propagating its uptake by FAT/CD36 at the plasma membrane of human umbilical vein endothelial cells (Kerkhoff et al., 2001). Notably, this transporter is known to be induced by endotoxin, TNF, and IL-1 (Memon et al., 1998). To summarize, the increased S100A8 expression strongly suggests infiltration of neutrophils into the liver of beef steers during the acute phase response to BRD pathogens. In addition, the fatty acid binding properties of S100A8 are suggestive of increased fatty acid uptake and accumulation into the liver.

Pathway analysis of the current experiment indicated that fatty acid metabolism was affected by bacterial and viral infection in steers challenged with BRD pathogens. Much research has been conducted examining the effects of inflammation on lipid metabolism. During acute inflammation, hepatic tissues shift priority from fatty acid oxidation and lipolysis to increasing fatty acid, triglyceride, and cholesterol content (Feingold and Grunfeld, 1986; Feingold et al., 1992). Much of the changes in intermediary metabolism brought about by the APR are due to changes in gene expression in hepatic tissues that alter fatty acid, cholesterol, and sphingolipid metabolism brought about by several cytokines including TNF-α, IL-1, and IL-6 (Khovidhunkit et al., 2004). The effects of these cytokines are mediated by changes in

transcription factors for genes involved in fatty acid metabolism including peroxisome proliferator-activated receptors α which leads to changes in expression of their target genes (Khovidhunkit et al., 2004; Kim et al., 2007).

Results from the current experiment indicated dramatic changes in the expression of *FADS2* gene suggesting changes in fatty acid desaturation congruent with the universal changes in hepatic lipid metabolism. This gene encodes the sequence for fatty acid desaturase 2 protein, more commonly referred to as Δ-6 desaturase (**D6D**), which is highly expressed in the liver (Cho et al., 1999). This enzyme catalyzes the desaturation of linoleic and α-linolenic acids to such polyunsaturated fatty acids as arachidonic (**AA**), eicosopentaenoic (**EPA**), and docosahexaenoic (**DHA**) acids (Nakamura and Nara, 2004). It has been well documented that these fatty acid products mediate the inflammatory response. Arachidonic acid promotes inflammation by its derivative eicosanoids, while the n-3 series fatty acids, EPA and DHA, can suppress the inflammatory response (Chapkin et al., 2009). However, it must be noted that these results are predicated upon dietary supplementation of EPA and DHA well above the levels that can be produced by the host tissues *in vivo* (Chapkin et al., 2009).

We were unable to find experimental results that describe the direct response of *FADS2* gene expression to the proinflammatory signaling. However, there is evidence that transcription factors for the gene of interest are affected by inflammatory signaling. Research demonstrates that *FADS2* gene possesses an enhancer element for both PPARα and sterol regulatory element binding protein 1 (**SREBP**) that when manipulated can affect mRNA levels for this gene in a fashion similar to their own expression (Matsuzaka et al., 2002; Tang et al., 2003). Proinflammatory signals associated with the acute phase

response, namely LPS, TNF-α, and IL-1, can suppress expression of PPARα and thereby decrease its transcriptional activity and mRNA levels of its target genes involved in fatty acid oxidation in hepatic cells (Beigneux et al., 2000; Kim et al., 2007). Moreover, SREBP-1c, a reciprocal transcription factor known to increase fatty acid synthesis, has been shown to respond to LPS and TNF- α by increased mRNA levels in mice and was associated with hepatic steatosis (Endo et al., 2007). Moreover, suppression of *FADS2* gene expression has been observed in rodents susceptible to fatty liver (Hall et al., 2010). Based on these results one could conclude that the SREBP-1 is being induced thereby leading to the suppression of *FADS2* expression. In addition, these previous studies reporting changes in *FADS2* expression less than 10-fold; our results indicate a much more pronounced suppression of polyunsaturated fatty acid metabolism in BRD challenged steers during the acute phase response.

In cattle, very little is understood about the *FADS2* gene expression in liver. Two microarray studies of hepatic tissues during experimentally induced ketosis in dairy cows revealed alterations in expression of genes involved in intermediary metabolism (Loor et al., 2007; McCarthy et al., 2010). Severe negative energy balance in high-yielding dairy cows decreased *FADS2* expression by 4.6 and 8.7 fold compared to lactating cows with mild negative energy balance in two independent studies (Loor et al., 2007; McCarthy et al., 2010). Hepatic lipidosis is often associated with ketosis in cattle and has similar pathognomonic characteristics, e.g. increased concentrations of non-esterified fatty acids and ketones in blood, increased triglyceride content, and proinflammatory cytokines, and can itself have prolonged deleterious consequences to animal health (Bobe et al., 2004). Therefore, it could be that the severe decrease in *FADS2* expression during the acute

phase response in steers challenged with BRD pathogens is indicative of changes in intermediary metabolism congruent with fatty liver syndrome.

One can conclude from the inflammatory markers induced during acute BRD infection that significant hepatocellular injury is occurring. It is possible that neutrophil infiltration and its cytotoxic products damage parenchymal cells during this time period. It is also of interest to note the relationship of a major neutrophil marker and shifts in fatty acid metabolism that occur during the APR. *S100A8* and *FADS2* genes both were significantly altered as a result of the experimentally induced inflammation in the liver of beef steers. The potential mobilization of fatty acids from the peripheral tissues, commonly associated with acute inflammation, leading to the suppression of fatty acid desaturation and binding proteins, such as S100A8, may lead to accumulation of fatty acids in the liver promoting lipidosis. This is of importance to beef producers because research has clearly established changes in the abundance of fat in peripheral tissues from calves treated for BRD. No research can be found evaluating the role of fatty liver in growing cattle, but research in dairy cows has shown fatty liver syndrome to promote morbidity and mortality in dairy cows and it can persist for weeks to months (Gerloff et al., 1986; Bobe et al., 2004). Further research is merited to evaluate fatty liver and bovine respiratory disease.

Table 4.1. Primer sequences, amplification efficiencies, and standard curve r^2 values

Gene	Forward Sequence	Reverse Sequence	Efficiency	r^2
18S	TTCGAACGTCTGCCCTATCAA	GATGTGGTAGCCGTTTCTCAGG	92.4%	0.998
BACE1	CCCTAGTGAAGCAGACCCACGT	ATGCTCCCTCCAACTGAGGCCA	98.7%	0.967
FADS2	GGAACCATCGCCACTTCCAGCA	TCTTGCCGTACTCAATGGGCTGC	103.7%	0.972
MRPS	GCTGTTTTCCGTGCGAAGCC	CAAGGCGGCTCCCGAAGATGTA	104.4%	0.995
MUSTN1	GCTCAGGAAGCCCCCATCAAGA	TGCTCACACTCCCGCATGACCT	106.5%	0.968
ORP	AATGACAACACCGCCACAGCCC	AGTGCTGCCTGAGCCCATGT	109.3%	0.997
QRSL1	AGCAGCGGCAACTGACCTTTCTG	CAACATGATCCTCGTGCCCCGT	98.8%	0.989
S100A8	AGGATGCGGACACTTGGTTCAAA	GCCCACCTTTATCACCAGCACG	97.4%	0.994
SEPN1	TGGCTACCTGTCCAACAACCGC	AGGGGCAAAACGGGTCTTCACG	102.1%	0.933
SIAE	AGTGCAGGGGGTTTTGGTCTGT	TGGCTCTGGGTGTGCCGTAA	110.2%	0.977
WDR91	AGGACCACGGCAAGGAACGGAA	AGCTTCGGGCTTCTTCTCCGCA	95.6%	0.995

Table 4.2. Comparison of microarray and RT-PCR results for selected genes in liver tissue of beef calves following 24 hours of joint infection with *Mannheimia haemolytica* and bovine viral diarrhea virus

Gene Symbol	Gene Name	Fold Change		
		Microarray	RT-PCR	Pr > 1*
BACE1	β-site APP-cleaving enzyme 1	-6.63	+1.81	0.41
FADS2	Fatty acid desaturase 2	-3.98	-184.27	0.10
MRPS2	Mitochondrial ribosomal protein S2	+2.79	+1.81	0.62
MUSTN1	Musculoskeletal, embryonic nuclear protein 1	+2.65	+30.26	0.13
ORP	Oxygen regulated protein	+10.71	+8.07	0.12
QRSL1	Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	-5.03	-0.66	0.70
S100A8	S100 calcium binding protein A8	+3.97	+42.13	< 0.01
SEPN1	Selenoprotein N, 1	+2.79	-2.34	0.06
SIAE	Sialic acid acetylesterase	-4.01	-3.23	0.18
WDR91	WD repeat domain 91	-3.71	0.71	0.79

*****Represents analysis of gene expression changes between PRE and 24H in INF calves obtained from RT-PCR.

Table 4.3. Comparison of RT-PCR results for selected genes in calves following 24 hours of joint infection with *Mannheimia haemolytica* and bovine viral diarrhea virus compared with calves unexposed to pathogens

	Control			Infected				P Values		
Gene	**PRE**	**12H**	**24H**	**PRE**	**12H**	**24H**	**SEM**	**TRT**	**TIME**	**TRT × TIME**
BACE1	-0.39	-0.31	-1.43	-0.69	2.96	2.52	1.25	0.03	0.35	0.23
FADS2	1.86	-4.40	-9.10	4.60	-54.66	-181.13	43.93	0.05	0.10	0.15
S100A8	0.04[a]	-1.40[a]	0.62[a]	-0.02[a]	43.99[b]	42.13[b]	7.37	< 0.001	< 0.01	< 0.01

[a,b] Values with different superscripts are considered significantly different ($P < 0.05$).

Figure 4.1. Categories of genes up-regulated in liver tissue of beef calves following 24 hours of joint infection with BVDV and *M. haemolytica*.

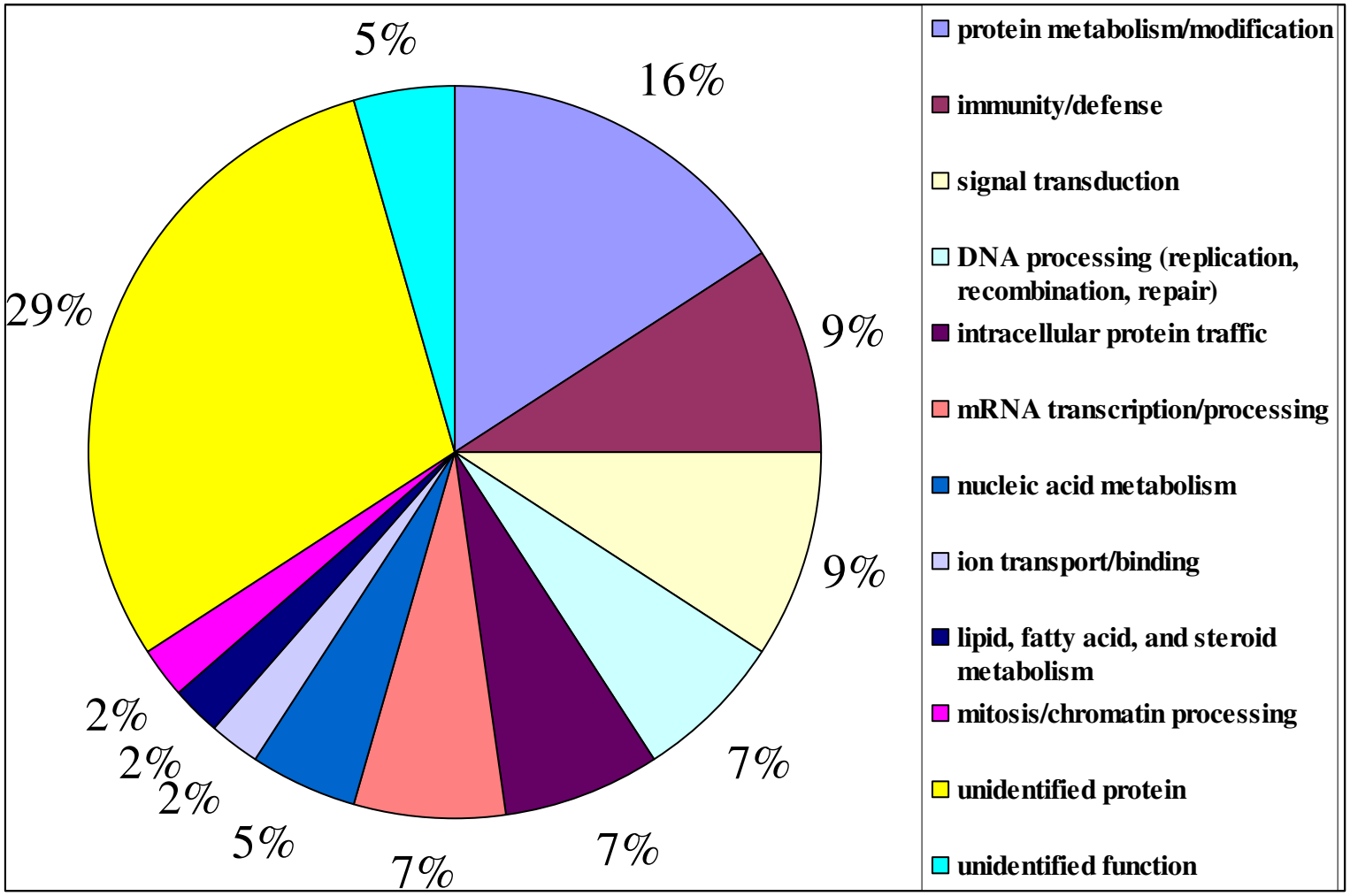
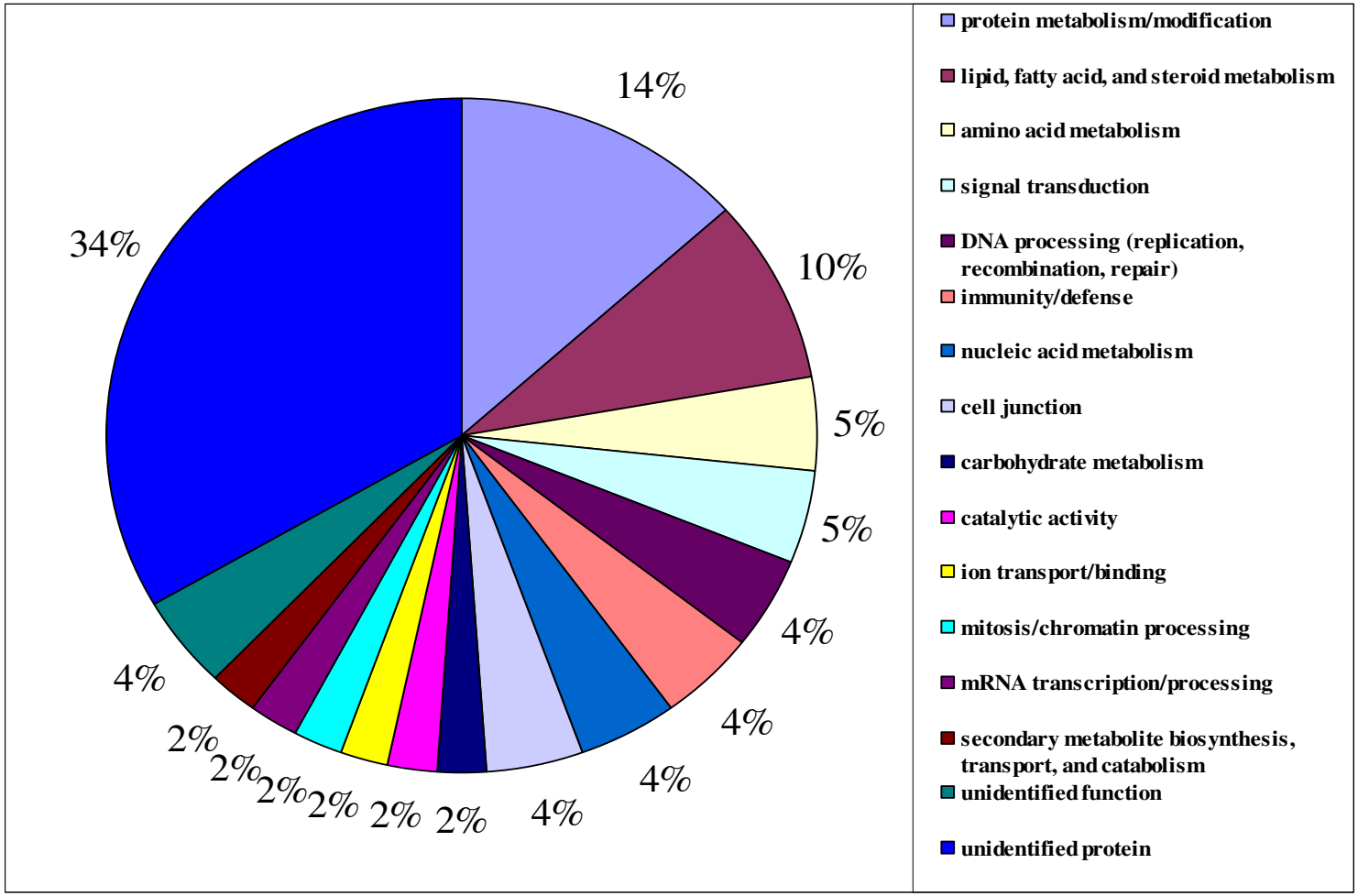


Figure 4.2. Categories of genes down-regulated in liver tissue of beef calves after 24 hours of joint infection with BVDV and *M. haemolytica*.



CHAPTER V

Gene expression changes in muscle and liver tissues of growing beef heifers at the time of treatment for bovine respiratory disease

ABSTRACT

Bovine respiratory disease (**BRD**) has been shown to have deleterious consequences on beef carcass characteristics by decreasing muscle and fat content. The objective of this study was to evaluate the effects of the number of BRD treatments on gene expression in muscle and liver tissues. We used 24 beef heifers that were identified as having nonspecific BRD. Muscle and liver biopsy samples were taken from untreated calves (**0X**), and calves treated once (**1X**), twice (**2X**), or thrice (**3X**) for BRD. RT-PCR was performed to evaluate gene expression. Fold change was determined using the ΔC_T method and was analyzed using the MIXED procedure. Orthogonal contrasts were performed to compare untreated and treated calves and for linear and quadratic effects. We observed differences ($P < 0.10$) among treatments for *ADIPOR1*, *BAD*, *FBXO32*, and *GLUL* in muscle tissues with inductions of 3.9, 3.6, 4.2, and 8.7 fold, respectively, in the treated calves compared to the controls ($P \leq 0.01$). Each of these genes increased in expression in a linear manner with number of treatments ($P < 0.05$). In liver tissues, significant differences were observed in *FASN* and *S100A8* expression ($P < 0.10$). *FASN* expression was reduced 6.8 fold in treated vs. control calves ($P = 0.02$) and had a quadratic response to number of treatments such that 1X, 2X, and 3X were suppressed 10.2, 4.0, and 6.1 fold, respectively, compared to 0X ($P = 0.10$). *S100A8* was increased 21.3 fold in 2X ($P < 0.10$). Results from the present experiment indicate gene expression changes in muscle characteristic of muscle atrophy and impaired growth signaling. These gene expression changes worsen with increased numbers of times treated for BRD. Gene expression changes in the liver, suggest a suppression of *de novo* fatty acid synthesis and inflammation.

INTRODUCTION

Bovine respiratory disease (**BRD**) continues to be the most prevalent ailment of cattle leading to detrimental effects on growth and performance (Snowder et al., 2006). Several groups have observed decrements in average daily gain (**ADG**) that approach 60% during the early feeding phase in calves treated for undifferentiated BRD (Montgomery et al., 2009; Schneider et al., 2009; Holland et al., 2010). This decrease in performance resulted in lighter final body weights and hot carcass weights when cattle were fed similar days on feed (Gardner et al, 1999; Schneider et al., 2009; Garcia et al., 2010).

While researchers have observed dramatic decreases in animal performance early in the feeding period, there is evidence to suggest that calves treated multiple times for BRD may be able to compensate for losses by increasing ADG and feed efficiency during the finishing phase by as much as 25% so that by the end of feeding, final body weights were similar to untreated calves (Montgomery et al., 2009; Schneider et al., 2009; Holland et al., 2010). In fact, some performance parameters actually increased linearly with the number of times treated except in chronically morbid calves (Montgomery et al., 2009; Holland et al, 2010). Indeed, researchers have found a significant correlation between the number of treatments and performance outcomes (Babcock et al., 2009).

However, little is known about the molecular basis for the disease outcomes in cattle with BRD. Previous research from our lab involving gene expression profiling of muscle and liver tissues in calves experimentally challenged with bovine viral diarrhea virus and *Mannheimia haemolytica* suggested inflammation in both tissues with atrophy

in muscle and lipidosis in liver (unpublished data). Based upon these previous results, we sought to examine the effects of the number of treatment events on the gene expression changes in these tissues in calves identified with clinical BRD.

MATERIALS AND METHODS

Cattle Management

All procedures performed with the animals were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (Protocol # AG0714). Twenty four commercial beef heifers (258.5 ± 29.2 kg) were selected from a larger study conducted at the Willard Sparks Beef Research Center that began in September 2009 (Wahrmund et al., 2011). Identification of calves for treatment was based upon 1) clinical signs of BRD by a trained professional using the following characteristics: lethargy, gauntness, altered gait, and nasal or ocular discharge; and/or 2) identification of sustained ruminal temperature elevation above 41° C. Calves received antimicrobial treatment if rectal temperature was ≥ 41° C or if calves were deemed severe or moribund as assessed by visual classification. Calves received tulathromycin (Draxxin; Pfizer Animal Health; Madison, NJ) at first treatment, enrofloxacin (4.5 ml/45.4 kg body weight; Baytril; Bayer Animal Health; Shawnee Mission, KS) at second treatment, and two doses of ceftiofur (2.0 ml/45.4 kg body weight; Excenel; Intervet/Schering Plough; Desoto, KS) at 48 h intervals for third treatment. Biopsy samples were taken for 3X at initial dosing for the third treatment.

Biopsy Procedures

Muscle and liver biopsies were taken at the time of treatment for calves at first (**1X;**n = 6), second (**2X;** n = 6), and third (**3X**; n = 6). A 10 day wait period was observed after sampling of all sick calves to insure that calves in the control group were indeed healthy (**0X**; n = 6). Calves were restrained while standing during biopsy procedures. Surgical areas were clipped and the sites were aseptically prepared by scrubbing thrice with iodine soap, ethanol (75%) solution, and rinsed with sterile water; scrubbing was performed in a circular motion beginning at the center of the focal area and moving outward. Following the third wash, the area was cleansed with iodine solution using the same scrubbing motion. Next, a local anesthetic (4 to 10 ml of lidocaine 2%) was injected subcutaneously in both sites and into the intercostal space for the liver area; incisions were made following a 3 to 5 minute pause to allow sufficient desensitization.

Muscle biopsy procedures were performed similarly to those described by Meijer and coworkers (1995). Samples were removed from a small area (5 x 5 cm) approximately 10 cm caudal to the last rib and approximately 8 cm lateral to the vertebrae on the right side. A stab incision (1 to 2 cm) using a scalpel blade was made through the skin, a sterile Bergstrom biopsy trochar (7 mm in diameter) was inserted into the *longissimus dorsi* muscle and 100 to 200 mg of tissue was removed, placed in a sterile vial, and immediately placed in liquid nitrogen (Dunn et al., 2003; Pampusch et al., 2003). Samples were stored at -80^{o}C until lab analysis was performed.

Liver biopsy sampling was performed similar to that described by Swanson et al., 2000. An area on the right cranial abdominal cavity was prepared in such a way as to

surround the intercostal space of the 11th and 12th ribs. Subsequently, the biopsy needle [12-gauge × 10 cm with a penetration depth of 22 mm; Bard® Monopty® Biopsy Instrument (C. R. Bard, Inc., Covington, GA)] was advanced through the intercostal muscle on the caudal side of the 11th rib, through the peritoneum and diaphragm, and into the liver. Upon collection of the sample, the instrument was retracted and the specimen was placed in a sterile vial and immediately placed in liquid nitrogen. All samples were stored at -80º C until analysis was performed.

RNA Isolation

Tissue samples were placed in 1-2 ml TRIzol Reagent™ (Invitrogen; Carlsbad, CA) and homogenized 15-30 s on ice (4º C). Supernatant was collected and phenol-chloroform extraction was performed to isolate total RNA according to the manufacturer's protocol with minor modifications. Briefly, samples were allowed to stand at room temperature (23º C) for 5 min. to promote protein dissociation. Chloroform was added to the samples at 0.2 ml per 1 ml of TRIzol reagent used and samples were allowed to stand for 10 min. at room temperature. Samples were then centrifuged at 12,000 x g for 15 min. in 4ºC. The aqueous phase was collected and RNA precipitated by adding an equal volume of absolute isopropanol. Samples were kept overnight in -20 º C followed by a second centrifugation as previously mentioned. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 12,000 x g for 2 min. The ethanol supernatant was removed and samples were allowed to dry at room temperature. RNA was suspended using diethylpyrocarbonate treated water (42º C) and concentration determined by spectrophotometry (NanoDrop ND-1000; Thermo Scientific; Wilmington, DE). Agarose gel (1%) electrophoresis was used to determine

RNA integrity. Samples were treated with DNase (DNase I, Amplification Grade; Invitrogen Corp., Carlsbad, CA) to eliminate contamination from genomic DNA according to the manufacturer's protocol.

Reverse Transcription Polymerase Chain Reaction

Reverse transcription was performed with 1800 ng RNA using QuantiTect reverse transcription kit (Qiagen Inc.; Valencia, CA) according to the manufacturer's protocol. Quantitative PCR was conducted with 90ng cDNA using the PerfeCTa® SYBR® Green FastMix® Kit (Quanta Biosciences Inc.; Gaithersburg, MD) according to the following reaction protocol: initial denaturation: 95ºC for 2 min. 30s; denaturation: 95ºC for 10s, annealing at 58ºC for 30s, and extension at 72ºC for 30s (forty cycles). The reaction was performed and fluorescent intensity measured using the BioRad MyiQ™ Real Time PCR Detection System (Bio-Rad Laboratories, Inc.; Hercules, CA). Amplification efficiency was assessed by performing qPCR with a standard curve of serial dilutions of cDNA (Table 5.1). Primer sequences are presented in Table 5.1.

Data Analysis

The experiment was conducted in a completely randomized design with number of times treated (TRT) as the main effect with animal(TRT) used as the residual error. Orthogonal contrasts were performed to compare untreated and treated calves as well as the linear and quadratic effects of increasing numbers of treatment. Data from RT-PCR was analyzed as the difference (ΔCt) between the threshold cycle (Ct) for *18S* and that for gene of interest. Fold change values were analyzed using the MIXED procedure (SAS v. 9.2, SAS Institute Inc.; Cary, NC). Fold change was determined using relative

quantification, otherwise known as the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene expression was considered significantly different at $P < 0.10$.

RESULTS

Muscle Samples

Muscle biopsy samples were collected to evaluate the differences in gene expression of selected genes involved in muscle growth and development. There were significant differences among the treatment groups for *ADIPOR1*, *FBXO32*, *BAD*, and *GLUL* ($P < 0.10$; Table 5.2). *ADIPOR1* encodes for the adiponectin receptor 1 and involved in signal transduction for growth promotion and its expression can be influenced by hormones such as adiponectin, insulin, and glucocorticoids. In this experiment, *ADIPOR1* was significantly induced 3.9-fold on average in the treated calves compared to the healthy control heifers ($P < 0.01$). There was a linear increase in *ADIPOR1* with increasing number of treatments with and an initial 3.3-fold increase in 1X compared to 0X and approximately 0.6-fold decrease in transcripts with each additional treatment above 1X ($P = 0.01$). *FBXO32* encodes for atrogin 1, an E3 ubiquitin ligase, involved in the ubiquitin-proteasome degradation of proteins. Its expression was higher in the treated group of calves compared to the healthy controls (4.2-fold; $P = 0.01$) and increased linearly as the number of treatments increased with a 2.8-fold induction in 1X and increasing further to 4.9-fold up-regulation in 2X and 3X ($P < 0.01$). *BAD* transcribes BCL2-associated agonist of cell death protein and is involved in apoptotic signaling. Transcription of *BAD* was induced an average of 5.2 fold in the treated heifers relative to the control heifers ($P < 0.01$). Its expression increased linearly with an increase in the

number of treatment events, such that expression in 1X, 2X, and 3X was 4.0, 4.4, and 7.4-fold, respectively, in comparison with 0X ($P < 0.001$). Lastly, *GLUL* is the gene for glutamine synthetase, an ATP-dependent enzyme that ligates ammonia to glutamate. It is a sensitive indicator of muscle atrophy. Expression of *GLUL* was induced an average of 8.7-fold in treated calves compared to healthy calves ($P = 0.01$). The greatest suppression occurred in the 2X group displaying 10.1-fold increase in expression compared to 0X, while 1X and 3X expression was 7.7 and 8.3-fold, respectively; yet a significant linear relationship existed among the groups ($P = 0.03$).

Liver Samples

Liver biopsy samples were taken to investigate the changes in gene expression as a result of prolonged BRD infection. We observed significant differences among the treatments for *FASN* and *S100A8* ($P < 0.10$, Table 5.3). We observed a significant suppression of *FASN* in treated calves compared to untreated calves (-6.78-fold, $P = 0.02$) There was a 21-fold up-regulation of *S100A8* in 2X compared to 0X, but differences between the control versus treated calves. There were also numeric differences for acetyl CoA carboxylase (*ACACA*) transcription between the treatment groups ($P = 0.11$). These differences resulted from a numeric decrease in *ACACA* expression in 1X and 3X heifers (+39.7 and +12.3-fold, respectively), Additionally, there was a trend for a decrease in fatty acid desaturase 2 (*FADS2*) expression in treated calves compared with controls (-52.8 fold, $P = 0.11$).

DISCUSSION

Results from our experiment indicate a linear expression of *ADIPOR1*, the gene that encodes for adiponectin receptor 1, as the number of treatments for BRD increases in beef heifers. This receptor is most abundantly expressed in skeletal muscle and mediates the effects of adiponectin, an adipokine (Yamauchi et al., 2003). The reason for the increased expression of *ADIPOR1* in the current experiment is difficult to explain. Glucocorticoids, which are often increased during morbidity, are capable of decreasing serum adiponectin levels (Shi et al., 2010). Inflammatory signaling may also play an indirect role in *ADIPOR1* expression. Researchers have found that TNF-α can decrease adiponectin secretion by adipocytes and alter serum concentrations of the adipokine (Degawa-Yamauchi et al., 2005; Lo et al., 2007). Therefore, it could be that a combination of stress and inflammation induced with BRD morbidity may result in decreased adiponectin levels in the blood and thereby suppress *ADIPOR1* expression in *longissimus dorsi* muscle tissues. However, expression of *ADIPOR1* has been shown to increase in response to stimulation by globular adiponectin in myotubes from lean human subjects (McAinch et al., 2006).In addition, *ADIPOR1* levels may be affected by insulin levels, however the data is equivocal. Research conducted using murine myocytes *in vitro* demonstrated insulin having a suppressive effect on *ADIPOR1* expression (Staiger et al., 2004; Tsuchida et al., 2004). On balance, in human subjects, *ADIPOR1* expression has been positively correlated with circulating insulin levels (Debard et al., 2004; Staiger et al., 2004). Infusion of insulin increased ADIPOR1 levels 2-fold in human skeletal muscle (Debard et al., 2004). The effect of insulin may play a role in BRD related induction of *ADIPOR1*, but it is unclear. Montgomery et al (2009) observed a linear decrease in

plasma glucose concentrations as number of treatments for BRD increased in feedlot heifers. Even though that group did not evaluate insulin levels, one may assume that with decreased glucose content insulin levels would concomitantly decrease. It may be that calves diagnosed with BRD in the present experiment decreased feed intake, thereby lowering insulin levels and its suppressive effects on *ADIPOR1* relative to healthy calves. It may be that a combination of stress, inflammation, and alterations in feed intake contribute to the changes in *ADIPOR1* expression seen in the beef heifers used in the current experiment.

We previously identified a network of genes that centered upon nuclear factor κB (**NF-κB**; data unpublished) in muscle from calves experimentally challenged with BRD pathogens. Nuclear factor κB signaling is known to mediate muscle catabolism in response to pro-inflammatory cytokines via the ubiquitin-proteasome pathway. To evaluate molecular markers of muscle degradation, we choose to evaluate the gene expression of *FBXO32*, which encodes for atrogin-1, an E3 ubiquitin ligase. Atrogin-1 and other atrogenes are tissue-specific and are often increased in atrophying muscle (Bodine et al. 2001; Gomes et al., 2001). Inflammatory ligands such as LPS, dexamethasone, and TNF-α have been shown to stimulate expression of these atrogenes with long-term effects, but interestingly inhibition of these pathways does not mitigate their expression (Frost et al., 2007). This group found that the anabolic hormone, insulin-like growth factor-1, could suppress atrogin-1 (Frost et al., 2007). In our study, we found a linear increase in *FBXO32* expression with calves treated two or more times having a near 5-fold induction of the gene. This would suggest that proteasomal muscle

degradation is increased in the muscle tissues of beef heifers treated multiple times for undifferentiated BRD.

This hypothesis is supported by the expression change in *GLUL*. This gene transcribes glutamine ammonia ligase, which is commonly referred to as glutamine synthetase. We found a linear increase in *GLUL* expression over 10-fold in calves treated twice for BRD. Inflammation is known to deplete intracellular glutamine content and increase expression of *GLUL* to ligate free ammonia from amino acid catabolism to glutamate. This is a hallmark of muscle atrophy (Calder and Yaqoob, 1999; Stipanik and Watford, 2000). Stimulation of skeletal muscle cell lines with TNF-α increased *GLUL* expression within hours and peak glutamine synthetase activity at 48 hours post-challenge (Chakrabarti, 1998). The stimulation of *GLUL* transcription in our study indicates that muscle atrophy is present and is increased in calves with more protracted BRD symptoms.

Lastly we found *BAD* expression to also be induced linearly with increasing numbers of treatment events for BRD calves. *BAD* encodes for the BCL2-associated agonist of cell death protein. This protein is a member of the B cell lymphoma-2 (BCL-2) family of proteins which are a group of oncogenes involved in cell survival. BCL-2 protein has been detected in rodent and human inflammatory myopathies, but absent in normal muscle tissues (Tews et al., 1997; Behrens et al., 1997). Further *in vitro* research revealed that increased expression of BCL-2 plays a role in myogenesis and protects muscle cells from apoptotic signaling (Dominov et al., 1998). In fact, this latter group observed in C2C12 murine muscle cell cultures that some BCL-2 expressing cells subjected to pro-apoptotic stimuli were able to survive and proliferate upon removal of

those stimuli (Dominov et al., 1998). Research has shown that BAD binds to BCL-2 essentially blocking pro-survival signaling and sensitizes cells to apoptosis (Zong et al., 2001; Kim et al., 2006). In addition, signaling with growth factors, such as platelet derived growth factor and insulin-like growth factor-1, lead to activation of protein kinase B/Akt activation which phosphorylates BAD and abrogates its apoptotic effects (Datta et al., 1997; Song et al., 2005). The increase in *BAD* mRNA could lead to increased BAD protein to promote apoptosis in existing myofibers and delay repair signaling by BCL-2 in myoblasts.

As expected, data from the present study indicate that muscle tissues from heifers treated multiple times for BRD are in a state of chronic inflammation. Genes involved in protein degradation and apoptosis are up-regulated allowing for the possibility that calves diagnosed with BRD have muscle atrophy proportional to the chronic nature and severity of infection. This is congruent with previous finding that calves treated multiple times for BRD have decreased weight gain and muscle accretion than calves not treated for BRD particularly during the early feeding phase (Gardner et al., 1999; Montgomery et al., 2009; Holland et al., 2010). It is of interest to note that three of the genes evaluated are a part of the Akt signal transduction pathway. Adiponectin receptor 1 mediates adiponectin signaling to the Akt pathway (Yamauchi et al., 2003) while atrogin-1 (Sandri et al., 2004) and BAD (Song et al., 2005) are downstream targets of this signal transduction pathway. Our results suggest that Akt signaling is being suppressed in muscle tissue of calves during treatment for BRD. Researchers have demonstrated Akt signaling pathway is involved in protein synthesis and that stimulation of such can enhance translation initiation, but such signaling is blunted under inflammatory conditions (Lang et al.,

2007). With pivotal effects in regulating protein synthesis and catabolism, further investigation into the role of Akt signaling in bovine muscle during morbidity may reveal therapeutic interventions capable of moderating the muscle loss associated with BRD.

We had previously identified gene targets that were affected during the acute phase response on the liver of steers experimentally challenged with *M. haemolytica* and bovine viral diarrhea virus. These genes were indicative of inflammation and dyslipidemia. Based upon those results, we sought to identify molecular changes in hepatic tissues in heifers with clinical BRD; however, because of variability in gene expression of the heifers in the study, we were unable to definitively characterize the prolonged effects of BRD on liver gene expression. There is evidence from the present study that *S100A8* expression is increased more than 20-fold in 2X calves compared with the other treatment groups. *S100A8* encodes S100 calcium binding protein A8 and acts as a chemoattractant for neutrophils (Xu and Geczy, 2000; Nacken et al., 2003; Gebhardt et al., 2006). It has been shown to increase during liver inflammation and promotes neutrophil infiltration, but its expression is inconsistent with the expression of other hepatic genes where we observed less effects as treatment number increased compared with healthy calves.

The magnitude of changes in gene expression seen in the present experiment in the liver, lend credence to our hypothesis that inflammation is continuing to affect lipid metabolism. Numerical differences in *ACACA*, *SCD* (stearoyl-CoA desaturase), and *FASN* expression suggest that fatty acid synthesis is inhibited in the liver during BRD infection; indeed there were significant differences between control and treated calves for *FASN* expression ($P = 0.02$). This is somewhat unexpected as previous research has

established that hyperlipidemia and increased fatty acid synthesis are characteristics of bacterial infections and cytokine stimulation (Feingold and Grunfeld,. 1986; Grunfeld and Feingold, 1991). However, the effects on gene expression are more varied. One group observed an increase in *FASN* transcripts in response to septic levels of TNF-α challenge in mice (Endo et al., 2007). On the other hand, lower doses of the cytokine and LPS are known to suppress transcription of *ACACA* and *FASN* by nearly 8-fold in previously fasted and re-fed mice, thereby effectively blocking the typical increase in these genes in realimentation (Feingold et al., 2011). In addition, gene expression profiling of liver tissues in mice injected with lipopolysaccharide showed decreased expression of genes involved in fatty acid synthesis and oxidation; notably *FASN* was suppressed more than 4-fold (Yoo and Desiderio, 2003).

Other factors may contribute to the changes in *FASN* expression in addition to inflammatory signaling. Nutritional effects have demonstrated effects on *FASN* synthesis. Fasting is known to suppress fatty acid synthase gene expression in rodents (Gosmain et al., 2005; Morgan et al., 2008). This suppression is mediated in part as a result of decreased blood glucose which works through insulin sensitization to positively regulate *FASN* expression (Prip-Buus et al., 1995). Heifers treated multiple times for undifferentiated BRD display a linear decrease in glucose concentrations in the blood as the number of times for treatment increases, but there is no reported measurement of insulin levels during such conditions (Montgomery et al., 2009). It has also been shown that calves treated for BRD have decreased feed intake (Holland et al., 2010). This combination of nutritional consequences of BRD morbidity may explain the decreased expression of *FASN* expression observed in the present experiment.

It may also be that increased fat mobilization, as a result of systemic inflammation and negative energy balance resulting from inappetence, leads to fatty acid accumulation in the liver thereby suppressing *de novo* fatty acid synthesis by a negative feedback mechanism. Interestingly this effect is mediated by polyunsaturated fatty acids (**PUFA**) which are demonstrated to suppress expression of several transcription factors and genes involved in lipogenesis (Pégorier et al., 2004). Omega-6 and omega-3 fatty acids are particularly effective in reducing *FASN* transcripts with some reductions approaching 8-fold (Blake and Clarke, 1990; Clarke et al., 1990a; Clarke et al., 1990b). Even though the history of these heifers was unknown, it may be that the linear improvement in *FASN* expression was a result of the decline in PUFA content in peripheral fat depots. Duckett et al. (1993) showed that calves coming off grass pastures have PUFA levels that steadily decline over time on feed with significant decline observed at 28 days. This could also be due to increased health status of these calves; it is possible that the most severe morbidity occurred at 1X and the calves at 3X are beginning to convalesce, however results from muscle tissue suggest an exacerbation of morbidity. The gene expression profile observed in the current study is similar to that observed in ketotic and/or energy deficient dairy cows (Loor et al., 2007; McCarthy et al., 2010). Dairy cows experiencing energy deficit have suppressed expression of *FADS2* which was also observed in the present experiment (Loor et al., 2007; McCarthy et al., 2010). This series of genes that are down-regulated indicates a generalized suppression in *de novo* fatty acid synthesis as a result of energy deficit. This suppression of fatty acid synthesis explains the decreased fat content that BRD treated calves have in subcutaneous, intramuscular, and perirenal fat depots.

In summary results from the present experiment show that gene expression changes in muscle tissues in heifers treated for BRD under field conditions are such that muscle atrophy is increased in a linear fashion as the number of treatment events increases. This indicates that BRD can have prolonged and detrimental consequences to muscle protein accretion such that proteolysis and inhibition of growth signaling are a result of chronic BRD infection. While the muscle gene expression was consistent, liver gene expression was more variable, but there is an indication that inflammation is present in the liver of calves treated 2X for BRD and that fatty acid metabolism is modified. *FASN* is the enzymatic regulator of fatty acid synthesis for a protracted period of time and its suppression demonstrates an overall decline in fatty acid synthesis, but shows improvement in expression levels as the treatment progresses. These results demonstrate the long term consequences of BRD and further underscore the need for early intervention and treatment of morbid calves.

Table 5.1. Primer sequences, amplification efficiencies, and standard curve r^2 values

Gene	Forward Sequence	Reverse Sequence	Efficiency	r^2
18S	TTCGAACGTCTGCCCTATCAA	GATGTGGTAGCCGTTTCTCAGG	92.4%	0.998
ACACA	TGCACGGCTGACTGGAGTTGA	TGTCCGTCTTCAGGTCGCGT	103.4%	0.971
ADIPOR1	CCTCCCTCACCCCTGTCCTGA	AGGCCCGAGAAAGATGGGACCAG	100.3%	0.922
BAD	TCCGGTGTGTGCGAGGACCA	TTCCGGCAGGTAAGGGCGGA	93.1%	0.947
FADS2	GGAACCATCGCCACTTCCAGCA	TCTTGCCGTACTCAATGGGCTGC	103.7%	0.972
FASN	ACCCCACAACTGCCATCCACCT	TGGCCTGTGTTGCCTTCGGA	95.7%	0.968
FBXO32	CCGTGAGGCTTGATGTCAGCT	GGGTTTTATCCGGGCCATGC	99.0%	0.982
GLUL	ACATTGAGGAGGCCATTGAG	TTGATGTTGGAGGTTTCGTG	100.9%	0.975
S100A8	AGGATGCGGACACTTGGTTCAAA	GCCCACCTTTATCACCAGCACG	97.4%	0.994
SCD	TCACATTGATCCCCACCTGCAA	TCGGTGACTCCACAGGCGAT	90.6%	0.987

Table 5.2. Least squares means of fold change of genes in muscle of beef heifers with bovine respiratory disease at time of treatment

	Number of Times Treated					P Values		
Gene	**OX**	**1X**	**2X**	**3X**	**SEM**	**TRT**	**UNTREATED VS. TREATED**	**LINEAR**
ADIPOR1	0.42	3.30	3.89	4.37	1.02	0.05	< 0.01	0.01
BAD	-0.26	3.97	4.36	7.37	1.19	< 0.001	< 0.001	< 0.001
FBXO32	0.29	2.84	4.89	4.86	1.20	0.04	0.01	< 0.01
GLUL	0.34	7.66	10.11	8.35	2.56	0.06	0.01	0.03

Table 5.3. Least squares means of fold change of genes in liver of beef heifers with bovine respiratory disease at time of treatment

	Number of Times Treated					P Values		
Gene	**OX**	**1X**	**2X**	**3X**	**SEM**	**TRT**	**UNTREATED VS. TREATED**	**LINEAR**	**QUADRATIC**
ACACA	0.15	-39.73	-1.03	-12.25	12.81	0.11	0.19	0.98	0.22
FADS2	0.05	-64.27	-40.83	-53.33	31.06	0.42	0.12	0.27	0.36
FASN	0.39	-10.19	-4.03	-6.13	2.72	0.06	0.02	0.22	0.10
S100A8	-0.17	-0.08	21.27	1.25	7.74	0.09	0.35	0.40	0.16
SCD	0.56	-18.05	-14.22	-13.15	9.88	0.50	0.14	0.34	0.28

CHAPTER VI

Summary Review of Gene Expression changes in Muscle and Liver Tissue of Growing Beef Calves with Bovine Respiratory Disease

Bovine respiratory disease continues to be the most prevalent and costly disease facing beef cattle in North America. Economic losses from the disease complex result not only from direct costs to the producer such as medication, labor, and mortality losses, but also extend indirect costs due to decreased animal weight gain, and decreased carcass value stemming from decreased marbling and ribeye area which cut into producers' profits.

In these experiments we sought to characterize gene expression changes in muscle and liver tissues during acute and chronic illness associated with BRD. Findings from these experiments showed that muscle tissue was less affected early in infection than liver tissues. However, we observed that gene expression in muscle over a more prolonged period of illness changed to indicate that muscle tissue is being catabolized. Protein degradation in muscle is used to supply amino acids for the immune response. Therefore, chronic morbidity can lead to significant reduction in muscle protein and invariably muscle content of the calf explaining in part the decreased muscle mass and yield that results from BRD infection in beef cattle.

In liver tissues, we observed an early and significant response to BRD infection. Significant inflammatory markers were observed in the liver indicative of immune cell infiltration and activity. In fact, in a companion study to these, our laboratory group observed significant increases in leukocytes and neutrophils within the first day of infection of BRD. In addition, the liver decreases expression of genes for fatty acid metabolism and specifically we observed a significant reduction in transcription of genes involved in fatty acid synthesis. This indicates that early during infection, the liver is decreasing synthesis of new fatty acids possibly due to mobilization from fatty acids from

peripheral tissues. These mobilized fatty acids are stored in the liver as an alternative energy source to carbohydrates to fuel the survival of the animal during acute infection when appetite is typically suppressed. On the other hand, it appears that the dramatic modifications that occurring in the liver during acute infection are attenuated with more prolonged infection. We observed that expression of inflammatory genes in the liver was only slightly changed over a prolonged period of infection. We further observed that expression of genes in fatty acid metabolism indicated less suppression of fatty acid synthesis as the length of morbidity increased. This may well result from a decrease in the amount of fatty acids mobilized from the periphery to the liver which could result in fatty liver. Utilization of these early mobilized fatty acids for energy by the calf over prolonged morbidity would lead to decreased fatty acid content stored the liver. This may explain the attenuation of the suppression of fatty acid metabolism.

Overall, it appears that muscle tissues are less affected by acute BRD infection than are liver tissues in growing beef calves. However, atrophy of muscle and depression of fatty acid synthesis in calves with BRD infection may explain the decreases in muscle yield and marbling often associated with calves treated from the disease.

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: GENE EXPRESSION CHANGES IN MUSCLE AND LIVER TISSUE OF GROWING BEEF CALVES WITH BOVINE RESPIRATORY DISEASE

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Completed the requirements for the Doctor of Philosophy/Education in Animal Science at Oklahoma State University, Stillwater, Oklahoma in December, 2011.

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Title of Study: GENE EXPRESSION CHANGES IN MUSCLE AND LIVER TISSUE OF GROWING BEEF CALVES WITH BOVINE RESPIRATORY DISEASE

Pages in Study: 156 Candidate for the Degree of Doctor of Philosophy

Major Field: Animal Nutrition

Scope and Method of Study:

Bovine respiratory disease is the most costly ailment in beef cattle. It leads to decreased carcass quality and profitability. It has been shown to decrease muscle and fat accretion. Research in other species has identified several gene targets in muscle and liver whose expressions are changed in response to inflammation, but there are few studies that address these molecular mechanisms in cattle. The objectives of our studies were to collect muscle and liver biopsy samples from beef cattle with bovine respiratory disease under experimental and field conditions and identify gene targets to understand the deleterious effects of morbidity on carcass quality and metabolism.

Findings and Conclusions:

We found that bovine respiratory disease modified gene expression in muscle tissue to indicate that atrophy was occurring. Several of the genes identified were involved in the Akt/protein kinase B signaling pathway and indicated a resistance to growth stimulation and pro-apoptosis. Expression of genes encoding for adiponectin receptor 1 (*ADIPOR1*), atrogin-1 (*FBXO32*), BCL2-associated agonist of cell death protein (*BAD*), glutamine synthetase (*GLUL*), and thioesterase superfamily member 4 (*THEM4*) were modified in muscle tissues in response to bovine respiratory disease conditions. In the liver, we found fatty acid metabolism and inflammation to be most affected. Expression of genes encoding for acetyl-CoA carboxylase (*ACACA*), β-site APP-cleaving enzyme 1 (*BACE1*), fatty acid desaturase 2 (*FADS2*), fatty acid synthase (*FASN*), and S100 calcium binding protein A8 (*S100A8*) were affected by bovine respiratory disease. The molecular targets identified in the muscle and liver help to explain the loss of muscle and fat that is often associated with this disease and future research is need to address way to manipulate their expression in ways to mitigate these losses.

ADVISER'S APPROVAL: Dr. Clint R. Krehbiel