The Effects of a Dual Challenge of Porcine Reproductive and Respiratory Syndrome Virus and Heat Stress on Metabolism of Growing Pigs

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ABSTRACT

Heat stress and immune challenge are costly issues to the swine industry causing significant loss in production and health including reduced efficiency in muscle accretion and energy utilization. Alterations to metabolism and immune response may participate in these shortcomings. The study objectives were to examine the metabolic profiles and immune status of swine subjected to a dual challenge of thermal stress and porcine reproductive and respiratory syndrome virus (PRRSV). To determine this, pigs were subjected to four treatments: thermo-neutral (22° C; TN), thermo-neutral PRRSV infected (TP), heat stress (HS), and heat stress PRRSV infected (HP), during two experimental phases. The first phase consisted of infecting half the experimental group with PRRSV while the rest remained infection free in thermo-neutral conditions. A second phase further divided infected and non-infected into heated conditions for three days of constant heat (35° C) or TN conditions. Venous blood was collected prior to each phase and before sacrifice to analyze for metabolites. At sacrifice liver and longissimus dorsi skeletal muscle samples were collected for gene expression analysis. Pigs in challenged conditions had increased body temperatures, reduced feed intake, and lighter body weights compared to controls, with greatest detriment to dual challenged pigs. In addition, challenged pigs had increased markers of muscle degradation. In challenged pigs, differences ($p<0.05$) were observed in the metabolic and cytokine gene expression profiles suggesting heat stress blunts the immune response of viral infection in muscle and liver. In conclusion, heat stress and immune challenge directly and indirectly affect metabolism and cytokine expression and both variables may contribute to decreased growth parameters.

Keywords: Porcine reproductive and respiratory syndrome virus, heat stress, metabolism

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ACC = ACETYLE CoA CARBOXYLASE

ADG = AVERAGE DAILY GAIN

ADP = ADENOSINE DIPHOSPHATE

AMP = ADENOSONE MONOPHOSPHATE

AMPK = AMP-ACTIVATED PROTEIN KINASE

ATP = ADENOSINE TRIPHOSPHATE

CBC = COMPLETE BLOOD COUNT

CK = CREATINE KINASE

DMI = DRY MATTER INTAKE

BUN = BLOOD UREA NITROGEN

BW = BODY WEIGHT

F-6-P = FRUCTOSE-6-PHOSPHATE

FFA = FREE FATTY ACID

FI = FEED INTAKE

G-6-P = GLUCOSE-6- PHOSPHATE

GH = GROWTH HORMONE

GHRH = GROWTH HORMONE RELEASING HORMONE

GI = GASTROINTESTINAL

HK = HEXOKINASE

HP = HEAT STRESS + PRRSV

HS = HEAT STRESS

HSP = HEAT SHOCK PROTEIN

IFN = INTERFERON

IGF-1 = INSULIN- LIKE GROWTH FACTOR-1

IL = INTERLEUKINS

JNK = C-JUN-TERMINAL KINASE

LD = LONGISSIMUS DORSI

LPL = LIPOPROTEIN LIPASE

LPS = LIPOPOLYSACCHARIDE

MAPK = MITROGEN ACTIVATED PROTEIN KINASE

mRNA = MESSANGER RNA

MSTN = MYOSTATIN

NADH = NICOTINAMIDE ADENINE DINUCLEOTIDE

NADPH = NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

NEBAL = NEGATIVE ENERGY BALANCE

NEFA = NON-ESTERIFIED FATTY ACID

NF-KB = NUCLEAR FACTOR –KB

NtMH = Nt-METHYLHISTIDINE

OAA = OXALOACETATE

PAMP = PATHOGEN ASSOCIATED MOLECULAR PATTERN

PC = PYRUVATE CARBOXYLASE

PDC = PYRUVATE DEHYDROGENASE COMPLEX

PDH = PYRUVATE DEHYDRGENASE

PDK = PYRUVATE DEHYDRGENASE KINASES

PDP = PYRUVATE DEHYDROGENASE PHOSPHOTASE

PEP = PHOSPHENOL PYRUVATE

PEPCK = PHOSPHOENOL PYRUVATE CARBOXYKINASE

PF = PAIR FEEDING

PFTN = PAIR-FED THERMO-NEUTRAL

PRR = PATTERN-RECOGNITION RECEPTOR

PRRSV = PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

REV = REVERSE ELECTRON TRANSFER

ROS = REACTIVE OXYGEN SPECIES

RR = RESPIRATION RATE

ST = SOMATOTROPIN

TCA = TRICARBOXYLIC ACID

TER = TRANSEPITHELIAL RESISTANCE

TLR = TOLL-LIKE RECEPTOR

TN = THERMO-NEUTRAL

TNF = TUMOR NECROSIS FACTOR

TP = THERMO-NEUTRAL + PRRSV

WBC = WHITE BLOOD CELL

Chapter I

Literature Review

Heat stress and the swine industry

Heat stress (HS) is a serious threat to the swine industry worldwide. As the global population increases, more people are living in tropical and sub-tropical climates (Renaudeau et al., 2012), which comes with an increased need for food production in warmer, more humid climates. However, the tropics are not the only area where heat stress is a problem. Temperate areas are experiencing hotter summer months and more severe weather, including more frequent and severe heat waves and droughts (U.S. EPA, 2010).

Heat stress occurs when an animal is unable to maintain their body temperature within a zone of thermal comfort due to excessive heat from the environment, resulting in increased body temperature and respiration rate and decreased feed intake and growth, (Renaudeau et al., 2012). The increased heat load experienced by these animals negatively impacts the U.S. swine industry by compromising production efficiency and jeopardizing animal welfare, costing millions of dollars annually. All phases of swine production are affected, but most notably the breeding and growing-finishing phases. Sow losses are estimated to be $113 million a year, while losses in the growing-finishing phase are estimated at $202 million a year (St-Pierre et al., 2003). These estimated

financial losses due to heat stress in the U.S. swine industry of over $300 million a year are multifaceted and dependent on phase of production.

In the breeding phase of production, both the male and female have decreased fertility during heat stress and up to five weeks after a heat episode (St-Pierre et al., 2003). Detrimental effects of heat stress on the sow include decreased dry matter intake (DMI), decreased milk production, and increased sow lactation body weight loss while reducing the weight gain of pre-weaned litters (St-Pierre et al., 2003). The extent of sow body weight loss combine decreased feed intake and increased costs of maintenance with a reallocation of energy away from growth toward lactation, ultimately affecting growth of the sow and survivability of the litter. Sows also display delayed or failure to return to estrus during heat stress conditions (St-Pierre et al., 2003; Renaudeau et al., 2012). This results in fewer litters per year and decreased productivity.

The growing-finishing phase is also heavily affected by heat stress due to increased time of production resulting from slowed growth rates, as well as decreased carcass quality. During heat stress conditions, feed intake is decreased, partially to reduce metabolic heat production; however, this results in less energy for growth. The consumed energy is partitioned toward maintaining euthermia. Research shows that growth during heat stress favors increased adipose retention and decreased muscle accretion (Verstegen et al., 1978; Heath, 1983). This results in decreased carcass value and less profitability to the industry.

Heat stress also causes the swine industry to suffer profit loss due to an increased occurrence and susceptibility to disease (Baumgard and Rhoads, 2013). As disease prevalence increases, health care costs are elevated, adding to the total cost incurred by

the swine industry. Additional costs to the industry are abatement strategies, which include increased ventilation, misters, etc (Renaudeau et al., 2012). Another strategy includes special farrowing crates that cool the sow while keeping the piglets warm, accommodating differences in thermoneutral zones between the two (Renaudeau et al., 2012). Minimal abatement strategies reduce national losses from $2.4 billion to $1.7 billion (St-Pierre et al., 2003). However upfront capital is required for installation and as populations move towards the tropics, typically developing countries, the financial means to implement abatement strategies may be more limited.

Effects of heat stress in pigs

Pigs that are unable to dissipate excess heat efficiently become heat stressed, resulting in multiple physiological responses. The immediate physiological effects include increased body temperature and respiration rates, as well as decreased feed intake (Renaudeau et al., 2008). The decreased feed intake is believed to reduce metabolic heat production, which is the sum of maintenance requirement and heat generated for production purposes (Renaudeau et al., 2012). Metabolic heat production is emphasized in rapid lean accretion by genetic selection. The decreased feed intake and increased energy demands toward maintenance of thermoregulatory homeostasis leads to decreased muscle growth. During heat stress the heat load becomes too intense and the pig is unable to rid itself of the excess heat, causing a change in set point of body temperature (Renaudeau et al., 2012). The effects are magnified due to the physiology of swine with a thick subcutaneous fat layer and lack of functional sweat glands (Curtis, 1983). To attempt to alleviate the heat load, swine increase panting to allow for evaporative cooling

in the oral cavity and esophagus (Patience et al., 2005) and utilize conductive cooling by becoming inactive and laying on their side to increase surface contact with the floor (Aarnink et al., 2006).

Metabolism

Carbohydrate metabolism

Animals require energy in the form of ATP throughout the body to maintain biological processes such as muscle contraction, cell movement, active transport, ion movement, and synthesis of macromolecules. Cellular energy status is one of the primary mechanisms for determining substrate utilization of glucose, fatty acids, lactate, and amino acids in integrated chemical reactions (Baumgard and Rhoads, 2012). Substrates can be converted into a number of metabolic intermediates and used in synthesis reactions or utilized to make ATP. The processes that generate the bulk of ATP include: glycolysis, tricarboxylic acid cycle (TCA) and oxidative phosphorylation via the electron transport chain. Regulation of these pathways is based on energy requirements involving synthesis and degradation rates of enzymes, allosteric interactions and covalent modification of enzymes, and regulation of substrate entry into the cells and intracellular compartments (Berg et al., 2007). The energy charge of the cell is reflected by the amounts of ATP, ADP, and AMP present (Berg et al., 2007).

Carbohydrates are readily available sources of energy that can be utilized in various ways throughout the body. Carbohydrate metabolism can alternate between the glycolytic pathway and the TCA cycle. The primary purpose of the glycolytic pathway is

to quickly produce 2 ATP and 2 pyruvate molecules, regardless of oxygen state (Berg et al., 2007) . In comparison, the TCA cycle generates large quantities of ATP more efficiently as well as metabolic intermediates that can be used in fatty acid synthesis, but oxygen must be present (Berg et al., 2007).

The glycolytic pathway has three rate limiting steps that commit the conversion of glucose to pyruvate. The first rate-limiting step is the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by the enzyme hexokinase (HK). The conversion to G-6-P by phosphorylation prevents glucose from exiting the cell and it can be used in glycolysis or glycogen synthesis. Increased levels of G-6-P in the cell inhibit HK, whereas insulin up regulates HK transcription and increases glucose uptake by cells (Printz et al. 1993).

The second rate-limiting step uses the enzyme phosphofructokinase to phosphorylate fructose-6-Phosphate (F-6-P) to fructose 1,6 bisphosphate (Berg et al., 2007). Fructose 1,6 bisphosphate can serve as an allosteric regulator to pyruvate kinase (final rate limiting step). Fructose 1,6 bisphosphate is the committed step to glycolysis. Prior to this reaction G-6-P could bypass glycolysis and enter the pentose phosphate pathway or be converted to glucose-1-phosphate for use in glycogen synthesis (Berg et al., 2007).

The final rate-limiting step occurs after a series of smaller reactions resulting in the final conversion of phosphoenol pyruvate (PEP) to pyruvate (Berg et al., 2007). A phosphorus molecule from PEP is transferred to ADP via the enzyme pyruvate kinase, to produce ATP. Net yield at the end of glycolysis is 2 ATP and 2 pyruvate molecules. The newly formed pyruvate molecules can then enter the TCA cycle or be released as lactate (Berg et al., 2007).

Pyruvate entry into the TCA cycle is dependent on a group of enzymes called the pyruvate dehydrogenase complex (PDC), which catalyze the irreversible oxidative phosphorylation of pyruvate to acetyl CoA, utilizing NADH, while transporting the molecule into the mitochondria (Sugden and Holness, 2003). Increases in available pyruvate and decreased acetyl CoA and NADH concentrations increases PDH activity (Sugden and Holness, 2003). Inactivation of skeletal muscle PDH shifts pyruvate toward lactate and alanine conversion for utilization in the liver and kidney for gluconeogenesis (Berg et al., 2007).

Regulation of PDC by activation or inactivation is important to the balance between glucose and fatty acid utilization. The two enzyme families that control this regulation are pyruvate dehydrogenase kinases (PDK) and pyruvate dehydrogenase phosphatases (PDP) (Sugden and Holness, 2003). Both enzymes can be regulated by acetyl CoA, NADH, transcriptional factors, and hormones (Baumgard and Rhoads, 2012).

The PDK enzyme family consists of four known isoforms, which can be expressed in different frequencies depending on tissue type (Gudi et al., 1995). The PDK4 isoform is highly expressed in muscle tissue (Sugden and Holness, 2003). PDK's are responsible for phosphorylation of PDC and, therefore, inactivation. Increases in fatty acids activate PDK's shifting metabolism toward fatty acid oxidation to conserve glucose. This may be due to decreased insulin action as it has been found that insulin has suppressive effects of PDK's (Sugden and Holness, 2003). Regulation of PDK is important during starvation states when substrate availability is decreased for glycolytic metabolism, as PDK's have been shown to increase during glucose deprivation. Fatty

acids become the preferred substrate, which have higher energy yields and are previously stored in the body.

The second family of enzymes regulating PDC is PDP. Pyruvate dehydrogenase phosphatase dephosphorylates PDC for activation (Sugden and Holness, 2003). Activation of PDC increases glucose utilization through the glycolytic pathway as well as resumption of the TCA cycle.

Mitochondria are organelles with a double membrane that function to provide the majority of the cellular energy in the form of ATP (Wallace et al., 2010) The conversion of pyruvate and fatty acid to acetyl-CoA takes place in the mitochondria and produced high-energy molecules, NADH and FADH2. Acetyl –CoA enters the TCA cycle to yield net NADH and FADH2 that are used for oxidative phosphorylation in the electron transport chain (Berg et al., 2007) Electrons from NADH and FADH2 are transferred between protein complexes I –IV in the ETC to reduces oxygen to water in a process that is coupled to the production of adenosine triphosphate (ATP) (Stephenson and Hawley, 2013). The NADH produced during pyruvate oxidation and B-oxidation transfer two electrons to NADH dehydrogenase (complex I), where as the FADH2 produced by B-oxidation transfers electrons to succinate dehydrogenase (complex II) to reduce ubiquinone. The most reduced form of ubiquinone is ubiquinol, which transfers electrons down to complex III and eventually to oxygen (Wallace et al., 2010). The energy released as electrons move down the chain are used to pump protons across the mitochondrial inner membrane through complexes I, III, and IV. The ATP synthetase complex uses the creation of an electrochemical proton gradient to generate ATP from ADP and phosphate (Wallace et al., 2010; Berg et al., 2007).

The mitochondria generate much of the endogenous cellular reactive oxygen species (ROS) through oxidative phosphorylation (Wallace et al., 2010). Reactive oxygen species can damage cellular proteins, lipids, and nucleic acids (Wallace et al., 2010). Under normal conditions, complex 1 regulates ROS production. However when there are ETC malfunctions, excess electrons accumulate with electron carriers and are passed directly to O_2 to generate superoxide anions. The superoxide anions generated by complex I are released into the mitochondrial matrix where they are converted to H_2O_2, whereas those produced by complex III are released in mitochondrial intermembrane space and converted to H_2O_2 (Wallace et al., 2010). Approximately 85-90% of oxygen radicals are produced through complexes I and III of the respiratory chain (Kembro et al., 2012). Substrate choice and concentration, physiological status, and mode of electron transport (forward or reverse), are critical factors affecting energetic status (Kembro et al., 2012). Forward electron transfer (FET) and reverse electron transfer (REV) occur in the presence of substrates of complex I (glutamate or pyruvate) and complex II (succiante), respectively. Emission rate of H_2O_2 are lower in FET, compared to REV (Kembro et al., 2012).

Glucose metabolism is regulated as described above but also via hormones: insulin and glucagon (Baumgard and Rhoads, 2013). Insulin is secreted by the pancreas in response to abundant glucose in the blood, which stimulates ATP production, fatty acid synthesis in adipose, and inhibits glycogen breakdown in muscle. However, when glucose levels are low, glucagon is secreted from the pancreas to increase circulating glucose levels in the blood by stimulating glycogenolysis and gluconeogenesis (Berg et al., 2007). Glycogenolysis is the breakdown of glycogen to glucose by way of glycogen

phosphorylase and G-6-P phosphatase in either the muscle or liver. Gluconeogenesis utilizes non-carbohydrate carbon sources like pyruvate, glucogenic amino acids, lactate, and glycerol to create glucose in the liver (Berg et al., 2007). The process of gluconeogenesis is essentially the process of glycolysis in reverse with a higher energy demand and enzyme variations. As previously mentioned glycolysis has three rate-limiting steps; these same rate-limiting steps occur in gluconeogenesis with an energy or hydrolysis component. The first major step is to convert pyruvate back to PEP, an energy demanding two step process. Pyruvate carboxylase (PC) plus ATP are needed to convert pyruvate to oxaloacetate (OAA). Next, OAA is converted to PEP via phosphoenol pyruvate carboxykinase (PEPCK) and ATP. After a series of smaller reactions, the second rate-limiting step occurs. Fructose 1, 6 bisphosphate must be hydrolyzed to fructose-6-phosphate. Finally, glucose-6-phosphate must also be hydrolyzed back to glucose for the process to be complete (Berg et al., 2007).

Lipid Metabolism

Lipid metabolism is dependent on B-oxidation, de novo fatty acid synthesis, fatty acid uptake, esterification, and fatty acid degradation. The cell's energy status, substrate availability, and hormones regulate the balance between synthesis and breakdown of adipose.

Fatty acid synthesis takes place in adipose and liver tissue when substrates are abundant and energy levels are elevated from oxidative phosphorylation. Increased substrates result from acetyl CoA accumulation after conversion from pyruvate (Berg et al., 2007). The accumulation of acetyl CoA regulates acetyl CoA carboxylase (ACC) to

produce malonyl CoA, a precursor to fatty acid synthesis. The process requires NADPH, which comes from the pentose phosphate pathway during the transfer of acetyl CoA into the cytosol, where fatty acid synthesis takes place (Berg et al., 2007). Acetyl CoA carboxylase is activated by dephosphorylation by AMP-activated protein kinase. Insulin also stimulates fatty acid synthesis (Stump et al., 2006).

Fatty acid degradation occurs when substrates are scarce and there is a demand for energy (Stump et al., 2006). The hormone glucagon facilitates the breakdown of triglycerides by stimulating hormone sensitive lipase (Berg et al., 2007). Lipoprotein lipase (LPL) hydrolyzes triglycerides from adipose into free fatty acids and monoacylglycerol. Glycerol can then be used during gluconeogenesis to form glucose to meet energy demands (Berg et al., 2007). Fatty acids are oxidized to acetyl-CoA by B-oxidation. The fatty acids must first be activated by conversion to acyl carnitine by a series of reactions before it can enter the mitochondria through carnitine acyltransferase (Berg et al., 2007). The goal of fatty acid oxidation is to generate acetyl-CoA and high-energy electrons. Fatty acids are broken down two carbons at a time to form acetyl-CoA and high-energy electron carriers such as NADH and FADH2. The NADH and FADH2 are used in the electron transport chain (ETC) for oxidative phosphorylation to generate ATP. Acetyl-CoA can also enter the TCA cycle to produce more high-energy electrons that will ultimately become ATP at the ETC (Berg et al., 2007).

Heat stress effects on metabolism

Carbohydrate metabolism

Carbohydrate metabolism is significantly altered during heat stress, which may contribute to altered carcass composition. This phenomenon has been observed in multiple species over the last 40 years (Verstegen et al., 1978; Heath, 1983; Pearce et al., 2013b). Heat stress causes a decrease in voluntary feed intake and a repartitioning of nutrients to maintain euthermia instead of growth. Typically during feed-restricted thermo-neutral conditions, animals experience decreased glucose utilization, decreased circulating insulin concentrations, and increased fatty acid mobilization, while still depositing protein at the expense of lipids (Baumgard and Rhoads, 2013; Pearce et al., 2013b). However, during heat stress, glucose utilization increases, circulating insulin concentrations are elevated, and fatty acid oxidation decreases, despite the decreased feed intake, indicating a shift in metabolism independent of decreased nutrition. To support this claim, heat stressed animals lose less body weight and condition compared to pair-fed counterparts, which had a continuous decrease in body weight and average daily gain (Pearce et al., 2013b).

During heat stress, carbohydrate metabolism shifts away from oxidation toward glycolysis to generate rapid ATP. Skeletal muscle shifts pyruvate away from the TCA cycle toward lactate, which acts to decrease lipolysis and decrease circulating non-esterified fatty acid (NEFA) concentrations (Brooks, 2009). Increased basal insulin concentrations during heat stress, may account for decreased adipose mobilization (Pearce et al., 2013b).

Human athletes exercising in heat have increased hepatic glucose production and enhanced carbohydrate oxidation at the expense of lipids (Baumgard and Rhoads, 2013). Muscle and hepatic glucose levels are elevated even after a meal during heat stress, and

not blunted by exogenous glucose (Angus et al., 2001). Increased glucose outputs originate from glycogenolysis and gluconeogenesis. Pyruvate carboxylase, an enzyme that controls lactate and alanine entry into the gluconeogenic pathway is increased during heat stress (Wheelock et al., 2008; Rhoads et al., 2008). Furthermore, studies demonstrate that lactate's contribution to gluconeogenesis is increased in heat stressed rodents (Collins et al., 1980; Hall et al., 1980). Therefore, increases in glycolysis and the Cori cycle may be an important mechanism to maintain glucose and energy homeostasis during heat stress in peripheral tissues (Baumgard and Rhoads, 2012).

Lipid metabolism

The effects of heat stress on lipid metabolism are intriguing and paradoxical. During heat stress, the enzyme lipoprotein lipase (LPL) increases in adipose tissue. Lipoprotein lipase is responsible for increased liberation of fatty acids from circulating hepatic-derived triglyceride storage (Berg et al., 2007). During a catabolic state, free fatty acid (FFA) circulation should be further emphasized by the release of hormones: epinephrine, cortisol, and glucagon (Baumgard and Rhoads, 2013). However, during a heat challenge adipose mobilization decreases, as evidenced by reduced lipolytic rates and enzyme activity seen in chickens and swine (Geraert et al., 1996; Heath et al., 1983). The lack of adipose mobilization means fatty acids are inaccessible fuel sources and carbohydrates must be utilized. Additionally, decreased circulating NEFA levels are seen, which are independent of plane of nutrition (Pearce et al., 2013b). The reduction of lipolytic activity may be due to the increased insulin secretion exhibited during heat

stress, which is an anabolic hormone. As lipid retention increases in heat stressed animals the carcass quality decreases, negatively impacting production.

Protein metabolism

Protein turnover is a balance between anabolism and catabolism. Anabolism is the formation of proteins utilizing dietary amino acids. Catabolism is protein breakdown to create amino groups, which are then used to synthesize urea, protein synthesis, or carbon skeletons for glucose and fatty acid synthesis.

Dietary amino acids are used to synthesize proteins for muscle growth, membrane glycoproteins, act as precursors for synthesis of DNA/RNA, and enzymes used in numerous biochemical processes (Young, 1976). Protein synthesis is regulated in part by amino acid availability, ATP levels, insulin, and IGF-1. Due to the mass of skeletal muscle, protein synthesis is an energetically expensive endeavor. Protein synthesis is a multistep process after the initiation of transcription. Messenger RNA (mRNA) contains a code for specific polypeptide sequences and acts as a template for synthesis of amino acid chains at the ribosome. The sequence leads to formation of a complex protein structure (Berg et al., 2007).

Heat stress appears to induce decreased muscle synthesis as well as increased muscle breakdown, which results in less lean accretion and poor production efficiency. The machinery for protein synthesis and RNA/DNA synthesis capacities are reduced by environmental hyperthermia (Streffer et al., 1982). Acute and chronic infection affects skeletal muscle, where protein synthesis is impaired and protein degradation is enhanced

(Escobar et al., 2004). Increased skeletal muscle catabolism during heat stress is indicated, in multiple species, by increased plasma markers such as: NtMH, BUN, and creatinine (Hall et al., 1980; Yunianto et al., 1997; Pearce et al., 2013b). Immediate and sustained increases in plasma creatine are seen in swine, independent of plane of nutrition. During hyperthermia, despite elevated insulin levels and its typical anti-proteolytic effects, skeletal muscle is still degraded (Pearce et al., 2013b).

Skeletal muscle makes up about 60-70% of body mass requiring energy for growth. Heat stress multiplies energy demand by mounting a heat shock response via heat shock proteins (HSP). Heat shock proteins are a cellular response to a wide range of stressors and are rapidly activated to protect against lethal injury (Dokladny et al., 2006). Hyperthermia results in the degradation and aggregation of extensive intracellular proteins, especially cytoskeleton (Yan et al., 2006). Heat shock proteins aid in protein refolding, targeting damaged proteins for degradation, protecting proteins against aggregation, maintaining overall integrity of cellular components, and modulating immune responses (Geiger and Gupte, 2011). An *in vitro* cell culture experiment using heat stressed Caco2 cells treated with quercetin, a HSP inhibitor, demonstrated decreased protective effects of HSP70 during heat-induced stress (Dokladny et al., 2006). HSP70 helps with protein folding during translation by stabilizing the proteins as they are synthesized (Moseley, 1998). HSP acclimation in cells is due to activation of HSP from a prior stressor, such as cytokines, rendering the cells resistant to further cytokine actions and acts to inhibit cytokine production (Moseley, 1998).

Heat stress effects on endocrine function

Somatotropin (ST) and insulin-like growth factor-1 (IGF-1) are potent lactogenic hormones (Baumgard and Rhoads, 2013). Somatotropin partitions nutrients toward the mammary gland, decreases nutrient uptake by extra-mammary tissues, and stimulates hepatic IGF-1 synthesis and secretion (Baumgard and Rhoads, 2013). During a negative energy balance (NEBAL), the somatotropic axis is uncoupled and hepatic IGF-1 synthesis is decreased despite elevated circulating ST concentrations (Rhoads et al, 2008). Individuals in NEBAL caused by heat stress tend to have reduced circulating ST concentrations (Rhoads et al, 2008). It is well established that fasting and severe malnutrition (energy and protein) result in a precipitous decline in circulating IGF-1 concentrations and an increase in circulating ST levels in most species (Rhoads et al, 2008). The inverse relationship is referred to as an uncoupling of the ST-IGF axis and is caused in part by a reduction in ST-dependant hepatic IGF-1 production. Heat stress reduces hepatic somatotropin receptor abundance, independent of nutrition, whereas, STAT-5 phosphorylation decreased during both heat stress and malnutrition (Rhoads et al., 2010). Hepatic IGF-1 mRNA abundance was lower in heat-stressed animals. Baumgard and Rhoads (2013) hypothesize that reduced IGF-1 may be a mechanism in which the liver and mammary tissues reduce milk synthesis and use nutrients for other purposes such as maintaining euthermia.

Growth hormone (GH) and IGF-1 interact with insulin to modulate its control of carbohydrate metabolism (Clemmons et al., 2004). During starvation periods, excessive GH secretion stimulates lipolysis, providing FFA's and glycerol as substrates for energy metabolism and inhibits insulin-induced suppression of hepatic gluconeogenesis by inducing resistance to downstream signaling molecules (Clemmons et al., 2004).

Glucose and insulin secretion dynamics result in counter-regulatory responses, and modifications in GH and IGF-1 function alters insulin's ability to maintain normal carbohydrate homeostasis. Low concentrations of serum IGF-1 and blocking GH action, results in improvement in insulin sensitivity (Clemmons et al., 2004). Growth hormone controls growth by regulating IGF-1 concentration (Clemmons et al., 2004).

Basal insulin is increased during heat stress conditions, which seems contradictory because heat stress is a catabolic condition whereas insulin is an anabolic hormone. Up regulation of insulin may play a key role in activating and up regulating HSP's, possibly causing a heat protective response (Baumgard and Rhoads, 2013). Increased insulin may also be a response to increased LPS released from damaged intestinal epithelia or due to a pro-inflammatory cytokine response.

Gut permeability

The function of the intestinal barrier is to serve as a first line of defense against the bacteria laden environment within the intestinal lumen. The two routes of movement for solutes across single-layered epithelia are 1) a transcellular route that consists of two barriers, an apical and a basolateral cell membrane; and 2) a paracellular pathway that can be considered as either a single barrier, the tight junction, or as two barriers, the tight junction and the intercellular space (Blikslager et al., 2007; Powell, 1981). Epithelial cells present a robust barrier to invasion by bacteria and their toxins, negating passive flow of luminal contents by their remarkably high trans-epithelial resistance (TER) compared with the paracellular space. Of the junctions that define the paracellular space, the apical-most tight junctions are principally responsible for regulating paracellular

permeability, although adherens junctions and desmosomes also play critical cellular functions. The structure of tight junctions is a series of pinpoint contacts between the apical-lateral membranes of adjacent cells forming a continuous belt of branching fibrils surrounding each cell (Blikslager et al., 2007). The tight junction fibrils seem to be composed of two rows of integral membrane proteins drawn from the plasma membranes of two adjacent cells (Powell, 1981), which bind to cytoplasmic plaque proteins anchoring the tight junction to the cytoskeleton (Blikslager et al., 2007). Tight junctions polarize the cell into apical and basolateral regions and regulate passive diffusion of solutes and macromolecules (Blikslager et al., 2007). The most sensitive mucosal barrier function measurement is TER, which measures the degree at which ions transverse tissue, predominately through the paracellular mode (Blikslager et al., 2007).

In leaky epithelium, the tight junction is the most important determinate of paracellular pathway resistance (Blikslager et al., 2007), probably due to tight junction proper when the lateral spaces are dilated and the intracellular spaces collapse (Powell, 1981). The swelling of the cells, an event that occurs during cyclic-AMP- induced secretion in small intestine, may close the intercellular space in a manner similar to that exerted by hypertonic mucosal solutions in leaky tissue (Powell, 1981). Tight junctions in crypts are leakier than those in the villus because crypts have higher linear density of paracellular pathways due to cell alignment and fewer, less organized fibril junctions than that of the complex villus structure. In fact, crypts are attributable to 63-73% of paracellular permeability in small intestine (Blikslager et al., 2007). Regardless of mechanism, increased intestinal permeation is devastating to the host's body.

Heat stress effects on gut permeability

During prolonged endurance training or heat stress, the body is forced to dissipate excess heat to maintain a euthermic homeostasis. To maximize radiant heat dissipation blood flow and nutrients are diverted from the splanchnic tissue of the intestine, via vasoconstriction, to the peripheral tissue near the skin (Pearce et al., 2012, 2013a; Lambert, 2009; Sawka et al., 2006). The reduced blood and nutrient flow to the intestinal epithelium compromises the intestinal barrier integrity (Pearce et al., 2013a). Reduced blood flow results in hypoxia to splanchnic tissue which leads to ATP depletion, acidosis, and altered ion pump activity (Lambert, 2009). Pearce and colleagues (2013a) speculate that ion pumps are up regulated during heat stress to help maintain osmotic homeostasis in the intestinal tract. Glucose transport and digestive capacity are altered during acute heat stress (Pearce et al., 2012). Heat stress increased Na+/K+ ATPase activity in the intestinal tract, which is an energetically expensive process. If ion balance and osmolarity are not tightly regulated it may result in perturbation of enzyme function, protein structure and membrane integrity, and water retention.

External stress, including physical stress like prolonged endurance exercise and heat stress, disrupts the tight junctions in the paracellular pathway between enterocytes of the intestinal epithelium. Damage to intestinal integrity may be, in part, to increase reactive oxygen species (ROS; Lambert, 2009). Pearce and colleagues (2013a) showed that jejunum morphology was significantly affected by prolonged heat exposure. For example, villus height and villus: crypt ratios were decreased (linear and quadratic manor) in heat stressed pigs when compared to thermo-neutral pigs (Pearce et al., 2013a).

However, intestinal morphological changes during heat stress can be confounded due to damages done by decreased feed intake.

The alterations in intestinal morphology leads to increased permeability within the gastrointestinal (GI) tract and potential lipopolysaccharide (LPS) release into the blood stream. LPS is an immuogenic component of the outer membrane of Gram-negative bacteria, including those found in high concentration in the intestinal tract (Lambert, 2009). The release of endotoxin (LPS) into the blood stream (via bacterial translocation) causes local and/ or systemic inflammatory responses (Lambert, 2009). Increased blood LPS levels are a function of both increased intestinal permeability and reduced LPS detoxification, neutralization or clearance from the body (Pearce et al., 2013a). The intestines of heat stressed pigs are more permeable to LPS than thermoneutral and their pair-fed thermoneutral counterparts. The heat stress induced intestinal permeability increases blood markers of endotoxemia, hypoxia, and inflammation (Hall et al., 2001). Inflammation is stimulated by pro-inflammatory cytokines like: TNF-α, IL-6, IL-1α, IL-1B, IFN-γ (Lambert, 2009). LPS is a potent stimulator of TNF-α release (Bouchama et al., 1991). TNF-α and IL-1α are endogenous pyrogens that produce fever and mediate shock (Kluger et al., 1997; Bouchama et al., 1991). Fever is a natural response to infection by the host (Dokladny et al., 2006). If the fever persists at an elevated temperature for a prolonged time it may result in multi system organ failure and death.

Heat stress effects on immune response

Heatstroke is a form of hyperthermia accompanied by acute physiological alterations, hyperthermia-caused cytotoxicity, systemic inflammation, oxidative damage,

and attenuated heat shock response and further multi-organ dysfunction (Yan et al., 2006). Hyperpyrexia or fever can induce cells secreting cytokines or promoting cytokine transcription, such as TNF-α, IL-1, Il-2, IL-6, IL-8, IL-10 (Yan et al., 2006). Cytokines are intercellular chemical messengers released by a variety of cell types, including macrophages, T and B cells, endothelial cells, and astrocytes (Leon, 2007). HSP play a protective role on cells from damage due to heat, hypoxia, endotoxin, and cytokines (Yan et al., 2006). Accumulation of HSP in a cell down regulates the production and secretion of TNF-α and IL-1 (Yan et al., 2006). HSP's ability to interact with pattern recognition receptors, such as TLR4, to induce cytokine release suggest that interactions between HSP's and the host immune system may be an important first line of defense against infection and inflammation (Leon, 2007). Proteins in the HSP70 family have been extensively studied for their protective function(s) against a variety of stressful insults including heat stress, ischemia, tissue injury, metabolic stress like glucose deprivation, and sepsis (Leon, 2007). HSP gene expression level increases as core temperature increases and modulates response by decreasing TNF-α during infection to increase febrile response (Leon, 2007). IL-6 may be produced in the skeletal muscle during hyperthermia, which has been shown in rats (Welc et al., 2013). Increases in IL-6 have been seen in the majority of heat stroke patients and positively correlate with mortality, post cooling elevated IL-6 relates to non-survival (Leon, 2007). Skeletal muscle responds to heat stress with a distinct "stress induced immune response" characterized by an early up regulation of IL-6, IL-10 and TLR4 and suppression of IL-1B and TNF-α mRNA, a pattern discrete from the classic innate immune response. Endotoxin induces the innate cytokine response with a down regulation of TLR4, up regulation of TNF-α and IL-1B

and then IL-6 and IL-10 also become up regulated (Welc et al., 2013) (Figure 1.1). Later heat stress response shows elevated TNF-α and IL-1B, which may be induced by late phase elevation of pathogen associated molecular patterns (PAMP) to stimulate toll-like receptors (TLR) to induce cytokine profile (Welc et al., 2013).

Heat stroke cause changes in immune status such as disturbances in leukocyte distribution, production of cytokines, and bacterial translocation following gut ischemia that may predispose to early infection (Leon, 2007). B and T lymphocyte synthesis was depressed during heat stress as well as phagocytic activity and number of blood leukocytes (Mashaly et al., 2004). Humoral immunity was also reduced during heat stress, which was seen by decreased antibody titers, this may be due to increased inflammatory cytokines during stress conditions (Mashaly et al., 2004). Endogenous cytokines may be important mediators in the systemic inflammatory response syndrome in heat stroke patients. Although elevated cytokine levels appear to be detrimental during a heat challenge, cytokine receptor knockout mice show that no response to the endogenous cytokine, specifically IL-6 and TNF−α, is more lethal (Leon, 2007). IL-10 appears to have protective effects on vascular permeability with endotoxemia due to inhibition on leukocyte recruitment evident in IL-10 knockout mice with greater leukocyte recruitment (Leon, 2007). IL-10 is an anti-inflammatory cytokine with potent inhibitory effects on pro-inflammatory cytokines IL-1, IL-6, IFN−γ, and TNF.

PRRS virus immune challenge in industry

Immune challenge is yet another factor that negatively impacts the swine industry in the U.S. and worldwide. An immune challenge that plagues the current swine industry

is Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). It was formerly know by many names, one of which was "mystery swine disease" when it was first seen in 1987 (Neumann et al., 2005). The mystery disease was characterized by respiratory disease (pneumonia), severe reproductive loss or failures, reduced growth, and increased mortality, with infection occurring regardless of age. The disease quickly spread to most pork producing countries in a short span of a few years (1988-1992). Once outbreak had occurred, the quick spread is believed to be due to management changes in the swine industry from small to larger herds, horizontal integration, larger companies in charge of breeding stock, increased transport of live pigs within and between countries, and greater use of artificial insemination (Zimmerman et al., 2003). In 1991, using retrospective serum samples, the disease was determined to be caused by a novel enveloped RNA virus from the genus Arterivirus, family Arteriviridae, termed Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) (Neumann et al., 2005; Zimmerman et al., 2003; ISU-CVM, 2013). The origin of the virus is unknown although it is believed to have transferred across species. Discovery of the RNA virus was difficult due to common co-infection and heterogeneity in the genome because of inherent errors common in transcription of RNA. There are two distinct forms that are genetically different but, clinically similar; LV –Leylstad in Europe and VR-2332 in the U.S. (ISU-CVM, 2013; Morgan Morrow et al., 2001). Multiple strains assure adaptability to changing environments (Morgan Morrow et al., 2001). Regardless of strain PRRSV has detrimental effects on the swine industry.

PRRSV is the most economically significant disease to affect US swine production since the eradication of classic swine fever (ISU-CVM, 2013). In 1961,

classic swine fever (hog cholera) cost US producers $57.4 million annually; $15.4 million for disease and $42 million for preventative measures. The economic impact adjusted for inflation is equivalent to $364.1 million in the year 2004 dollars (Neumann et al., 2005). A study based on USDA surveys and multiple farms, of varying production types and sizes, estimated total annual economic impact of PRRSV on swine producers to be $560.32; $66.75 million in breeding herds and $493.57 million in growing pig populations. Economic estimates are measured by a decrease in reproductive health, increased death, and reduction in the rate and efficiency of growth, but do not include increased prevention and treatment costs (Neumann et al., 2005).

Sows and gilts of breeding age infected with PRRSV can experience periods of anorexia, fever, lethargy, depression, respiratory distress, and vomiting. In some cases bluing (cyanosis) of the ears, abdomen, and vuvla have been seen. However, in breeding operations infected with PRRSV, reproductive problems are most obvious, including decreased number of sows that conceive or farrow, increased premature farrowing, late term abortions, stillborn, mummified fetuses, or weak piglets, but can also have unexpected returns to estrus. Rate of stillborns can reach up to 75%, as an outbreak progresses more mummified fetuses and less stillbirths are observed (ISU-CVM, 2013). Decreased litter size can also be due to embryonic death during early gestation or preweaned pig mortality. Between 3 studies of PRRSV outbreak, economic impact average was $255 per breeding sow, some as high as $302 per sow (Neumann et al., 2005; Zimmerman et al., 2003). The primary impacts arising from reduced number of piglets produced and a 3.8 reduction from baseline of pigs weaned per pig per year (Neumann et al., 2005). In breeding herds reproductive failure mainly affect sows and

gilts but can also affect boars. Due to infection, boars can have decreased libido and semen quality as well as shedding in semen (ISU-CVM, 2013). Outbreak typical duration is 2-3 months, but signs may be cyclical in large operations.

In nursery to finishing pigs the average cost attributed to infection is due to increased morbidity and mortality, decreased growth and feed efficiency, and decreased carcass quality. According to a study done by Neumann et al. (2005), nursery pigs ADG decreased by 25.29%, daily feed intake along with feed efficiency also decreased, and an 11.7% increase in amount of feed needed per kilogram of gain, and increased mortality rate by 10.7%. Grow finisher decrease ADG 12%, 7.6% lose of feed efficiency, mean mortality increase 6%. The clinical signs of infection in grow- finish pigs include: fever, depression, lethargy, stunting due to systemic disease, sneezing, and pneumonia, followed by expiratory dyspenea. Peak age for respiratory disease is 4-10 weeks. Piglets that contract PRRSV in late gestation are typically persistently infected, and carry the virus up to 11 weeks in the bloodstream, while intermittently shedding for up to 30 weeks (Zimmerman et al., 2003). Increased cost per pig in endemic infection with PRRS for nursery pigs is $11.50 per pig marketed, with a further increase in costs due to health care for prevention and treatment. Animal health expenses during the peak of the outbreak were 4 times greater than the costs before the PRRSV outbreak, and expenses related to secondary infections increased by 60% during the 12 month period after the outbreak (Zimmerman et al., 2003). Infection leads to lesions in the lungs and lymph nodes, other infections commonly coincide with PRRSV (ISU-CVM, 2013). In the growing pig PRRSV is most harmful in combination with other pathogenic viruses, bacteria, and

mycoplasma hyponeumonia (Zimmerman et al., 2003). It can be difficult to separate the financial impacts of PRRSV from dual infections.

The financial losses continue with the persistence of an outbreak, which due to PRRSV ability to be both an epidemic and endemic within a herd have the potential to be devastating (ISU-CVM, 2013). PRRSV has a slow infection, or rolling period of symptom display, meaning not every animal becomes infected at once, unlike most viruses that quickly spread to all animals (Zimmerman et al., 2003). The severity of the disease can range from lack of clinical signs to severe symptoms, variations are due to 1) virulence of virus, 2) whether it is an initial infection or ongoing, 3) age, 4) other disease causing agents present in the population, and 5) herd size and management practices (Zimmerman et al., 2003). Viral infection has the ability to persist in long-term carrier pigs (greater than 200 days), but pigs typically become immune by a steady decline in antibody titers over a period of time after infection, and then stop shedding by day 60 post-infection (ISU-CVM, 2013).

The virus is highly infectious with a dose as small as 10 virions when exposed intranasally or intramuscularly, but not highly contagious (Morgan Morrow et al., 2001). PRRSV spreads via direct contact. Nasal secretion, urine, semen, mammary secretion and feces all contain transmissible virions. Post-infection, pigs will shed the virus for extended periods in salvia (42 days), semen (68 days), fecal shedding can be continuous for ~40 days or intermittent (Morgan Morrow et al., 2001). Transmission is most common during the acute phases where the virus is being shed in high quantities compared with chronic stages where the virus replicates in susceptible cells for months (Zimmerman et al., 2003). Biosecurity between swine production phases is important, as

it is easily transferable by vertical infection, from one generation to the next, by the dam in utero to the fetus or between the boar and sow during mating. Infection can also be passed horizontally between pen mates and in-between production phase transitions in the environment (ISU-CVM, 2013).

Although there are no known replication-competent insect vectors, mosquitoes and houseflies can act as fomites to spread the virus mechanically. PRRS is only moderately resistant to environmental degradation and easily inactivated by most disinfectants (ISU-CVM, 2013). If conditions are suitable (4°C, pH 7.5) the virus can survive days to weeks (Morgan Morrow et al., 2001). The virus can survive 11 days in water but is highly susceptible to drying out, only surviving about one day on typical fomites such as wood, steel, and straw.

PRRSV is transmitted to the tonsil or upper respiratory system by infection of alveolar macrophages then migrates to regional lymphoid tissue to replicate (Escobar et al., 2004). Once the virus reaches circulation in the bloodstream and monocytes, it can persist for several weeks. Success of the PRRS virus as a pathogen is complex but may be ascribed to the ability of the virus to replicate for months in monocyte- derived cells in the face of an active immune response (Neumann et al., 2005). Recovered sows are resistant to the same strain but may show clinical signs to a heterologous strain if encountered (ISU-CVM, 2013). Once a herd is infected it will indefinitely cycle through the herd, persisting 2.5 years after a PRRSV outbreak.

Detection of PRRSV on a farm can be difficult to discern between other stressors, therefore must be confirmed in a lab using tissue or serum samples. The best tissues for viral detection include: brochoalveloar lavage, serum, lung, lymph nodes, tonsil, and

spleen (ISU-CVM, 2013). Virus can be isolated from serum 4-6 weeks post infection in young pigs but as little as 1-2 weeks post infection in sows and boars (Morgan Morrow et al., 2001). PRRSV antigens can be detected using fluorescent antibody tests, immunohistochemistry, or PCR to detect PRRS virus genome. An advantage of the PCR method is the test method is independent or not influenced by the presence of either maternal antibodies or vaccine induced antibodies (Duinhof et al., 2011).

Immune challenge effects on swine

Immunological stress from bacteria or viruses negatively impact swine in multiple physiological responses. Initially there is a decrease in voluntary feed intake and an increase in body temperature and respiration rates. The reduced feed intake decreases weight gain and feed efficiency as seen in pigs and poultry (Hevener et al., 1999) When considering the decreased growth performance of a LPS-induced immune challenge approximately two thirds of the decrease was directly due to reduced nutrient intake, while one third was due to decreased efficiency of nutrient use in growth (Dritz et al., 1996). Immunological stress directs metabolism away from growth to provide energy to mount an immune response (Robert et al., 2003). Inflammation and acute phase response play an integral part in immune system with the nonspecific responses impacting growth and nutrient partitioning, mediated by cytokines (Dritz et al., 1996). The immune system activates macrophages to initiate fever and reduce appetite. The induction of a febrile response is necessary to initiate countermeasures to control and eliminate invading pathogens and to prevent overstimulation of the immune system (Carroll et al., 2012).

Immunity and cytokines

The immune system has two types of response to a challenge, the innate immune system and the adaptive immune system. The innate immune system is an immediate response with no cell specific recognition and no memory, where as the adaptive immune system is cell specific and takes 4-7 days to mount a response. The innate immune system has a humoral complement system that opsonizes and kills pathogens through the pathogen associated molecular patterns (PAMP) recognition mechanism. These highly conserved soluble membrane bound proteins are collectively called pattern-recognition receptors (PRR). The PAMP/ PRR interaction triggers the innate immune response (Jin and Flavell, 2013). Once activated the innate immune system acts as a first line of defense by activating macrophages which release cytokines to produce a pro-inflammatory response to destroy invaders.

A specific type of PRR is the toll-like receptors (TLR), which are found on macrophages, mast cells, and dendritic cells, all essential to the innate immune system (Leaver et al., 2007). Toll-like receptor's are transmembrane proteins, which recognize invading microbes when TLR ligands bind to the receptor and activate signal pathways that alter the pattern of gene expression in the cells, launching immune and inflammatory responses to destroy the pathogen (Leaver et al., 2007). The pattern of activation for all surface TLR's are general, but the subsequent intracellular signal cascade, which include a number of transcription factor activations, are unique for each TLR to mount the best response to a specific pathogen (Jin and Flavell, 2013). Toll-like receptors are specific for diverse ligands including: bacterial cell wall components, viral double stranded RNA, and small molecule anti-viral compounds. Specifically TLR4 is outer membrane

associated and responds to LPS from gram-negative bacteria (Jin and Flavell, 2013). Toll-like receptor three is found on the endosome surface and is activated by double stranded viral RNA (Miller et al., 2009). Toll-like receptors leads to activation of the transcription factor nuclear factor-KB (NF-KB), mitogen activated protein kinase (MAPK) p38, and c-Jun N-terminal kinase (JNK) (Jin and Flavell, 2013).

The pro-inflammatory cytokines responsible for metabolic effects to maintain homeostasis during immune challenge are TNF-α, IL-1, and IL-6 in rodents and most species, which are activated by macrophages (Webel et al., 1997). TNF-α, IL-1, and IL-6 interact in multiple ways to produce inflammation and fever to destroy invading pathogens. TNF-α can increase lethargy and sickness behavior (Carroll et al., 2012). IL-1 and other pro-inflammatory cytokines elicit the syntheses of acute-phase proteins, which increase rapidly during inflammation (Dritz et al., 1996). Elevated IL-6 and IL-1B are associated with reduction in feed intake and growth (Escobar et al., 2004). During an immune challenge cytokines can be responsible for reduced appetite and feed intake, inhibit nutrient absorption, increase metabolic rate, and alter nutrient utilization (Escobar et al., 2004). However, excessive cytokine numbers can cause tissue damage and potentially more harm to the host.

Immunological cytokine effect on metabolism

Immunological stress due to infection, inflammation, and/or trauma can stimulate a variety of cells, including lymphocytes and macrophages, to secrete cytokines responsible for altering the host's metabolism (Johnson, 1997). Specifically IL-1, IL-6,

and TNF-α have been found to: mediate metabolism of carbohydrates, fats, and proteins, regulate hypothalamic-pituitary outflow, and act on the brain to suppress appetite (Johnson, 1997). During an immune challenge voluntary feed intake is reduced and nutrient partitioning is shifted away from skeletal muscle accretion toward mounting an immune response (Robert et al., 2003). Growth rate of animals affected by immune challenge are believed to be a result of the pro-inflammatory cytokines, which increase muscle degradation and increase lipolysis.

Immune response increases muscle degradation apart from feed deprivation by inducing cytokines. The pro-inflammatory cytokines act directly on the skeletal muscle to inhibit protein accretion and accelerate protein degradation. Lipopolysaccharide endotoxemia changes protein synthesis by altering the ratio of RNA to protein and protein synthesis efficiency. Proteins are lost from muscle and the amino acids are utilized in the liver for gluconeogenesis and synthesis of acute phase proteins to support immune function. IL-1 and TNF-α both stimulate amino acid release from muscle, increased uptake of amino acids by the liver, and increase hepatic synthesis of acute phase proteins (Argiles et al., 1990). IL-6 stimulates hepatocytes to increase amino acid uptake. Plasma urea nitrogen, an indicator of amino acid catabolism, increases 8 to 12 hrs after cytokine administration in pigs (Webel et al., 1997). Cytokines can induce a variety of endocrine responses that are also likely to induce proteolysis, such as IL-1 stimulating glucocorticoids, which typically has a negative feedback on overeating (Webel et al., 1997). Glucocorticoids can also produce increased circulating glucose via glycogenolysis as well as, tissue specific metabolic effects such as in liver to increase gluconeogenesis and protein synthesis, while in muscle and adipose they induce proteolysis and lipolysis

(Webel et al., 1997). Infection can also be associated with alterations in lipid and glucose metabolism. In rodents TNF-α, IL-1, and IL-6 induce hypertriglyceridemia by decreasing muscle and adipose lipoprotein lipase (LPL) activity and increasing fatty acid synthesis (Argiles et al., 1990). TNF-α has been shown to inhibit synthesis of LPL in transformed mouse fibroblast and adipocyte cells and stimulate lipolysis (Argiles et al., 1990).

Immune challenge effects on growth hormone

Immunological stress can negatively impact animal growth in multiple ways. Immune response depresses feed intake, which will decrease growth. Insulin-like growth factor-1 (IGF-1), which is important to increase growth rates, can be influenced by feed intake and changes in metabolic demand (Hevener et al., 1999). During an immune challenge IGF-1 is reduced for a short time but no long-term effects have been seen (Hevener et al., 1999). Rats challenged with LPS or IL-1B have reduced IGF-1 in plasma, liver, skeletal muscle, and pituitary, as well as reduced IGF-1 mRNA abundance in liver and skeletal muscle (Spurlock, 1997). The anabolic action of GH is accomplished in part by the induction of IGF-1 in liver and skeletal muscle. IGF-1 and GH form the somatotropin axis and are positively correlated under normal body conditions. However, during immune challenge the linkage between GH and IGF-1 becomes uncoupled, evident by GH returning to baseline and IGF-1 still being depressed (Spurlock, 1997). Immunological stress has been shown to suppress GH concentrations and pulsatile release and is linked to cytokines IL-1 and IL-1B by multiple reasons: 1) intravenous delivery of IL-1 receptor antagonist neutralizes the effect of endotoxin in GH concentrations 2) release of GH releasing hormone (GHRH) from medial basal

hypothalamus explants is decreased by IL-1B where as somatotropin release is increased cytokines act directly on skeletal muscle to reduce the efficacy of anabolic hormones such as IGF-1 and insulin. Insulin is typically reduced during immune challenge (Spurlock, 1997).

Inflammation and muscle wasting in several conditions have been characterized by elevated myostatin (MSTN). Myostatin is a negative regulator of muscle mass. An increase of MSTN might partially explain why infection inhibits growth (Escobar et al., 2004). "Inflammatory cytokines may activate MSTN transcription directly via NF-KB activation or indirectly through glucocorticoid release (Escobar et al., 2004)." Reduced appetite, long term, also stimulates MSTN expression with similar decrease in IGF-1 (Escobar et al., 2004). Over expression of myostatin attenuates the IGF-1-mediated increase in myotube or fiber diameter as well as IGF-1 stimulated Akt phosphorylation stimulating muscle growth in C2C12 cell lines (Morissette et al., 2009). Myostatin infection in cells could block IGF-1 hypertrophy, but increase Akt activity could overcome MSTN inhibition of hypertrophy (Morissette et al., 2009).

Summary

The swine industry is sensitive to environmental and immune challenges such as heat and PRRS virus. As described above, each challenge causes detrimental losses in profit, production, and health status to the animals. A major contributor to these losses is the decreased feed intake experienced in animals challenged with either heat or PRRSV. As expected, the animal's growth is inhibited directly by reduced intake but further challenged as nutrients are diverted away from growth in response to a challenge. In heat

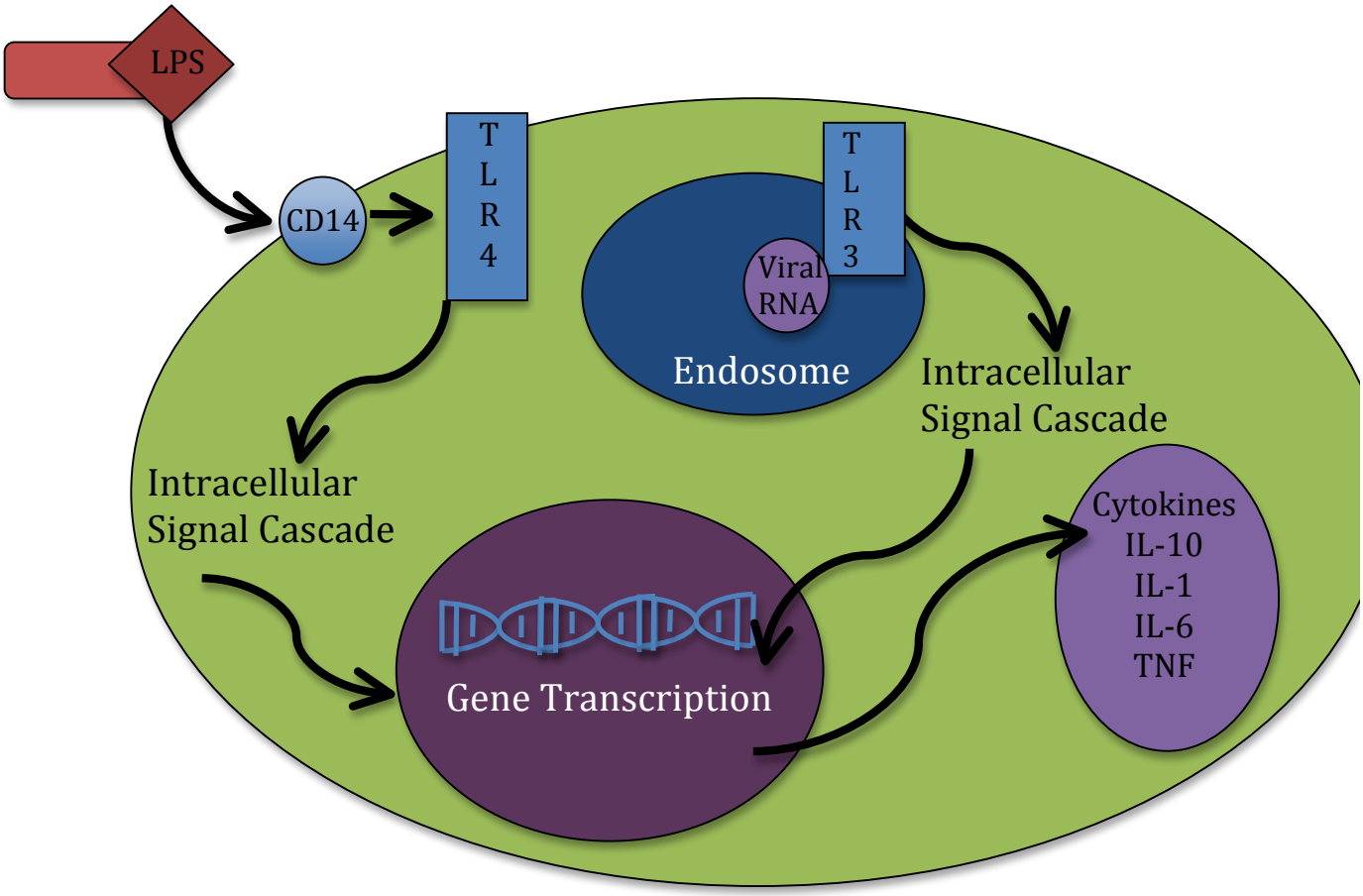
stressed pigs nutrients are partitioned toward maintaining euthermia and producing pro-inflammatory cytokines. The cytokine production has not been well defined but is believed to be due to decreased intestinal integrity as blood is diverted away from the splanchnic tissue toward to skin to dissipate excess heat (Lambert, 2009). During an immune challenge such as PRRSV, nutrients are diverted toward mounting an innate immune response, which includes a febrile response and inflammatory cytokine production (Johnson, 1997).

Decreased nutrient availability in both heat stress and PRRSV challenged pigs occurs as a result of an increased energy demand to maintain body function and reduced feed intake. Clearly, metabolism is affected by energy intake, energy demand and availability of either a carbohydrate or fatty acid source, which can alter production. Typically, when plenty of food is consumed, the body preferentially uses carbohydrates as a source of energy. However, when carbohydrates like glucose are scarce, the body must breakdown fatty acids as a source of energy. Fatty acid breakdown occurs during an immune challenge. Interestingly, during heat stress fatty acids are not mobilized as expected and a shift to glycolytic metabolism is likely to occur (Baumgard and Rhoads, 2012). Both challenges are marked by muscle protein degradation as the amino acids are needed to synthesis acute phase proteins, cytokines, and glucose, via gluconeogenesis, in the liver and kidneys (Webel et al., 1997; Baumgard and Rhoads, 2013).

Metabolism and growth can be further affected by cytokine and/or hormonal changes seen during either heat stress or PRRSV challenge. The overall aim is to determine the effect that a dual challenge of heat stress and PRRS viral challenge has on

metabolism, cytokine production, and hormone changes on the production of growing pigs.

Figure 1.1: Endotoxin and Viral RNA initiation of Toll-like receptors and cytokine production. The innate immune system is activate by pathogen associated molecular patterns (PAMPs) which attach to pattern-recognition receptors, specifically Toll-like receptors to initiate a downstream intracellular signal cascade that alters the pattern of gene expression in the cells, launching immune and inflammatory responses to destroy the pathogen. Specific PAMPs trigger specific Toll-like receptors, such as LPS activating TLR4, while TLR3 is activated by viral RNA.



Chapter II

Effects of a dual heat stress and immune challenge on whole body metabolism in growing pigs

Abstract

Heat stress and immune challenges negatively impact nutrient allocation and metabolism in swine, especially due to elevated heat load. In order to assess the effects of a dual heat and immune challenge on metabolism, 9 week old crossbred barrows were individually housed, fed ad libitum, divided into four treatments: thermo-neutral (TN), thermo-neutral PRRSV infected (TP), heat stress (HS), and heat stress PRRSV infected (HP), and subjected to two experimental phases. Phase one occurred in TN conditions (22°C) were half the animals were infected with PRRS virus (n=12), while the other half (n=11) remained uninfected. Phase two began, after 10 days with half of the uninfected (n=6) and infected groups (n=6) transported to heated rooms (35°C) for three days of continuous heat, while the rest remained in TN conditions. Blood samples were collected prior to each phase and at trial completion before sacrifice. PPRS viral load indicated only infected animals were infected. Individual rectal temperature (T_r), respiration rates (RR), and feed intakes (FI) were determined daily. Pigs exposed to either challenge had an increased T_r, ($P < 0.0001$) whereas RR increased ($P < 0.0001$) with heat stress, compared to TN. Average daily gain (ADG) and body weight (BW) decreased with challenges compared to TN, with the greatest loss to dual challenged pigs. Markers of muscle degradation such as creatine kinase, creatinine, and urea nitrogen were elevated during challenges. Blood glucose levels tended to decrease in HS pigs. Metabolic flexibility tended to decrease in PRRS infected pigs as well as HS pigs. Fatty acid

oxidation measured by CO_2 production decreased in dual challenged pigs, whereas it increased in HS compared to TN. Taken together, these data indicate dual challenged pigs experienced the most detrimental physiological impacts, than either PRRSV or heat stress alone.

Introduction

While global populations continue to increase more people are living in tropical or subtropical locations were the temperatures are much warmer (Renaudeau et al., 2011). Not only are increased temperatures an issue in the tropics but also in temperate areas where weather patterns are changing toward hotter summer months and more frequent heat waves (U.S. EPA, 2010). The increased temperature index is detrimental to animal agriculture, especially in pigs that lack functional sweat glands to alleviate the heat load resulting in heat stress. Heat stress causes an annual loss of over $300 million to the United States swine industry (St-Pierre et al., 2003). The economic burden induced by heat is due to suboptimal growth rate, inefficient nutrient utilization, decreased carcass composition, and poor reproductive performance (St-Pierre et al., 2003).

Heat stress however, is not the only detrimental factor to the United States swine industry. Pigs often suffer from immune challenges that can be either bacterial or viral infections. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) plagues the current swine industry and has an estimated annual economic impact of over $560 million. Economic estimates account for decreased reproductive health, increased death, and reduction in the rate and efficiency of growth (Neumann et al., 2005).

Developing a strategy to mitigate the effects immune challenge and heat stress on production performance requires a better understanding of how a dual challenge may

affect nutrient allocation, physical production parameters, and protein and lipid metabolism. During both immune and heat challenges feed intake is reduced, with further nutrient repartitioning toward immune responses and heat alleviation respectively. The decreased nutrient availability reduces muscle growth and increases production length. Muscle accretion decreases and muscle degradation increases during both challenges in order to provided amino acids for new molecules to form, such as acute phase proteins and heat shock proteins (Johnson, 1997; Rhoads et al., 2012). Opposite effects are seen during heat stress than during immune challenge with respect to increased lipid retention (Rhoads et al., 2008) verse increased lipid mobilization respectively (Webel et al., 1997). This implies that immune challenge and heat stress could potentially be affecting metabolism in an opposite manner.

The study objectives were to determine effects of a dual challenge of heat stress and PRRS virus on production parameters and metabolism in growing pigs. We hypothesized the dual HS and immune challenge would be more detrimental to swine production in regard to growth and metabolism compared to either challenge alone.

Materials and Methods

Animals and Treatment

Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee approved all procedures involving animals. Uninfected crossbred barrows (n=24) were brought to Litton Reaves Hall at 6 weeks of age and housed in individual pens (with individual feeders and waters) in one of two rooms (12 pens/room) at thermo-neutral conditions (22°C) with a 12hr light and 12hr dark cycle. An antibiotic

free commercial diet was fed ad libitum and provided adequate levels of all essential nutrients (Table 2.1). Animals were allowed to acclimate to the controlled environment in the Virginia Tech facility for three weeks, during which time they were all prescreened for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV; IDEXX, Westbrook, Maine). During the acclimation phase one pig was deemed unfit for trial due to injury, resulting in one less pig (n=5) in the TN control group, whereas all other treatments (TP, HS, HP) had n=6. Prior to initiation of treatments animals (n=23) were weighed and randomly assigned to one of four treatments based on average body weights. The treatments consisted of 1) thermo-neutral (TN) conditions (22°C), 2) thermo-neutral + PRRSV infection (TP) conditions, 3) heat stress (HS) conditions, and 4) heat stress + PRRSV infection (HP) conditions. The experiment was divided into two periods based on experimental conditions: 1) infection (day 1-11) and 2) HS (day 11-14). Day one marked the beginning of the trial with the differentiation of the two TN rooms into non-infected and infected. The pigs designated for infection were inoculated with 1mL of PRRS virus (VR-2385) intramuscularly behind the ear. The virus was kindly provided by Dr. X.J. Meng (CRC, Virginia Tech). After ten days of viral incubation part two of the experiment was implemented (day 11) with the removal of heat stress designated pigs from their respective TN or TP environment into preheated rooms (35°C) while the rest remained in thermo-neutral conditions. . On day 14 after three days of continuous heat all animals, independent of treatment were sacrificed for sample collection.

Each room's temperature and humidity were measured (Acurite® model Thermo Hygro, Bellingham, WA) and recorded at 6AM and 5PM. An electric forced air heater (Model F3F551QT, TPI Corporation) with constant circulation maintained the heat stress

room temperatures. All animals were monitored for signs of distress throughout the experiment. Body temperature and respiration rates were measured twice daily (6AM and 5PM) and thrice daily during heat stress (6AM, 12PM, 5PM). Rectal temperatures were taken with a digital thermometer (Welch Alleyn Sure Temp PLUS, Skaneateles Falls, NY) and respiration rates (breaths/min) measured by visual observation with a stopwatch. If body temperature rose above 40.5°C the pigs were hosed down with cool water for 2 minutes then temperature was retaken. If the temperature was still elevated pigs were removed from the room for 5 minutes or until temperature decreased below 40.5°C. During this cooling period the pigs had access to water. Pigs were fed ad libitum and feed intake was recorded once daily (6AM) by a weigh back method. To calculate feed intake feeders were weighed each morning, the feeder weight from the previous day was subtracted from the current day and then total amount of feed given the current day was added. Body weights were taken (6AM) every three days (Way Pig 300, Raytec Manufacturing, Schuyler, NE), including day 1, 11, and 14.

Blood was collected on day 1 prior to inoculation, day 11 prior to environmental treatment, and day 14 prior to sacrifice. Jugular vein blood was obtained (two 4mL BD vacutainers containing lithium heparin and 10mL BD vacutainers containing silicone coat, Franklin Lakes, NJ) and centrifuged at 4°C for 20 minutes at 1425 x g (IEC Centra-8R Centrifuge, Needhan Heights, MA). On day 14 jugular vein blood was obtained (10mL BD vacutainer containing liquid K_2EDTA) and sent to Virginia Maryland Regional College of Veterinary Medicine for complete blood count (CBC) analysis. After blood samples were collected on day 14 the pigs were euthanized with Beuthanasia, followed by exsanguination.

All tissues were harvested within 10 minutes of death and included: Longissimus Dorsi muscle and liver. The Longissimus Dorsi was excised using aseptic technique, a sample was taken for metabolic analysis and the rest was snap frozen in liquid nitrogen and stored at -80°C until analysis. A liver sample was taken and snap frozen in liquid nitrogen and stored at -80°C until analysis.

Metabolite Assays

Plasma was aliquoted into three 2mL tubes, two stored at -20°C and one was sent to the Virginia Maryland Regional College of Veterinary Medicine for analysis with a large animal blood panel. The large animal blood panel included analyses for glucose, urea nitrogen, creatinine, total protein, albumin, globulin, creatine kinase, aspartate aminotransferase, gamma-glutamyl transpeptidase, total, direct, and indirect bilirubin, phosphorus, calcium, magnesium, sodium, potassium, chloride, CO2, and anion gap. All plasma samples were analyzed with the Beckman Coulter AU480 (Brea, CA). Serum was allowed to coagulate at room temperature for 1 hour before centrifuging at 4°C for 20 minutes at 1425 x g. Serum was aliquoted into 2mL micro-centrifuge tubes and stored: 1 sample at -80°C for qPCR and the rest at -20°C. Day 14 jugular vein blood was obtained (10mL BD vacutainer containing liquid K2EDTA) and sent to VT vet school for complete blood count (CBC) analysis, which measured: red blood cells, hematocrit, hemoglobin, white blood cells (WBC), segmented neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Serum samples were analyzed with a Siemens Advia 2120 (Malvern, PA).

Viral Load Determination

Porcine reproductive and respiratory syndrome virus plasmid pACYC VR2385 was kindly provided by Dr. X.J. Meng (CRC, Virginia Tech). The plasmid was transformed into *E. coli* MACH-1 cells (Invitrogen, Carlsbad, CA) with Kanamycin antibiotics and incubated at 37°C for 16 hrs. Colonies were picked and incubated in LB Broth in the orbital shaker (Thermo Scientific MaxQ4450, Rockford, IL) at 37°C for 16hrs. Plasmid DNA was extracted using the Qiagen Mini Prep Kit (Qiagen) according to manufacturers protocol. A Nanophotometer Pearl (Implen, Westlake Village, CA) was used to determine purity and concentration of DNA. DNA was stored in a -20°C freezer.

The plasmid DNA was linearized and serial diluted in order to create a standard curve to determine the PRRS viral load using the Promega (Madison, WI) buffers and XBA1 enzymes. Briefly, 3uL Buffer D, .3uL BSA100x, 1.2uL RNAse free water, .5uL XBA1 and 25uL of Plasmid DNA (5-10ug) totaling 30uL were mixed and incubated at 37°C for 16hrs. Seventy uL of RNAse free water was added along with 100uL of phenol/chloroform/isoamyl alcohol (25:24:1) (Ambion, Grand Island, NY) to each tube, then vortexed for 30 seconds and centrifuged at high speed for 5 minutes. The top aqueous layer was transferred into a sterile 1.5mL tube with the addition of 30uL of 7.5 M NH_4OAc (Ammonium acetate) and 130uL of cold (4°C) 100% isopropanol mixed and placed at -20°C for 35 minutes. The tubes were centrifuged at high speed in 4°C for 30 minutes then the supernatant was aspirated. Ice cold (-20°C) 80% ethanol (500uL) was gently added to the DNA pellet and centrifuged for 10 minutes at 4°C then the pellet was aspirated and dried until all ethanol had evaporated. The pellet was re-suspended in 10uL of sterile RNase free water and stored at -20°C.

RNA extraction

RNA extraction was performed on serum in continuation for determining viral load and on liver and muscle samples for gene expression analysis. Serum samples were transferred from -80°C freezer to ice to thaw. Serum sample (200uL) were transferred to 2mL tubes containing 1mL of Trizol cell lysis reagent (5 Prime, Gaithersburg, MD). Serum was mixed with Trizol reagent by vortexing for approximately 15 seconds then centrifuged for 10mins. at 8,000 x g in the 4°C centrifuge. Longissimus dorsi (LD) muscle and liver samples were retrieved from the -80°C and transferred to liquid nitrogen. Samples were kept frozen as they were crushed in the original foil package, transferred to new foil packages, and placed back into liquid nitrogen for extraction. Muscle and liver samples (100mg) were transferred to a 5 mL plastic centrifuge tube containing 1 mL of Trizol cell lysis reagent (5 Prime, Gaithersburg, MD). Tissue was homogenized in the Trizol reagent using a Power Gen 125 homogenizer from Fisher Scientific set at six speed for two passes of approximately 15 seconds each. The homogenizer was cleaned with deionized water, and primed with Trizol between each sample. The samples were transferred to 2mL tubes and centrifuged for 10mins. at 8,000 x g in the 4°C centrifuge. The process is now the same for both serum and tissue samples. The top aqueous portion was transferred to a phase lock gel tube from 5 Prime (Gaithersburg, MD) and incubated at room temperature for 5 minutes. Chloroform (200uL) was added to the cell lysate, shaken vigorously for approximately 5 seconds to create an emulsion, and centrifuged for 5 minutes at 12,000 x g, separating the clear aqueous phase and the pink colored organic phase. The clear aqueous phase, on the top of

the gel, was transferred to a new 1.5mL centrifuge tube along with 500ul of isopropanol. Tubes were inverted to mix, and incubated on ice for 10 minutes in order to allow the RNA to precipitate. Tubes were transferred to a 4°C centrifuge and centrifuged for 10 minutes at 12,000 x g. The supernatant was poured off leaving the RNA pellet at the bottom of the tube. One mL of 70% ethanol was then added to the RNA pellet. Tubes were centrifuged again at 7500 x g for 5 minutes in the 4°C centrifuge. The supernatant was poured off. Excess supernatant was gently pipetted from around the RNA pellet in order to allow it to dry thoroughly and exposed to air for 5 minutes. RNAse free water was added to the pellet and mixed in order to re-dissolve the total RNA. Once the RNA pellet was dissolved a Nanophotometer Pearl (Implen, Westlake Village, CA) was used to determine purity and concentration of total RNA in each sample, after which the tubes were transferred to the -80°C freezer to be stored.

RNA clean-up

Total RNA samples for muscle and liver were retrieved and thawed from the -80°C freezer. Samples exceeding 1000ng/uL in concentration were diluted with RNAse free water to approximately 800ng/uL for clean up. All samples used in the clean up procedure were 100uL by volume. The Qiagen RNEasy CleanUp Kit was used for this procedure. β-mercaptoethanol (350 uL) was diluted in RLT Buffer (10uL β-mercaptoethanol to 350uL RLT Buffer) was added to each sample. 100% ethanol (250uL) was added to each sample and mixed by pipetting. Each sample was then transferred to an RNEasy spin column and centrifuged for 15 seconds at 10,000 x g. The flow through collected at the bottom of the column was discarded. RW1 Buffer (350uL)

was added to each column, and columns were centrifuged again for 15 seconds at 10,000 x g. DNAse (80uL) was diluted in RDD Buffer (10uL DNAse to 70uL RDD buffer) and was added to each column and incubated for 15 minutes at room temperature. RW1 Buffer (350uL) were then added to each column, and columns were centrifuged for 15 seconds at 10,000 x g. Flow through was discarded. RPE Buffer (500uL) was added to each spin column. Columns were centrifuged for 15 seconds at 10,000 x g. RPE Buffer (500uL) were added to each spin column. Columns were centrifuged for 2 minutes at 10,000 x g. After this wash procedure, the flow through tube was discarded, and the spin column was transferred to a new flow through tube. The column was centrifuged once more at 15,000 x g for 1 minute to get rid of any excess ethanol that was left behind. Columns were then transferred to a 1.5mL centrifuge tube. RNAse free water was added to the column, and columns were centrifuged for 1 minute at 10,000 x g. The flow through was carefully pipetted from the centrifuge tube back into the column. Columns were centrifuged once more for 1 minute at 10,000 x g. Samples in the centrifuge tubes were examined using the nanophotometer for concentration and purity, and then stored in the -80°C freezer.

cDNA Synthesis

The Bio-Rad iScript cDNA Synthesis Kit (Hercules, CA) for RT-qPCR was used for this procedure. Total RNA samples (serum, muscle, and liver) were thawed from the -80°C freezer and 1ug/uL RNA was used for each cDNA synthesis reaction. Samples were diluted with RNAse free water to a total volume of 15uL. A master mix containing the iScript reaction mix (1uL) and the iScript reverse transcriptase (4uL) were added to

each diluted sample and mixed by reverse pipetting. The total volume for each reaction was 20uL. Sample tubes were incubated using a Bio-Rad T100 thermal cycler (Hercules, CA) set for 5 minutes at 25°C followed by 30 minutes at 42°C followed by 5 minutes at 85°C and a holding period set for 4°C. cDNA samples were then frozen and stored at -20°C until RT-qPCR.

Real-time qPCR

The SsoAdvanced SYBR Green Supermix Kit from Bio-Rad (Hercules, CA) was used for this procedure consisting of 10ul SYBR Green, 5ul (1ng/ul) of cDNA, 0.5ul (10uM) of forward primer, and 0.5ul of (10uM) of reverse primer were mixed with 4ul RNAse free water for a total volume of 20uL per reaction. Samples (serum, liver and muscle) were incubated in triplicate using a Bio-Rad CFX96 Real Time System set for enzyme activation at 95°C for 30 seconds (1 cycle), denaturation at 95°C for 5 seconds (40 cycles), annealing and extension at 65°C for 30 seconds (40 cycles), and a melt curve from 65-95°C in 5°C increments. The samples were analyzed against a standard curve and the average SQ mean was taken for each sample. The geometric mean of the houskeeper genes (18S, EEFIA1, and B-actin) was divided by the SQ mean of each sample to accommodate for any variation between samples.

Metabolic Flexibility

Mitochondrial isolation from longissmus doris muscle. Mitochondria were isolated from longimssimus dorsi muscle as previously described with modifications [22]. Tissue samples were collected in buffer containing 67mM sucrose, 50mM

Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation.

Respiration in isolated mitochondria. Respirometry measures of isolated mitochondria were performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Immediately following mitochondrial isolation, protein was quantified using a Pierce bicinchoninic acid assay (Thermo Scientific, Rockford, IL) and mitochondria were plated on Seahorse cell culture plates at a concentration of 5 ug/ well in the presence of 10 mM pyruvate (P5280; Sigma-Aldrich, St. Louis, MO) and 5 mM malate (P5280; Sigma-Aldrich, St. Louis, MO). Experiments consisted of 25 second mixing and 4-7 minute measurement cycles. Oxygen consumption was measured as previously described (Brand, 2011; Brand, 1998) under basal conditions, ADP (5 mM, Sigma-Aldrich, St. Louis, MO) state 3 stimulated respiration (State 3), oligomycin (2μM) induced state 4 respiration State 4_O), and uncoupled, maximal respiration in the presence of FCCP (0.3 μM) to assess respiratory capacity (State 3_u). Respiratory control ratio (RCR) was calculated as the ratio of ADP stimulated state 3 and oligomycin induced state 4 respiration. Oligomycin induced state 4 respiration was used to account for any contaminating ATPase activity that may prevent the restoration of a low respiration (Brand, 2011; Brand, 1998). Data are expressed as pmol/min. All experiments were performed at 37 °C.

Fatty acid, glucose, and pyruvate oxidation. Palmitate ([1-14C]-palmitic acid), glucose ([U-14C]-glucose), and pyruvate ([1-14C]-pyruvate) oxidation were performed

as previously described (Reyna et al., 2008; Hulver et al., 2005). Pyruvate oxidation was used to assess the activity of pyruvate dehydrogenase (PDH), the enzyme that catalyzes the oxidation of pyruvate resulting in the provision of glucose-derived acetyl-CoA to the TCA cycle. This is a direct measure of pyruvate dehydrogenase activity as the number one carbon of pyruvate is liberated as CO_2 in the oxidation of pyruvate to acetyl-CoA. Metabolic flexibility was assessed by comparing pyruvate oxidation with and without 100uM palmitic acid. The degree to which pyruvate oxidation decreased in the presence of FFA was used as an index of metabolic flexibility.

Reactive Oxygen Species

Reactive Oxygen Species measures in isolated mitochondrial. Amplex Red Hydrogen Peroxide/Peroxidase assay Kit was used for measures of ROS production. To measure ROS production from complex 1, complex 3, and reverse electron transfer (REV), isolated mitochondria were plated on a 96-well black plate at a concentration of 5ug/well under three different conditions, respectively. The three conditions were pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/rotenone (200nM) for complex 1, pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/SOD (400U/ml)/antimycin A (2μM) for complex 3, and succinate (20mM)/oligomycin (2μM) for reverse electron flow to complex 1 (REV). Experiments were conducted in sucrose/mannitol solution in order to maintain the integrity of the mitochondria. Experiments consisted of 1 minute delay and 1 minute reading cycles, followed by a 5 second mixing cycle performed every third reading. All experiments were performed at 37°C. Measures for ROS levels were conducted on a microplate reader (Biotek synergy 2, Winooski, VT). Fluorescence of

Amplex Red, added prior to plate reading, was measured using a 530nm excitation filter and a 560nm emission filter.

Data Analysis

All data were statistically analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc. Cary, NC). Data are reported as LSmeans and considered significant if $P<0.05$. Daily measurements (rectal temperature, respiration rates, and feed intake) and blood samples for each animal's respective parameter were analyzed using repeated measures with day as the repeated effect. The model included treatment, day, and interaction. Analysis also tested the difference between all environmental temperature and immune status (TN, TP, HS, HP) on day 14.

Results

Immune challenged pigs had an increased PRRS viral load ($P < 0.04$) on day 11 compared to day 1. In the non-infected group there was no difference between days. Temperature had no significant effect on the viral load (Figure 2.1).

Pigs exposed to immune challenge, HS, or both had increased rectal temperatures compared to TN ($P < 0.0001$), and this increase was maintained for the remaining 11 days of the experiment (Figure 2.2A). The dual challenge had the greatest maintained increase in rectal temperature. There was a significant ($P<0.0001$) treatment by day interaction and a significant ($P<0.0001$) temperature effect with increased respiration during heat stress compared to TN conditions (Figure 2.2B). Following initiation of heat

stress, a tendency of increased ($P < 0.1$) respiration rate in dual challenged animals compared to either challenge individually was observed during the last three days of the experiment. In comparison to the thermo-neutral environment pigs, the heat-stressed pigs exhibited an increased ($P < 0.0001$) respiration rate (105/bpm vs. 67/bpm).

Irrespective of day, immune challenge decreased ADG ($P = 0.05$) compared to TN, with the greatest decrease seen in the dual challenged animals (Figure 2.3). Independent of day, body weights decreased ($P < 0.006$) in immune challenged, HS, and dual challenged pigs, 35.4kg, 36.4kg, 35.2kg vs. 38.7kg, compared to TN animals (Figure 2.4). During the immune challenge, although BW still increased in infected animals during the duration of the experiment these pigs had decreased growth ($P < 0.02$) (34.3kg vs. 26.2 kg) compared to TN. Final BW tended to decrease ($P < 0.09$) in HS and decreased ($P < 0.05$) during immune challenge compared to TN.

Blood metabolite analysis indicated an effect on protein metabolism by both immune challenge and HS (Table 2.3, 2.4). Creatine kinase (CK) decreased ($P < 0.005$) during the dual challenge compared to all TN. During the dual challenge phase of the experiment, creatinine levels increased ($P < 0.01$) on dual challenged pigs compared to heat stress and immune challenge. Creatinine in dual challenged pigs increased ($P < 0.01$) between day 11 and 14 and was elevated ($P < 0.003$) on day 14 compared to TP. Blood urea nitrogen (BUN) levels are different by day ($P < 0.02$) where BUN increased on day 11 and 14 compared to day 1.

Plasma glucose concentration tended to be different between treatments ($P < 0.06$) as concentration decreased during HS (Table 2.3, 2.4). Plasma glucose concentration was

different by day ($P < 0.01$), concentration decreased ($P<0.02$) between day 1 and 11 and increased ($P < 0.03$) between day 11 and 14.

Muscle metabolism responded negatively to each challenge. Metabolic flexibility measured by percent change in PDH in response to FFA, tended to decrease ($P < 0.08$) in PRRS infected pigs (3.98 vs. 19.09) compared to non-infected (Table 2.5, 2.6). Metabolic flexibility tended to decrease ($P = 0.1$) during HS. Fatty Acid oxidation via CO_2 production decreased ($P < 0.04$) in dual challenged pigs (0.108 vs. 0.156, 0.161) compared to PRRSV and HS challenge respectively. Heat stress tended to increase ($P < 0.09$) FA oxidation via CO_2 production in HS pigs compared to TN. No significant difference was observed in the metabolic expression of pyruvate oxidation, pyruvate +FA oxidation, fatty acid oxidation via acid soluble metabolite and total fatty acid oxidation (Table 2.5, 2.6). Reverse electron transfer (REV) and electron transport chain complexes I and III (Table 2.7, 2.8) had no significant difference in production of mitochondrial reactive oxygen species, compared to controls.

Skeletal muscle PDK2 mRNA abundance was down regulated ($P = 0.001$) in HS compared to TN conditions, but not affected by immune status (Table 2.10). However, skeletal muscle PDK4 gene expression was reduced ($P < 0.03$) by HP challenges pigs compared to infection and HS. No significant differences were observed in metabolic gene expression in the liver of PC, PEPCK1, and PEPCK2 (Table 2.10).

Discussion

Immune challenge and heat stress reduces production and economic gain. Determining the mechanism(s) by which the dual immune challenge and HS

detrimentally impacts homeostasis and energy metabolism in growing pigs may allow for future nutritional or pharmaceutical interventions to ameliorate the negative effects of increased environmental temperatures and immune challenge.

Gene expression of PRRS viral load significantly increased in infected pigs, indicating that the infected animals were indeed infected with the PRRS virus and that there was no cross contamination to non-infected pigs. The viral load varied by day, displaying a dramatic increase from day 1 to day 11 as viral infection occurred, and then decreased day 11 to 14 as the pigs recovered.

Heat stress and PRRSV dual challenge has not been studied previously, therefore basic characterization of the physiological responses were measured in the growing pigs. An initial effect of both challenges is increased body temperature (Rhoads et al., 2008; Pearce et al., 2013b; Carroll et al., 2012). In this study the experimental protocol resulted in marked pyrexia as all infected animals had increased body temperatures three days after inoculation compared to TN and this was sustained throughout the experimental period. The induction of a febrile response to infection is necessary to control and eliminate invading pathogens, by way of an inflammatory response (Carroll et al., 2012). Hyperthermia ensued after initiation of HS as all body temperatures were elevated above that of TN. The HS regiment was constant and lacked a cyclical or diurnal pattern of ambient temperature, which prevented the pigs from returning to euthermia during the cooler hours of the night. Consequently our HS protocol more closely resembles tropical or semitropical regions and southern regions of the United States.

Heat stress conditions caused a dramatic increase in respiration rate compared to TN, although infection did not play a significant role. As the pigs heat load increases the

pigs respire more to dissipate heat via esophageal evaporative cooling (Patience et al., 2005). The lack of functional sweat glands in pigs does not permit normal skin evaporative cooling (Curtis, 1983). HS plus infection (HP) numerically appear to have an additive effect on respiration rate, possibly due to increased stress from a respiratory virus.

A conserved response to challenged animals is a reduced feed intake (Renaudeau et al., 2012; Neumann et al., 2005). The decreased ingested nutrients leads to negatively impacted growth. This is supported in the current study with decreased body weights (BW) in challenged animals, with the most detriment to the dual challenged pigs in comparison to TN. However, a study in heat stressed pigs showed that decreased gross BW was not just a result of decreased fed, as pair-fed counterparts actually had less BW gain than HS animals (Pearce et al., 2013b). Once heat stress was initiated the dual challenged pigs ceased growth compared to individually challenged pigs. Analysis of ADG showed similar results to BW. However, on day 5 of the immune challenge the ADG was reduced and by day 11 had recovered to pre-infection status. However, ADG was reduced between day 11 and day 14 with the initiation of heat stress. From this, ADG indicates that infection lowers growth with reduced feed intake, but recovers with time. However, the initiation of heat stress reduces the feed intake again and slows growth, therefore reducing ADG. The physical parameters measured in this study indicate dual challenged pigs had the most detrimental impacts on production, than either PRRSV or heat stress alone.

Muscle growth is dependent on the occurrence of more protein accretion than protein degradation. However, due to muscle accounting for ~60-75% of BW it is a good

source of amino acids and energy (Frost and Lang, 2008). A response to under nutrition is skeletal muscle degradation and increased circulating BUN and creatinine, which can be used to assess proteolysis (Pearce et al., 2013b). Interestingly, BUN was not affected by treatment, but was increased on day 11 and day 14 compared to day 1. Although an increased BUN has been observed in heat stress conditions in pigs, cows, and heifers (Rhoads et al., 2013), Pearce et al. (2013b) observed a decline in BUN levels after 3 days of heat stress. This suggests that the BUN in HS pigs may have been elevated at initial onset of treatment and decline over time. In LPS infected pigs, BUN was observed to increase 8 to 12 hours after infection, but only trended toward elevation by 24hrs (Webel et al., 1997). It is possible that BUN is a short-term measure of proteolysis and blood sampling was not frequent enough to see a change. In our experiment, plasma creatinine was elevated in heat stress, immune, and dual challenged pigs compared to thermo-neutral controls. Creatinine can be an indicator of muscle proteolysis as it is produced from the breakdown of creatine phosphate. Creatine phosphate is converted to creatine by creatine kinase (CK) during muscle catabolism (Berg, 2007). Increased plasma creatinine results agree with previous data in heat stressed swine (Pearce et al., 2013b). Plasma CK levels were elevated on day 14, suggesting that over the challenged pigs an increase in muscle catabolism is occurring. The increased creatinine and CK levels during heat stress and immune challenge support the notion of increased muscle degradation.

Phosphorylation of the pyruvate dehydrogenase complex by PDK causes inactivation and a shift toward fatty acid oxidation to spare glucose (Sugden and Holness, 2003). Expression of PDK4 increases when glucose is low (Sugden and Holness, 2003). An elevated PDK4 expression has also been observed during HS above control levels

(Sanders et al., 2009). In this study, we observed reduced PDK2 mRNA expression during heat stress in skeletal muscle. However, PDK4 was significantly reduced during the dual heat and immune challenge. This may suggest there is an increased carbohydrate demand during the dual challenge.

Fatty acids are degraded two carbons at a time to provide acetyl-CoA to the TCA cycle and NADH and FADH2 to the ETC for generation of energy by the process of B-oxidation (Berg et al., 2007). Fatty acid oxidation measured by CO_2 production decreased during a dual challenge. This observation is in line with decreased PDK4 during a dual challenge, suggesting there is an increased carbohydrate demand. Analysis of fatty acid oxidation by temperature showed an increase during heat stress. This finding is interesting as it has previously been observed that lipid retention increases and fatty acids are not mobilized during HS (Verstegen et al., 1987; Heath, 1983). Partial fatty acid oxidation measured by acid soluble metabolite and total fatty acid oxidation had no significant difference between treatments.

During this study plasma glucose had decreased in heat stressed animals, possibly indicating a shift in metabolism. Metabolism alternates between fuel sources of glucose, which is converted to pyruvate through glycolysis, or fatty acids. The oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase complex, converts pyruvate in the cytosol to actely-CoA in the mitochondria (Berg et al., 2007). The shear volume of body muscle mass has a major impact on metabolism. Metabolic flexibility is the ability to switch between carbohydrates and lipids as fuel sources (Stump et al., 2006). In this study metabolic flexibility was measured by the degree to which pyruvate oxidation decreased in the presence of FFA. Metabolic flexibility decreased in PRRSV challenged

animals compared to noninfected. In addition, when evaluated solely on temperature, metabolic flexibility had a tendency to decrease during HS compared to TN conditions. A decrease in metabolic flexibility in PRRS infected and HS pigs suggests the muscle is inflexible in its ability to alternate between fuel sources. The may be part of the reason a lack of adipose mobilization has been seen during heat stress (Verstegen et al., 1987; Heath, 1983).

Reactive oxygen species (ROS) are produced by oxidative phosphorylation in the mitochondria. Under normal physiological conditions, ROS production is regulated by complex 1 and electron scavengers. However, when mitochondria are malfunctioning there are excessive amounts of electrons that accumulate in the electron carriers that increase ROS production (Wallace et al., 2010). The mitochondrial oxidative capacity is typically reduced and ROS production elevated during metabolic inflexibility (Stump et al., 2006). Interestingly, analysis of mitochondrial ROS produced by complex 1, complex 3, and reverse electron transfer (REV) was not significantly affected by any challenge condition. Reduction of substrate oxidation by inactivation of PDC by increased PDK4 expression may serve to reduce mitochondrial ROS production and prevent cellular damage (Baumgard and Rhoads, 2012).

During states of reduced nutrient intake the body produces glucose via gluconeogenesis (Webel et al., 1997; Wheelock et al., 2008; Rhoads et al., 2008). It is surprising that the enzymes PC, PEPCK1, and PEPCK2 involved in gluconeogenesis are not significantly different from the control pigs. The repartitioning of nutrients away from growth leave the body with decreased glucose availability and increased energy demand. It is possible that PC, PEPCK1, and PECK2 enzymes require more time to

mobilize a response then three days in thermal stress. However, a previous study in lactating dairy cows observed cytosolic PEPCK (2) mRNA abundance increased for pair feeding (PF) but not heat stress (HS), while PC increased in PF and HS (Rhoads et al, 2010). The lack of elevation of gluconeogenic enzymes during immune challenge may be due to more efficient fatty acid oxidation.

In conclusion, dual challenge appears to have the most detrimental impacts on physiological parameters with respect to body temperature and body weight gain in pigs. Dual challenged animals also displayed the highest degree of muscle degradation as indicated by creatinine, CK, and blood urea nitrogen. During heat stress, decreased blood glucose concentration, increased fatty acid oxidation, and decreased metabolic flexibility were observed. In the dual challenged pigs, decreased PDK4 gene expression, fatty acid oxidation, and metabolic flexibility was observed. Collectively, these data may indicate that the dual challenged pigs have a shift in metabolism toward a greater carbohydrate demand.

Table 2.1: Ingredients (DM basis) and chemical composition of diet.

Ingredient	**%DM**
Corn Meal	60.02
Hi-Pro Soybean Meal	28.30
Distiller Dried Grains	5.00
Lard	1.50
R.D. Blood	1.50
Ground Limestone	1.19
Phosphate Dical	1.18
Lysine 78.8%	0.36
Salt (Plain)	0.35
Sow Premix 3	0.15
D.L. Methionine	0.15
L-Threonine 98.5%	0.10
Choline CL 60%	0.08
Maxi-MIL HP Binder	0.05
.06% Selenium Premix	0.04
Ronozyme P-CT 540%	0.03

Table 2.2: PRRS virus Primer

Gene	Direction	Sequence 5’ to 3’
PRRSV pACYC	Forward	GGCAACTCAGACGACCGAAC
PRRS pACYC	Reverse	AGTAGTAATTGGACAGCGAGAAGG

The pair of primers was designed based on the nsp2 region by the Beacon software.

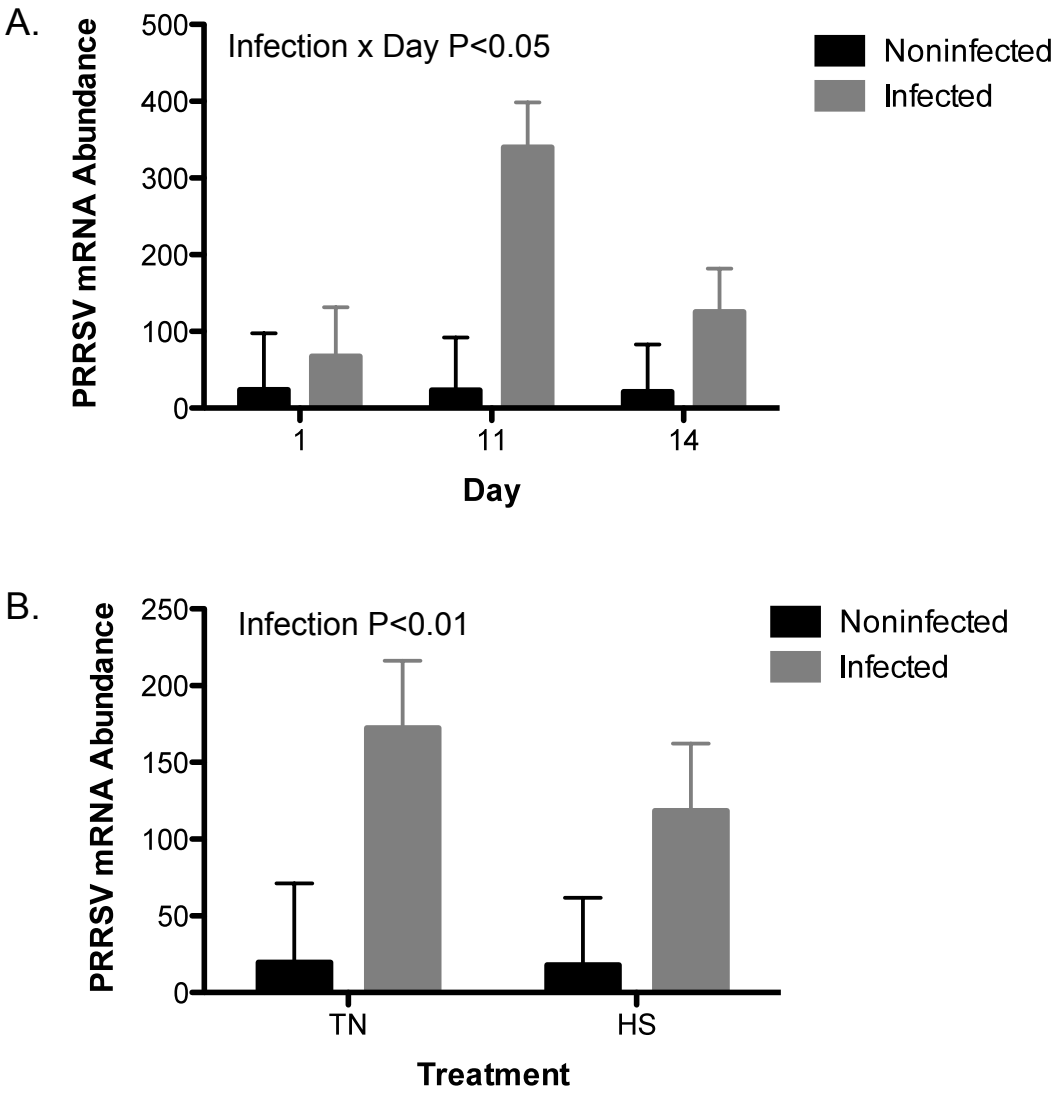


Figure 2.1: Dual challenge effects of PRRS virus and heat stress on temporal change of PRRS viral load. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) temporal change of PRRS viral load and (B) effects of TN, TP, HS, and HP on change in viral load at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

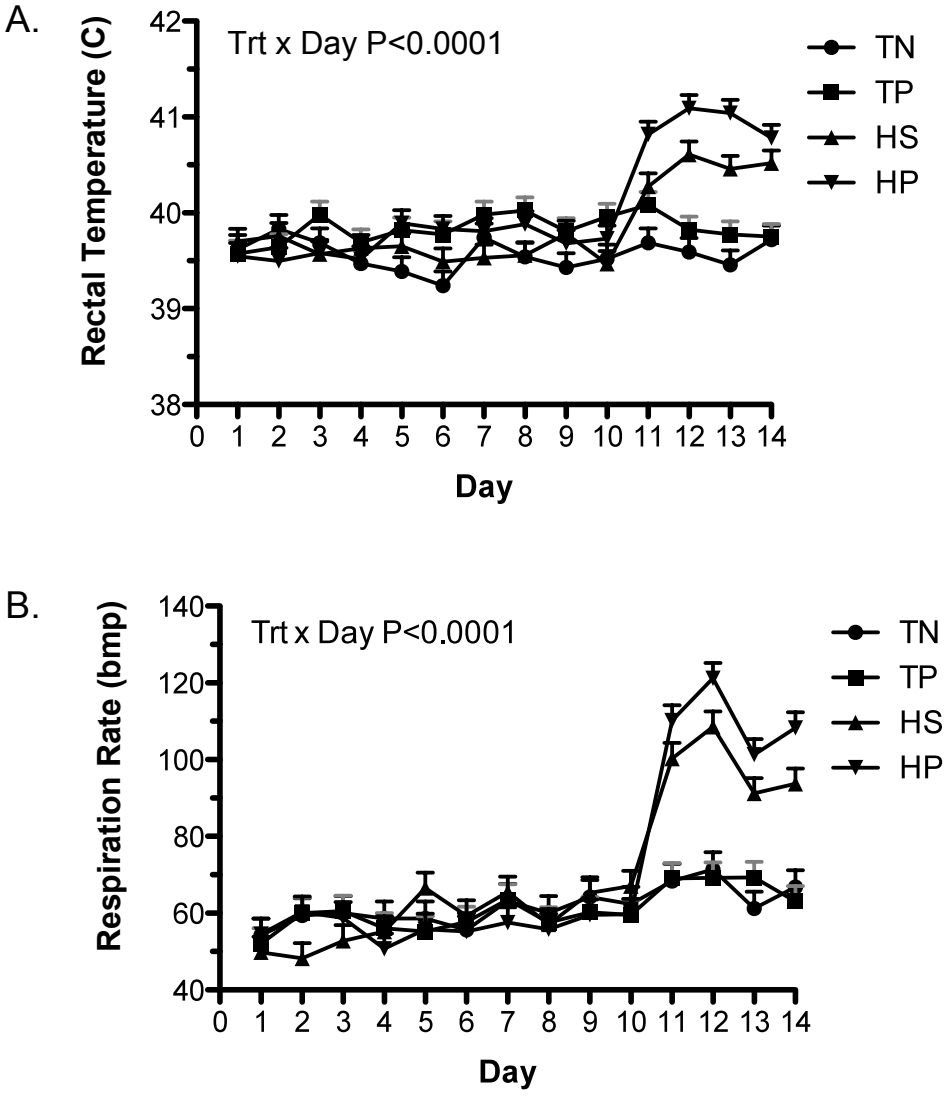


Figure 2.2: Dual challenge effects of PRRS virus and heat stress on temporal patterns of rectal temperature and respiration rate. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) Rectal temperature and (B) Respiration rates in growing pigs. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

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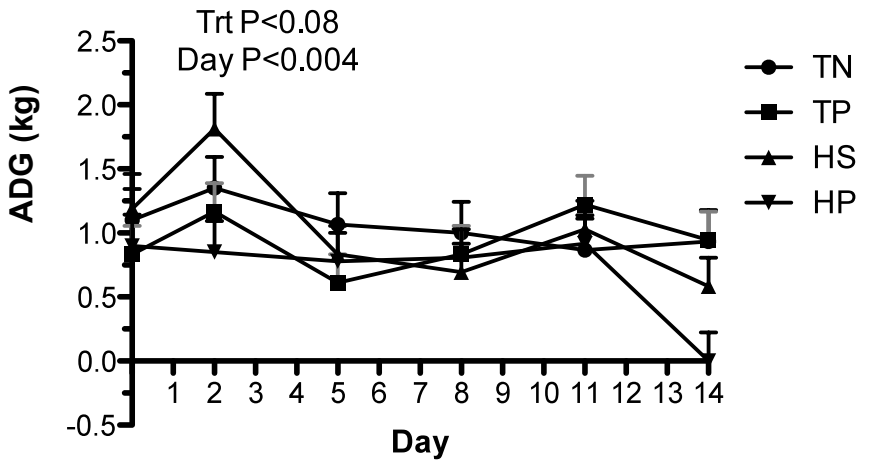

Figure 2.3: Dual challenge effects of PRRS virus and heat stress on average daily gain. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on average daily gain. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

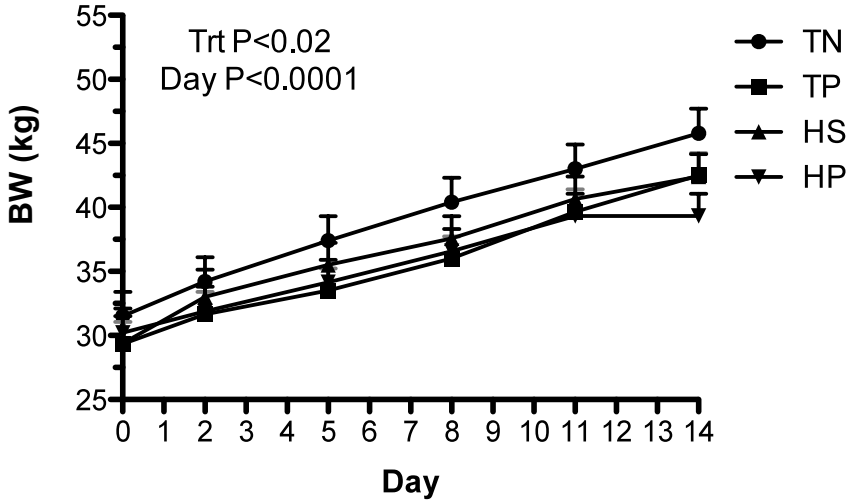

Figure 2.4: Dual challenge effects of PRRS virus and heat stress on temporal change in body weights. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on on temporal change in body weights in growing pigs. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

Table 2.3: Effects of PRRS virus and heat stress (HS) on plasma energetic variables in growing pigs

	Days of Experiment[1]															
	1				11				14					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	TN	HS	TP	HP	TN	HS	TP	HP	SEM	Trt	Day	T x D
CK, UL[5]	4680	1710	2979	2015	8349	2456	3326	1417	2618	15119	3818	4699	2101	0.16	0.05	<0.00
Creatinine, mg/dL	0.78	0.82	0.85	0.83	1.0	0.92	1.0	1.0	0.98	1.1	1.0	1.4	0.05	<0.02	<.0001	<0.01
Urea Nitrogen, mg/dL	10.8	11.0	10.8	11.5	14.2	12.0	13.3	11.3	13.6	14.0	12.5	12.6	1.0	0.71	0.02	0.58
Glucose, mg/dL	120.8	122.3	127.7	119.0	117.2	108.7	112.5	109.2	134.6	114.2	125.8	112.7	5.1	0.06	0.01	0.45

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN: 2301; 0.06; 1.1; 5.6. Significant difference at P<0.05. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Creatine Kinase

Table 2.4: Effects of PRRS virus and heat stress (HS) on plasma energetic variables in growing pigs.

	Environment[1]					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	INF	T x I
CK, UL[5]	2617	15119	3818	4698	3087	0.05	0.16	0.08
Creatinine, mg/dL	0.98	1.13	1.02	1.37	0.06	<0.001	<0.04	0.12
Urea Nitrogen, mg/dL	13.6	14.0	12.5	12.7	1.2	0.82	0.33	0.92
Glucose, mg/dL	134.6	114.2	125.8	112.7	6.3	<0.02	0.43	0.58

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN: 3382; 0.07; 1.29; 6.8. Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Creatine Kinase

Table 2.5: Effects of PRRS virus and heats stress (HS) on metabolic flexibility in growing pigs.

	Environment[1]					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	Immune	T x I
Pyruvate Oxidation	324.53	366.02	359.82	344.53	47.39	0.79	0.89	0.57
Pyruvate + FA Oxidation	249.64	312.33	325.92	306.26	33.94	0.54	0.33	0.25
Metabolic Flexibility	22.87	15.31	3.43	4.52	7.92	0.69	<0.08	0.59
FAO Oxidation[5]	0.086	0.161	0.157	0.108	0.027	0.63	0.76	<0.04
FAO ASM[6]	2.23	2.25	2.18	1.47	0.26	0.21	0.14	0.19
FAO Total[7]	2.31	2.41	2.34	1.58	0.28	0.27	0.18	0.16

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN: Pyruvate (51.91); Pyruvate +FA (37.18); Flex (8.67); FAO CO (0.03); FAO ASM (0.29); FAOtotal (0.31). Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Fatty acid oxidation by CO_2 Production
[6]Fatty acid oxidation by Acid soluble metabolites
[7]Total fatty acid oxidation

Table 2.6: Effects of heats stress (HS) on metabolic flexibility in growing pigs.

	Environment[1]			P-value
Variable	TN[2]	HS	SEM	Trt
Pyruvate Oxidation	324.53	366.02	42.14	0.52
Pyruvate + FA Oxidation	249.64	312.33	37.02	0.28
Metabolic Flexibility	22.87	15.30	2.86	0.1
FAO Oxidation[5]	0.086	0.161	0.02	<0.09
FAO ASM[6]	2.23	2.25	0.25	0.95
FAO Total[7]	2.31	2.41	0.27	0.81

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions or heat stress (HS; 35°C) conditions. SEM TN: 46.16; 40.55; 3.13; 0.029; 0.28; 0.30. Significant difference at $P<0.05$. TN n=5 and HS n=6.
[2]Thermo-neutral
[3]Fatty acid oxidation by CO_2 Production
[4]Fatty acid oxidation by Acid soluble metabolites
[5]Total fatty acid oxidation

Table 2.7: Effects of PRRS virus and heats stress (HS) on mitochondrial ROS production in growing pigs.

	Environment[1]					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	Immune	T x I
ROS Complex 1[5]	104.4	95.8	76.2	88.5	14.1	0.90	0.23	0.48
ROS Complex 3[6]	171.6	177.0	158.3	153.2	25.5	1.0	0.49	0.84
ROS REV[7]	693.6	777.67	766.8	746.2	116.0	0.79	0.86	0.66

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM HP: 15.4; 28.0; 127.1. Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Mitochondrial reactive oxygen species Complex 1
[6]Mitochondrial reactive oxygen species Complex 3
[7]Mitochondrial reactive oxygen species REV receptor

Table 2.8: Effects of heats stress (HS) on mitochondrial ROS production in growing pigs.

	Environment[1]			P-value
Variable	TN[2]	HS	SEM	Temp
ROS Complex 1[3]	104.4	95.8	18.0	0.75
ROS Complex 3[4]	171.6	177.0	30.9	0.9
ROS REV[5]	693.6	777.67	108.9	0.62

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions or heat stress (HS; 35°C) conditions. SEM TN: 19.7; 33.8; 119.2. Significant difference at $P<0.05$. TN n=5 and HS n=6.
[2]Thermo-neutral
[3]Mitochondrial reactive oxygen species Complex 1
[4]Mitochondrial reactive oxygen species Complex 3
[5]Mitochondrial reactive oxygen species REV receptor

Table 2.9 Primers used in RT-PCR for the liver and skeletal muscle samples.

Genes	Direction	Sequence 5’ to 3’
18S rRNA	Forward	GTAACCCGTTGAACCCCAT
18S rRNA	Reverse	CCATCCAATCGGTAGTAGCG
B-actin	Forward	CCAGCACCATGAAGATCAAGATC
B-actin	Reverse	ACATCTGCTGGAAGGTGGACA
EEFIA1	Forward	TCATTGATGCTCCAGGACACA
EEFIA1	Reverse	CCGTTCTTGGAAATACCTGCTT
PDK2	Forward	ACTGCAACGTCTCTGAGGTG
PDK2	Reverse	AGGTGGGAGGGGACATAGAC
PDK4	Forward	CTGGGAGCACCACCCCACCT
PDK4	Reverse	GCGCATCACAAAGCGAGCCG
PC	Forward	CAGGAGAACATCCGCATCAAC
PC	Reverse	ACCAGCAGGGAATCGTAGTG
PEPCK1	Forward	AGGAGAGAAAACGTAGGCGA
PEPCK1	Reverse	GACTTTGGCCGAGTGGTTGA
PEPCK2	Forward	ACCAATGTGGCTGAGACGAG
PEPCK2	Reverse	AGTTAGGATGTGCACAGGGC

Table 2.10: Effects of PRRS virus and heat stress (HS) on metabolic gene expression

	Environment[1]					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	Immune	T x I
PC (liver)[5]	0.0011	0.0030	0.0012	0.0013	0.0008	0.22	0.34	0.25
PEPCK1 (liver)[6]	0.007	0.010	0.012	0.006	0.004	0.67	0.89	0.19
PEPCK2 (liver)	0.009	0.013	0.012	0.008	0.003	0.98	0.75	0.23
PDK2 (muscle)[7]	0.00037	0.00024	0.00044	0.00021	0.00004	0.001	0.66	0.26
PDK4 (muscle)	0.00023	0.00049	0.00124	0.00025	0.00023	0.17	0.15	<0.03

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN (liver): PC (0.00085); PEPCK1 (0.0039); PEPCK2 (0.0032); (muscle) PDK2 (0.000045); SEM TP (muscle): PDK2 (0.00005); PDK4 (0.00029). Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Pyruvate carboxylase
[6]Phosphoenol pyruvate carboxykinase
[7]Pyruvate dehydrogenase kinase

Chapter III

Effects of a dual heat stress and immune challenge on immune and growth factor response in growing pigs

Abstract

Heat stress and immune challenges activate an immune response, which reduces the animal's ability to grow efficiently. In order to assess the effects of a dual heat and immune challenge on cytokine production and growth, 9 week old crossbred barrows were individually housed, fed ad libitum, divided into four treatments: thermo-neutral (TN), thermo-neutral plus PRRSV infection (TP), heat stress (HS), and heat stress plus PRRSV infection (HP), and subjected to two experimental phases. Phase one occurred in thermo-neutral conditions (22°C) where half the animals were infected with PRRS virus (n=12) and allowed to incubate for 10 days, while the other half (n=11) remained infection free. For phase two, half of each the non-infected (n=6) and infected groups (n=6) were moved to heated rooms (35°C) for three days of continuous heat, while the rest remained in thermo-neutral conditions. Blood samples were collected prior to the onset of each phase and at trial completion before sacrifice. Liver and skeletal muscle (longissmus dorsi) samples were collected at sacrifice. Real time-PCR was used to determine mRNA abundance within tissue samples. Heat stress tended to decrease WBC and lympocytes and increase monocytes and eosinophils during heat stress. However, neutrophils were significantly increased ($P < 0.01$) during a dual challenge. Plasma IL-6 significantly decreased ($P<0.02$) in response to the dual challenge. Significant decreases ($P < 0.05$) were observed in abundance of skeletal muscle TLR3, TLR4, IL-1B, IL-6, and TNF-α gene expression in response to the dual infection. Similarly, liver IFN- α and IL-6 mRNA abundance also decreased during the dual challenge. Skeletal muscle myostatin gene expression increased ($P <$

0.01) during the dual challenge, compared to TN. Both muscle and liver IGF-1 were not significant affected. These data indicate a decreased cytokine and growth factor response to dual heat and immune challenges.

Introduction

Animals affected by challenges such as viral infections and heat stress can alter cytokine and growth factor production. Challenged animals elicit an immune response to destroy invading pathogens. The pathogens contain portions of the cell called pathogen associated molecular patterns (PAMPs) that are recognized by highly conserved membrane bound proteins called pattern-recognition receptors (PRRs) found on immune and other cell types (Leaver et al., 2007). A specific type of PRR is the toll-like receptors (TLR). There are greater than a dozen different toll-like receptors that respond to specific antigens. Viral RNA activates a response through TLR3 whereas (Miller et al., 2009), TLR4 responds to lipopolysaccharide (Frost and Lang, 2008). Once the appropriate PAMP engages the corresponding TLR an intracellular signal cascade is initiated, which leads to gene transcription and production of cytokines (Leaver et al., 2007). The cytokines produced include IL-1, IL-6, IL-10, and TNF-α and each acts differently to prevent infection. Interleukin-1 and TNF- α induce fever by changing the body's internal temperature set point (Kluger et al., 1997; Bouchama et al., 1991). IL-1 and other pro-inflammatory cytokines elicit the syntheses of acute-phase proteins, which increase rapidly during inflammation (Dritz et al., 1996). During a stress challenge such as immune or heat stress, heat shock proteins (HSP) are up regulated to provide protective effects against protein damage and may play a role in cytokine regulation (Moseley, 1998).

Skeletal muscle tends to be degraded during challenges, such as heat stress and immune responses, to provide amino acids for the production of acute phase proteins, cytokines, and protective heat shock proteins. Each type of challenge may also affect growth factors such as IGF-1 and MSTN expression and impact the ability of the animal to grow efficiently (Escobar et al., 2004; Clemmons, 2004).

This study examines blood profiles, plasma cytokine concentrations, and gene expression in both liver and skeletal muscle to determine the effects of a dual immune and heat stress challenge on cytokine expression and growth factors. We hypothesized the dual immune and heat challenge would blunt the effects of cytokine expression and growth compared to either challenge individually.

Materials and Methods

Animals and Treatment

Animal immune challenge and heat stress experiment was performed at Virginia Polytechnic Institute and State University in a manner identical to that described in Chapter II.

Cytokine Analyses

Blood was collected and plasma harvested as described previously. Plasma was aliquoted into 2mL tubes and stored at -20°C for ELISA kits (NeoBio Lab, Park Woburn, MA). Briefly, 50uL of sample (plasma) or standard was added to the appropriate wells in the supplied microtiter plate. Note that wells have been pre-blocked and no additional blocking steps are required. The plate was incubated for 1 hour at room temperature. The plates were washed 4X with 1x wash solution (provided by kit) 350uL each time per well. Wells were emptied between washes by physically striking against paper towels in order to remove residual solution. Next,

100uL of conjugate was added to each well and the plate shaken. The plate was then incubated for 1 hour at 37°C. After incubation, the plate was washed again as previously described. Then, 50uL of substrate A is added to each well followed by the addition of 50uL of substrate B. The plate was then covered and incubated for 10 minutes at room temperature. 50uL of stop solution was added to each well and the plate shaken. At this time the plate was read at 450nm using a Power Wave XS (Bio Tek, Winooski, VT) Values were calculated by subtracting the mean blank value from each sample or standard and calculating the mean for triplicate wells.

RNA extraction

RNA extraction was performed on muscle and liver samples in a manner identical to that described in Chapter II.

RNA clean-up

RNA clean-up was performed on muscle and liver samples in a manner identical to that described in Chapter II.

cDNA synthesis

cDNA synthesis was performed on muscle and liver samples in a manner identical to that described in Chapter II.

Real time-qPCR

Real time-qPCR was performed on muscle and liver samples in a manner identical to that described in Chapter II.

Data Analysis

All data were statistically analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc. Cary, NC). Data are reported as LSmeans and considered significant if $P<0.05$. Plasma analysis used repeated measures with day as the repeat effect, with a model that included

treatment, day, and interaction. The model analyzed the differences between environmental temperature, immune status, and interaction (TN, TP, HS, HP) on day 14.

Results

Analysis of a complete blood count (CBC) indicates the white blood cell (WBC) counts had a tendency to decrease ($P < 0.1$) with HS (19.7 vs. 23.0) compared to TN (Table 3.1). The percentage of lymphocytes also decreased ($P < 0.002$) during HS compared to TN animals. However, increased monocyte ($P < 0.05$) and eosinophil ($P < 0.02$) percent was observed during HS compared to TN. Percent segmented neutrophils tended to increase ($P < 0.1$) during dual challenge compared to HS and immune challenge. No significant difference in red blood cells and platelets was observed

Skeletal muscle toll-like receptor 3 and 4 gene expression decreased ($P < 0.01$) in dual challenged pigs, while HS and infection were both increased. Plasma interleukin-1B, independent of treatment, tended to decrease ($P < 0.06$) between day 1 and day 11 then increased ($P < 0.0001$) between day 11 and 14 (Figure 3.1). Plasma IL-1B concentration was lower during HS on day 14 then TN counterparts. Interleukin-6 concentration, independent of treatment, decreased on day 11 compared to day 1 (Figure 3.2). However, a significant interaction ($P < 0.02$) was observed on day 14 indicating reduced concentration in dual challenge animals compared to heat stress or immune challenge alone.

Skeletal muscle IL-1B mRNA abundance decreased ($P < 0.03$) in response to a dual challenge compared to immune challenge and HS (Table 3.2). Liver, IL-6 gene expression tended to decrease ($P < 0.08$) during dual challenge as compared to immune challenge and HS. Skeletal muscle IL-6 decreased ($P < 0.004$) during dual challenge compared to either challenge

individually. Skeletal muscle TNF- α gene expression decreased ($P<0.05$) during dual challenge compared to infection and HS. Liver IFN- α mRNA abundance tended to decrease ($P = 0.06$) in dual challenged pigs in comparison to HS and infection alone. No significant differences were observed in immune response in liver to IL-1B, TNF- α, TLR3, TLR4, and HSP70 or in muscle to IFN- α and HSP70 gene expression.

Skeletal muscle MSTN mRNA abundance increased ($P < 0.006$) in HP pigs, and decreased during each single challenge alone. Gene expression of IGF-1 did not significantly differ in either liver or muscle during the study.

Discussion

Elicitation of an immune response, from heat stress or PRRS virus, decreases growth efficiencies and impacts expression of growth factors. Although in heat stress, the origin of the immune response is not definitively known, it is suspected to arise from both the skeletal muscle and in the gastrointestinal tract, as damage is done to both tissues (Pearce et al., 2013a,b). The reduced growth may be a partial result of increased muscle protein breakdown, mentioned in chapter 2, to provide amino acids for production of cytokines, acute phase proteins, and protective heat shock proteins. Growth may also be impacted by alterations in the expression of cytokines and various growth factors such as IGF-1 and myostatin. The objective of this study was to determine how a dual heat and immune challenge would affect growth based on cytokine and growth factor expression.

The immune system is made up of two distinct responses, the innate immune response that occurs rapidly as a first line of defense against invading pathogens and the adaptive immune response that takes a few days to initiate but uses specific antibodies to recognize the invading

pathogens. Further the innate immune response utilizes inflammation and cytokines as its method of attack. It appears that blood counts change as a result of challenge. Infection with a highly pathogenic PRRSV strain showed elevated levels of macrophages and neutrophils (Han et al., 2014). Complete blood counts in heat stressed chickens show decreased WBC counts and antibody production (Mashaly et al., 2004). In heat stressed pigs, increased neutrophil numbers are seen whereas antibody-producing cells decreased (Morrow-Tesch et al., 1994). In support of this, we observed decreased lymphocytes and a trend toward decreased WBC counts during heat stress as well as increased monocytes and eosinophils. Also neutrophil numbers were elevated during the dual challenge in comparison to individual challenges. It is tempting to speculate that heat stress requires more of an innate immune response verse an adaptive response.

An innate immune response stimulates cytokine production when a pathogen associated molecule pattern (PAMP) attaches to a pattern-recognition receptor (PRR) protein and elicits an intracellular cascade that promotes gene transcription of cytokines (Frost and Lang, 2008). Toll-like receptors are specific PRR on macrophages that function to detect distinct microbial pathogen-associated molecular patterns (PAMPs) (Leaver et al., 2007). Viral RNA activates TLR3 (Miller et al., 2009), whereas TLR4 is activated by LPS of Gram-negative bacteria cell membranes (Frost and Lang, 2008). In support of this, TLR3 was increased most in response to PRRSV and TLR4 was increased most during HS. However we observed a decrease in both TLR3 and TLR4 during the dual challenge. The decrease seen in the dual challenged pigs may be due to the heat stress increasing the pigs stress levels further and blunting the immune response. An LPS challenge in mice indicated a direct muscle responses to PAMPs after up regulated expression of TNF- α and IL-1B mRNA within 30 minutes (Lang et al., 2003). Another study injected LPS directly into muscle, which strongly induced IL-6 mRNA

transcription (Frost et al., 2006). A muscle cells ability to respond to a wide variety of PAMPS is consistent with muscle wasting and stunted growth associated with a wide variety of infectious insults (Frost and Lang, 2008).

Cytokine production has been recognized to not only regulate immune function but also modifying the growth process (Spurlock, 1997). Pro-inflammatory cytokines, such as TNF- α and IL-1B, seem to be in part responsible for decreased protein synthesis due to cytokine anatagonists and neutralizing antibodies ameliorate the decrease in protein synthesis (Lang et al., 1996). Accelerated protein degradation during immune challenge provides amino acids for synthesis of acute phase proteins (Spurlock, 1997), specifically IL-1 (Dritz et al., 1996). In this study muscle IL-6 expression was increased due to heat stress, while IL-1B was decreased due to heat stress in plasma. Elevated skeletal muscle IL-6 and reduced IL-1B has been previously reported in acute heat stressed rats at maximum temperature (42.4ºC) by Welc et al. (2013). However, the general cytokine response observed during this study was a decreased expression during the dual challenge compared to either heat stress or PRRSV infection individually, in plasma IL-6, muscle IL-1B, IL-6, and TNF- α, and liver IFN- α. It appears that the dual challenged animals have a unique cytokine response that suggests immune response is blunted by heat stress, compared to either individual challenge alone.

Heat shock proteins ability to interact with pattern recognition receptors, such as TLR4, to induce cytokine release suggest the interactions between HSPs and the host immune system may be an important first line of defense against infection and inflammation (Leon, 2007) In fact, HSP may play a role in the inhibition of cytokine response, such as TNF- α and IL-1B (Kluger et al., 1997). Surprisingly, HSP70 was not significantly affected by immune challenge, heat stress or the combination. Heat shock proteins are known to increase during stress

conditions such as heat stress, immunological stress, tissue injury, and metabolic stress like glucose deprivation, as a protective mechanism (Leon, 2007). It is possible that the mRNA expression of HSP70 may be lower than that observed if protein expression were analyzed (Kamanga-Sollo et al., 2011). There is also evidence suggesting that heat shock proteins depend on the activation of the mTOR pathway in order to be synthesized (Choe et al., 2012). An unpublished study in our lab found that heat stress decreased two major downstream enzymes activated by the mTOR complex giving a potential for decreased HSP70 expression.

The ability of an animal to grow efficiently relies on growth factors such as insulin-like growth factor-1 (IGF-1) and myostatin (MSTN), which can be affected by immune responses. Insulin-like growth factor -1 functions as an endocrine hormone to initiate intracellular signaling via the AKT pathway for cell growth and proliferation. Infection elicits differing responses in GH secretion, but universally found to produce GH resistance (Lang et al., 2005) while simultaneously affecting IGF-1 expression in plasma and tissue and partially mediate muscle wasting (Frost and Lang, 2008). Growth hormone resistance has historically been induced in adipose and skeletal muscle by TNF- α and IL-6 (Jacobi et al., 2006). Muscle and liver production of IGF-1 is reduced and circulating concentrations of IGF-1 decrease in response to immune challenge (Jacobi et al., 2006). IGF-1 gene expression in this study did not differ in either liver or skeletal muscle. However, in a previous study heat stress and pair feeding alone were not enough to alter hepatic IGF-1 gene expression during moderate malnutrition (Rhoads et al., 2010). Another study showed that hepatic IGF-1 production decreased during HS (Baumgard and Rhoads, 2013).

Myostatin is a negative regulator of muscle mass. There was a significant increase in MSTN mRNA expression in the muscle of dual challenged pigs, while MSTN expression was

not significantly different for individual challenges compared to controls. It is interesting that myostatin was not up regulated by PRRSV infection alone, as seen in a previous study (Escobar et al., 2004). The increase in MSTN in dually challenged pigs may be due to the indirect effect of reduced feed intake over multiple days. It is also possible that the inflammatory cytokines may activate MSTN transcription directly via nuclear factor-kB activation (Escobar et al., 2004). The increased myostatin expression may partly explain the growth restriction during the dual challenge.

In conclusion, heat stress and immune challenge negatively impact pig growth, due in part to altered cytokine and myostatin response. Similarly, toll-like receptor expression appears to change in a challenge-specific manner, for example TLR3 was elevated in PRRS infected animals whereas TLR4 was increased during HS. However. Both TLR3 and TLR4 were decreased in dual challenge as were the majority of cytokine expression in plasma, muscle, and liver. The decreased cytokine expression during the dual challenge may be partly due to decreased TLR activation. The impact of cytokines on skeletal muscle may be worsened by specific cytokines such as IL-1, which stimulate muscle degradation (Dritz et al., 1996). Dual challenged animals appear to have a blunted immune response to heat stress. Further the growth restriction may be in part due to increased myostatin expression. The exact mechanism(s) responsible for the arrested growth specifically during the dual challenge warrants further investigation.

Table 3.1: Effects of PRRS virus and heat stress (HS) on complete blood count in growing pigs

	Day 14 of Experiment [1]					P-value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	Immune	T x I
RBC[5]	7.66	7.36	7.35	7.10	0.32	0.30	0.27	0.92
WBC[6]	23.0	19.7	25.0	20.4	2.9	0.10	0.56	0.78
LYMPH%[7]	0.65	0.52	0.70	0.42	0.06	0.002	0.58	0.17
SEG%[8]	0.30	0.37	0.25	0.48	0.05	0.005	0.48	<0.1
MONO%[9]	0.04	0.08	0.05	0.06	0.02	0.04	0.65	0.33
EOS%[10]	0.012	0.025	0.005	0.040	0.011	<0.02	0.65	0.22
PLATELETS	397.4	324.0	446.8	276.5	151.8	0.32	0.99	0.69

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN: 0.2; 1.81; 0.04; 0.03; 0.01; 0.01; 96.0. Significant difference at P<0.05. TN n=5, TP n=6, HS n=6, HP n=6.

[2]Thermo-neutral

[3]Thermo-neutral and PRRSV

[4]Heat stress and PRRSV

[5]Red blood cells

[6]White blood cells

[7]Lymphocytes percent of WBC

[8] Segmented neutrophils percent of WBC

[9]Monocytes percent of WBC

[10]Eosinophils percent of WBC

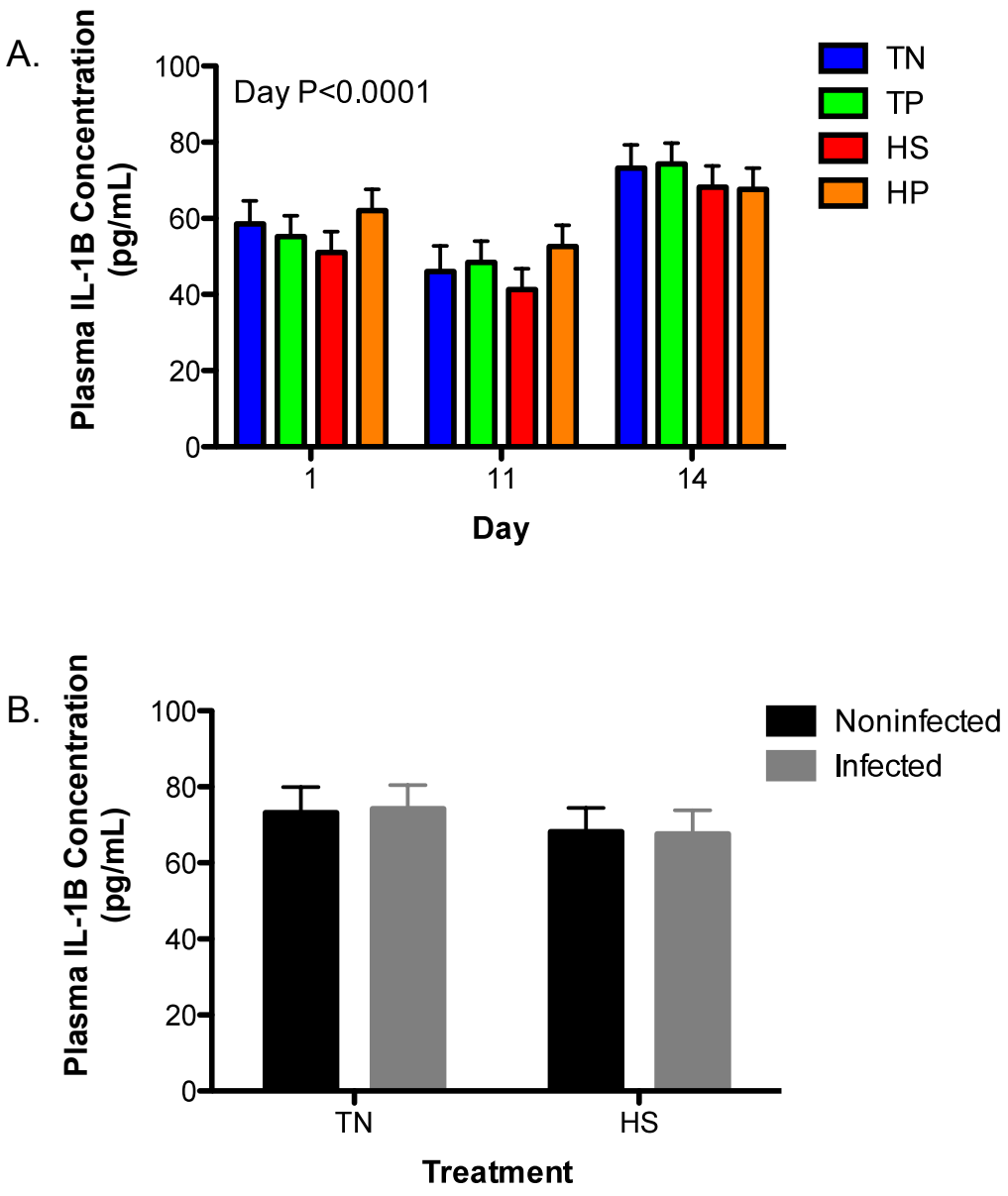

Figure 3.1: Dual challenge effects of PRRS virus and heat stress on plasma IL-1B abundance. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) IL-1B expression by day and (B) IL-1B expression by treatment at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

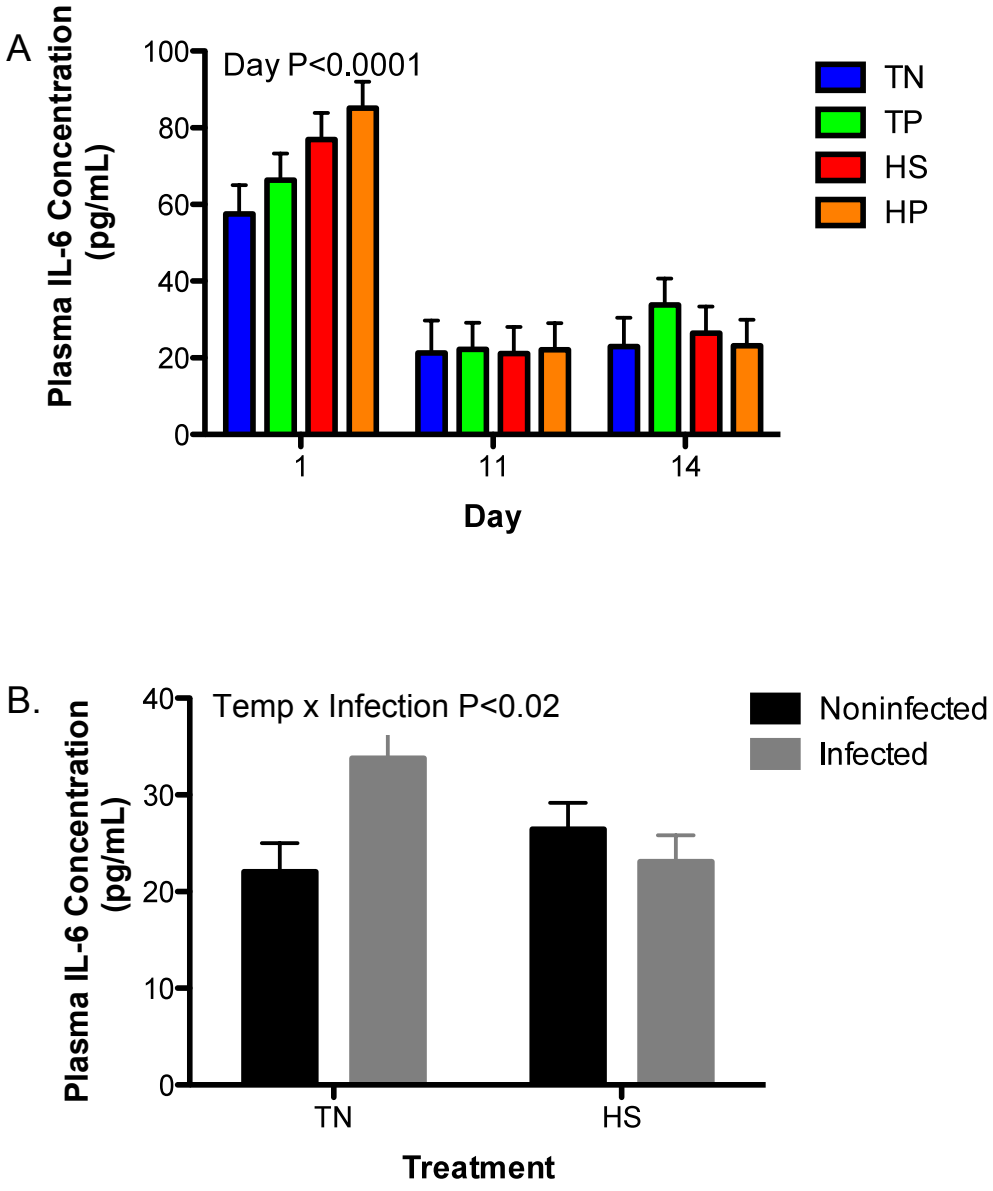

Figure 3.2: Dual challenge effects of PRRS virus and heat stress on plasma IL-6 abundance. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) IL-6 expression by day and (B) IL-6 expression by treatment at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

Table 3.2 Primers used in RT-PCR for the liver and skeletal muscle samples.

Genes	Direction	Sequence 5' to 3'
18S rRNA	Forward	GTAACCCGTTGAACCCCAT
18S rRNA	Reverse	CCATCCAATCGGTAGTAGCG
B-actin	Forward	CCAGCACCATGAAGATCAAGATC
B-actin	Reverse	ACATCTGCTGGAAGGTGGACA
EEFIA1	Forward	TCATTGATGCTCCAGGACACA
EEFIA1	Reverse	CCGTTCTTGGAAATACCTGCTT
IL-1B	Forward	TCTGCCCTGTACCCCAACTG
IL-1B	Reverse	CCCAGGAAGACGGGCTTT
IL-6	Forward	GCGCAGCCTTGAGGATTTC
IL-6	Reverse	CCCAGCTACATTATCCGAATGG
IFN- α	Forward	ACCTTCCAGCTCTTCAGCAC
IFN- α	Reverse	CAGGGGCTGTAGCTCTTCTC
TNF- α	Forward	AACCCTCTGGCCCAAGGA
TNF- α	Reverse	GGCGACGGGCTTATCTGA
TLR3	Forward	CTGTTGTCAGAGTAAATGAATCACC
TLR3	Reverse	GACTATGCCGTTGACAAAACAC
TLR4	Forward	CTACTCCAGCCAGGACGAAGA
TLR4	Reverse	GCACCCCCTCCTCCAAGTT
IGF-1	Forward	GCACATCACATCCTCTTCGC
IGF-1	Reverse	GCCTCCTCAGATCACAGCTC
HSP70	Forward	AGCACAAGAAGGACATCAGC
HSP70	Reverse	GAAGTCGATGCCCTCGAACA
MSTN	Forward	CGCTACGACGGAAACGATCA
MSTN	Reverse	TGCCTGGGTTCATGTCAAGT

Table 3.3: Effects of PRRS virus and heat stress (HS) on gene expression during an immune response.

	Day 14 of Experiment [1]					P-Value		
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	Temp	Immune	T x I
IL-1B[5] (liver)	0.000071	0.000039	0.00017	9.2E-6	0.000082	0.26	0.68	0.45
IL-1B (muscle)	7.5E-6	0.000041	0.000023	0.000014	8.0E-6	0.17	0.53	<0.03
IL-6[6] (liver)	0.00011	0.00019	0.00034	0.000087	0.000087	0.37	0.49	0.08
IL-6 (muscle)	0.000042	0.00012	0.000041	0.000032	0.000012	<0.02	<0.003	<0.004
TNF-α [7] (liver)	4.8E-6	0.000028	0.000011	6.4E-6	0.000010	0.40	0.48	0.22
TNF- α (muscle)	0.000012	0.000029	0.000022	0.000016	5.1E-6	0.33	0.74	<0.05
IFN- α [8] (liver)	0.00035	0.00083	0.00063	0.00043	0.00017	0.41	0.72	0.06
IFN- α (muscle)	0.00042	0.00026	0.00033	0.00038	0.000083	0.52	0.88	0.26
TLR3[9] (liver)	0.000048	0.00039	0.00019	0.00012	0.00014	0.33	0.65	0.15
TLR3 (muscle)	0.00017	0.00024	0.00036	0.00017	0.00004	0.18	0.16	<0.008
TLR4[10] (liver)	0.000019	0.000097	0.000042	0.000022	0.000032	0.39	0.44	0.15
TLR4 (muscle)	0.00012	0.00029	0.00016	0.000096	0.000038	0.22	0.08	0.01
HSP70[11] (liver)	0.015	0.053	0.025	0.023	0.012	0.15	0.42	0.13
HSP70 (muscle)	0.054	0.076	0.041	0.023	0.022	0.92	0.19	0.41
MSTN[12] (muscle)	0.001	0.0006	0.0007	0.0011	0.0001	0.82	0.40	<0.006
IGF-1[13] (liver)	0.00009	0.0005	0.0003	0.0002	0.0002	0.52	0.74	0.15
IGF-1 (muscle)	0.002	0.002	0.002	0.001	0.0003	0.15	0.40	0.30

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN (liver, muscle): IL-1B (0.000089, 8.744E-6); IL-6 (0.000095, 0.000013); TNF- α (0.000011, 5.6E-6); IFN- α (0.00018, 0.000091); TLR3 (0.00015, 0.000044); TLR4 (0.000035, 0.000041); HSP70 (0.013, 0.024); SEM TP (muscle): IL-1B (9.8E-6); IL-6 (0.000015); TNF- α (6.2E-6); IFN- α (0.000102); TLR3 (0.000049); TLR4 (0.000046); HSP70 (0.027). Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.

[2]Thermo-neutral

[3]Thermo-neutral and PRRSV

[4]Heat stress and PRRSV

[5]Interleukin-1B

[6]Interleukin-6

[7]Tumor Necrosis Factor- α

[8]Interferon- α

[9]Toll-like receptor 3

[10]Toll-like receptor 4

[11]Heat shock protein 70

[12]Myostatin

[13]Insulin-like growth factor-1

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Appendix

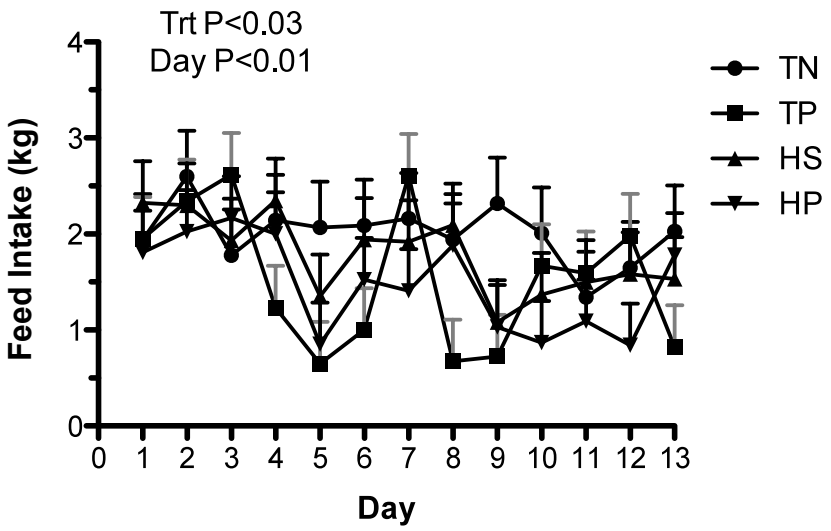

Figure A-2.1: Dual challenge effects of PRRS virus and heat stress on feed intake. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on daily feed intake. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

Table A-2.1 Effects of PRRS virus and heat stress (HS) on plasma energetic variables in growing pigs

Variable	Days of Experiment[1]															
	1				11				14					P-value		
	TN[2]	HS	TP[3]	HP[4]	TN	HS	TP	HP	TN	HS	TP	HP	SEM	Trt	Day	T x D
Albumin, mg/dL	3.52	3.57	3.55	3.53	3.96	3.62	3.43	3.48	3.86	3.73	3.57	3.47	0.12	<0.05	0.42	0.39
AnionGap, mEqL	18.4	18.4	22.6	18.8	15.7	17.4	19.6	19.1	23.4	20.8	25.0	18.4	2.1	0.13	0.04	0.64
AST, UL[5]	35.2	32.5	49.2	33.0	79.2	33.0	63.2	38.8	42.8	55.5	45.5	41.5	11.0	0.26	0.14	0.22
Calcium, mg/dL	11.3	11.2	11.5	11.3	11.4	10.7	10.9	10.7	11.4	10.9	10.9	10.2	0.2	<0.01	<0.002	0.04
Chloride, mEqL	101.6	101.3	102.8	102.2	105.6	100.7	101.0	101.0	100.4	101.3	101.2	102.5	1.1	0.50	0.61	0.07
CO_2, mEqL[6]	27.4	28.2	25.0	27.7	25.4	28.8	28.0	28.3	24.2	22.3	23.5	22.3	1.5	0.89	<0.001	0.59
Globin, g/dL	2.16	2.22	2.20	2.30	2.30	2.25	2.47	2.47	2.36	2.62	2.58	2.82	0.17	0.39	<0.02	0.94
Magnesium, mg/dL	1.80	1.73	1.82	1.73	2.04	1.77	1.85	1.80	1.92	1.82	1.92	1.82	0.07	0.05	0.08	0.73
Phosphorous, mg/dL	9.10	9.25	9.78	9.42	9.70	8.70	9.07	8.68	9.46	8.20	9.35	7.47	0.34	<0.01	0.013	0.05
Potassium, mEqL	6.36	6.52	6.88	5.97	5.68	6.35	6.30	6.27	6.38	6.27	6.38	5.55	0.45	0.41	0.60	0.84
Sodium, mEqL	141.0	141.3	143.5	142.7	147.0	140.5	142.3	142.2	141.6	138.2	143.3	137.7	1.2	<0.01	<0.01	<0.02
Total Bilirubin, mg/dL	0.08	0.10	0.10	0.10	0.22	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.02	0.31	0.11	<0.07
Total Protein, g/dL	5.68	5.77	5.75	5.82	6.26	5.87	5.90	5.95	6.22	6.35	6.15	6.28	0.16	0.84	<0.001	0.71
GGT, UL[7]	17.8	22.7	20.3	25.0	19.8	20.5	27.7	24.0	18.6	22.0	28.0	25.0	2.2	<0.01	0.44	0.34

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN 0.13; 2.3; 12.1; 0.2; 1.2; 1.8; 0.19; 0.07; 0.37; 0.49; 1.3; 0.03; 0.17; 2.4. Significant difference at P<0.05. TN n=5, TP n=6, HS n=6, HP n=6.

[2]Thermo-neutral

[3]Thermo-neutral and PRRSV

[4]Heat stress and PRRSV

[5]Aspartate Amino Transferase

[6]Carbon dioxide
[7]Gamma-glutamyl transferase

Table A-2.2: Effects of PRRS virus and heat stress (HS) on blood chemistry variables in growing pigs at the end of experiment.

	Environment[1]					
Variable	TN[2]	HS	TP[3]	HP[4]	SEM	P-value
Albumin, mg/dL	3.86	3.73	3.57	3.47	<0.1	<0.90
AnionGap, mEqL	23.4	20.77	25.0	18.4	1.7	0.27
AST, UL[5]	42.8	55.5	45.5	41.5	8.7	0.36
Calcium, mg/dL	11.4	10.9	10.9	10.2	0.2	0.46
Chloride, mEqL	100.4	101.3	101.2	102.5	1.2	0.87
CO_2, mEqL[6]	24.2	22.3	23.5	22.3	1.4	0.81
Globin, g/dL	2.36	2.62	2.58	2.82	0.18	0.95
Magnesium, mg/dL	1.92	1.82	1.92	1.82	0.06	0.98
Phosphorous, mg/dL	9.46	8.20	9.35	7.47	0.31	0.34
Potassium, mEqL	6.38	6.27	6.38	5.55	0.31	0.27
Sodium, mEqL	141.6	138.2	143.3	137.7	0.9	0.24
Total Bilirubin, mg/dL	0.13	0.10	0.10	0.10	0.01	0.27
Total Protein, g/dL	6.22	6.35	6.15	6.28	0.15	0.99
GGT, UL[7]	18.6	22.0	28.0	25.0	2.16	0.17

[1]Pigs were exposed to thermo-neutral (TN; 22°C) conditions; thermo-neutral infected (TP; 22°C) conditions; heat stress (HS; 35°C) conditions; or heat stress infected (HP; 35°C). SEM TN: 0.1; 1.9; 9.6; 0.2; 1.3; 1.5; 0.20; 0.06; 0.34; 0.3; 1.0; 0.016; 0.17; 2.37. Significant difference at $P<0.05$. TN n=5, TP n=6, HS n=6, HP n=6.
[2]Thermo-neutral
[3]Thermo-neutral and PRRSV
[4]Heat stress and PRRSV
[5]Aspartate Amino Transferase
[6]Carbon dioxide
[7]Gamma-glutamyl transferase

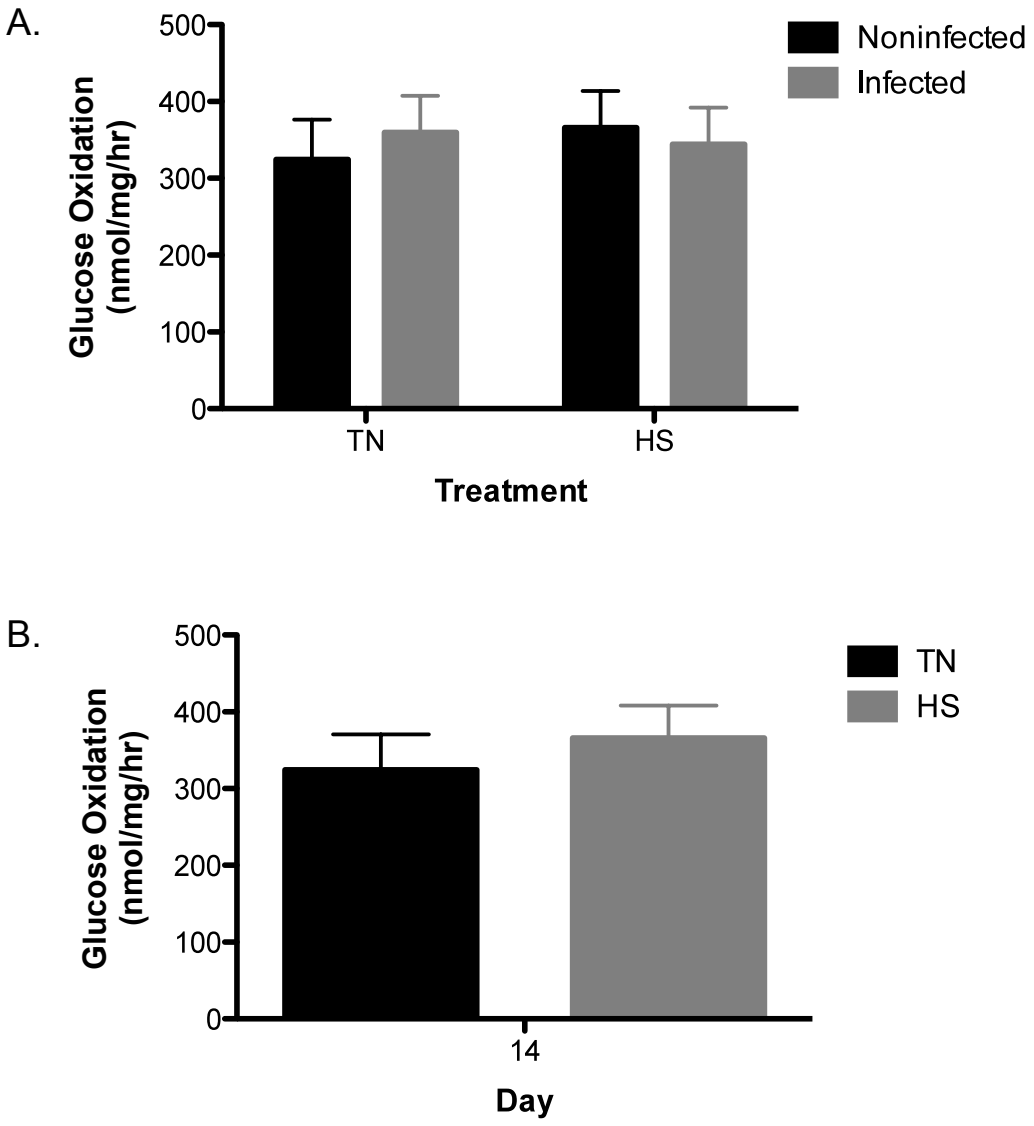

Figure A-2.2: Dual challenge effects of PRRS virus and heat stress on glucose oxidation. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on glucose oxidation measured by CO_2 production at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6. and (B) effects of TN and HS on glucose oxidation measured by CO_2 production at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

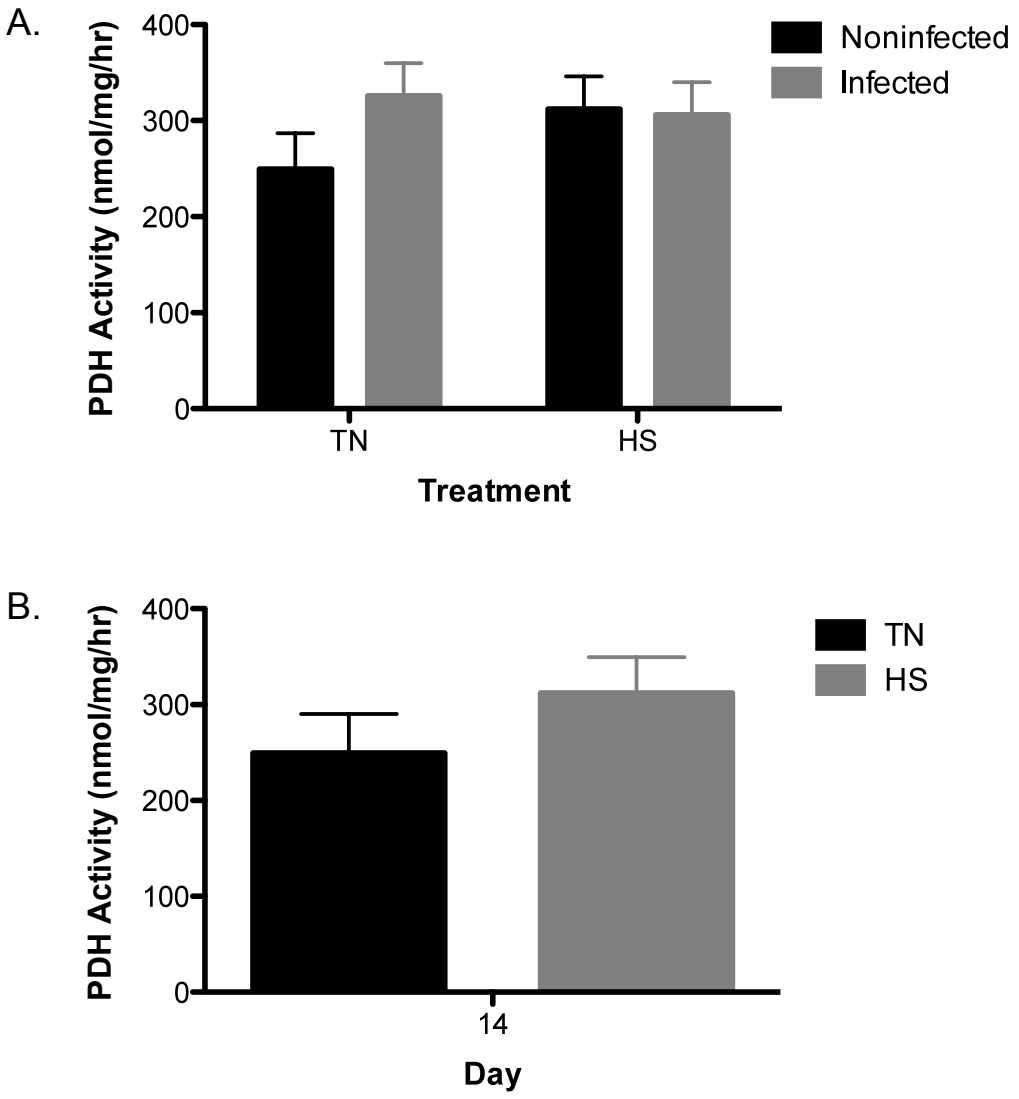

Figure A-2.3: Dual challenge effects of PRRS virus and heat stress on PDH activity. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on PDH activity measured by CO_2 production at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on PDH activity measured by CO_2 production at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

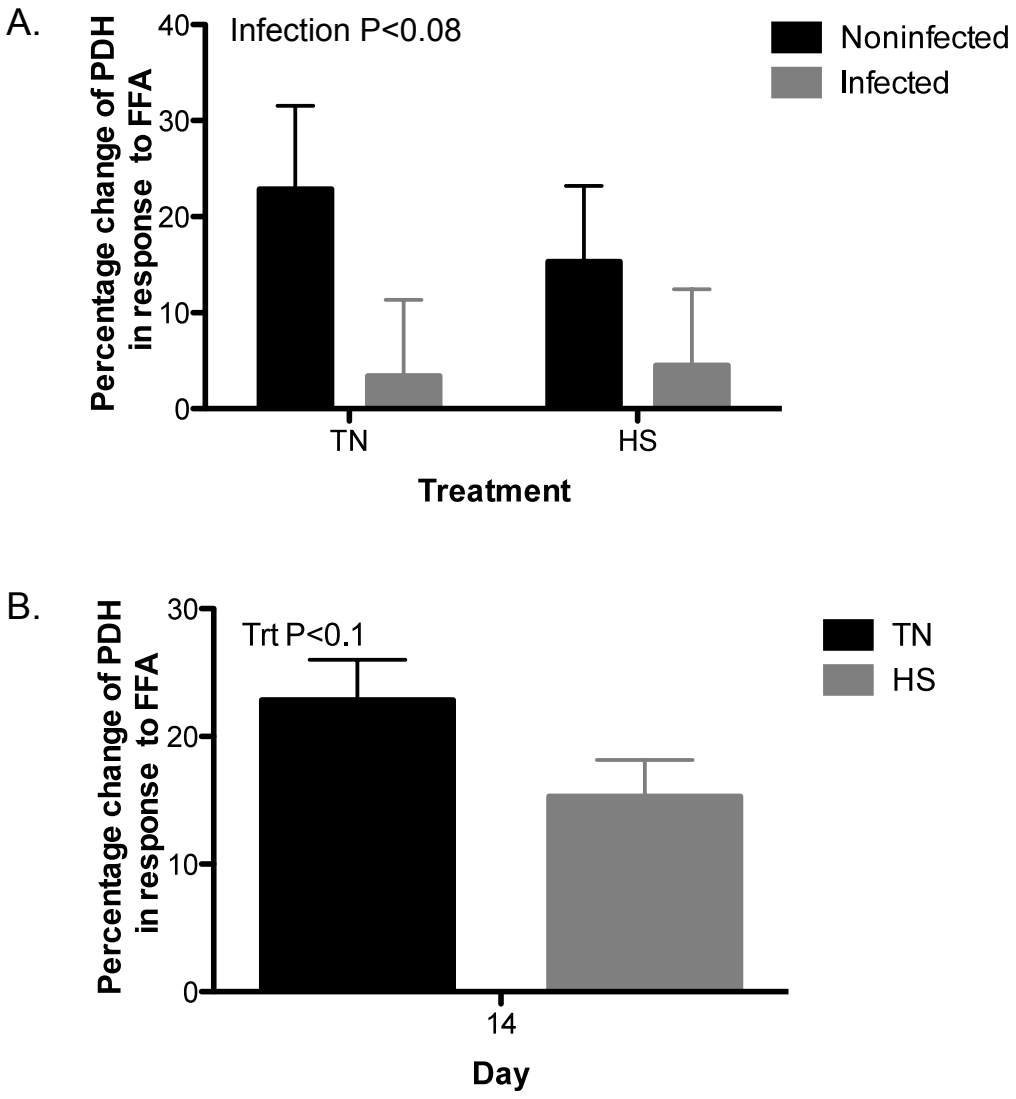

Figure A-2.4: Dual challenge effects of PRRS virus and heat stress on metabolic flexibility. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on metabolic flexibility measured by percent change in PDH in response to FFA at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on metabolic flexibility measured by percent change in PDH in response to FFA at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

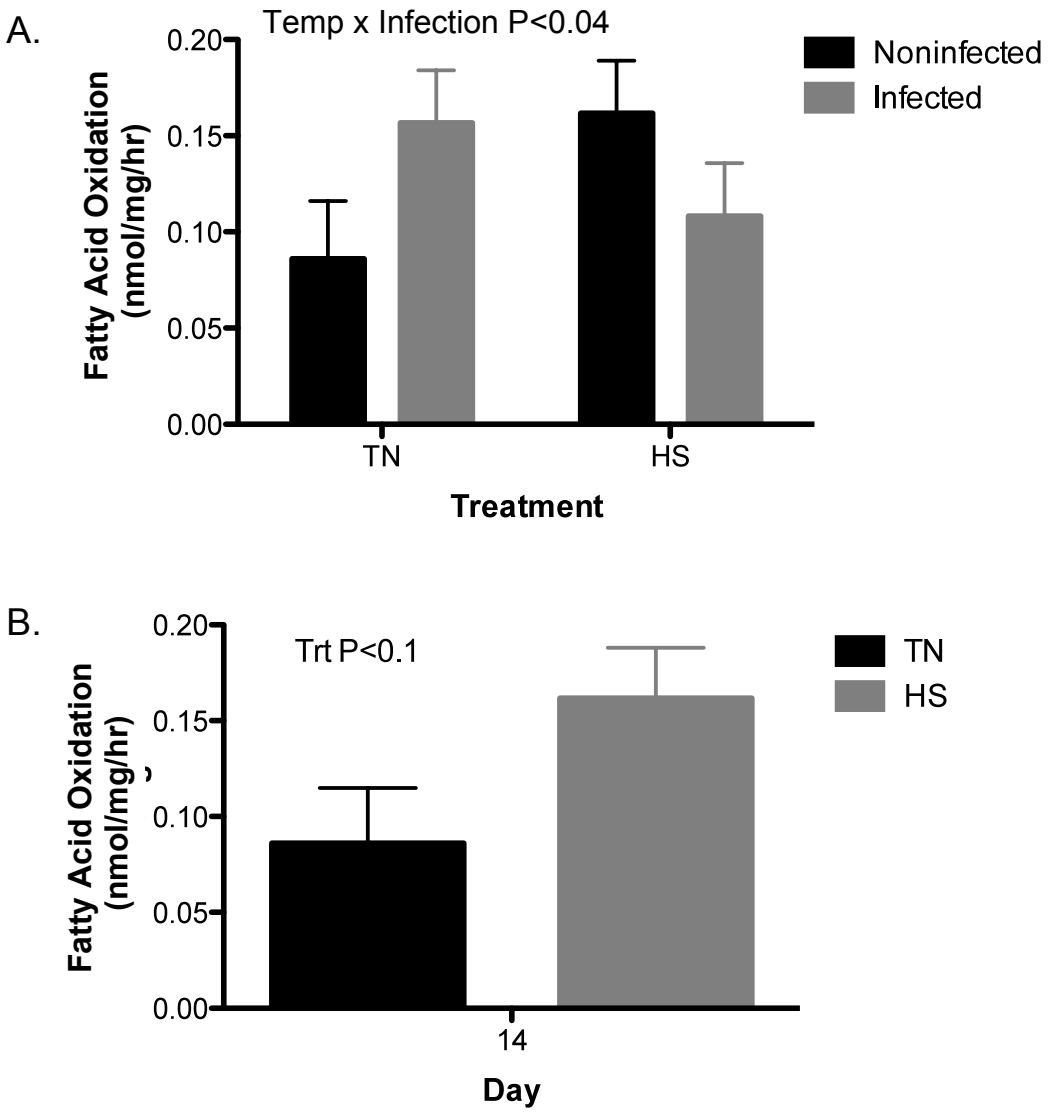

Figure A-2.5: Dual challenge effects of PRRS virus and heat stress on fatty acid oxidation. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on fatty acid oxidation by CO2 production at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on fatty acid oxidation by CO2 production at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

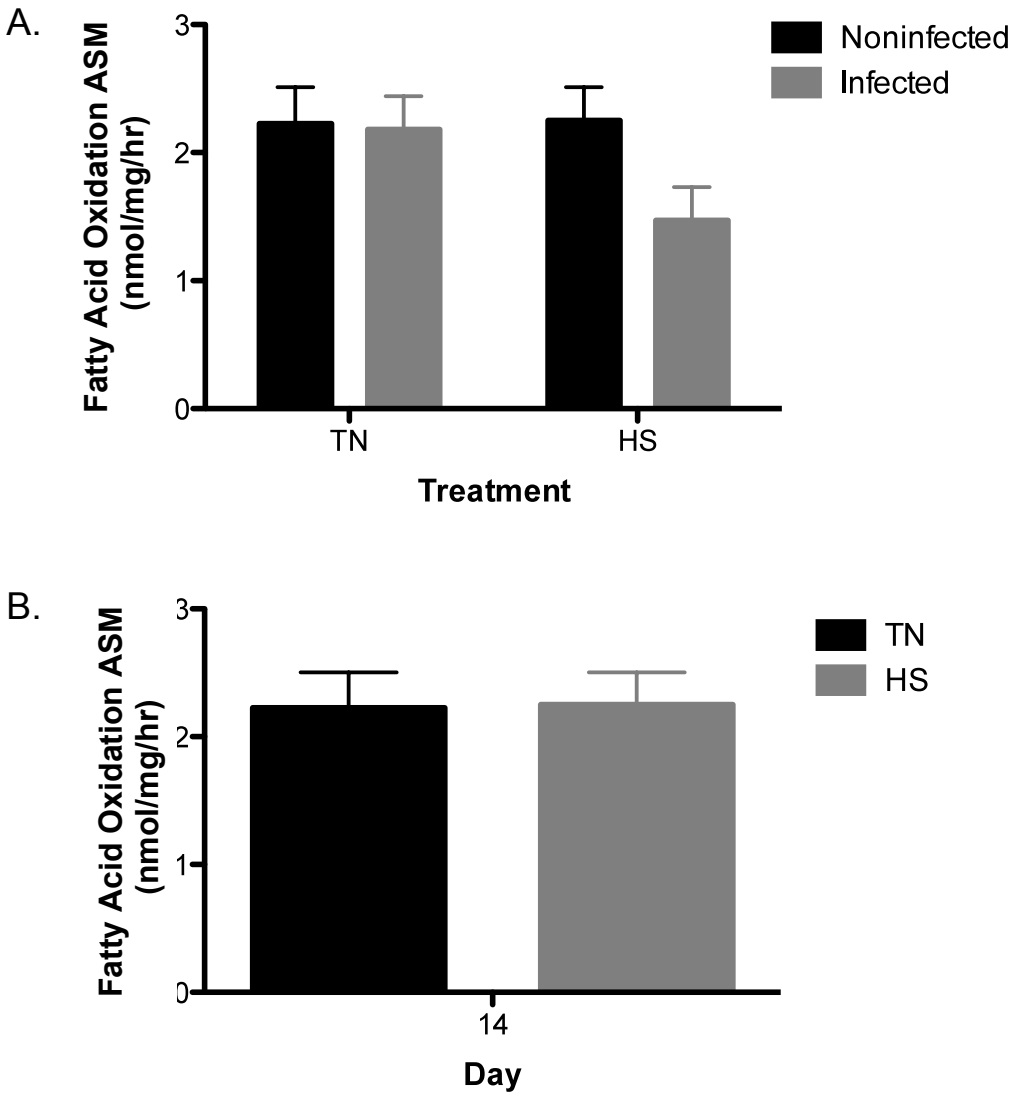

Figure A-2.6: Dual challenge effects of PRRS virus and heat stress on partial fatty acid oxidation. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on fatty acid oxidation by acid soluble metabolites at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on fatty acid oxidation by acid soluble metabolites at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

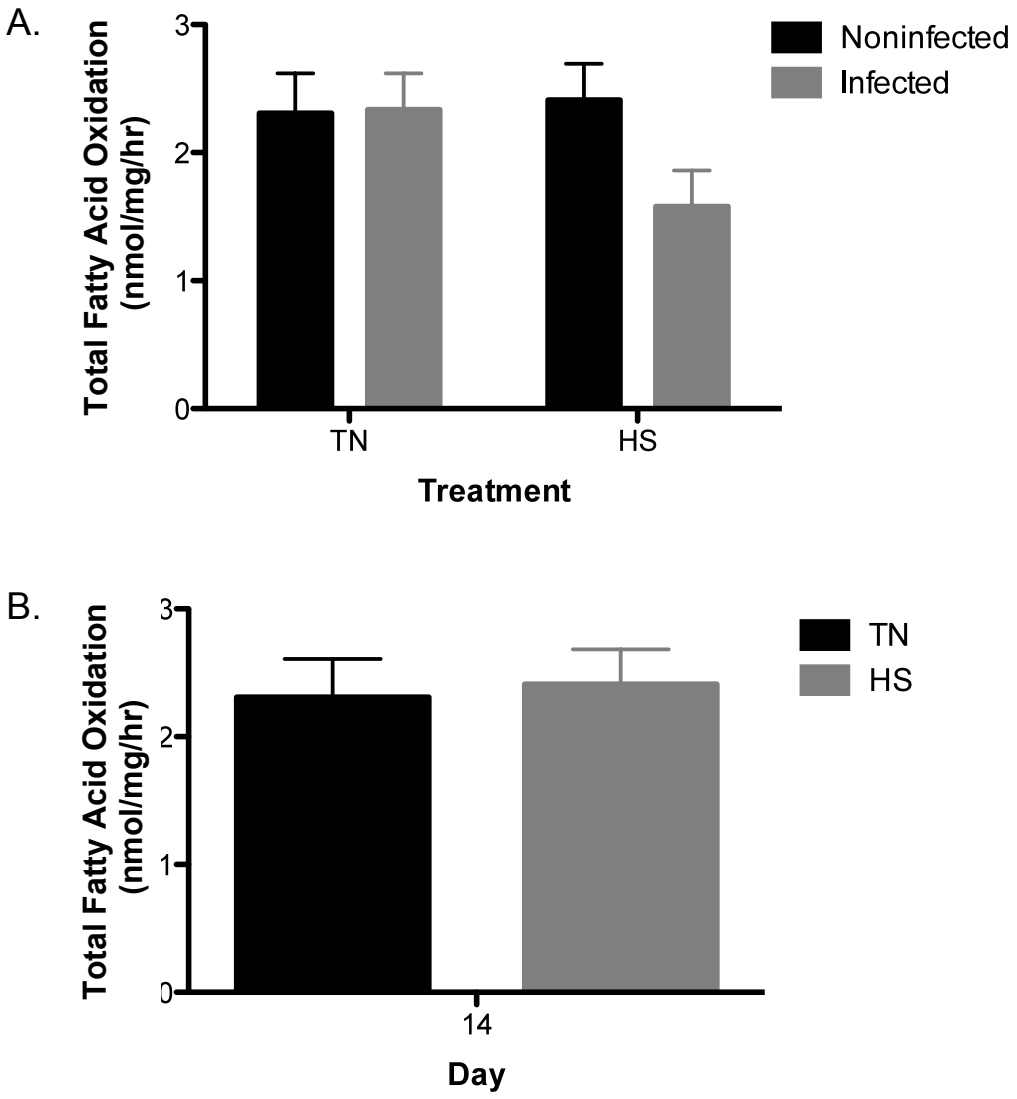

Figure A-2.7: Dual challenge effects of PRRS virus and heat stress on total fatty acid oxidation. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on ((A) effects of TN, TP, HS, and HP on fatty acid oxidation by acid soluble metabolites at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on fatty acid oxidation by acid soluble metabolites at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

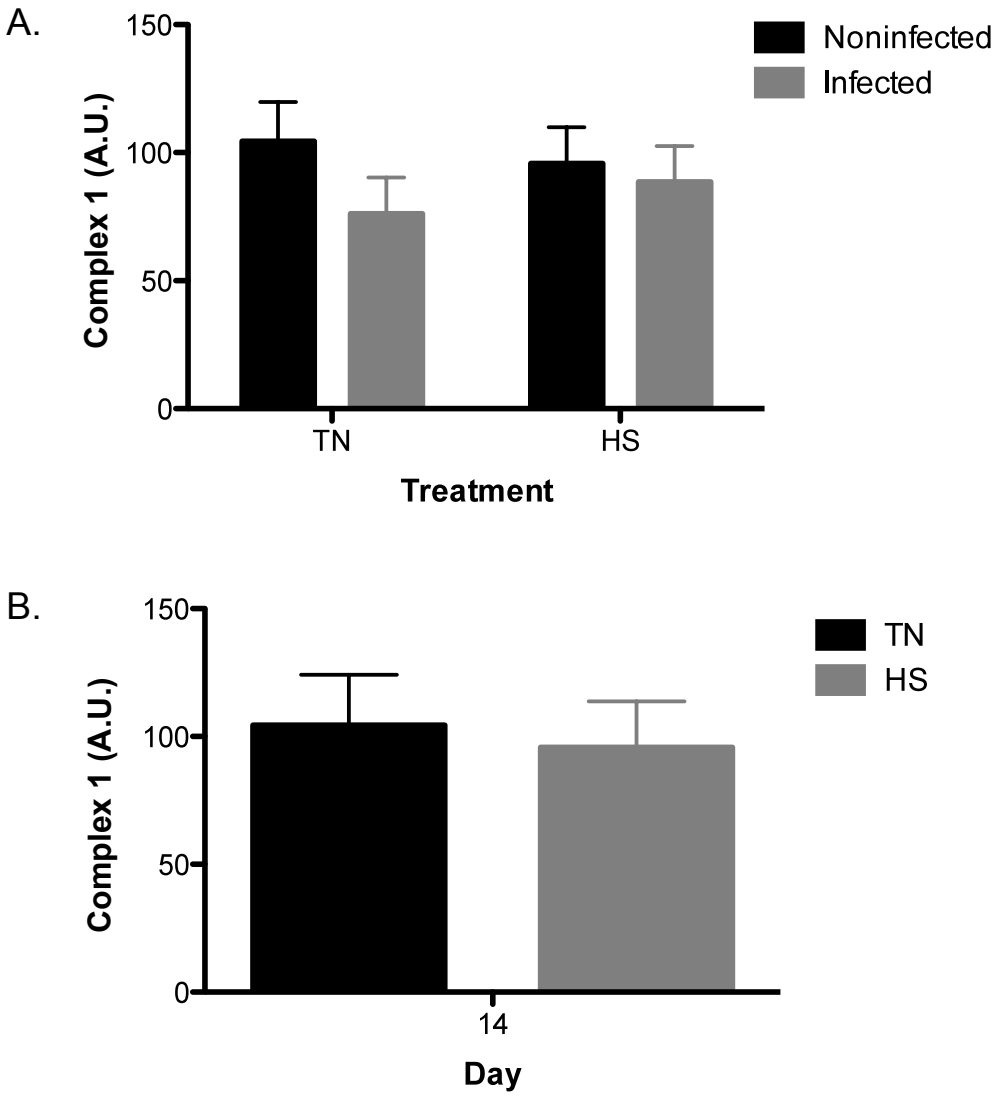

Figure A-2.8: Dual challenge effects of PRRS virus and heat stress on mitochondrial reactive oxygen species (ROS) complex 1. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on mitochondrial reactive oxygen species production by complex 1 at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on mitochondrial reactive oxygen species production by complex 1 at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

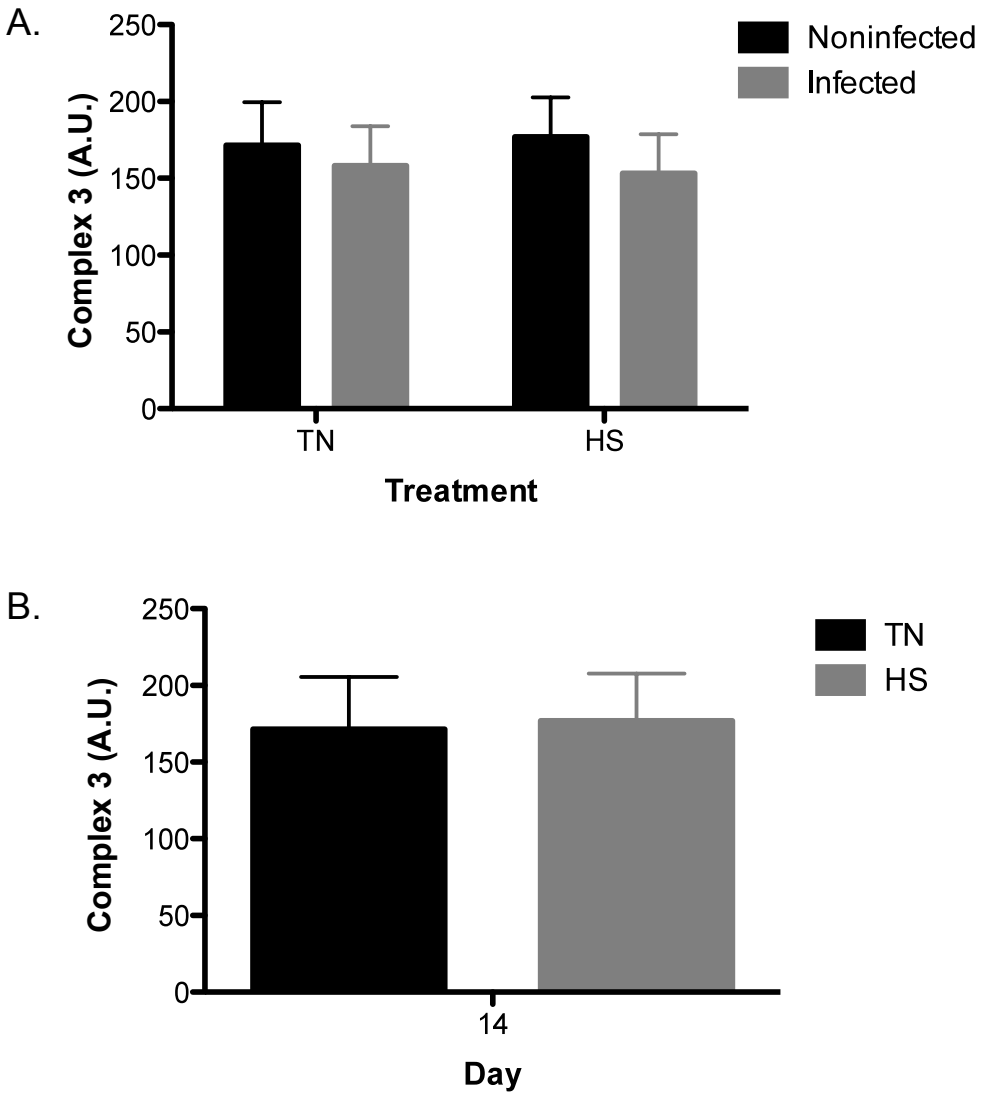

Figure A-2.9: Dual challenge effects of PRRS virus and heat stress on mitochondrial reactive oxygen species (ROS) complex 3. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on mitochondrial reactive oxygen species production by complex 3 at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on mitochondrial reactive oxygen species production by complex 3 at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

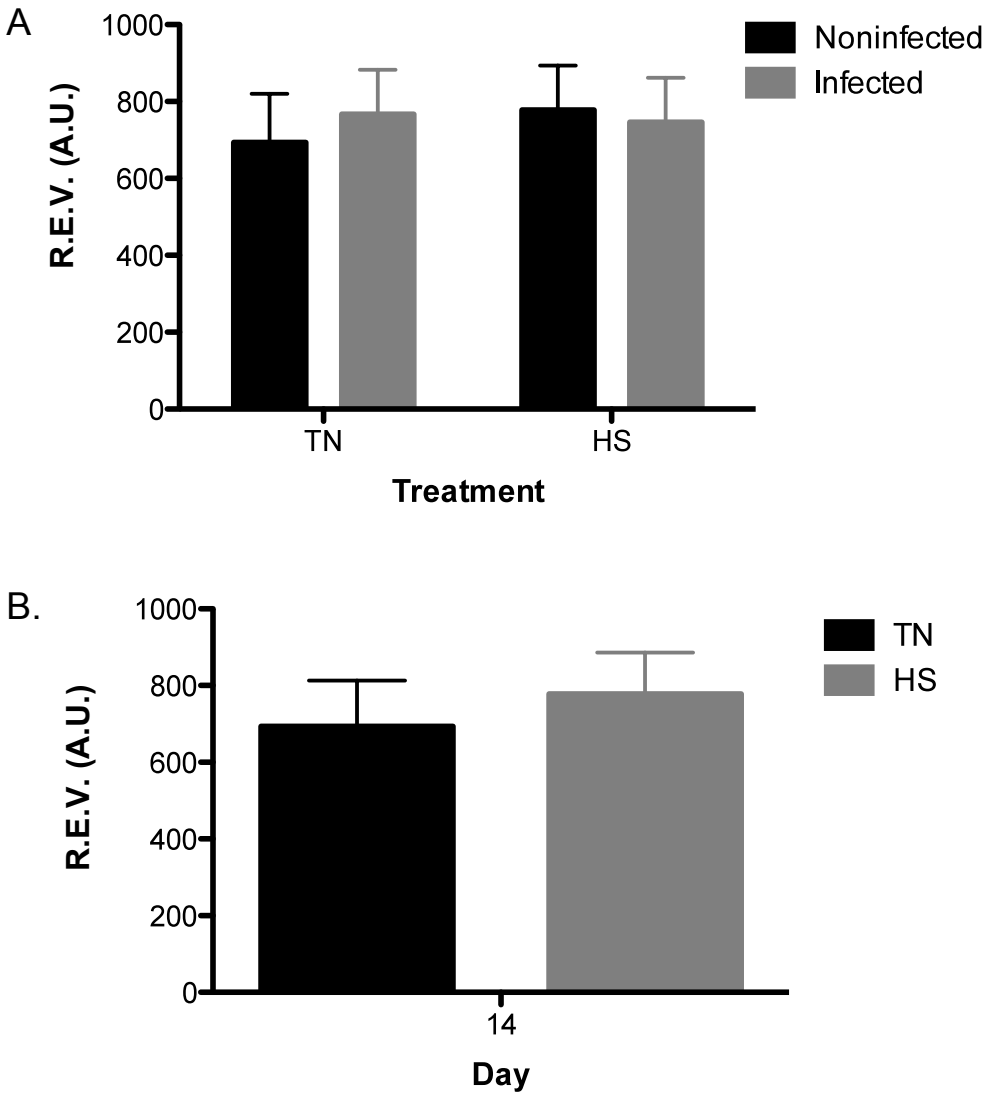

Figure A-2.10: Dual challenge effects of PRRS virus and heat stress on mitochondrial reactive oxygen species (ROS) REV. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on (A) effects of TN, TP, HS, and HP on mitochondrial reactive oxygen species production REV at the end of the experiment TN n=5, TP n=6, HS n=6, HP n=6 and (B) effects of TN and HS on mitochondrial reactive oxygen species production REV at the end of the experiment. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

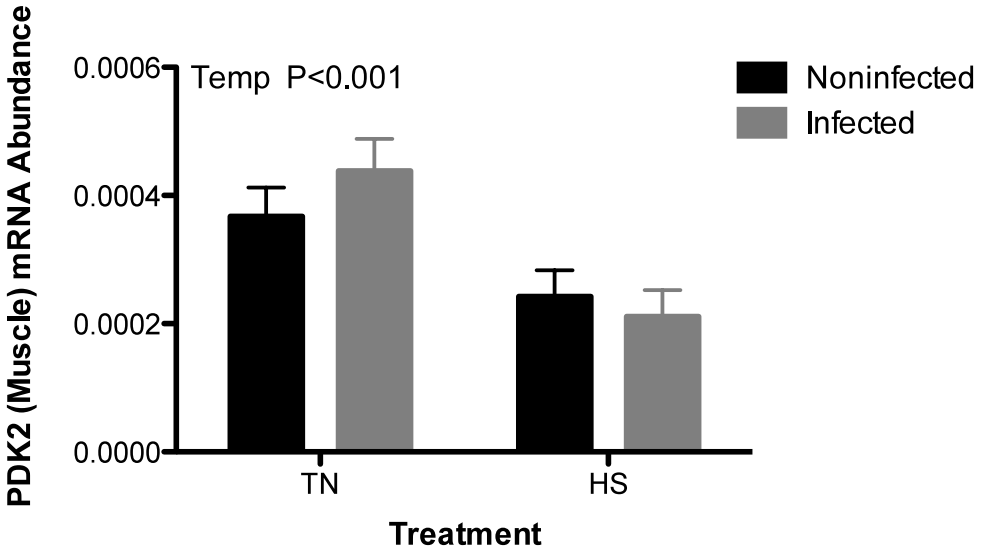

Figure A-2.11: Dual challenge effects of PRRS virus and heat stress on PDK2 gene expression in skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on PDK2 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

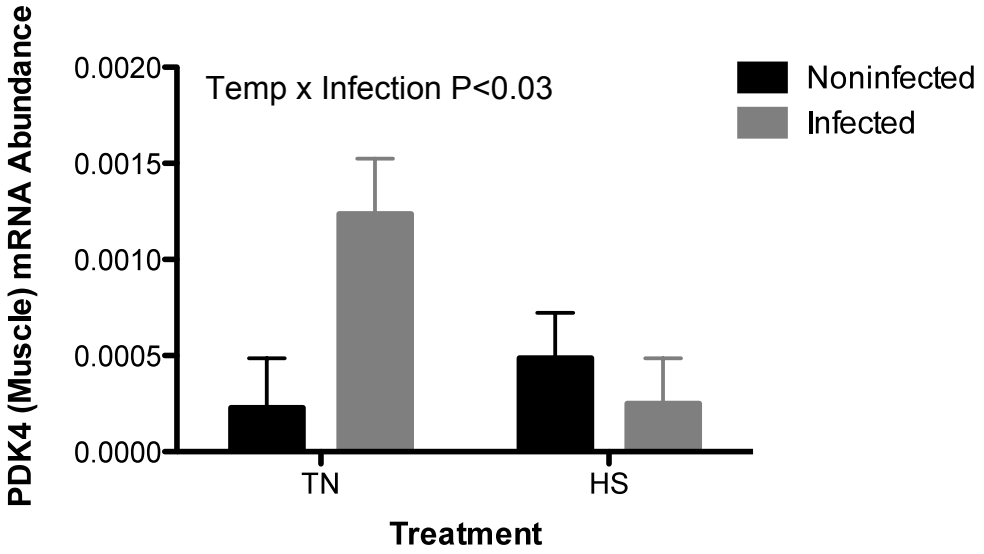

Figure A-2.12: Dual challenge effects of PRRS virus and heat stress on PDK4 gene expression in skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on PDK4 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

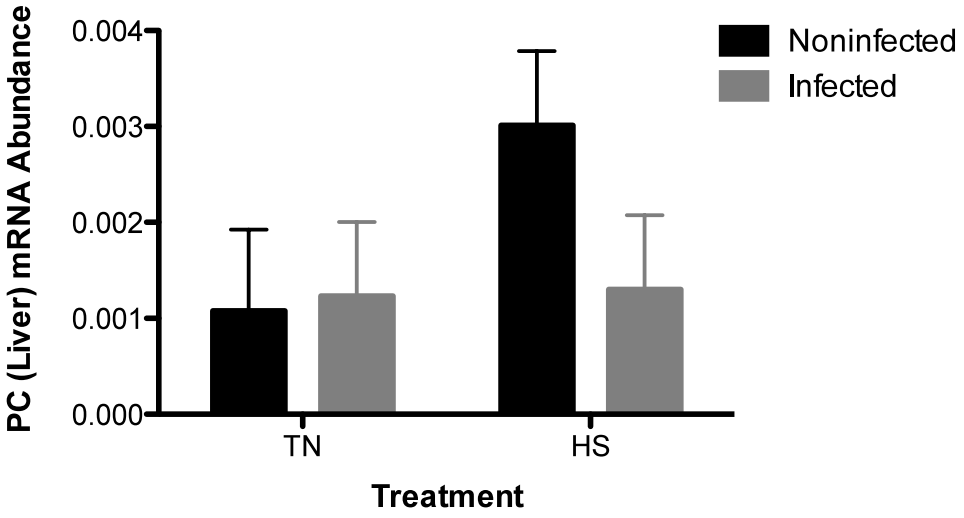

Figure A-2.13: Dual challenge effects of PRRS virus and heat stress on PC gene expression in liver. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on PC mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

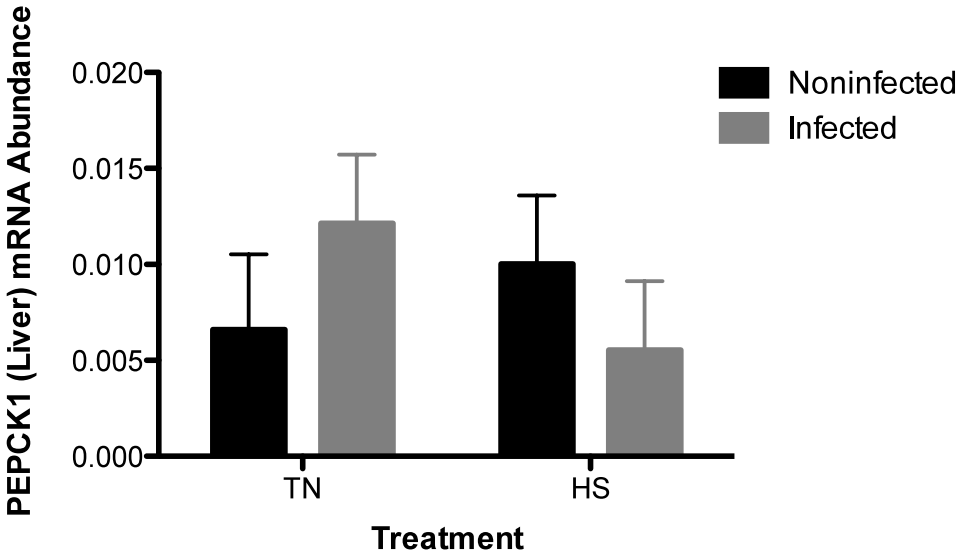

Figure A-2.14: Dual challenge effects of PRRS virus and heat stress on PEPCK1 gene expression in liver. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on PEPCK1 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

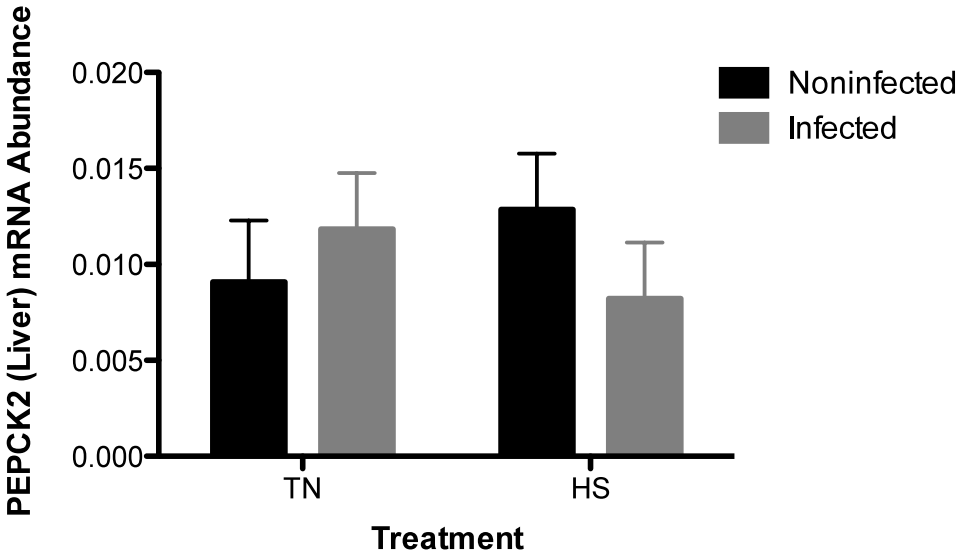

Figure A-2.15: Dual challenge effects of PRRS virus and heat stress on PEPCK2 gene expression in liver. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on PEPCK2 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

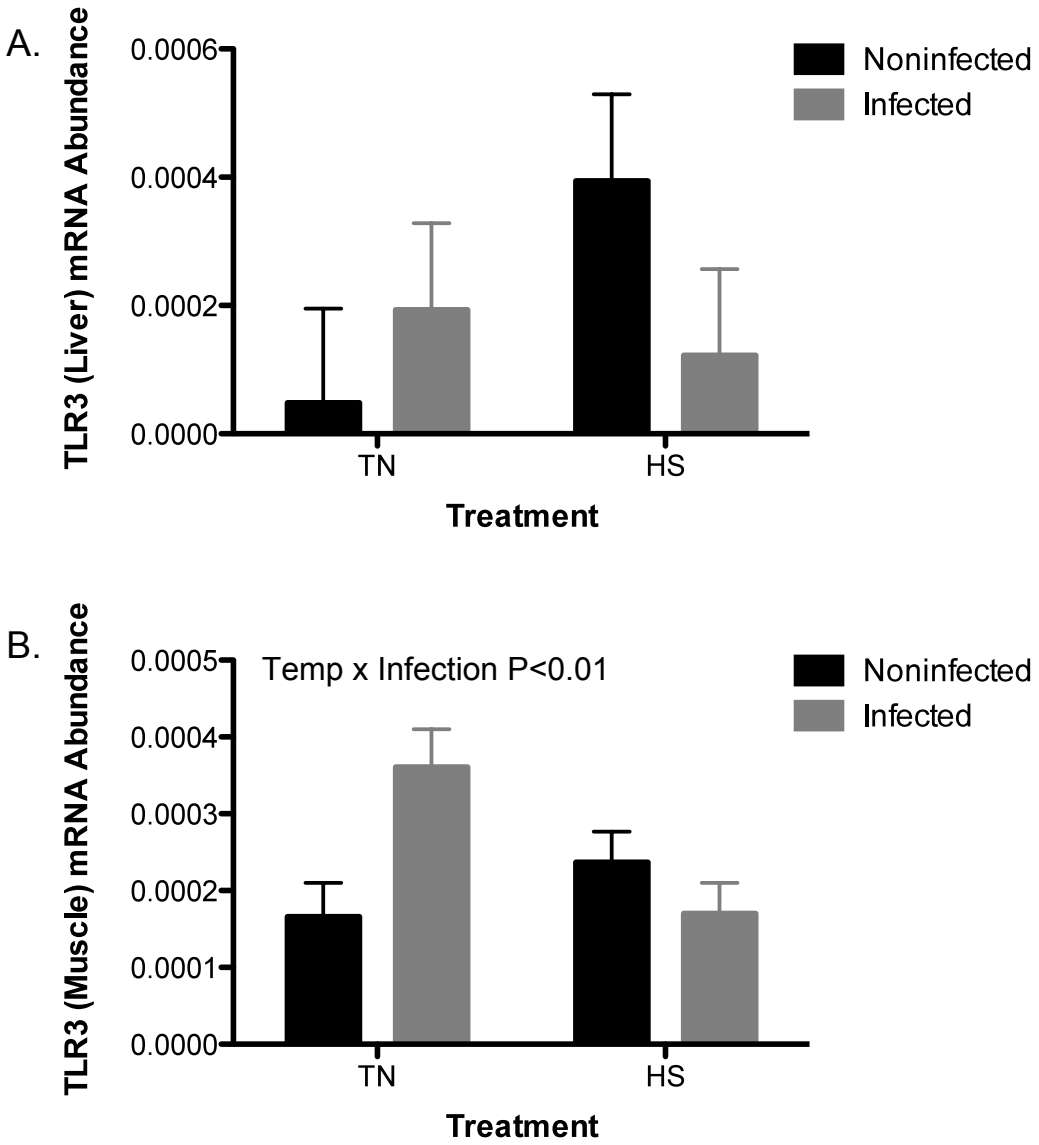

Figure A-3.1: Dual challenge effects of PRRS virus and heat stress on TLR3 gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on TLR3 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

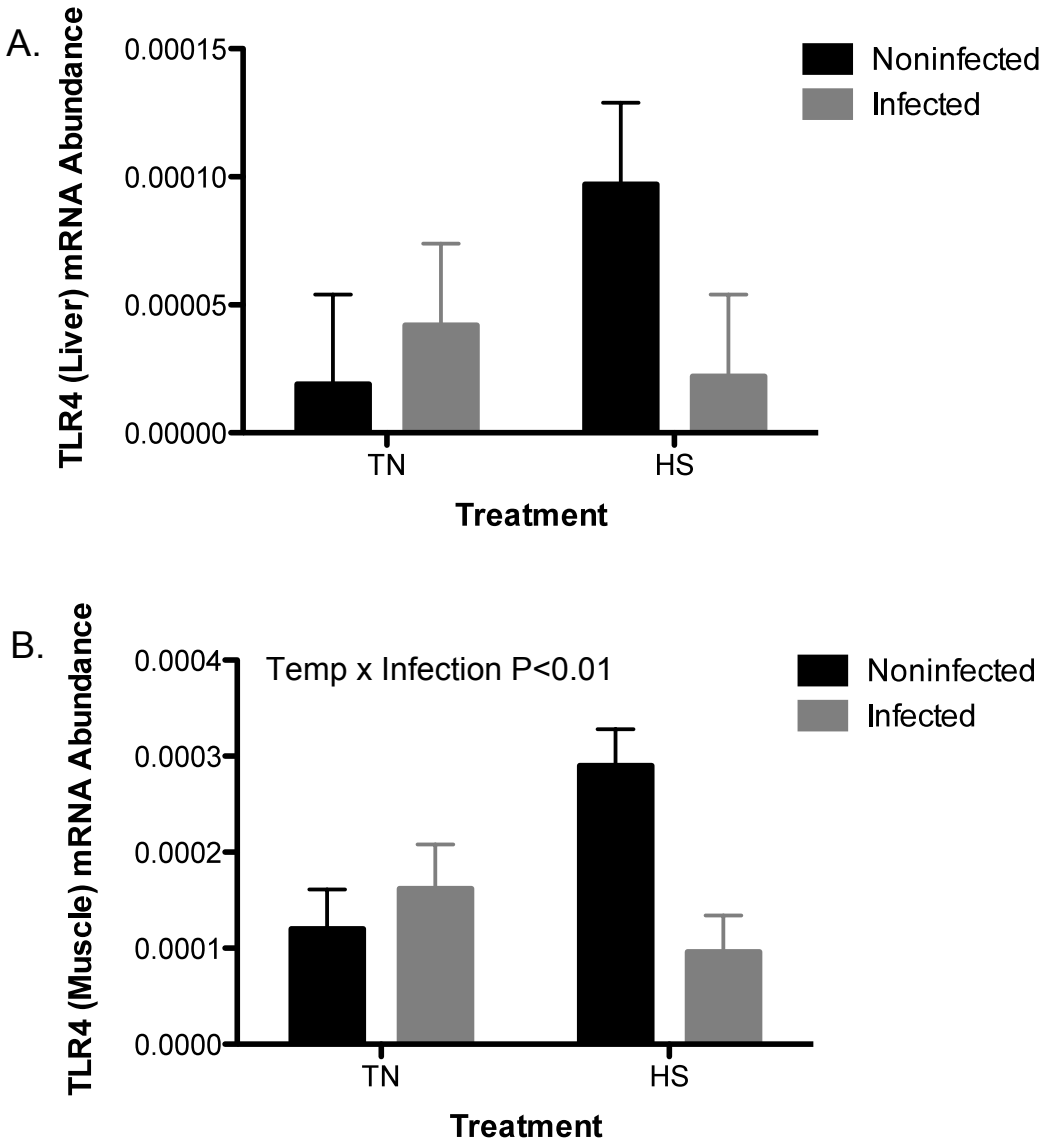

Figure A-3.2: Dual challenge effects of PRRS virus and heat stress on TLR4 gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on TLR4 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

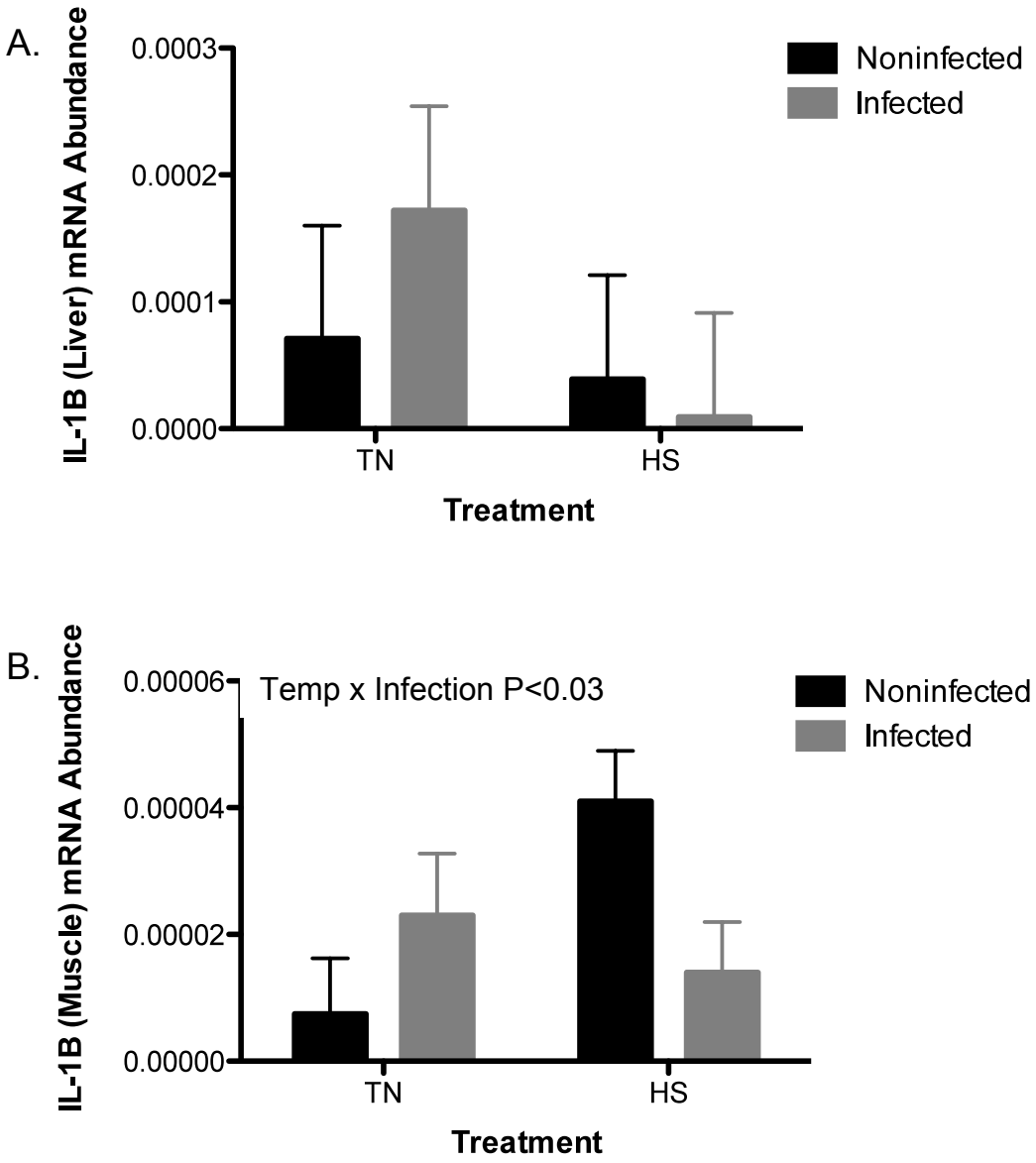

Figure A-3.3: Dual challenge effects of PRRS virus and heat stress on IL-1B gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on IL-1B mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

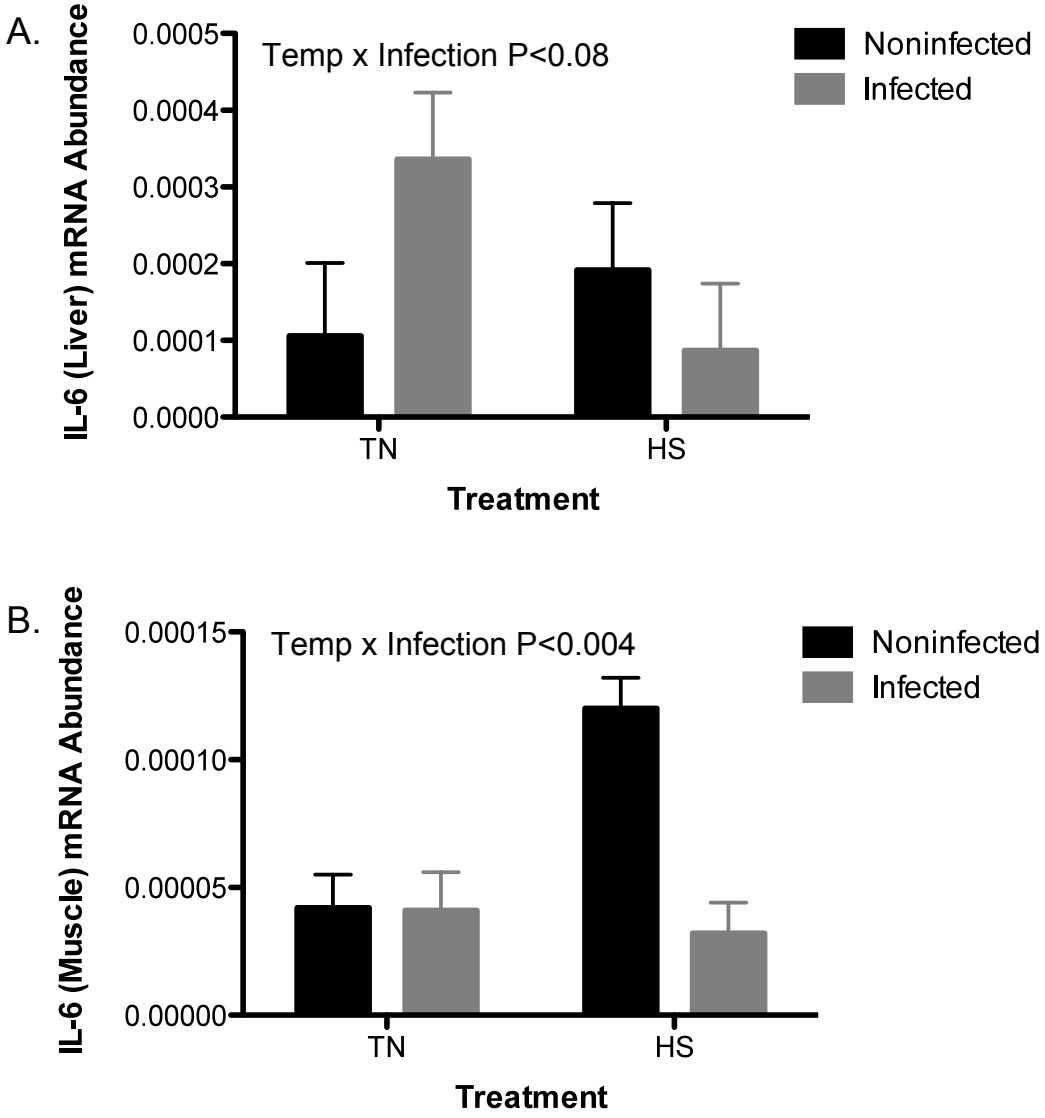

Figure A-3.4: Dual challenge effects of PRRS virus and heat stress on IL-6 gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on IL-6 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

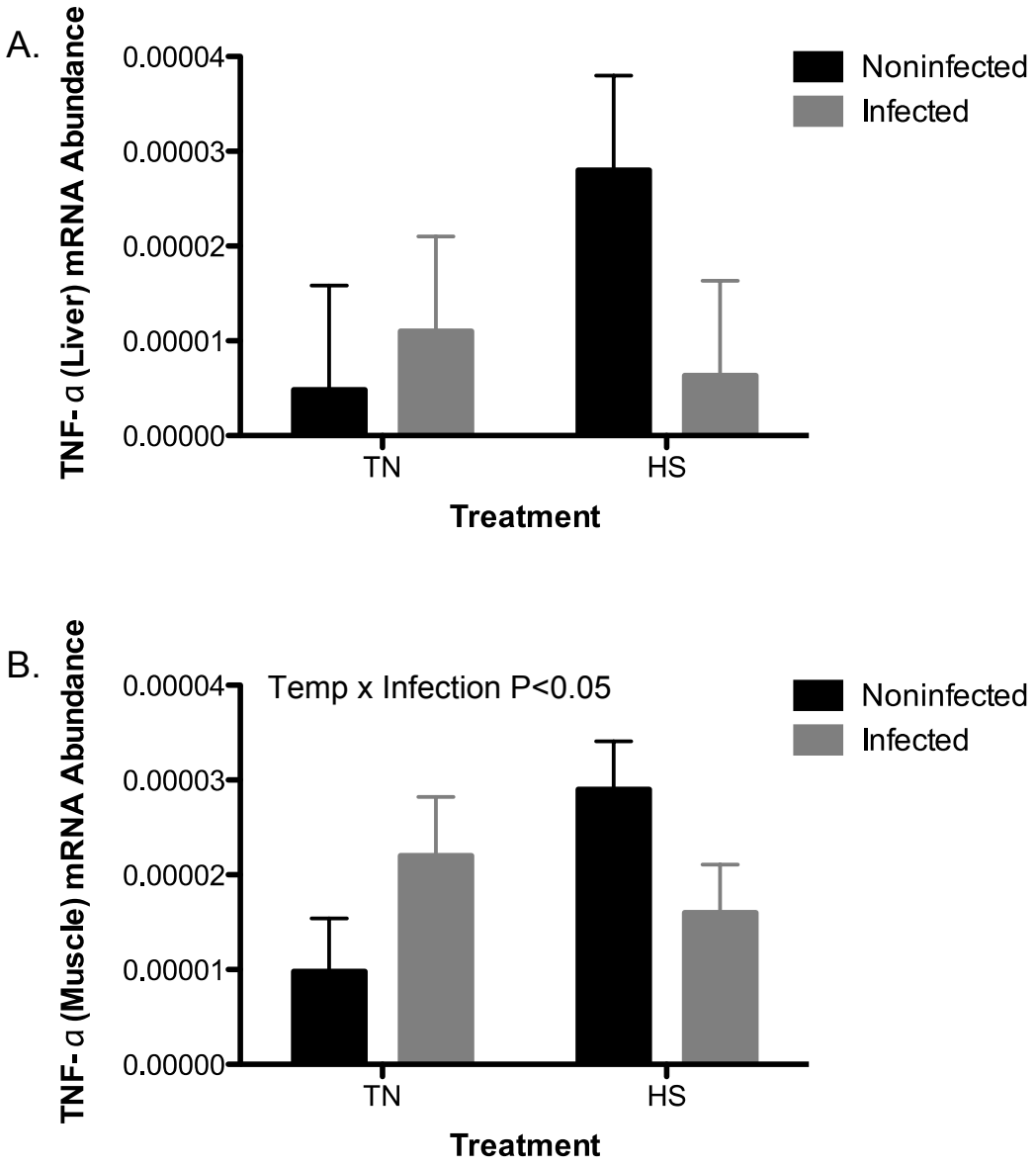

Figure A-3.5: Dual challenge effects of PRRS virus and heat stress on TNF-α gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on TNF- α mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

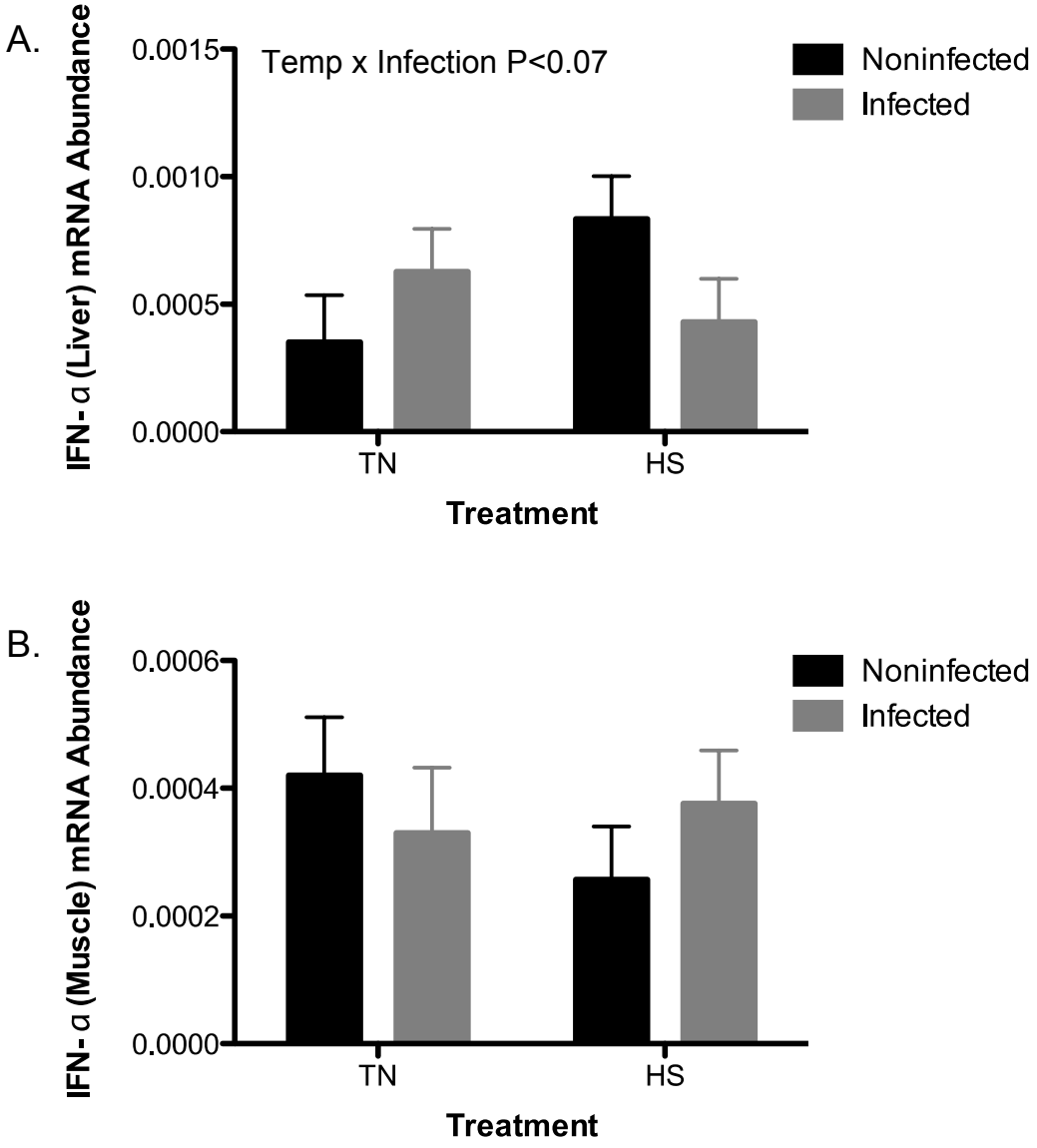

Figure A-3.6: Dual challenge effects of PRRS virus and heat stress on IFN-α gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on IFN-α mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

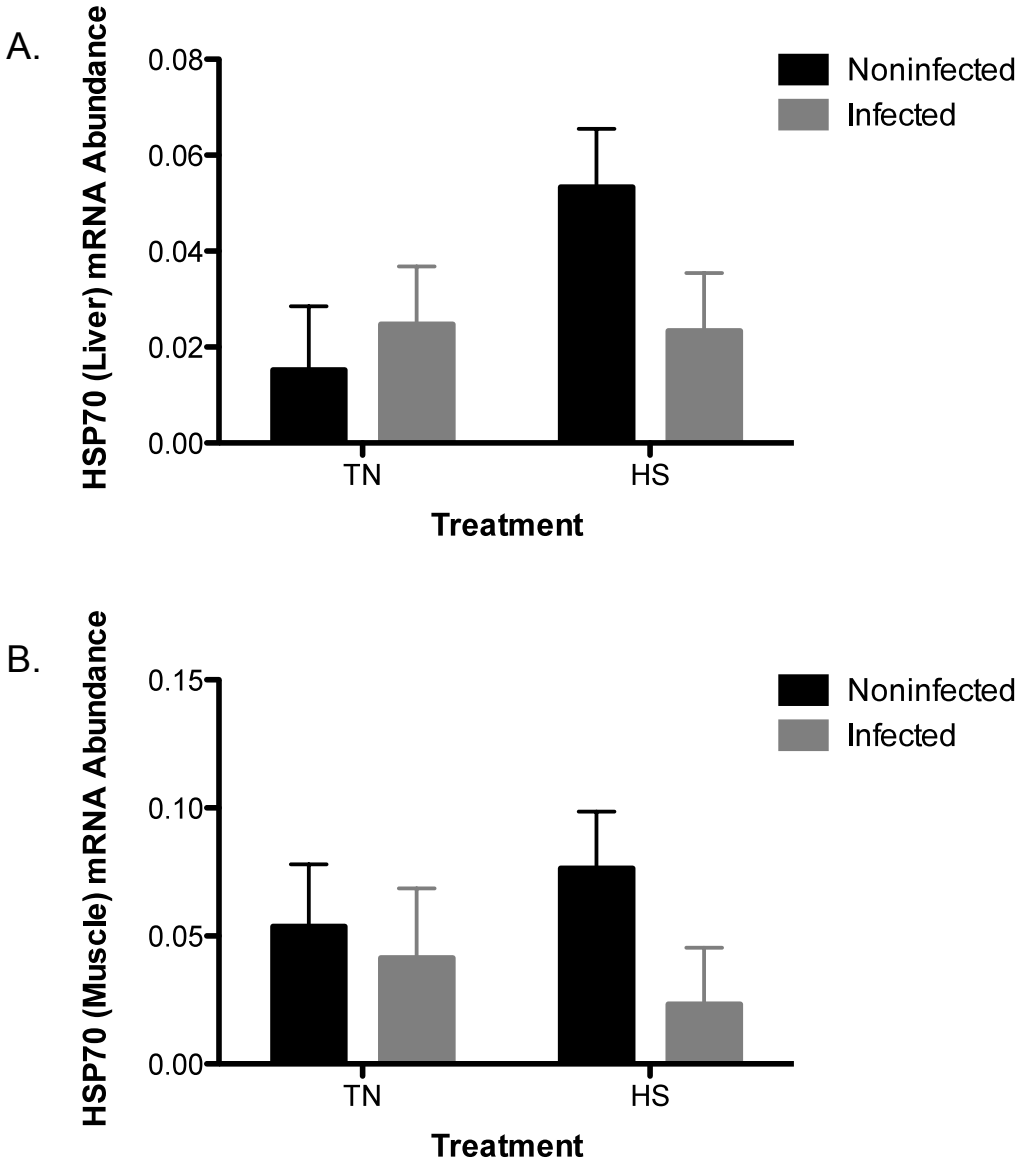

Figure A-3.7: Dual challenge effects of PRRS virus and heat stress on HSP70 gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on HSP70 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

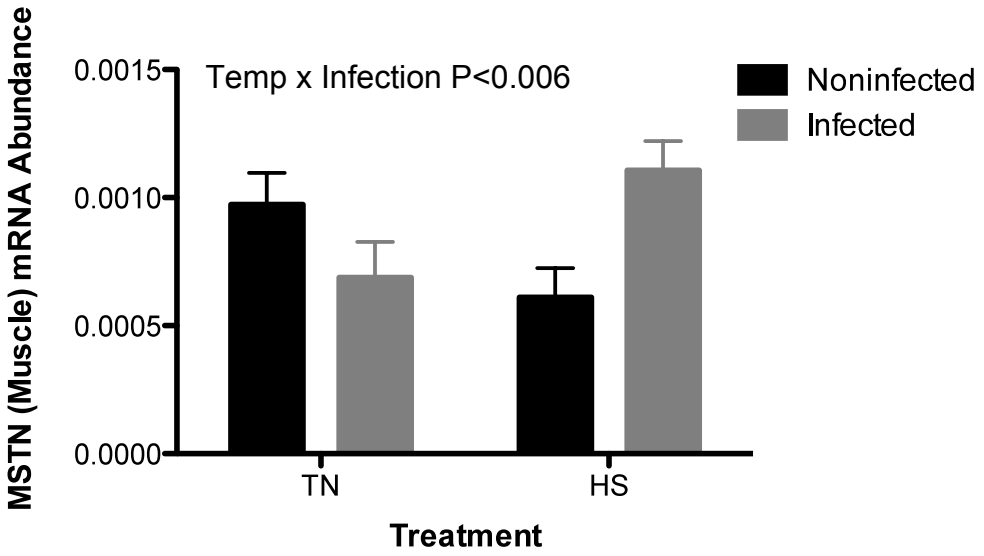

Figure A-3.8: Dual challenge effects of PRRS virus and heat stress on myostatin (MSTN) gene expression in skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on MSTN mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.

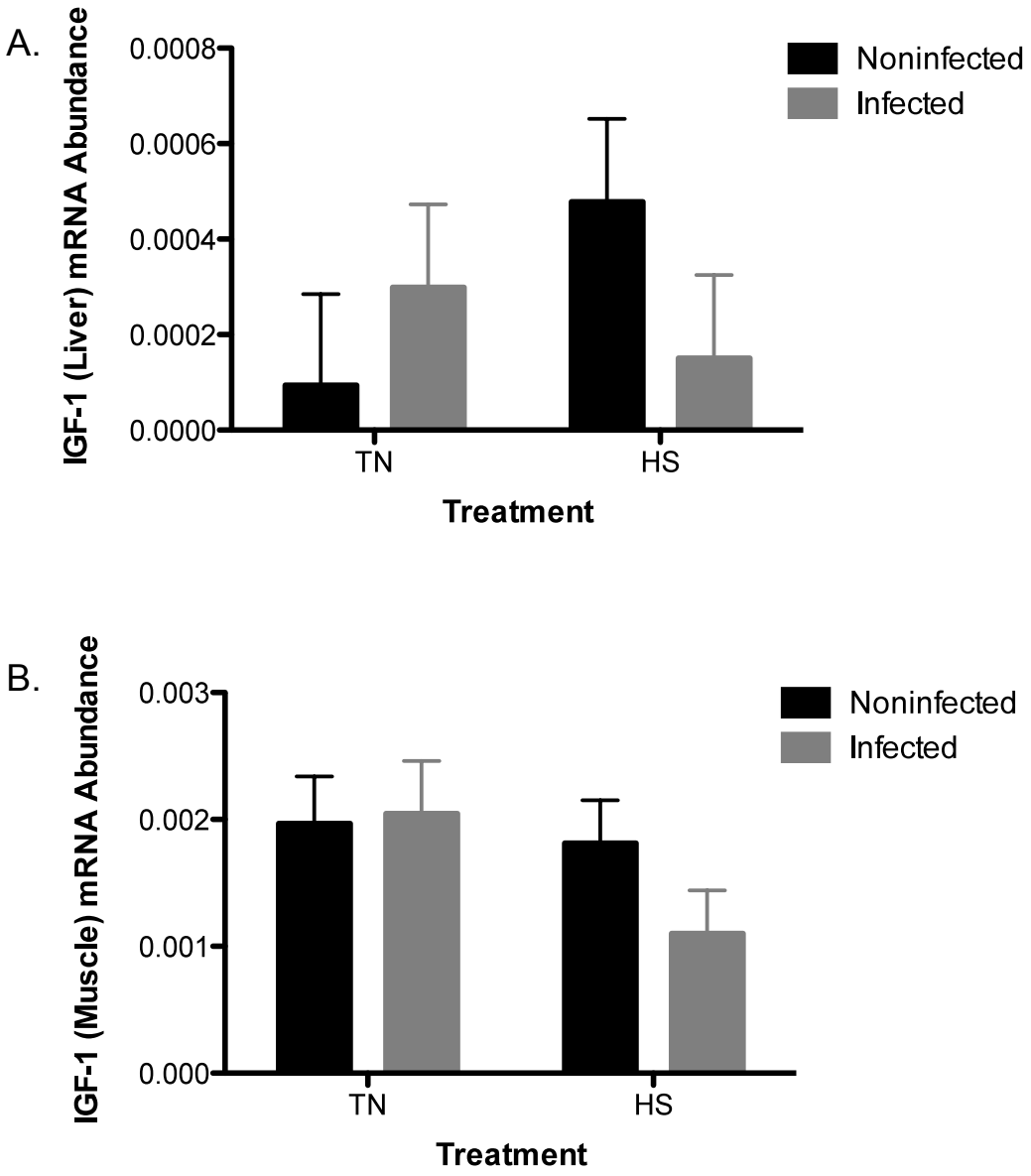

Figure A-3.9: Dual challenge effects of PRRS virus and heat stress on IGF-1 gene expression in (A) liver and (B) skeletal muscle. A PRRS viral challenge in thermo-neutral infected (TP; 22°C) conditions or uninfected thermo-neutral (TN; 22°C) conditions from day 1-11 and heat stress, uninfected (HS; 35°C) conditions or heat stress infected (HP; 35°C) conditions from day 11-14 on IGF-1 mRNA abundance measured at the end of the experiment by RT-PCR. Data represent LS means ± S.E.M. TN n=5, TP n=6, HS n=6, HP n=6. Statistical significance was set at $P < 0.05$.