THE EFFECTS OF MATERNAL AND POSTNATAL INFECTIONS WITH PORCINE REPRODUCTIVE AND RESPIRATORY VIRUS ON MUSCLE GROWTH AND DEVELOPMENT IN PIGLETS

BY

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THESIS

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ABSTRACT

Infection can reduce feed intake, limit weight gain, and decrease muscle growth by directing nutrients away from protein accretion and towards an inflammatory response; however, it is unclear how maternal infection affects offspring skeletal muscle development. Additionally, it is unclear how viral infections mechanistically reduce skeletal muscle growth and protein accretion. Pregnant gilts were inoculated with PRRSV (P piglets) or sterile culture medium (C Piglets) at 80 days of gestation. Offspring were euthanized at birth (d0) or at four weeks of age (d28). Samples from the longissimus dorsi (LD) and psoas major (PM) were used for gene expression, while the semitendinosus (ST) at d28 was used to determine muscle fiber area and number. At d0 relative LD weight was decreased (P=0.01) while PM was increased (P=0.01) in P piglets compared with C piglets. At d28, whole LD (P=0.01) and relative LD weight (P=0.03) decreased in P piglets compared with C piglets. Cell number decreased (P=0.04) while muscle fiber cross sectional area within ST increased (P=0.02) in P piglets compared with C piglets. At d0 in the LD, maternal PRRSV infection resulted in a shift towards more oxidative fiber type expression and a decrease in negative growth factors as myosin heavy chain (MHC) *2b* (P=0.02), *MHC2x* (P=0.01), and myostatin (P=0.01) expression decreased in P piglets compared with C piglets. However, at d28, there were no differences in MHC expression. A shift towards more oxidative fiber types also occurred in the PM at d0, as *MHC1* expression was increased (P=0.03) in P piglets compared with C piglets. Furthermore, an increase in positive growth factors was observed as myogenic differentiation 1 (P<0.01) and myogenin (P<0.01) increased while myostatin expression decreased (P=0.04) in P piglets compared with C piglets. These data suggest maternal PRRSV infection restricts in utero muscle fiber development.

Postnatal infection was evaluated in pigs inoculated with PRRSV or sterile culture medium (control) at 35 days of age. Pigs were maintained on similar diets and euthanized at 49 days of age. Samples from the LD and PM were used for gene expression, while the ST was used to determine muscle fiber cross sectional area and number. From 35 to 49 days of age, body weight gain ($P<0.01$) and feed intake ($P\leq0.04$) were reduced in PRRSV inoculated pigs compared with control pigs. However, feed to gain ratio was only reduced ($P<0.01$) the first week after inoculation in PRRSV inoculated pigs compared with control. Longissimus dorsi and ST weights were decreased ($P<0.01$) and relative PM weight was reduced ($P<0.01$) in pigs inoculated with PRRSV compared with control pigs. In addition, muscle fiber size of PRRSV inoculated pigs tended to be reduced ($P=0.09$) compared with control pigs, but muscle cell number was unaffected. In the PM, myostatin expression tended to be increased ($P=0.08$) while myogenic factor 5 ($P=0.01$) and insulin-like growth factor 1 ($P=0.04$) were decreased in pigs inoculated with PRRSV compared with control pigs. Infection had minimal effects on myogenic growth factors in the LD as myogenic differentiation factor 1 (*MYOD*) was increased ($P<0.01$) and *IGF* 2 was decreased ($P=0.04$) in pigs inoculated with PRRSV compared with control pigs. Porcine reproductive and respiratory virus infection decreased muscle weights and cell size through a decrease in myogenic growth factor expression. However, differences between muscles suggest muscles may respond differently to PRRSV infection depending on their fiber type composition.

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CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

In order to feed a population of 9 billion people in 2050, the world will need to produce more food with fewer resources. New efficient, practical, and profitable technologies need to be used in order to reach these demands. Current technologies used in animal agricultural production focus on postnatal growth, improving live performance and lean muscle growth have led to more efficient animal production. Relatively less attention, however, is focused on prenatal growth, despite the fact that market animals spend roughly one-third of life in gestation. During gestation, formation of primary and secondary muscle fibers determines overall postnatal growth potential of skeletal muscle. Primary muscle formation is controlled by genetic factors, but secondary muscle formation can be altered by external influences such as maternal nutrition or maternal stress (Buckingham et al., 2003) Therefore, manipulations can occur in gestation to enhance or limit muscle growth efficiency.

Stress is defined as a disruption in homeostasis resulting in positive or negative adaptations (Moberg, 2000). In response to immunological stress, an inflammatory response occurs in order to adapt to a pathogen (Black, 2003). While inflammation aids in destroying pathogenic structures through enhanced permeability of membranes (Watkins et al., 1995), inflammation can also be perceived as a negative adaptation as production of inflammatory cytokines (interlukin-6; IL-6, tumor necrosis factor alpha; TNF-α) directs nutrients away from tissue growth to support an immune defense (Johnson, 2002). The most dramatic repartitioning is observed in skeletal muscle (Escobar et al., 2004). While effects of inflammation on skeletal

muscle growth has been determined, the influence of inflammatory cytokines on prenatal muscle development is less clear.

Unfortunately, maternal inflammatory responses due to infection are typical in swine production and can be linked to multiple diseases. The most noted has been porcine reproductive and respiratory syndrome virus (PRRSV). In the United States, PRRSV causes a $299 million loss in productivity per year within breeding herds alone (Zimmerman et al., 2012). Yet, it is unclear how PRRSV infection in pregnant dams affects developing offspring. Furthermore, PRRSV leads to lean growth deficiencies in growing pigs, causing them to finish with more fat cover at lighter weights (Stahly et al., 1997). But, it is unclear how postnatal PRRSV infection mechanistically down-regulates muscle development and decreases lean yield production. Without a better understanding of prenatal and postnatal infection, improvements in growth efficiency will be limited. This review of the current literature will lay the foundation to further investigate prenatal animal growth potential while detailing how immunological stress affects the molecular mechanisms that reduce growth. It will also discuss muscle growth and development, immunological stress, inflammation and its effects on muscle development.

MUSCLE GROWTH AND DEVELOPMENT

Embryogenesis is the process of embryo formation from fertilization and cleavage to fetal formation. There are three main events that occur during embryogenesis: gastrulation, neurulation, and segmentation/somitogenesis. During gastrulation, cells in the epiblast begin to replicate in Hensen's Node, causing a thickened structure (primitive streak) to form parallel to the long axis of the embryo, proceeding from head to tail. The primitive streak then begins to deepen into a depression forming the primitive groove (Bachvarova et al., 1998). Epithelial mesenchymal transformation, transformation from epithelial cells into mesenchymal through

characteristic alterations, is necessary for proper formation of the primitive groove. Epithelial mesenchymal transformation is regulated by fibroblast growth factor (FGF), which aids in alterations of cell adhesion (Radisky, 2005). As migration of cells continues, three distinct germ layers form: ectoderm, mesoderm, and endoderm (Mundlos, 2010).

Next, the edge of primitive groove becomes more pronounced, initiating neurulation (Copp et al., 2003). The two edges of the groove converge, resulting in an essential neural tube that acts as a structural unit to the notochord, which forms ventral to and outside of the neural tube through hedgehog signaling (Choi and Harfe, 2011). Along the neural tube, mesodermal cells begin to condense into blocks (somites). Somitogenesis is tightly governed by Hox genes, which produce transcription factors that define positional identity of tissues (Christ and Ordahl, 1995). These transcription factors regulate cell adhesion, migration, cycle, and apoptosis. Positional identity of tissues derived from somites is specified by the expression pattern of Hox genes, which are regulated by an overlap in gradient of growth factors: Wingless-Int (Wnt) proteins (regulates 3' Hox gene expression), FGF (regulates 5' Hox gene expression), and retinoic acid (antagonizes FGF and enhances anterior 3' Hox gene expression; (Maconochie et al., 1996)). These genetically regulated pathways are essential to specify function of each cell. Muscle development is a highly regulated system and any minimal alterations could affect proper and optimal formation of muscle.

COMMITMENT TO MATURATION

Limb bud formation is essential for skeletal muscle development. Each limb arises from a small bud of mesodermal cells covered by surface ectoderm. In the progress zone of proliferating cells, FGF maintains proliferation and delays differentiation of mesoderm. As limb buds grow, medial cells are exposed to less FGF, resulting in mesenchymal condensation that becomes

cartilage (Mariani and Martin, 2003). During limb bud formation, cells become specified and enter myogenesis.

Myogenesis consists of six well-defined steps: commitment/determination, migration, proliferation, differentiation, fusion, and maturation/hypertrophy. Cells begin myogenesis by expressing myogenic regulatory factor (MRF) genes, thus committing cells to become muscle (Ott et al., 1991). Muscle regulatory factors are transcription factors that interact with DNA and activate transcription of muscle specific genes such as myosin, actin, and titin (Buckingham, 1992). An essential characteristic of muscle cells are Myf5 and MyoD as they determine muscle lineage. An increase in Myf5 and MyoD expression will stop cell proliferation and increase synthesis of myogenin. Expression of myogenin biochemically changes characteristics of differentiation and allows myoblast fusion to occur (Wang and Jaenisch, 1997).

Muscle cell proliferation and migration are directed by FGF signaling. After migration, myoblasts continue to divide and increase in cell number through proliferation. Proliferation is regulated by peptide growth factors in the cell cycle. During G1-phase, myoblasts are in growth arrest and are most sensitive to external growth factors. Timing and concentrations of certain growth factors encourage cells to continue through the cell cycle and their absence will cause cells to exit the cell cycle and develop into specific tissues (Allen and Boxhorn, 1989). Growth factors that encourage muscle cell proliferation are insulin-like growth factor 1 (IGF-1) and FGF, while myostatin and tumor-necrosis factor alpha (TNF-α) inhibit proliferation (Glass, 2005).

Myostatin, in particular, is a negative regulator specifically of skeletal muscle mass (Zhu et al., 2004). Myostatin inhibits proliferation by binding to activin type II receptors through divergent signaling cascades. First, Smad3 phosphorylation will increase, inhibiting production

of MyoD and myogenin. This inhibition stops differentiation within cells, restricting precursor cells from becoming myoblasts. Second, myostatin binding to activin type II receptor will activate and increase p21. This leads to inhibition of cyclin/CDK complex which is needed to move into S phase of the growth cycle (Lee, 2004).

Regulation of myogenic differentiation is regulated by Notch/Hes pathway (Kitamura et al., 2007). Active Notch signaling suppresses MyoD transcription, preparing cells for fusion as they halt cellular division (Shawber et al., 1996). Myoblast fusion is promoted by IGF-1 and integrin (Yun and Wold, 1996). Myotube fusion results in increased transcription of muscle specific genes: myogenin, MRF4, muscle creatine kinase (MCK), and the thick and thin filament proteins, myosin and actin, respectively (Sabourin and Rudnicki, 2000). After myoblasts fuse, they develop more muscle characteristics as they mature to reach full functionality. During maturation, there is an up-regulation of myofibril proteins and assembly of myofibrils into functional muscle units. Muscle development is a complex system that is highly dependent on specific growth promoter and repressor signaling during fetal development (Butler-Browne et al., 1990). The fetus relies on maternal health and nutrient regulation to maintain proper balance and signaling of muscle growth.

PRIMARY AND SECONDARY FIBER FORMATION

Muscle fiber fusion occurs in a series of waves with a portion of myoblasts fusing to form myotubes while other myoblasts continue to proliferate. Primary myotubes (fibers) develop first and act as scaffolding for secondary myotubes to form, organizing their alignment, and aiding in proper fusion (Choi et al., 2008). Primary myotubes are thought to mature into slower contracting, oxidative muscle fibers expressing myosin heavy chain (MHC) 1 in adult skeletal muscle, while secondary muscle fibers form the faster contracting, more glycolytic fibers that

express MHC II isoforms (Alnaqeeb and Goldspink, 1987). In swine, there may be as many as 20 secondary muscle fibers formed for each primary muscle fiber (Gerrard and Grant, 2006), therefore secondary muscle fibers have a large effect on growth potential of an animal. Primary muscle fiber development is independent from environmental cues, meaning maternal factors such as immunological stress may not affect their development (Qu et al., 1998; Yaffe, 1968).

Conversely, secondary muscle fiber formation is responsive to external influences; therefore, external factors can manipulate total muscle fiber number (Wigmore and Stickland, 1983). Secondary muscle fibers develop as innervation begins, making their development dependent on innervation (Fredette and Landmesser, 1991). In an aneural system, a myoblast cannot differentiate into a secondary myofiber; however, myotubes can degenerate and be replaced with connective tissue through atrophy (Jirmanová and Zelená, 1970). Furthermore, maternal nutrition is crucial for fetal muscle development because organogenesis and other tissue development takes precedence over skeletal muscle development (Zhu et al., 2010). If maternal undernutrition occurs during mid to late gestation, secondary muscle fiber formation is decreased in offspring in order to repartition necessary nutrients to organogenesis, reducing postnatal growth rate (Dwyer et al., 1994).

Similar to maternal under-nutrition, in an overfed, obese maternal nutrition model, secondary muscle fiber formation was decreased in offspring (Du et al., 2010). This reduction in secondary fiber formation was attributed to chronic inflammation associated with production of inflammatory cytokines (Tong et al., 2009), and not to a direct effect of nutritional excess on the fetus. Given this reduction in muscle fiber formation in obese dams, it can be speculated that maternal infection will induce inflammation symptoms on the fetus and reduce muscle fiber number.

FIBER TYPES AND POSTNATAL GROWTH

Muscle fiber types can be defined by their metabolic, contractile and morphological characteristics and divided into four different fiber types based on MHC isoform; MHC 1, MHC 2a, MHC 2x, and MHC 2b (Schiaffino et al., 1989). Among the 4 types, MHC 1 fibers are slowest contracting, have many mitochondria, are darker in color, and have the greatest capacity for oxidative metabolism, while MHC 2b fibers are the fastest contracting fibers, have fewer mitochondria, are lightest in color, and have the greatest capacity for glycolytic metabolism. Myosin heavy chain 2a and 2x fibers are also fast contracting fibers, however, they are intermediate in many characteristics and color, and have mixed capacities for both oxidative and glycolytic metabolism (Ausoni et al., 1990). Muscles are developmentally comprised of different fiber types; however, each may be altered by neural, endocrine and physical stimuli.

Postnatal muscle growth is achieved through hypertrophy or an increase in muscle cell size (Goss, 1966). Both primary and secondary muscle fibers can increase in size; however, secondary muscle fibers or MHC 2 fibers experience more hypertrophic growth than primary, MHC 1, fibers (Ashmore et al., 1973). When growth inhibitors (e.g., myostatin) were deleted, the proportion of MHC 2x fibers increased 66 $\pm$ 9% ($P<0.01$) in the semitendinosus, a muscle with mixed MHC fibers. In fact, the MHC 1 fiber population was reduced in the semitendinosus (Bouley et al., 2005). This suggests MHC 2 fibers are more responsive to hypertrophic growth signals. Therefore, fewer MHC 2 fibers at birth will limit postnatal growth potential.

Postnatal growth is highly dependent upon satellite cells as they supply new sources of DNA to support new protein synthesis during growth (Allen and Rankin, 1990). During satellite cell activation, Notch-1 translocates to the nucleus and causes proliferation of paired box (Pax) 3 and Pax7 expressing satellite cells. Without Pax3 and Pax7, these cells die or exhibit cell-cycle

arrest. Through expression of Pax3 or Pax7, MyoD and myogenin increase expression, allowing a new myoblast to form and fuse (Buckingham, 2007).

IMMUNOLOGICAL STRESS AND FETAL PROGRAMMING

During immunological stress, such as infection, an innate immune response occurs (Aderem and Ulevitch, 2000). Pattern-recognition receptors (PRR) such as toll-like receptors (TLR) recognize the molecular finger print of a pathogen and activate inflammatory responses. (Medzhitov, 2007; Sabroe et al., 2008). Macrophages and other immune cells express specific TLR. Once specific TLR bind a pathogen-associated molecular pattern, a cascade of events releases nuclear factor-kappa beta (NF-κβ), which translocates to the nucleus to increase expression of inflammatory cytokines, including TNF-α and IL-6 (Effros et al., 1991).

Cytokines are synthesized in the cytoplasm, secreted by the endoplasmic reticulum, and induce a cast array of biological effects (Zhang and Kaufman, 2008). Specifically, TNF-α increases vascular permeability and stimulates local inflammation through increased membrane receptor function. Cytokines also induce increased internal temperature by mobilizing protein and energy in muscle and fat tissue; this decreases infectious replication (Moldawer and Copeland, 1997). In general, these cytokines increase permeability in blood vessels, allowing more serum proteins to be transferred to infection sites to destroy pathogenic structures (Watkins et al., 1995).

Fetal programming is defined as developmental changes in structure or metabolism triggered by maternal stimuli during fetal development (Godfrey and Barker, 2001). Nutritional changes during gestation have been well-studied in terms of fetal programming but the effect of maternal infection is less well-understood. When *Escherichia coli* lipopolysaccharide, a well-

defined model of immune system activation in rodents, was administered to pregnant mice, an increase in maternal circulation of IL-6 and TNF-α led to increased IL-6 and TNF-α levels in amniotic fluid, and decreased fetal neurodevelopment and survival (Collins et al., 1994). Furthermore, maternal inflammation due to infection has been correlated with low birth weight and higher incidence of premature birth (Rogers and Velten, 2011). Therefore, maternal infection can alter fetal development, but its effect on fetal muscle development is less clear. Maternal obesity models demonstrate low-grade inflammation including production of TNF-α and IL-6 (Wei et al., 2008). These models have been shown to shift commitment of fetal mesenchymal stem cells away from a myogenic lineage (Du et al., 2010). However, mechanistic pathways to how inflammation decreases fetal myogenesis have yet to be defined. The cytokines IL-6 and TNF-α can negatively affect muscle development as they inhibit myogenic differentiation (Langen et al., 2001; Tong et al., 2009).

INTERLEUKIN-6 AND MUSCLE GROWTH

Specifically, IL-6 has been related to growth deficits in children and sarcopenia in elderly as it directly or indirectly mediates catabolic effects on skeletal muscle (DeBenedetti et al., 1997; Grimble, 2003; Haddad et al., 2005). In transgenic mice exhibiting a 5-fold increase in circulating IL-6 concentrations, body weight was decreased 50-70% compared with non-transgenic littermates due to reduced circulating IGF-1 levels. It has been suggested that IL-6 may inhibit liver IGF-1 production or decrease ternary binding complex IGF-1 traveling in circulation. More importantly, decreased IGF-1 levels found in transgenic mice cannot be attributed to nutritional disorders as all mice maintained normal feed intake, therefore suggesting decreases in growth were due to increased IL-6 directly (DeBenedetti et al., 1997). Furthermore, IL-6 infusion in rats resulted in muscle atrophy characterized by a loss of myofibrillar protein of

up to 17% (Haddad et al., 2005) compared to non-infused rats. In addition, when direct effects of IL-6 on skeletal muscle were examined, IL-6 reduced phosphorylation of ribosomal S6 kinase (60%) and signal transducer and activator of transcription 5 (33%); key regulators of cell growth (Dufner and Thomas, 1999).

TUMOR NECROSIS FACTOR ALPHA AND MUSCLE GROWTH

Interveneous administration of TNF-α accelerated muscle protein degradation (García-Martínez et al., 1993). Muscle growth can be directly and indirectly inhibited by TNF-α (Figure 1.1). Directly, TNF-α has a catabolic effect on differentiated muscle through activation of NF-kB (Reid and Li, 2001). Binding of TNF-α to type 1 TNF-α receptors (TNFR1), activates NF-kB and up-regulates ubiquitin/proteasome pathway that is responsible for degradation of most intracellular protein (Mani and Gelmann, 2005). Proteasomes break down protein to peptides, which will be degraded into amino acids (Reid and Li, 2001). In addition, active NF-kB translocates to the nucleus to initiate transcription of cytokines and growth regulatory factors (Langen et al., 2001).

Moreover, TNF-α can indirectly reduce muscle development through insulin resistance (Reid and Li, 2001). Under normal conditions, insulin binds to insulin receptors on skeletal muscle stimulating intrinsic tyrosine kinase activity which auto-phosphorylates insulin receptor substrates. This leads to activation of phosphatidylinositol 3-kinase (PI3K), which increases phophoinositides that activate downstream kinases such as protein kinase B/Akt (Goodyear et al., 1995). Phosphorylation of Akt substrate 160 inhibits glycogen synthase kinase 3, which stimulates translocation of glucose transporter type 4 (GLUT4) to cell membranes to allow glucose to enter cells where it is used in glycogen synthesis pathways (Kurth-Kraczek et al., 1999). In addition to its role in glucose metabolism, insulin is also a potent stimulator of muscle

protein accretion. Insulin functions in skeletal muscle by causing an up-regulation of protein synthesis via PI3K–mammalian target of rapamycin (mTOR) signaling pathway. When insulin binds to insulin receptors on the sarcolemma, insulin receptor substrate (IRS)-PI3K-Akt pathway is activated phosphorylating mTOR (Bolster et al., 2002). Activation of mTOR results in phosphorylation of downstream targets that ultimately increase the number of ribosomes to support protein synthesis during hypertrophy (Nader et al., 2005).

Cytokines, however, have the ability to disrupt insulin signaling through the IRS-PI3K-Akt pathway, there by leading to insulin resistance (Hotamisligil, 2006). Because insulin is a strong stimulator of growth and shares a pathway with IGF, disruption could cause growth deficits. When specific skeletal muscle receptors (e.g., TNFR1 and TNFR2) bind to TNF-α tyrosine phosphorylation of insulin receptor substrate (IRS-1) is decreased. Furthermore, IRS-1 serine phosphorylation increases (Bouzakri and Zierath, 2007). These two events result in increased degradation of IRS-1, reduced activation of PI3K, and reduced glucose uptake into cells (Pederson et al., 2001). Furthermore, when TNFR1 binds TNF-α, AMP-activated protein kinase (AMPK), a regulator of muscle metabolism and gene expression, is suppressed reducing skeletal muscle acetyl CoA carboxylase phosphorylation, fatty acid oxidation, glycogen metabolism, and glucose uptake decrease, all of which are linked to insulin resistance (Wei et al., 2008).

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Increased expression of inflammatory cytokines in skeletal muscle has been investigated in many scenarios (Späte and Schulze, 2004), but the effects of viral infection are less well-known. Furthermore, minimal research has shown lasting effects of maternal infection during pregnancy on a developing fetus. In the United States, PRRSV causes a $664 million loss in

productivity annually; with 45% of production loss occurring in breeding herds (Zimmerman et al., 2012). Animals infected with PRRSV exhibited reduced feed intake and growth due to immunological stress response (Sandberg et al., 2006). Immunological stress caused by PRRSV generates an inflammatory response consisting of increased production of inflammatory cytokines IL-6 and TNF-α production (Black, 2003). Escobar et. al. (2004) reported increased inflammatory cytokines from PRRSV infection led to increased myostatin expression that contributes to decreased protein accretion and reduced myofiber area in muscles of mixed MHC fiber composition. However, muscle inflammation targets a reduction in MHC 2 fiber number and size with minimal changes in MHC 1 fibers (Deschenes, 2004). Therefore, more information is needed to determine if growth restricted during immunological stress depends on fiber type composition.

OBJECTIVES

Maternal infection is common in animal pregnancy, but with minimal research in this area, expected outcomes and preventative measures to protect progeny are scarce. Information gained in this area is critical for preventing and treating developmental and health related problems. Furthermore, these results can help increase production efficiency by gaining a greater understanding of the importance of prenatal growth in livestock production.

As reviewed, inflammatory cytokines inhibit myogenic differentiation through multiple pathways. Through this study we will determine if myogenic differentiation is inhibited in offspring of maternally infected dams to create reductions in muscle growth. Furthermore, we will establish a fundamental knowledge of the influence of maternal infection during pregnancy concerning the molecular basis of muscle growth and development. In addition, we can understand how maternal infection will affect overall production potential of animals.

Furthermore, defining mechanisms involved in inhibiting postnatal skeletal muscle protein accretion during infection is necessary to understand how specific muscles are growth restricted. With a thorough understanding of muscle growth inhibition during infection, nutrient requirements can be adjusted or new marketing techniques can be applied to reduce production loss.

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FIGURES

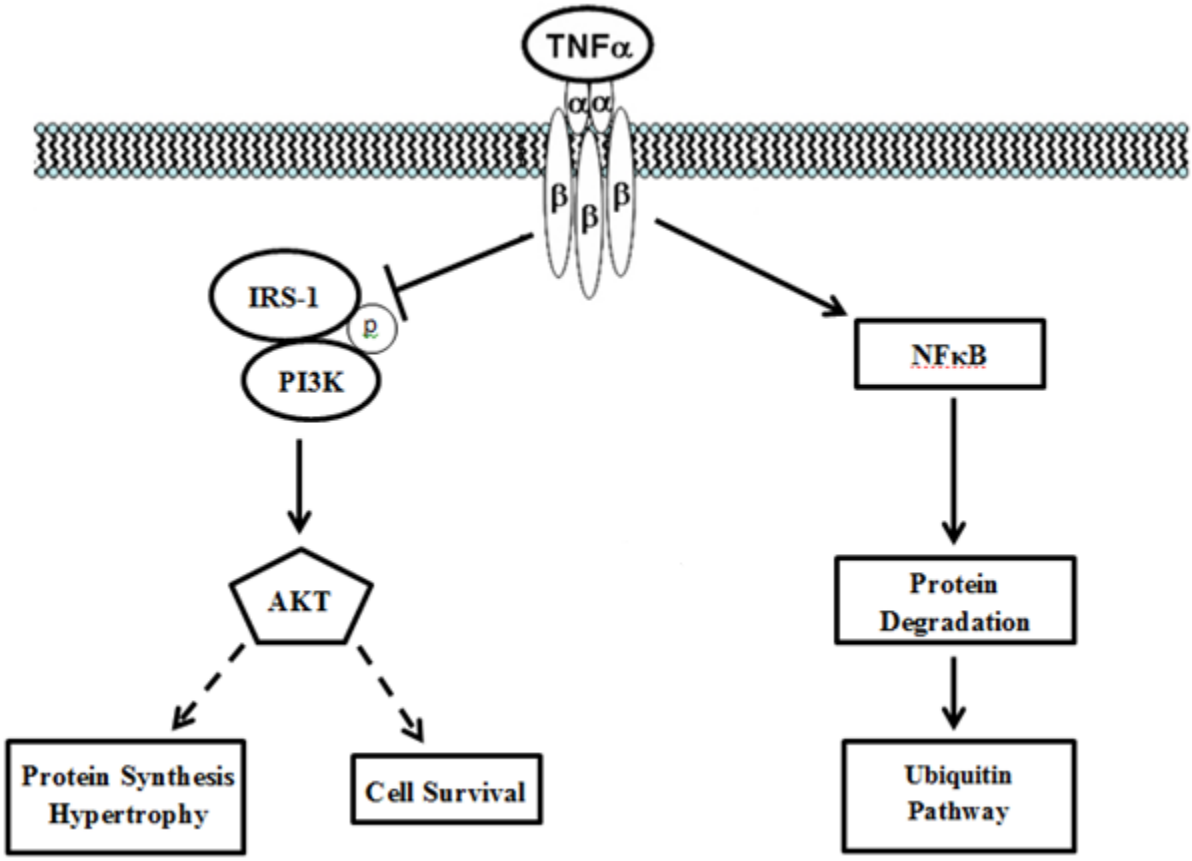

Figure 1.1. Tumor necrosis factor alpha (TNF-α) directly and indirectly inhibits skeletal muscle growth. Directly, TNF-α activates nuclear factor- κβ (NF- κβ), causing protein degradation through the ubiquitin/proteasome pathway. Indirectly, TNF-α binds to specific muscle receptors and decreases tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), decreasing activation of phosphoinositide 3-kinase (PI3K). Adapted from Grounds et al. (2008).

CHAPTER 2

EFFECTS OF MATERNAL INFECTION WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON MUSCLE GROWTH AND DEVELOPMENT IN PIGLETS

ABSTRACT

Infection stimulates nutrient repartitioning away from skeletal muscle to support the immune system, which can limit weight gain and decrease muscle growth. The effects of maternal infection and inflammation during pregnancy on offspring skeletal muscle development, however, are not well understood. Therefore, pregnant gilts were inoculated with porcine reproductive and respiratory syndrome virus (PRRSV) or sterile culture medium at 80 days of gestation. Offspring were euthanized at birth (d0, n=58) or at four weeks of age (d28, n=24). Data were analyzed as a 2-way ANOVA with the fixed effects of inoculation and sex, with piglet serving as the experimental unit. At each age, offspring were weighed and longissimus dorsi (LD), psoas major (PM) and semitendinosus (ST) muscles were removed and weighed. Samples from the LD and PM were used for gene expression, while the ST at d28 was used to determine muscle fiber area and number. At d0, relative LD weight decreased (P=0.01) while relative PM increased (P=0.01) in piglets from PRRSV infected gilts (P) compared with piglets from control gilts (C). At d28, whole LD (P=0.01) and relative LD weight (P=0.03) decreased in P piglets compared with C piglets. Muscle fiber number decreased (P=0.04) while muscle fiber cross sectional area of ST increased (P=0.02) in P piglets compared with C piglets. At d0 in the LD, maternal PRRSV infection reduced expression of the glycolytic fiber types myosin heavy chain (MHC) *2b* (P=0.02) and *2x* ($P \leq 0.01$). Furthermore, myostatin expression decreased (P=0.01) in P piglets compared with C piglets. However, at d28, there were no differences in MHC expression. A shift towards more oxidative fiber types occurred in the PM at d0, as *MHC1* expression was

increased (P=0.03) in P piglets compared with C piglets. Furthermore, insulin-like growth factor (*IGF*) 2, myogenic differentiation 1 (MyoD) and myogenin expression increased (P<0.01) while myostatin expression decreased (P=0.04) in P piglets compared with C piglets. At d28, no differences in myogenic growth factors were observed in the PM and LD. Overall, increased *IGF2* expression and decreased myostatin expression at d0 in the PM suggests increased hypertrophy in line with the increased muscle fiber size observed in the ST. However, maternal PRRSV infection restricted in utero muscle fiber development in piglets as evidenced by reduced muscle fiber number in P piglets, and therefore may limit skeletal muscle growth potential.

Key words: maternal infection, muscle development, porcine reproductive and respiratory syndrome virus

INTRODUCTION

Infections are common in animal production and result in lost production and efficiency due to reduced feed intake (Sandberg et al., 2006). In addition, inflammatory cytokines such as interlukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) direct nutrients away from tissue growth toward an inflammatory response (Johnson, 2002) with the most dramatic repartitioning away from skeletal muscle (Hasselgren et al., 2002; Strassmann et al., 1992). Inflammation can be extremely taxing on muscle growth in an infected animal; however, it is unclear how immunological stress in pregnancy affects skeletal muscle development and subsequent growth in offspring.

In rodents, *Escherichia coli* lipopolysaccharide (LPS) is a well-defined model of immune system activation that results in synthesis of inflammatory cytokines (Urakubo et al., 2001). When LPS was administered to pregnant mice, increased maternal circulation of IL-6 and TNF-α increased IL-6 and TNF-α levels in amniotic fluid, and decreased fetal neurodevelopment and

survival. (Collins et al., 1994). As inflammatory cytokines IL-6 and TNF-α inhibit myogenic differentiation (Langen et al., 2001; Tong et al., 2009) and maternal cytokines have the ability to alter fetal development, skeletal muscle development of offspring may be limited by maternal infection. Prenatal development is especially crucial in skeletal muscle because muscle fiber number is set at or near birth (Dwyer et al., 1994). Animals born with fewer muscle fibers have slower, less efficient lean muscle growth (Dwyer et al., 1993). Therefore, the objective of this study was to determine the effect of maternal PRRSV infection on offspring skeletal muscle development and growth.

MATERIALS AND METHODS

Animal Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Illinois and followed guidelines recommended in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). Ten PRRSV-free pregnant gilts from the University of Illinois Swine Research Center were delivered to the Edward R. Madigan Laboratory in five sets of two gilts each on day 70 of gestation between January and September 2013 (Table 2.1). Gilts were housed in isolated standard farrowing crates and fed 2.27 kg/d of a standard diet for gestating gilts (Table 2.2). Room temperature was maintained at 26.7ºC and a continuous water source was provided. Starting on day 73 of gestation, voluntary feed intake and rectal temperature of gilts were measured daily until farrowing. Feed intake was measured by weighing remaining feed in bowl from previous day. On day 80 of gestation, gilts were inoculated intranasally with PRRSV (5 mL of tissue culture infectious $dose_{50}$ of strain P-129, Purdue University, West Lafayette, IN) or sterile culture medium (control, 5 mL Dulbecco's phosphate buffered saline). Blood (10 mL) was collected

from an ear catheter 10ml/d for 14 consecutive days, then weekly until farrowing and immediately after farrowing to determine the presence of PRRSV with a qualitative test. The presence of PRRSV antibodies in the serum of all gilts was analyzed by the Veterinary Diagnostic Laboratory (University of Illinois, Urbana, Illinois) using a PRRSV-specific ELISA kit (IDEXX Laboratories, Westbrook, ME). This assay has 98.8% sensitivity and 99.9% specificity, with an S/P ratio of >0.4 indicating a positive sample. Serum samples from piglets, detailed below, were tested for PPRSV in the same manner.

Six gilts were anesthetized on day 114 of gestation via intravenous injection with a combination of TKX (telazol:ketamine:xylazine, 4.4 mg/kg BW, Fort Dodge Animal Health, Fort Dodge, IA) followed by euthanasia via intravenous administration of an overdose of sodium pentobarbital (390 mg/ml Fatal Plus - administered at 1 ml/5 kg BW, Vortech Pharmaceuticals, Dearborn, MI). Caesarean sections were performed after euthanasia to collect piglets. From the 3 control gilts, 34 piglets (20 males, 14 females) were collected. All tested PRRSV negative. From the 3 PRRSV infected gilts, 24 piglets (14 males, 10 females) were collected. Of these, 17 piglets were PRRSV negative (11 males, 6 females) and 7 piglets were PRRSV positive (3 males, 4 females).

Additionally, 4 gilts were allowed to farrow naturally. Piglets were subjected to normal processing that included trimming needle teeth, umbilical trimming and sanitation, and iron dextran injection (intramuscular injection at 100 mg, penicillin at 0.2 cc., Betadine Surgical Scrub, Purdue Products L.P, Stamford, CT). Testicles of male piglets remained intact. From the 2 control gilts, 12 piglets (8 males, 4 females) were collected. All were PRRSV negative at birth and remained so throughout the study. From the 2 PRRSV infected gilts 12 piglets (7 males, 5 females) were collected. Of these, 9 piglets were PRRSV negative (5 males, 4 females) and 3

piglets were PRRSV positive (2 males, 1 female) at birth. The piglets were weaned from gilts at 2 days of age and housed in individual isolated cages. At this time, all piglets from gilts inoculated with PRRSV tested positive for PRRSV. Piglets were weighed daily and provided 360 ml/kg body weight of a commercially available milk replacer (Advance Liqui-Wean, Milk Specialties, Prairie, MN). Milk replacer was delivered in 14 equal aliquots using an automatic delivery system as previously reported by Elmore et. al. (2014). At 28 days of age, piglets were anesthetized and euthanized using procedures similar to gilts.

Growth Measurements and Sampling

At each time point (d0 and d28), body weight and crown-to-rump length was recorded for each piglet. The semitendinosus (ST), longissimus dorsi (LD), and psoas major (PM) were removed from the left side of the piglet and weighed. Three 100mg samples were collected from the LD and PM, placed in RNAlater (Ambion-Life Technologies, Grand Island, NY) and held at -80˚C until gene expression analysis. Samples of the LD were collected posterior to the spinalis dorsi muscle.

Muscle cell number and size

To determine muscle cell number and size, the ST from each piglet at d28 was anchored by string to a wooden stick to prevent shortening and held at 4˚C until rigor mortis was complete (approximately 24 hours). After 24 hours, the ST was submerged in approximately 8 mL of 10% neutralized buffered formalin (37% formaldehyde, Fischer Scientific, Fair Lawn, New Jersey). After 48 hours of submersion, the ST was sliced in half, perpendicular to the direction of the muscle fibers, and the cut face of the ST was traced on acetate paper. To determine the cross sectional area of the muscle the outlines were traced using an Intuos 4 Wacom tablet (PTK-840; Tokyo, Japan) into Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, San Jose,

CA) and measured. Approximately 1 cm^3 samples were collected from each ST to determine cross sectional area and muscle fiber number. Using these samples, 2 to 3, 7-µm sections per animal were cut perpendicular to the muscle fibers with a microtome, fixed to a glass slide and stained with hematoxylin and eosin. Five random images were captured for each section using a total magnification of 564X on an AMG Exos XL Microscope (EVOS, Bothell, WA). The area of all fibers in each of five images were measured using Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, San Jose, CA) to determine an average cross sectional area of individual muscle fibers. Images were standardized to 100 µm scale bar; 440 pixel length was set equivalent to 100 µm. Total fiber number was estimated by dividing total cross sectional area of the ST by the average cross sectional area of individual muscle fibers.

Gene Expression Analysis

Total RNA was isolated from LD and PM muscles after tissue disruption using the Tri-Reagent method (Sigma-Aldrich, St. Louis, MO) according to Clark et al. (2011). One µg of RNA was reverse transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) according to manufacturer's instructions. Then, cDNA was stored at -20° C until further analysis.

Expression of ribosomal protein L19 (RPL 19) was unaffected by treatment, age and sex and therefore, was chosen as the reference gene. Quantitative real-time PCR was completed in accordance with qScript PerfeCTa FastMix II, Rox (Quanta Biosciences, Gaithersburg, MD) master mix procedures, using a Step One Plus qPCR machine (Applied Biosystems by Life Technologies; Grand Island, NY). Each gene of interest was normalized to RPL 19 expression and calibrated to the male control (calibrator group). The ΔΔCT method was used to calculate fold change between treatments and sexes. Expression of *TNF-α*, *IL-6*, *PRRSV*, myostatin,

insulin-like growth factor 1 and 2, *MYOD*, *MYF5*, myogenin, *PAX7*, uncoupling protein 3, and the myosin heavy chain genes (Table 2.3) was quantified. All primers had efficiency between 90 and 100%.

Statistical analysis

All data were analyzed using 2-way ANOVA in the MIXED procedure of SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of treatment, sex, and their interactions. Piglet was the experimental unit for all data analysis. Treatment consisted of two groups: piglets born from control gilts (C) and, due to minimal differences and limited sample size, piglets born PRRSV positive and PRRSV negative data were pooled and analyzed as piglets born from PRRSV infected gilts (P). For gene expression data, ΔΔCt were analyzed and then displayed as fold changes standardized to male control piglets. Many piglets had *TNF-α*, and *IL-6* expression below detectable limits; therefore, in order to analyze these data categorical variables were assigned. All piglets with expression under detectable limits (CT > 40) were categorized as 0 (no expression) and others were split into tertiles with the least expression assigned a value of 1, mid-range expression assigned 2, and greatest expression assigned 3. These data were analyzed using a 2-way ANOVA in the GLIMMIX procedure of SAS. The model included the fixed effects of treatment, sex, and their interactions. All residuals were tested for normality using the Univariate procedure of SAS with normal probability plots. Least square means were separated with the PDIFF option. Statistical differences were accepted as significant at $P<0.05$ and trends were noted at $0.05<P<0.10$.

RESULTS

Effects of PRRSV Inoculation in Gilts

All gilts inoculated with PRRSV at day 80 of gestation tested positive for PRRSV infection one week after inoculation. Prior to infection, average daily feed intake was not different (P=0.47) between PRRSV inoculated gilts and control gilts (Figure 2.1). Average daily feed intake decreased (P=0.04) approximately 36% in PRRSV inoculated gilts compared with control gilts between 0 and 11 days after inoculation. From 11 days after inoculation until farrowing, average daily feed intake, however, was similar between treatments (P>0.21). Prior to infection, average daily body temperature was not different (P=0.80) between PRRSV inoculated gilts and control gilts. Average daily body temperature increased (P=0.04) approximately 0.5°C in PRRSV inoculated gilts compared with control gilts between 0 and 11 days after inoculation. From 11 days after inoculation until farrowing, average daily body temperatures were similar between treatments (P>0.21).

Body and Muscle Weights of Offspring

At d0, there were no differences (P≥0.17) in body weight, absolute muscle weights, and body length between treatment groups (Table 2.4). There tended to be an interaction between treatment and sex as LD weight tended to be reduced (P≤0.09) in female P piglets compared with male P piglets and female C piglets, but LD weights were not different from male C piglets. Furthermore, there was an interaction between treatment and sex as PM weight was heavier (P≤0.08) in male P piglets compared with all other treatments. Relative LD weight was reduced (P=0.01) and relative ST weight tended to be reduced (P=0.08) in P piglets compared with C piglets. Furthermore, there was an interaction between treatment and sex as relative PM weight

was increased (P<0.01) in male P piglets compared with all other treatment and sex combinations.

At d28, body weight tended to be decreased (P=0.07) in P piglets compared with C piglets, but there were no differences in body length between treatments. Whole LD (P=0.01) and relative LD (P=0.03) weights were reduced in P piglets compared with C piglets, but whole and relative weights of PM and ST muscles were not different (P≥0.29) between treatment groups.

Muscle Fiber Number and Area, and Inflammatory Cytokine Expression

At d28, average fiber area within the ST was increased (P=0.02) by 30% in P piglets compared with C piglets (Figure 2.2). However, muscle cell number of the ST was reduced (P=0.04) by 33% in P piglets compared with C piglets.

At d0 in the PM, there were no differences in *IL-6* or *TNF-α* expression between P piglets and C piglets within muscle (Table 2.5). There was an interaction between treatment and sex as *TNF-α* expression increased (P≤0.02) in LD of male P piglets compared with male C piglets, but expression did not differ between treatment in females (P=0.14). However, at d28, *IL-6* (P<0.01) and *TNF-α* (P<0.01) expression in muscle tissue (LD and PM) were increased in P piglets compared with C piglets.

Longissimus Dorsi – Gene Expression

At d0, *MHC2b* (P=0.02) and *MHC2x* (P=0.01) expression was reduced in LD of P piglets compared with C piglets, but there were no differences (P≤0.15) in *MHC1* and *MHC2a* expression between treatments (Table 2.6). Myostatin expression decreased (P=0.01) in P piglets compared with C piglets. However, Myf5 expression, a myogenic regulatory factor, was also decreased (P=0.03) in P piglets compared with C piglets. There were no differences (P≥0.11) in

MYOD, myogenin, *IGF1*, *IGF2*, or *PAX7* expression between treatments, however, *UCP3* expression was increased (P=0.05) in P piglets compared with C piglets.

At d28, *MHC2a* expression tended to decrease (P=0.08) in P piglets compared with C piglets (Table 2.7); however, there were no differences (P≥0.44) in *MHC 2b*, or *2x* expression. In addition, there was an interaction between treatment and sex as *MHC1* expression increased (P≤0.08) in female P piglets compared with male P piglets and C piglets. There were no differences (P≥0.13) in positive or negative growth factors, except *PAX7* expression was increased (P=0.02) in P piglets compared with C piglets.

Psoas Major – Gene Expression

At d0, *MHC1* expression was increased (P=0.03) in PM of P piglets compared with C piglets; however there were no differences (P≥0.18) in *MHC2a*, *2x*, or *2b* expression (Table 2.8). Myostatin and *MYF5* expression decreased (P≤0.04) in P piglets compared with C piglets while *MYOD*, myogenin, and *IGF2* expression were increased (P≤0.02). In addition, *UCP3* expression was increased (P<0.01) in P piglets compared with C piglets. Additionally, there tended to be an interaction between treatment and sex as *PAX7* expression was reduced (P≤0.08) in female C piglets compared with all other treatment and sex combinations. At d28, there were no differences (P≥0.11) in myosin heavy chain and myogenic growth factor expression in PM between treatments (Table 2.9). However, there was an interaction between treatment and sex as *MYOD* expression was reduced (P≤0.02) in female C piglets compared with all other treatment and sex combinations.

DISCUSSION

Immunological stress or infection causes a disruption in homeostasis through production of inflammatory cytokines that direct nutrients away from muscle protein synthesis (Fredette and

Landmesser, 1991). However, the effect of and mechanisms by which maternal infection influences skeletal muscle development remain unclear. Thus, the objectives of this study were to determine how skeletal muscle growth was altered in offspring from gilts subjected to immunological stress through maternal PRRSV infection during pregnancy and understand the molecular mechanisms that regulate muscle growth as a result of maternal infection.

Maternal infection caused a 33% reduction in ST muscles fiber number in P piglets compared with C piglets. Previous studies demonstrate reduction in muscle fiber number may have been due to maternal nutrient restriction (Zhu et al., 2006). In this study, however, the decrease in gilt feed intake due to PRRSV infection was both mild (36%) and transient (< 11 d). Therefore, while possible, it is unlikely that nutrient restriction alone explains the reduction in muscle fiber number. On the other hand, piglets from maternally over-fed sows had a reduction in muscle fiber number due to a decrease in secondary fiber population (Dwyer et al., 1994; Wigmore and Stickland, 1983). These over-fed sows also had increased inflammatory cytokines, IL-6 and TNF-α, similar to PRRSV infection (Black, 2003; Thanawongnuwech et al., 2004). Although inflammatory cytokine (*TNF-α* or *IL-6*) expression was not greater in fetal skeletal muscle of P piglets compared with C piglets, P piglets may have been exposed to increased circulating TNF-α or IL-6 or had increased expression in another tissue. Maternal inflammatory cytokines can pass directly to the fetus or they can stimulate the production of fetal inflammation cytokines (Ashdown et al., 2006; Fortunato et al., 1996; Urakubo et al., 2001). More importantly, in an equal nutrient intake trial, transgenic mice expressing increased levels of circulating inflammatory cytokines had a 50-70% decrease in weight compared with non-transgenic littermates suggesting decreases in growth was due to inflammatory cytokines (DeBenedetti et al., 1997). We, therefore, hypothesize maternal inflammatory cytokines reduced secondary fiber

populations. Reductions in muscle fiber number correlate with reduced growth rates and ultimately, lighter weight pigs with decreased lean growth efficiency (Dwyer et al., 1993). Therefore, maternal infection may result in impaired muscle development, reduced growth efficiency, and decreased overall lean muscle yield of progeny.

In our study, the effect of maternal PRRSV infection on muscle growth and gene expression differed between muscles. The PM was chosen as a representative slow, oxidative muscle; MHC type 1 is its predominant fiber type (Leseigneur-Meynier and Gandemer, 1991). Myosin type 2 fibers experience more hypertrophic growth than MHC type 1 fibers (Ashmore et al., 1973). Furthermore, in muscle atrophy studies, MHC type 2 fibers were more prone to reductions in muscle fiber area while little effect was observed in MHC type 1 fibers (Balagopal et al., 2001; Lexell et al., 1988). This suggests that MHC type 2 fibers are more sensitive to both hypertrophic and atrophic signals than type 1 fibers. Therefore, restriction in muscle growth due to maternal infection should be more prominent in muscles with a greater degree of MHC type 2 fibers.

In our study, this was, in fact, the case. At d0, PM and ST muscle weight were increased in P piglets compared to C piglets, and at d28, PM and ST weights were unaffected by maternal PRRSV infection. On the other hand, LD weight was reduced in P piglets compared with C piglets at both d0 and d28. This reduction in weight was accompanied by reduced *MHC 2x* and *2b* expression at d0, as well as reduced expression of the myogenic regulatory factor *MYF5*. Contrary to these findings was increased myostatin expression at d0 in LD muscle, which may suggest compensatory hypertrophy by the muscle to overcome a reduction in muscle fiber number.

Compensatory growth can be defined as a process by which an animal accelerates its growth after a period of restricted development (Hornick et al., 2000). Compensatory gain is also suggested in the PM and ST muscles. Increased hypertrophy of ST fibers was observed. Furthermore, myostatin expression was reduced and *IGF2*, *MyoD*, myogenin and *PAX7* expression were increased in PM muscles at d0 in P piglets compared with C piglets. Myostatin is known to be an inhibitor of muscle growth, therefore, a decrease in myostatin expression and elevated *IGF2* expression can lead to protein synthesis and increase lean tissue deposition (Haidet et al., 2008; Lin et al., 2002; Whittemore et al., 2003). Furthermore, an increase in *MYOD* expression causes an increase in myoblasts and satellite cell activation, which leads to an increase in myogenin expression (Adams et al., 1999; Smith et al., 1994). Myogenin expression is associated with terminal differentiation, suggestion PM of P piglets was still undergoing muscle formation. Moreover, an increase in *PAX7* suggests the PM of P piglets had an increase in satellite cell population for further growth or regeneration of skeletal muscle (Olguin and Olwin, 2004). Due to the nature of PRRSV infected samples, data regarding fiber area of specific fiber types were unavailable, but such data may provide greater insight to this phenomenon.

CONCLUSION

Maternal PRRSV infection restricts in utero muscle fiber development as evidenced by the reduced muscle cell number. Increased *IGF2* expression and decreased myostatin expression at d0 in the PM and increased muscle fiber size observed in the ST suggests compensatory muscle growth. Although large differences were not observed in muscle weights between treatments, we predict efficiency would be lost during the finishing phases of production as a decrease in muscle fiber number would likely lead to slower growing pigs. It is essential to know the infection status of a breeding herd as visual cues (decrease feed intake, increase body

temperature) may be subtle, but maternal infection during the prenatal period may limit muscle development and have long term effects on lean growth efficiency.

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TABLES

Table 2.1 Number of infected and non-infected gilts per set and age of piglets at the time of euthanization.

Gilt Set	Control	PRRSV Inoculated	Age of Piglets when Euthanized
1	1	1	0 days
2	0	2	0 days
3	1	1	28 days
4	1	1	28 days
5	2	0	0 days

Table 2.2 Composition of diet fed to gilts[1]

Ingredient	% Diet
Soybean Meal	11.55
Dicalcium Phosphate	1.90
Lime	0.75
Corn	77.15
Beet Pulp	7.00
Choice White Grease	1.00
Swine Trace Mineral Salt[1]	0.35
Sow Pac[2]	0.10
Vitmix ADEK[3]	0.20

[1]Swine trace mineral salt included the following percentage per kilogram added: Salt, 80-85%; Iron, 2.57%; Zinc, 2.86%; Manganese, 0.57%; Copper, 0.23%; Iodine, 0.01%; Selenium, 0.01%.
[2]Sow Pac included the following per kilogram added: Biotin, 221 mg; Choline; 661,387 mg; Folic Acid, 2,205 mg.
[3]Vitmix ADEK included the following per kilogram added: Vitamin A (retinyl acetate), 3,306,937 IU; Vitamin D3 (cholecalciferol), 330,694 IU; Vitamin E (dl-α tocopheryl acetate), 44,093 IU; Vitamin K (menadione nicotinamide bisulfite), 2,205 mg; Riboflavin, 4,409 mg; Vitamin B12, 17.64 mg; d-Pantothenic acid (d-calcium pantothenate), 12,125 mg; Niacin (nicotinamide and nicotinic acid), 16,535 mg; Choline, 143,190 mg.

Table 2.3 Taqman primers and probe sequences used for quantitative real-time PCR.

Gene Symbol	Gene Name	Gene Bank Reference Sequence	Forward Sequence Reverse Sequence Probe Sequence
IGF1[1]	insulin-like growth factor 1	NM_214256.1	
IGF2[2]	insulin-like growth factor 2	NM_213883.2	CCGTGCTTCCGGACAACT AGGTGTCATAGCGGAAGAACTTG CCCCAGATACCCCGTGG
IL-6[2]	interleukin-6	NM_001252429.1	AACAACCTGAACCTTCCAAAAAT TCCACAAGACCGGTGGTGAT CAATCAGGAGACCTGCT
MHC1[2]	myosin heavy chain 1	NM_001104951.1	AATGTCCAGCAGGTGATGTATGC ATCCGTGTCACCATCCAGTTG AGGCCGTGTATGAGAAG
MHC2a[2]	myosin heavy chain 2a	NM_214136.1	GCAAAAGCGTAATGCTGAAGCT CCTCTTCCGTCTGGTAGGTGAGT TGCGCAAACATGAGAGG
MHC2b[2]	myosin heavy chain 2b	NM_001123141.1	GGGTCTTCGGAAACATGAGAGA TCCTGCAGCCTGAGAACATTC TTACCAGACTGAGGAGGAC
MHC2x[2]	myosin heavy chain 2x	NM_001104951.1	GGGTCTACGCAAACACGAGAGA CAGATCCTGGAGCCTGAGAATG AAGGAACTCACTTACCAAAC
MYF5[2]	myogenic factor 5	NM_001278775.1	GTCCAGAAAGAGCAGCAGTTTTG AGGAGCTTTTATCCGTGGCATAT ATCTACTGTCCGGATGTAC
MYOD[1]	myogenic differentiation 1	NM_001002824.1	
Myogenin[2]	Myogenin	NM_001012406.1	GTCCAGAAAGAGCAGCAGTTTTG AGGAGCTTTTATCCGTGGCATAT ATCTACTGTCCGGATGTAC
Myostatin[2]	Myostatin	NM_214435.2	CGACGGAAACGATCATTACCA AAGCAGCATTTGGGTTTTCCT TACAGAGTCTGATCTTCTAATG
PAX7[2]	paired box 7	XM_005659088.1	AGGCAGCAAGCCCAGACA CCCTCTTGTACTCCTCAATCTTTTTC CGACTCCGGATGTGG
PRRSV[2]	PRRSV	AY150312	CGCACCAGATGGGACCTACTT ACGGTGTTCAGTGAGGGCTTT CGCTGCGTTGACTGG
RPL 19[2]	ribosomal protein L19	XM_003131509.3	TGACCGCCACATGTATCACAGT TGTGGATGTGCTCCATGAGAA TGTACCTGAAAGTGAAGGG
TNF-α[2]	tumor necrosis factor	NM_214022.1	TGACCGCCACATGTATCACAGT TGTGGATGTGCTCCATGAGAA TGTACCTGAAAGTGAAGGG
UCP3[1]	uncoupling protein 3	NM_214049.1	

[1] ABI Assay ID: *IGF1* = Ss03394499_m1, *MYOD* = Ss03378464_u1, *UCP3* = Ss03391403_m1

[2]Custom design assay ID

Table 2.4 Effects of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring body weight, length, and muscle weights at birth.

	Treatment		Sex			P-Values		
Item	Control	PRRSV	Male	Female	SEM[1]	T	S	T X S[2]
Day 0								
Piglets, n	34	25	35	24				
Body Weight, kg	1.27	1.23	1.29	1.20	0.07	0.65	0.35	0.11
Body Length, cm	25.68	27.08	26.11	26.64	0.79	0.17	0.59	0.13
Longissimus Dorsi, g	13.46	12.15	13.37	12.24	0.98	0.30	0.37	0.08
Relative weight[3]	1.09	0.96	1.04	1.01	0.03	0.01	0.56	0.20
Psoas Major, g	2.58	3.00	2.98	2.60	0.25	0.19	0.24	0.01
Relative weight[3]	0.20	0.24	0.23	0.22	0.01	0.01	0.44	<0.01
Semitendinosus, g	2.41	2.67	2.73	2.34	0.23	0.37	0.19	0.20
Relative weight[3]	0.19	0.22	0.21	0.20	0.01	0.08	0.43	0.51
Day 28								
Piglets, n	12	12	15	9				
Body Weight, kg	5.30	4.87	5.08	5.09	0.08	0.07	0.97	0.85
Body Length, cm	42.27	40.72	40.92	42.06	1.17	0.19	0.33	0.98
Longissimus Dorsi, g	87.75	73.23	72.59	87.75	5.11	0.01	0.14	0.50
Relative weight[3]	1.66	1.50	1.51	1.65	0.05	0.03	0.06	0.51
Psoas Major, g	16.15	13.55	15.08	14.62	1.40	0.11	0.82	0.71
Relative weight[3]	0.30	0.28	0.29	0.30	0.01	0.29	0.67	0.57
Semitendinosus, g	18.10	15.98	16.63	17.45	1.76	0.38	0.38	0.69
Relative weight[3]	0.34	0.35	0.33	0.36	0.01	0.71	0.19	0.70

[1] SEM reported as maximum standard error of mean.

[2]Trt = treatment, T X S = interaction between treatment and sex.

[3]Muscle weight as a percentage of body weight.

Table 2.5 Effect of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring PRRSV and inflammatory cytokine status in muscle.[1]

	Treatment				Sex				P-value		
Item[2]	Control		PRRSV		Male		Female		T	S	TxS
Longissimus Dorsi											
Day 0											
IL-6	0.87	(0.75-0.98)	0.94	(0.80-1.07)	0.97	(0.85-1.08)	0.84	(0.70-0.98)	0.44	0.15	0.15
TNF-α	1.10	(0.95-1.24)	1.22	(1.05-1.40)	1.21	(1.07-1.36)	1.11	(0.94-1.28)	0.26	0.36	0.01
Day 28											
IL-6	1.06	(0.83-1.30)	1.52	(1.30-1.75)	1.32	(1.12-1.52)	1.26	(1.00-1.52)	0.01	0.71	0.26
TNF-α	1.06	(0.87-1.24)	1.45	(1.28-1.63)	1.20	(1.04-1.36)	1.31	(1.11-1.51)	<0.01	0.37	0.99
Psoas Major											
Day 0											
IL-6	1.13	(0.98-1.28)	1.25	(1.07-1.43)	1.03	(0.98-1.29)	1.23	(1.07-1.43)	0.29	0.34	0.22
TNF-α	0.88	(0.76-1.01)	0.86	(0.71-1.01)	0.78	(0.66-0.90)	0.96	(0.81-1.11)	0.80	0.07	0.58
Day 28											
IL-6	1.06	(0.89-1.23)	1.49	(1.32-1.65)	1.23	(1.09-1.37)	1.31	(1.13-1.50)	<0.01	0.48	0.79
TNF-α	1.15	(0.96-1.33)	1.78	(1.61-1.96)	1.34	(1.19-1.49)	1.59	(1.39-1.79)	<0.01	0.05	0.71

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2]*IL-6* = interleukin-6, *TNF-α* = tumor necrosis factor, T = treatment, S = sex, T X S = interaction between treatment and sex

Table 2.6 Effect of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring myosin heavy chain and myogenic growth factors gene expression in the longissimus dorsi at birth.[1]

	Treatment				Sex				P-value		
Item[2]	Control		PRRSV		Male		Female		T	S	TxS
MHC1	1.03	(0.40-2.66)	1.38	(0.57-3.31)	1.20	(0.46-3.13)	1.19	(0.50-2.83)	0.15	0.96	0.71
MHC2a	1.04	(0.77-1.40)	0.89	(0.62-1.26)	0.93	(0.69-1.25)	0.99	(0.69-1.41)	0.48	0.79	0.93
MHC2x	0.93	(0.63-1.38)	0.51	(0.34-0.75)	0.79	(0.53-1.19)	0.60	(0.40-0.90)	0.01	0.21	0.55
MHC2b	0.74	(0.38-1.42)	0.36	(0.19-0.68)	0.75	(0.38-1.48)	0.35	(0.18-0.67)	0.02	0.02	0.62
MYF5	0.95	(0.80-1.13)	0.71	(0.58-0.87)	0.85	(0.72-1.00)	0.79	(0.64-0.97)	0.03	0.58	0.84
MYOD	0.78	(0.35-1.74)	1.26	(0.59-2.67)	1.36	(0.60-3.11)	0.72	(0.33-1.53)	0.11	0.03	0.62
Myogenin	1.11	(0.73-1.68)	1.71	(1.02-2.86)	1.44	(0.97-2.15)	1.31	(0.78-2.22)	0.19	0.77	0.37
Myostatin	0.93	(0.79-1.10)	0.65	(0.53-0.79)	0.86	(0.73-1.00)	0.71	(0.58-0.86)	0.01	0.14	0.69
IGF1	0.87	(0.43-1.76)	0.64	(0.33-1.25)	0.85	(0.41-1.75)	0.66	(0.34-1.28)	0.25	0.35	0.93
IGF2	0.91	(0.19-4.33)	0.95	(0.21-4.18)	0.96	(0.20-4.62)	0.90	(0.21-3.91)	0.87	0.80	0.61
PAX7	0.95	(0.35-2.61)	0.89	(0.33-2.38)	1.00	(0.36-2.76)	0.85	(0.32-2.24)	0.59	0.18	0.62
UCP3	0.68	(0.46-1.00)	1.26	(0.80-2.00)	1.17	(0.81-1.70)	0.73	(0.46-1.18)	0.05	0.13	0.32

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] MHC = Myosin Heavy Chain, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *IGF1* = insulin-like growth factor 1, *IGF2* = insulin-like growth factor 2, *PAX7* = paired box 7, *UCP3* = uncoupling protein 3, T = treatment, S = sex, T X S = interaction between treatment and sex.

Table 2.7 Effect of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring myosin heavy chain and myogenic growth factors expression in the longissimus dorsi at 28 days of age.[1]

	Treatment				Sex				P-value		
Item[2]	Control		PRRSV		Male		Female		T	S	TxS
MHC1	1.00	(0.77-1.29)	1.13	(0.91-1.42)	0.89	(0.73-1.08)	1.28	(0.96-1.69)	0.44	0.04	0.04
MHC2a	0.95	(0.59-0.95)	0.70	(0.41-1.17)	0.91	(0.44-1.88)	0.73	(0.45-1.17)	0.08	0.24	0.52
MHC2x	1.17	(0.28-4.95)	1.29	(0.27-6.06)	1.10	(0.17-7.25)	1.38	(0.40-4.77)	0.59	0.22	0.63
MHC2b	0.94	(0.51-1.75)	0.77	(0.43-1.40)	1.11	(0.66-1.88)	0.65	(0.33-1.29)	0.63	0.21	0.33
MYF5	1.02	(0.85-1.23)	0.97	(0.80-1.16)	1.03	(0.88-1.22)	0.95	(0.78-1.17)	0.67	0.54	0.36
MYOD	1.17	(0.88-1.54)	1.27	(0.98-1.66)	1.06	(0.84-1.34)	1.40	(1.03-1.90)	0.64	0.15	0.87
Myogenin	0.82	(0.09-7.47)	0.69	(0.06-7.45)	0.83	(0.04-15.97)	0.68	(0.10-4.54)	0.56	0.51	0.56
Myostatin	1.32	(0.80-2.19)	1.81	(0.53-1.59)	1.81	(1.18-2.78)	1.32	(0.75-2.34)	0.80	0.84	0.36
IGF1	1.13	(0.43-2.94)	1.64	(0.58-4.66)	1.22	(0.30-4.99)	1.52	(0.63-3.64)	0.13	0.42	0.92
IGF2	1.07	(0.90-1.28)	1.15	(0.97-1.37)	1.00	(0.86-1.16)	1.24	(1.02-1.51)	0.54	0.08	0.51
PAX7	0.98	(0.86-1.12)	1.23	(1.09-1.41)	1.05	(0.33-3.36)	1.15	(0.52-2.52)	0.02	0.28	0.14
UCP3	1.00	(0.57-1.74)	0.92	(0.53-1.59)	0.83	(0.51-1.37)	1.09	(0.59-2.02)	0.83	0.48	0.47

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] MHC = Myosin Heavy Chain, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *IGF1* = insulin-like growth factor 1, *IGF2* = insulin-like growth factor 2, *PAX7* = paired box 7, *UCP3* = uncoupling protein 3, T = treatment, S = sex, T X S = interaction between treatment and sex.

Table 2.8 Effect of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring myosin heavy chain and myogenic growth factors gene expression in the psoas major at birth.[1]

	Treatment				Sex				P-value		
Item[2]	Control		PRRSV		Male		Female		T	S	TxS
MHC1	0.98	(0.78-1.24)	1.47	(1.11-195)	1.16	(0.93-1.46)	1.24	(0.93-1.65)	0.03	0.72	0.59
MHC2a	0.90	(0.63-1.30)	0.98	(0.71-1.37)	0.98	(0.68-1.41)	0.91	(0.66-1.27)	0.42	0.52	0.23
MHC2x	0.78	(0.54-1.13)	0.70	(0.49-0.98)	0.83	(0.57-1.21)	0.65	(0.46-0.93)	0.42	0.11	0.11
MHC2b	1.18	(0.25-5.64)	1.61	(0.37-6.97)	1.18	(0.25-5.62)	1.62	(0.38-6.96)	0.18	0.17	0.94
MYF5	0.97	(0.84-1.12)	0.73	(0.62-0.88)	0.86	(0.74-0.99)	0.83	(0.69-0.99)	0.02	0.79	0.77
MYOD	0.87	(0.62-1.22)	1.77	(1.27-2.48)	1.28	(0.90-1.82)	1.20	(0.85-1.68)	<0.01	0.69	0.23
Myogenin	0.95	(0.68-1.31)	1.57	(1.13-2.18)	1.21	(0.87-1.70)	1.23	(0.88-1.71)	<0.01	0.95	0.49
Myostatin	0.97	(0.81-1.15)	0.73	(0.59-0.90)	0.92	(0.77-1.08)	0.77	(0.62-0.95)	0.04	0.19	0.42
IGF1	0.99	(0.85-1.15)	0.90	(0.74-1.09)	0.98	(0.85-1.14)	0.90	(0.74-1.10)	0.44	0.48	0.60
IGF2	1.03	(1.24-1.83)	1.50	(0.87-1.21)	1.11	(0.94-1.30)	1.40	(1.14-1.70)	<0.01	0.07	0.16
PAX7	0.85	(0.69-1.03)	1.14	(0.93-1.41)	1.03	(0.84-1.27)	0.94	(0.76-1.16)	0.02	0.46	0.08
UCP3	0.84	(0.43-1.67)	2.89	(1.52-5.50)	1.61	(0.81-3.24)	1.51	(0.79-2.87)	<0.01	0.78	0.32

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] MHC = Myosin Heavy Chain, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *IGF1* = insulin-like growth factor 1, *IGF2* = insulin-like growth factor 2, *PAX7* = paired box 7, *UCP3* = uncoupling protein 3, T = treatment, S = sex, T X S = interaction between treatment and sex.

Table 2.9 Effect of maternal porcine reproductive and respiratory virus (PRRSV) inoculation on offspring myosin heavy chain and myogenic growth factors expression in the psoas major at 28 days of age.[1]

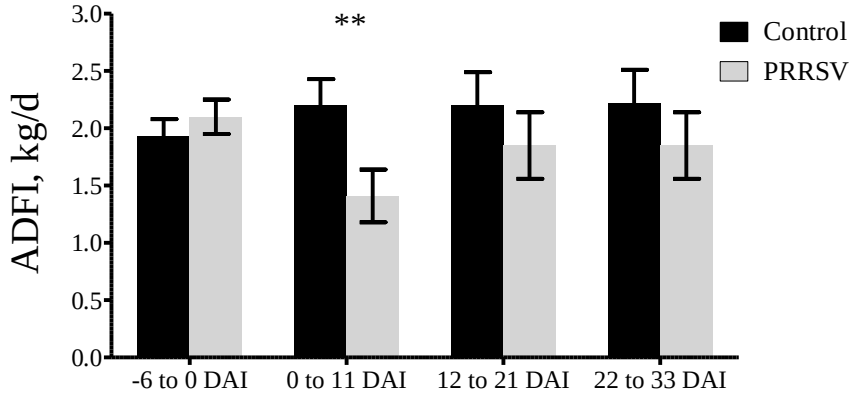
	Treatment				Sex				P-value		
Item[2]	Control		PRRSV		Male		Female		T	S	TxS
MHC1	0.94	(0.59-1.50)	0.83	(0.52-1.34)	1.02	(0.69-1.51)	0.77	(0.45-1.32)	0.71	0.40	0.64
MHC2a	0.93	(0.67-1.29)	0.98	(0.72-1.33)	1.07	(0.81-1.41)	0.84	(0.60-1.20)	0.83	0.29	0.68
MHC2x	0.93	(0.13-6.89)	0.76	(0.09-6.29)	0.86	(0.08-9.55)	0.82	(0.14-4.77)	0.18	0.68	0.56
MHC2b	0.77	(0.16-3.73)	0.48	(0.07-3.21)	0.79	(0.05-12.58)	0.47	(0.09-2.50)	0.40	0.43	0.99
MYF5	1.04	(0.77-1.40)	0.85	(0.85-1.13)	0.86	(0.67-1.11)	1.02	(0.74-1.41)	0.32	0.39	0.64
MYOD	0.59	(0.42-0.83)	0.66	(0.48-0.89)	0.79	(0.60-1.05)	0.49	(0.34-0.71)	0.63	0.04	0.02
Myogenin	1.00	(0.78-1.28)	0.76	(0.60-0.96)	0.82	(0.66-1.01)	0.93	(0.70-1.22)	0.11	0.46	0.45
Myostatin	0.71	(0.18-2.88)	0.74	(0.09-6.09)	0.96	(0.07-12.83)	0.55	(0.15-2.06)	0.51	0.03	0.74
IGF1	0.89	(0.33-2.43)	0.70	(0.24-2.05)	0.87	(0.24-3.13)	0.72	(0.27-1.90)	0.45	0.56	0.91
IGF2	0.97	(0.48-1.93)	0.94	(0.44-1.98)	1.01	(0.39-2.63)	0.90	(0.49-1.63)	0.78	0.33	0.68
PAX7	0.79	(0.59-1.07)	0.72	(0.56-0.94)	0.82	(0.65-1.03)	0.70	(0.51-0.97)	0.64	0.41	0.12
UCP3	0.88	(0.36-2.13)	1.03	(0.46-2.28)	1.33	(0.35-5.11)	0.68	(0.28-1.63)	0.52	0.04	0.22

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] MHC = Myosin Heavy Chain, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *IGF1* = insulin-like growth factor 1, *IGF2* = insulin-like growth factor 2, *PAX7* = paired box 7, *UCP3* = uncoupling protein 3, T = treatment, S = sex, T X S = interaction between treatment and sex.

FIGURES

A



B

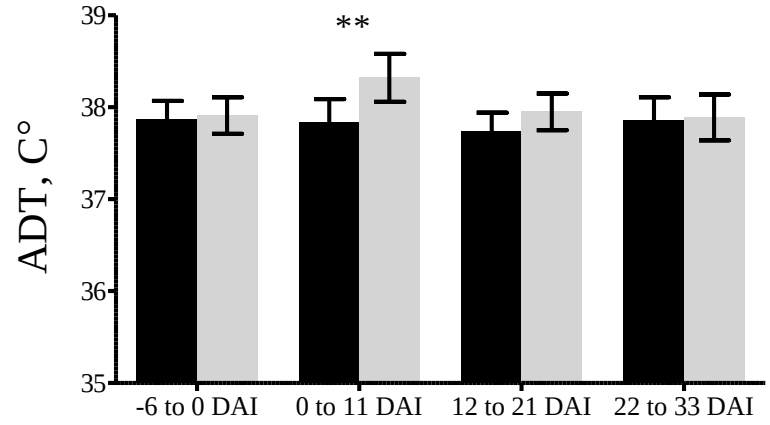
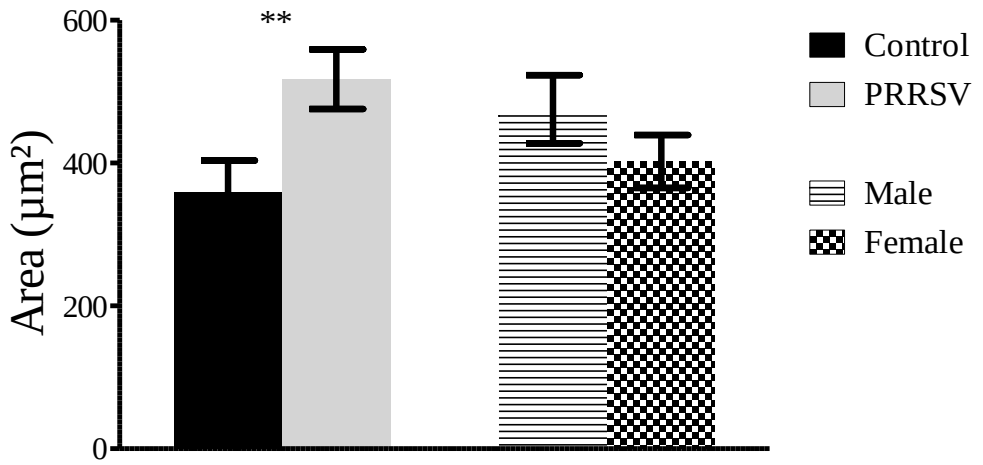


Figure 2.1 Effects of PRRSV inoculation on feed intake (A) and body temperature (B) of gilts. ** represent values that are significantly different at P<0.05. Each value is the mean and the bars represent the standard error of the mean. DAI = days after inoculation, ADFI = average daily feed intake, ADT = average daily temperature.

A



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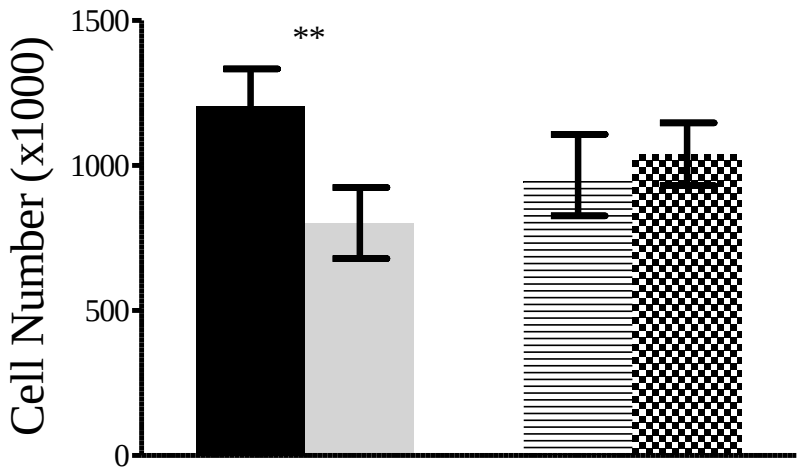

Figure 2.2 Muscle cell number and fiber area of the semitendinosus in 28 d old piglets from control and PPRSV infected gilts from PRRSV inoculated gilts. ** represent values that are significantly different at α=0.05. Each value is the mean and the bars represent the standard error of the mean.

CHAPTER 3

EFFECTS OF POSTNATAL INFECTION WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON MUSCLE GROWTH AND GENE EXPRESSION IN PIGS

ABSTRACT

In swine, viral infections decrease growth in part by directing nutrients away from skeletal muscle; however, the effect of viral infections on myogenic factors that regulate muscle hypertrophy are not clearly defined. Therefore, the objective of this study was to delineate alterations in gene expression profiles that govern muscle growth in response to porcine reproductive and respiratory syndrome virus (PRRSV) during a rapid growth period. Differential responses dependent on fiber type were also investigated. Pigs were inoculated with PRRSV or sterile culture medium (control) at 35 days of age, maintained on similar diets and euthanized 14 days after inoculation. Upon sacrifice, body weight and weights of longissimus dorsi (LD), psoas major (PM) and semitendinosus (ST) muscles were assessed. Samples from the LD (fast glycolytic muscle) and PM (slow oxidative muscle) were used for gene expression, while the ST was used to determine muscle fiber cross sectional area and number. From 0 to 14 days after inoculation, body weight ($P<0.01$) and feed intake ($P\leq0.04$) were reduced 27% and 37%, respectively, in PRRSV-inoculated pigs compared with control pigs. Longissimus dorsi, ST, and PM weights were reduced ($P<0.01$) in pigs inoculated with PRRSV compared with control pigs. In addition, muscle fiber size of PRRSV-inoculated pigs tended to be reduced ($P=0.09$) compared with control pigs, but muscle cell number was unaffected. In the PM, myostatin expression tended to be increased ($P=0.08$) while *MYF5* ($P=0.01$) and insulin-like growth factor 1 ($P=0.04$) were decreased in pigs inoculated with PRRSV compared with control pigs. Also in the PM, myosin heavy chain (MHC) *2b* and *2x* decreased ($P<0.01$) in pigs inoculated with

PRRSV compared with control pigs, suggesting a reduction in muscle hypertrophy. In the LD, myogenic differentiation factor 1 (*MYOD*) was increased ($P<0.01$) and *IGF* 2 was decreased ($P=0.04$) but there were no differences ($P \geq 0.22$) in *MHC* expression in pigs inoculated with PRRSV compared with control pigs. Overall, PRRSV infection decreased muscle weights and cell size through a decrease in muscle hypertrophy. However, gene expression differences between LD and PM muscles suggest muscles with opposing fiber types may respond differently to PRRSV infection.

Key words:

Muscle, Infection, Immunological Stress, Porcine Reproductive and Respiratory Syndrome Virus

INTRODUCTION

An immunological stress response can be defined as a shift in nutrient partitioning away from tissue growth and towards the immune system in an effort to reach homeostasis following infection (Klasing and Johnstone, 1991). Although this immunological stress response is beneficial to the survival and recovery of the host, this response results in undesirable effects on the growth and production performance of the animal. Immunological stress elicits an inflammatory response through the production of inflammatory cytokines, including interlukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), to inhibit the spread of a virus (Black, 2003). However, IL-6 and TNF-α inhibit muscle cell proliferation and differentiation partially through increased expression of myostatin (Roubenoff, 2007) thereby reducing the capacity for muscle growth (Zhu et al., 2004).

Furthermore, muscles are comprised of different fiber types that can be classified by their metabolic properties among other biochemical and physiological characteristics. Inflammatory cytokines alter tissue metabolism through energy redistribution (Wisse, 2004), by decreasing protein synthesis and increasing catabolism (Wellen and Hotamisligil, 2005). Inflammation is also a potential mechanism of sarcopenia, or age related loss of muscle mass. Sarcopenia targets a reduction in MHC 2 fiber number and size with minimal changes in MHC 1 fibers (Deschenes, 2004). Therefore, fiber type composition may be a determinant of which fibers are targeted to obtain nutrients required to fight infection during an immunological stress response. A more thorough understanding of how immunological stress affects the molecular mechanisms that reduce growth is needed to determine if nutritional programs can prevent growth restriction. The objectives of this study were to delineate how the molecular mechanisms that govern muscle growth are altered in pigs infected with porcine reproductive and respiratory syndrome

virus (PRRSV) during a rapid growth period, and to determine how muscles comprised of different fiber types differ in their response to infection.

MATERIALS AND METHODS

Animal Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Illinois and followed guidelines recommended in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). Sixteen castrated male weanling pigs from the University of Illinois Swine Research Center were delivered to the Edward R. Madigan Laboratory at 21 days of age. When received, each pig was housed individually in a disease containment chamber and received three intramuscular injections of lincomycin (11 mg/kg of body weight; Zoetis, Florham Park, NJ) for preventative care. To acclimate to the facility, pigs were fed a common diet that met or exceeded the Nutritional Research Council (2012) nutrient requirements and had ab libitum access to feed and water for one week. At 28 days of age, pigs were weighed and divided into 2 uniform groups based on body weight and litter of origin. Once divided, both groups were fed a diet (Table 3.1) that contained 17.5% soybean meal. Pigs were given a one week adaptation period to the diet prior to inoculation. Feed intake was recorded daily by weighing remaining feed in the feeder from the previous day. Body weight was also recorded daily and subtracted from pig weight at 28 days of age to calculate body weight gain.

At 35 days of age, one group (n=8) was intranasally inoculated with a 1×10^5 50% tissue culture infective dose of PRRSV (P-129 isolate, Purdue University, West Lafayette, IN) diluted in 2 mL of Dulbecco's phosphate buffered saline (PBS) while the control group (n=8) received 2

mL of Dulbecco's PBS intranasally. To avoid cross-contamination, PRRSV infected pigs were housed in a separate hall from controls. At 14 days after inoculation, pigs were anesthetized by intramuscular injection of TKX (mixture of Telazol®, ketamine, and xylazine; 4.4 mg/kg BW, Fort Dodge Animal Health, Fort Dodge, IA) and euthanized by intracardiac administration of a lethal dose of sodium pentobarbital (390 mg/ml Fatal Plus - administered at 1 ml/5 kg BW, Vortech Pharmaceuticals, Dearborn, MI). Serum PRRSV load was measured at 0, 3, 7, and 14 days after inoculation by extracting PRRSV RNA and analyzing with real-time PCR (University of Illinois Veterinary Diagnostic Laboratory, Urbana, IL). Viral load was expressed as cycle threshold (Ct) values, where a higher Ct value represents a lower amount of PRRSV RNA.

Growth Measurements and Sampling

At sacrifice, body weight and crown-to-rump length was recorded for each piglet. The semitendinosus (ST), longissimus dorsi (LD), and psoas major (PM) were removed from the left side of each piglet and weighed. Three samples (100mg) were collected from the LD and PM, placed in RNAlater (Ambion-Life Technologies, Grand Island, NY) and held at -80˚C until gene expression analysis. Samples of the LD were collected posterior to the spinalis dorsi. Ten mL blood samples were also collected for TNF-α detection by ELISA.

Muscle cell number and size

To determine muscle cell number and size, the ST from each piglet was anchored by string to a wooden stick to prevent shortening and held at 4˚C until rigor mortis was complete (approximately 24 hours). After 24 hours, the ST was submerged in approximately 8 mL of 10% neutralized buffered formalin (37% formaldehyde, Fischer Scientific, Fair Lawn, New Jersey). After 48 hours of submersion, the ST was sliced in half, perpendicular to the direction of the

muscle fibers, and the cut face of the ST was traced on acetate paper. To determine the cross sectional area of the muscle, outlines were traced using an Intuos 4 Wacom tablet (PTK-840; Tokyo, Japan) into Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, San Jose, CA) and measured. Next, approximately 1 cm^3 samples were collected from each ST to determine cross sectional area and muscle fiber number. Using these samples, approximately 3 7-µm sections per animal were cut perpendicular to the muscle fibers with a microtome, fixed to a glass slide and stained with hematoxylin and eosin. Five random images were captured for each section using a total magnification of 564X on an AMG Exos XL Microscope (EVOS, Bothell, WA). The area of all fibers in each of five images were measured using Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, San Jose, CA) to determine an average cross sectional area of individual muscle fibers. Images were standardized to a 100 µm scale bar where 440 pixel length was set equivalent to 100 µm. Total fiber number was then estimated by dividing total cross sectional area of the ST by the average cross sectional area of individual muscle fibers.

Gene Expression Analysis

Total RNA was isolated from LD and PM muscles after tissue disruption using the Tri-Reagent method (Sigma-Aldrich, St. Louis, MO) according to Clark et al. (2011). One µg of RNA was reverse transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) according to manufacturer's instructions. cDNA was then stored at -20° C for further analysis.

Expression of ribosomal protein L19 (*RPL 19*) was unaffected by treatment and therefore, was chosen as the reference gene. Quantitative real-time PCR was completed in

accordance with qScript PerfeCTa FastMix II, Rox (Quanta Biosciences, Gaithersburg, MD) master mix procedures, using a Step One Plus qPCR machine (Applied Biosystems by Life Technologies; Grand Island, NY). Each gene of interest was normalized to *RPL 19* expression and then calibrated to the control pigs (calibrator group). The ΔΔCT method was used to calculate fold change between treatments. Expression of myostatin, insulin-like growth factor 1 and 2, *MYOD*, *MYF5*, myogenin, *PAX7*, and the myosin heavy chain genes (Table 2.3) was quantified. All primers had efficiency between 90 and 100%.

Statistical analysis

Data were analyzed using a one way ANOVA in the MIXED procedure of SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA). Pigs was the experimental unit for all data analyses, and the model included fixed effect of treatment. Feed intake and body weight gain were analyzed as average weekly measurements. For gene expression data, ΔΔCT were analyzed and presented as fold change. All residuals were assessed for normality using normal probability plots with the Univariate procedure of SAS. Least square means were separated with the PDIFF option. Statistical differences were accepted as significant at $P<0.05$ and trends were noted as $0.05<P<0.10$.

RESULTS

PRRSV Inoculation

All pigs inoculated with PRRSV demonstrated clinical signs of infection as body weight ($P<0.01$) and feed intake ($P=0.04$) were reduced from d 0 to 7 after inoculation in pigs inoculated with PRRSV compared with control pigs (Figure 3.1 and Table 3.3). In the second week following inoculation, body weight ($P<0.01$), feed intake ($P<0.01$) and gain to feed ($P<0.01$) were reduced 27%, 37%, and 49% respectively, in pigs inoculated with PRRSV compared with

control pigs; however, gain to feed ratio was unaffected by PRRSV infection from 7 to 14 days after inoculation. Furthermore, serum TNF-α expression was increased ($P<0.01$) in PRRSV-inoculated pigs compared with control pigs 3 days after inoculation, and remained elevated ($P<0.01$) until 14 days after inoculation (Figure 3.1).

Muscle Weight, Cell Size and Number

Body length, LD, PM and ST weights were reduced ($P<0.01$) 11.6%, 34.9%, 54.6% and 40.5%, respectively, in pigs inoculated with PRRSV compared with control pigs (Table 3.4). Relative PM and ST weights expressed as a percentage of body weight of PRRSV infected pigs were reduced ($P\leq0.03$) 16 and 7 percentage units, respectively, compared with control pigs.

Muscle fiber number within the ST was not different ($P = 0.99$) between PRRSV-inoculated and control pigs. However, cross sectional area of the muscle fibers tended to decrease ($P=0.09$) approximately 25% in PRRSV-inoculated pigs (571.0 ± 67.47 μm²) compared with control pigs (747.7 ± 67.47 μm²; Figure 3.2).

Gene Expression

In the LD, there were no differences in moystatin or myosin heavy chain expression between pigs in either treatment group (Table 3.5). However, *IGF* 2 expression ($P=0.04$) was decreased in pigs inoculated with PRRSV compared with control pigs. Conversely, *MYOD* ($P<0.01$) and myogenin ($P=0.05$) expression were increased in pigs inoculated with PRRSV compared with control pigs. Expression of all other positive growth factors (*MYF5*, *IGF1*, and *PAX7*) in the LD was unaffected by PRRSV infection.

In the PM, *MHC2b* ($P=0.01$) and *2x* ($P<0.01$) expression were decreased in pigs inoculated with PRRSV compared with control pigs, while *MHC1* and *2a* were unaffected by

PRRSV inoculation (Table 3.6). Furthermore, *MYF5* (P=0.01) and *IGF1* (P=0.04) expression was decreased as myostatin expression tended to increase (P=0.08) in pigs inoculated with PRRSV compared with control pigs. There were no differences in *MYOD*, myogenin, *IGF2*, and *PAX7* expression between treatments.

DISCUSSION

In the United States, annual loss of productivity due to PRRSV was estimated at $664 million (Zimmerman et al., 2012) mainly due to loss in production efficiency as immunological stress response reduces lean tissue growth (Sandberg et al., 2006). Defining mechanisms involved in inhibiting skeletal muscle protein accretion during infection is necessary to mitigate effects of the disease. The objectives of this study were to determine 1) measure gene expression that inhibit protein accretion in pigs infected with PRRSV during a rapid growth period, and 2) differential effects of PRRSV on skeletal muscle growth based on fiber type composition.

Reductions in muscle weight due to postnatal PRRSV infection are assumed to be primarily the result of limited hypertrophy. This was confirmed in our study as PRRSV infection decreased muscle through a reduction in muscle fiber size in PRRSV-inoculated pigs compared with control pigs. PRRSV infection also elevated serum TNF-α and decreased IGF 2 expression in the LD and IGF 1 expression in the PM, reducing LD and PM muscle weights. These findings suggest that PRRSV infection restricts muscle growth by stimulating protein degradation, decreasing protein synthesis or a combination of both. Implicated in these processes are inflammatory cytokines, such as TNF-α and IL-6, which activate nuclear factor- κβ (NF- κβ) causing protein degradation through the ubiquitin pathway (Grounds et al., 2008). Increased circulating TNF-α concentrations in PRRSV infected pigs of our study is suggestive of increased muscle protein degradation. Additionally, increased circulating inflammatory cytokines may

also decrease circulating IGF1 concentrations and reduce overall body weight (DeBenedetti et al., 1997). Decreased muscle IGF expression is associated with increased muscle atrophy and decreased muscle protein synthesis (Grounds et al., 2008).

Another possible mechanism limiting hypertrophy in PRRSV-infected pigs is myostatin, which was increased in PM in our study. Myostatin was previously implicated in the inhibition of muscle growth during PRRSV infection (Escobar et al., 2004). Myostatin is a known negative regulator of myoblast proliferation and skeletal muscle growth, and is thought to reduce growth in part by inhibiting the mammalian target of rapamycin (mTOR) signaling pathway (Trendelenburg et al., 2009). Reduced mTOR activity decreases myoblast differentiation through a decrease in myogenic regulatory factors (Haidet et al., 2008; Lin et al., 2002; Whittemore et al., 2003). Therefore, increased myostatin may be responsible for reduced *MYF5* expression in pigs inoculated with PRRSV compared with control pigs in the PM. Furthermore, myogenic regulatory factors act as transcription factors for several muscle specific genes including the myosin heavy chains (Noden et al., 1999). Therefore, reduced *MYF5* expression may have precipitated the reduction of *MHC2b* and *2x* expression in pigs inoculated with PRRSV compared with control pigs. Together these data suggest that postnatal PRRSV infection increased myostatin expression in the PM, possibly driving decreases in *MYF5*, *MHC2b* and *2x* expression.

In muscles of mixed metabolic profiles, myostatin has been associated in the inhibition of muscle growth (Escobar et al., 2004); however, increased myostatin expression led to muscle atrophy specifically in MHC type 2 muscle fibers (Reardon et al., 2001). The PM is metabolically different from the LD as the PM is comprised primarily of oxidative fibers, while the LD is comprised primarily of glycolytic fibers (Leseigneur-Meynier and Gandemer, 1991). It

can be hypothesized that muscle catabolic events such as infection may have a more profound effect on fast, glycolytic muscles than slow, oxidative muscles. As discussed previously, a catabolic effect was evident in the PM through an increase in myostatin and reduced *MHC2b* and *2x* expression. However, in the LD *MYOD* and myogenin expression were increased. An increase in *MYOD* and myogenin expression is correlated with an increase in myoblasts, satellite cell activation, and terminal differentiation (Adams et al., 1999; Smith et al., 1994). This increase in myogenic growth factors would stimulate MHC expression supporting the lack of differences in MHC expression in the LD of pigs inoculated with PRRSV compared with control pigs. Typically, after two to three weeks of acute illness, PRRSV-infected pigs display compensatory growth (Benfield et al., 1999). Compensatory growth can be defined as a process by which an animal accelerates its growth after a period of restricted development (Hornick et al., 2000). We hypothesize the LD displayed signs of compensatory growth, leading to no differences in MHC expression while the PM was still inhibited through myostatin production and continued to experience a decrease in hypertrophic growth. This suggests that while glycolytic muscle may be more susceptible to atrophy, it may also recover more rapidly.

CONCLUSION

Similar to previous studies, we concluded that PRRSV infection elicited an immunological stress response of increased circulating inflammatory cytokines and decreased feed intake and growth. Infection caused a reduction in muscle hypertrophy that resulted in a decrease of muscle and body weight. Furthermore, reduced hypertrophy may result from increased myostatin expression that reduced myogenic growth factors and decreased *MHC 2x* and *2b* expression. In addition, muscles comprised of primarily glycolytic fibers may recover more quickly while muscles with a larger percentage of oxidative fibers may be slower to attain

compensatory gain after infection. The expected decrease in growth efficiency suggests marketing PRRSV infected pigs at lighter weights to minimize feed costs.

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TABLES

Table 3.1 Composition of the diet fed to all pigs from 7 days before inoculation to 14 days after inoculation (as-fed basis)[1]

Item	Diet (%)
Corn	46.13
Soybean meal	17.50
Dried whey	14.95
Distiller's dried grains with solubles	10.00
Poultry by-product meal[2]	7.00
Choice white grease	1.50
Ground limestone	0.68
Monocalcium phosphate	0.27
Sodium chloride	0.40
Vitamin and mineral premix[4]	0.30
Choline chloride	0.07
L-Lys HCl	0.60
DL-Met	0.27
L-Trp	0.08
L-Thr	0.15
L-Val	0.10
Calculated energy and nutrient composition	
ME, kcal/kg	3,402
CP, %	22.75
Standardized ileal digestible AA, %	
Lys	1.38
Met + Cys	0.83
Trp	0.26
Thr	0.86
Val	0.91
Ca, %	0.80
Available P, %	0.40

[1]All pigs received a common diet for 7 d immediately following weaning at 3 wk of age. Then pigs were provided this diet for the remainder of the trial.

[2]Low ash, pet-food-grade poultry by-product meal, American Proteins, Inc., Hanceville, AL

[3]AP 920, APC, Inc., Ankeny, IA

[4]Vitamin-mineral premix included the following per kilogram of complete diet: Vitamin A (retinyl acetate), 11,128 IU; Vitamin D3 (cholecalciferol), 2,204 IU; Vitamin E (dl-α tocopheryl acetate), 66 IU; Vitamin K (menadione nicotinamide bisulfite), 1.42 mg; Thiamine (thiamine mononitrate), 0.24 mg; Riboflavin, 6.58 mg; Pyridoxine (pyridoxine hydrochloride), 0.24 mg; Vitamin B12, 0.03 mg; d-Pantothenic acid (d-calcium pantothenate), 23.5 mg; Niacin (nicotinamide and nicotinic acid), 44 mg; Folic acid, 1.58 mg; Biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.

Table 3.2 Taqman primers and probe sequences used for quantitative real-time PCR.

Gene Symbol	Gene Name	Gene Bank Reference Sequence	Forward Sequence Reverse Sequence Probe Sequence
IGF1[1]	insulin-like growth factor 1	NM_214256.1	
IGF2[2]	insulin-like growth factor 2	NM_213883.2	CCGTGCTTCCGGACAACT AGGTGTCATAGCGGAAGAACTTG CCCCAGATACCCCGTGG
IL-6[2]	interleukin-6	NM_001252429.1	AACAACCTGAACCTTCCAAAAAT TCCACAAGACCGGTGGTGAT CAATCAGGAGACCTGCT
MHC1[2]	myosin heavy chain 1	NM_001104951.1	AATGTCCAGCAGGTGATGTATGC ATCCGTGTCACCATCCAGTTG AGGCCGTGTATGAGAAG
MHC2a[2]	myosin heavy chain 2a	NM_214136.1	GCAAAAGCGTAATGCTGAAGCT CCTCTTCCGTCTGGTAGGTGAGT TGCGCAAACATGAGAGG
MHC2b[2]	myosin heavy chain 2b	NM_001123141.1	GGGTCTTCGGAAACATGAGAGA TCCTGCAGCCTGAGAACATTC TTACCAGACTGAGGAGGAC
MHC2x[2]	myosin heavy chain 2x	NM_001104951.1	GGGTCTACGCAAACACGAGAGA CAGATCCTGGAGCCTGAGAATG AAGGAACTCACTTACCAAAC
MYF5[2]	myogenic factor 5	NM_001278775.1	GTCCAGAAAGAGCAGCAGTTTTG AGGAGCTTTTATCCGTGGCATAT ATCTACTGTCCGGATGTAC
MYOD[1]	myogenic differentiation 1	NM_001002824.1	
Myogenin[2]	Myogenin	NM_001012406.1	GTCCAGAAAGAGCAGCAGTTTTG AGGAGCTTTTATCCGTGGCATAT ATCTACTGTCCGGATGTAC
Myostatin[2]	Myostatin	NM_214435.2	CGACGGAAACGATCATTACCA AAGCAGCATTTGGGTTTTCCT TACAGAGTCTGATCTTCTAATG
PAX7[2]	paired box 7	XM_005659088.1	AGGCAGCAAGCCCAGACA CCCTCTTGTACTCCTCAATCTTTTTC CGACTCCGGATGTGG
PRRSV[2]	PRRSV	AY150312	CGCACCAGATGGGACCTACTT ACGGTGTTCAGTGAGGGCTTT CGCTGCGTTGACTGG
RPL 19[2]	ribosomal protein L19	XM_003131509.3	TGACCGCCACATGTATCACAGT TGTGGATGTGCTCCATGAGAA TGTACCTGAAAGTGAAGGG
TNF-α[2]	tumor necrosis factor	NM_214022.1	TGACCGCCACATGTATCACAGT TGTGGATGTGCTCCATGAGAA TGTACCTGAAAGTGAAGGG

[1] ABI Assay ID: *IGF1* = Ss03394499_m1, *MYOD* = Ss03378464_u1, *UCP3* = Ss03391403_m1

[2]Custom design assay

Table 3.3 Effects of porcine reproductive and respiratory virus (PRRSV) infection on growth performance of pigs.[1]

Item	Control	PRRSV	SEM	P-Value
Day -7 to -1				
ADFI, g	446.43	430.65	58.37	0.85
G:F, kg	0.45	0.29	0.09	0.24
Day 0 to 7				
ADFI, g	726.75	576.88	47.04	0.04
G:F, kg	0.73	0.37	0.07	<0.01
Day 8 to 14				
ADFI, g	1025.13	532.13	42.14	<0.01
G:F, kg	0.67	0.63	0.06	0.69

[1]Values represent least square means of 8 pigs. Pigs were inoculated on day 0.

Table 3.4 Effects of porcine reproductive respiratory syndrome virus (PRRSV) on body length and muscle weights 14 d after inoculation.[1]

Item	Control	PRRSV	SEM[a]	P-Value
Pigs, n	8	8		
Body Length, cm	61.67	54.53	1.40	<0.01
Longissimus Dorsi, g	343.17	223.36	22.93	<0.01
Relative weight[2]	1.98	1.75	0.13	0.20
Psoas Major, g	71.37	32.39	5.40	<0.01
Relative weight[2]	0.41	0.25	0.02	<0.01
Semitendinosus, g	72.44	43.12	4.67	<0.01
Relative weight[2]	0.41	0.34	0.02	0.03

[1] SEM reported as maximum standard error of mean

[2] Muscle weight as a percentage of body weight

Table 3.5 Myosin heavy chain and myogenic growth factors gene expression in longissimus dorsi muscle of pigs 14 days after porcine reproductive respiratory virus (PRRSV) inoculation.[1]

Item[2]	Control		PRRSV		P-Value
MHC1	1.00	(0.33-2.99)	0.83	(0.28-0.49)	0.80
MHC2a	1.00	(0.26-3.83)	1.67	(0.44-6.41)	0.57
MHC2x	1.00	(0.47-2.12)	1.23	(0.58-2.60)	0.68
MHC2b	1.00	(0.48-2.07)	0.54	(0.26-1.11)	0.22
IGF1	1.00	(0.53-1.90)	0.92	(0.50-1.67)	0.84
IGF2	1.00	(0.65-1.54)	0.52	(0.34-0.80)	0.04
MYF5	1.00	(0.43-2.35)	0.45	(0.19-1.06)	0.18
MYOD	1.00	(0.66-1.52)	2.71	(1.78-4.12)	<0.01
Myogenin	1.00	(0.64-1.57)	1.86	(1.19-2.91)	0.05
Myostatin	1.00	(0.41-2.41)	0.98	(0.40-2.36)	0.97
PAX7	1.00	(0.60-1.67)	0.95	(0.59-1.53)	0.87

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] Abbreviations: MHC = Myosin Heavy Chain, *IGF1* = insulin-like growth factor 1,
IGF2 = insulin-like growth factor 2, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *PAX7* = paired box 7, UCP3 = uncoupling protein 3

Table 3.6 Myosin heavy chain and myogenic growth factors gene expression in psoas major muscle of pigs 14 days after porcine reproductive respiratory virus (PRRSV) inoculation.[1]

Item[2]	Control		PRRSV		P-Value
MHC1	1.00	(0.63-1.58)	0.92	(0.58-1.45)	0.78
MHC2a	1.00	(0.68-1.47)	0.66	(0.45-0.97)	0.13
MHC2x	1.00	(0.72-1.39)	0.35	(0.25-0.49)	<0.01
MHC2b	1.00	(0.38-2.66)	0.13	(0.05-0.34)	0.01
IGF1	1.00	(0.61-1.65)	0.47	(0.28-0.77)	0.04
IGF2	1.00	(0.60-1.66)	1.01	(0.61-1.68)	0.98
MYF5	1.00	(0.65-1.55)	0.39	(0.25-0.60)	0.01
MYOD	1.00	(0.74-1.35)	1.10	(0.83-1.47)	0.61
Myogenin	1.00	(0.75-1.33)	0.98	(0.74-1.30)	0.92
Myostatin	1.00	(0.69-1.44)	1.59	(1.10-2.29)	0.08
PAX7	1.00	(0.69-1.45)	0.74	(0.51-1.08)	0.25

[1]Values are least squares means of fold changes with 95% confidence intervals in parentheses. All values of gene expression were compared to that of male control pigs.

[2] Abbreviations: MHC = Myosin Heavy Chain, *IGF1* = insulin-like growth factor 1,
IGF2 = insulin-like growth factor 2, *MYF5* = Myogenic factor 5, *MYOD* = myogenic differentiation 1, *PAX7* = paired box 7, UCP3 = uncoupling protein 3

FIGURES

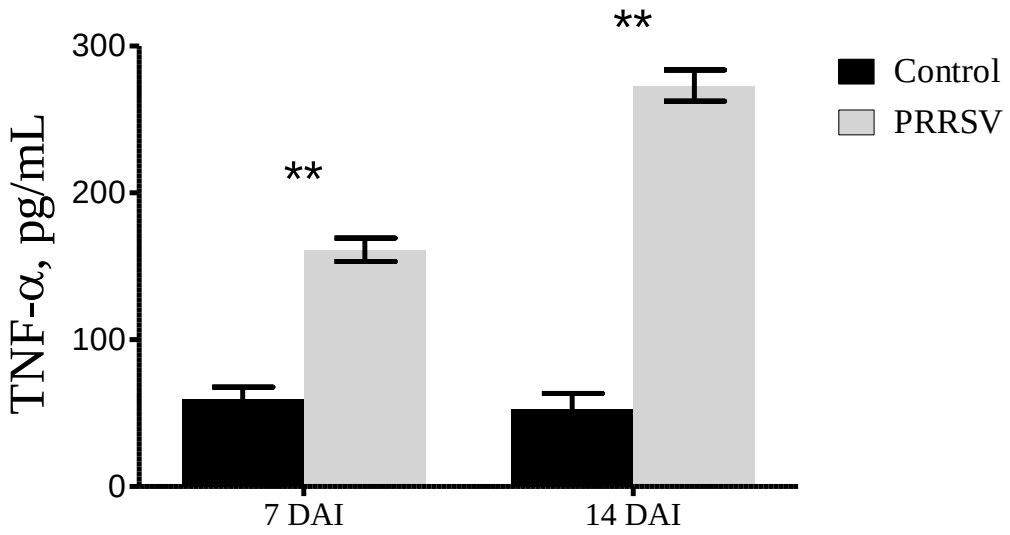


Figure 3.1 Serum TNF-α of PRRSV-inoculated and control pigs at 7 and 14 days after infection (DAI). Pigs were subjected to intranasal PRRSV inoculation at 35 days of age (PRRSV), while control pigs were intranasal inoculated with a saline solution (Control). Pigs were sacrificed 14 days after inoculation. Bars represent the standard error of the mean. The main effect of PRRSV was significant (**, $P<0.05$) for TNF-α concentrations after the inoculation.

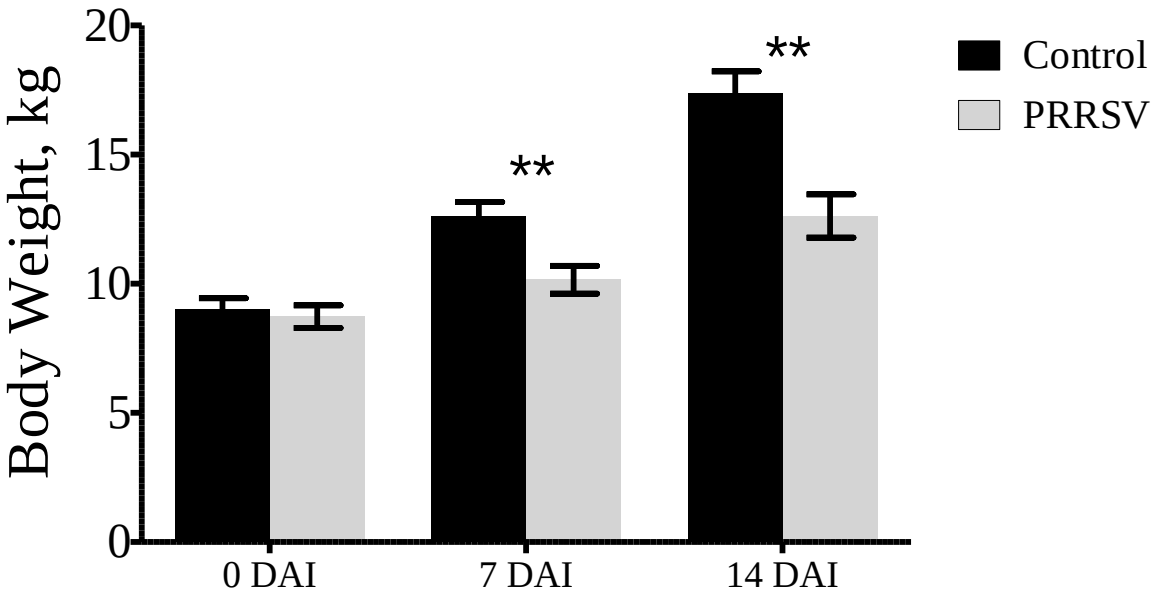
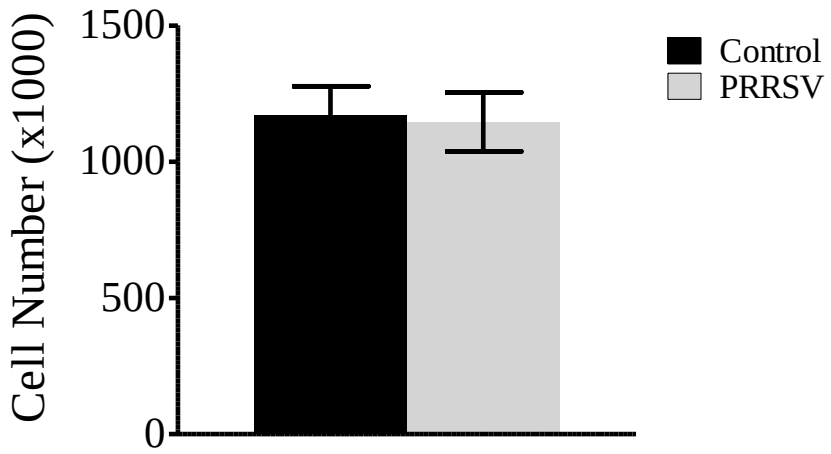


Figure 3.2 Body weight of PRRSV-inoculated and control pigs at 0, 7, and 14 days after infection (DAI). Pigs were subjected to intranasal PRRSV inoculation at 35 days of age (PRRSV), while control pigs were intranasal inoculated with a saline solution (Control). Pigs were sacrificed 14 days after inoculation. Bars represent the standard error of the mean. The main effect of PRRSV was significant (**, $P<0.05$) for weight gain after the inoculation.

A



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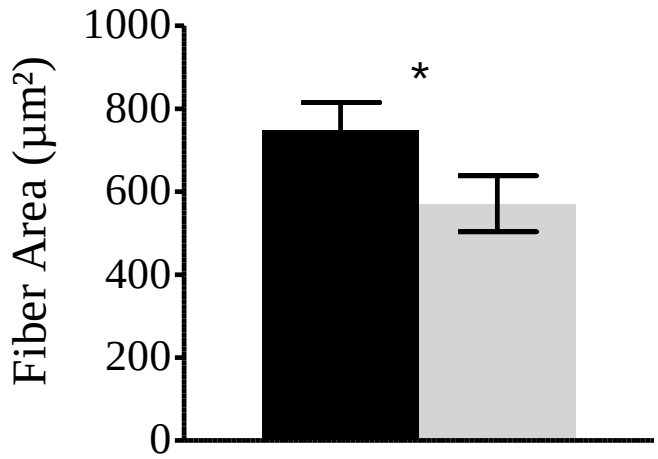

Figure 3.3 Semitendinosus muscle cell number and size of the semitendinosus in PRRSV-inoculated pigs and control pigs, 14 d after inoculation. The main effect of PRRSV tended to be significant (*, $P<0.10$) for area of muscle fibers.