Investigation of Growth Performance and Immunomodulatory Factors Associated with the Hepatic Gene Expression of the Insulin-like Growth Factor System in Nursery Pigs

by

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ABSTRACT

INVESTIGATION OF GROWTH PERFORMANCE AND IMMUNOMODULATORY FACTORS ASSOCIATED WITH THE HEPATIC GENE EXPRESSION OF THE INSULIN-LIKE GROWTH FACTOR SYSTEM IN NURSERY PIGS

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The insulin-like growth factor (IGF) system is an important determinant of growth in animals and recent findings suggest that immunomodulatory activity may regulate the IGF system independent of nutritional parameters. A cross-sectional study of the hepatic gene expression of the IGF system (IGF-1, IGFBP-3, GHR) and immunomodulatory agents (CRP, Hp, SAA, IL-1β, IL-6, IL-10, IL-18, IFN-α, IFN-γ, TNF-α) in nursery pigs (n=168) from 8 farms was completed. Production parameters and swine pathogens (*Salmonella* spp., *L. intracellularis*, *Brachyspira* spp., ETEC, MRSA, SIV, and PRRSV) were also assessed in relation to the IGF system. Hepatic expression of IGF-1, IGFBP-3, and GHR was associated with growth performance and was inversely associated with several immunomodulatory agents including Hp, SAA, IL-1β, IL-18, and TNF-α. These findings suggest that elevated levels of proinflammatory agents may dysregulate the transcriptional expression of the IGF system in hepatic tissue and consequently affect the growth and metabolism of nursery pigs.

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CONTRIBUTIONS

Mackenzie Slifierz assisted with the field work, sample collection, and the autopsies, completed the extraction, quantification, and qualification of RNA, DNase digestion, and primer design, assisted with the RT-qPCR, analyzed the data, and disseminated the results.

Dr. Kees de Lange led the research project.

Dr. Vahab Farzan designed the study and survey, coordinated the bacteriological and virological laboratory work, helped with the autopsies, and assisted with the data analysis.

Dr. Bob Friendship helped coordinate the field work, interpreted the results, and provided critical feedback on the logistics of the study.

Marko Rudar assisted with primer design, RNA extraction, and DNase digestion, in addition to helping with the autopsies and assisting with interpreting the results.

Jing Zhang coordinated and completed the RT-qPCR laboratory work at the University of Guelph's Advance Analysis Centre.

Dr. Durda Slavic coordinated the bacteriological testing of the samples at the Animal Health laboratory.

Dr. Helena Grgic completed the isolation and detection of SIV from the nasal samples in the Department of Population Medicine.

Dr. Susy Carman performed the detection of PRRSV at the Animal Health Laboratory.

Joyce Rousseau assisted with culturing and detecting MRSA from the nasal samples at the University of Guelph's Centre for Public Health and Zoonoses Laboratory.

Brian Bloomfield, Sara Malison, Kristina Dekroon, and Rachel Poppe assisted with collecting samples, weighing pigs, and managing data at all stages of the study.

Doug Wey helped with the autopsies and sample collection.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADG – average daily gain

APP – acute-phase protein

APR – acute-phase response

CRP – C-reactive protein

CT – cholera toxin

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GH – growth hormone

GHR – growth hormone receptor

GLS – generalized least squares

Hp – haptoglobin

IFN- interferon

IGF – insulin-like growth factor

IGFBP – insulin-like growth factor binding protein

IL – interleukin

LPS – lipopolysaccharide

LSDV – least squares dummy variable

RLP4 – receptor-like protein 4

SAA – serum amyloid A

TNF – tumor necrosis factor

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Herd health is an important determinant of productivity for swine production systems, especially in the nursery phase when pigs are particularly susceptible to disease. Rapid diagnosis and intervention is critical for minimizing the negative impacts of swine disease which cost the swine industry hundreds of millions of dollars in lost productivity each year (Duhamel and Joens, 1994; McOrist et al., 1997; Neumann et al., 2005; Holtkamp et al., 2013). Recent advances in proteomics and transcriptomics have progressed the development and utilization of biomarkers as clinical tools for rapidly assessing herd health (Petersen et al., 2004). Biomarkers are measurable biological compounds that convey information about the physiological state of an animal, and provide an objective, unbiased, and rapid method for detecting disease and evaluating interventions (Mayeux, 2004).

Biomarkers can also be used to contrast desirable traits and assess productive performance of livestock species (Ibeagha-Awemu et al., 2008). Evaluating the insulin-like growth factor (IGF) system is of particular interest because it is an important determinant of growth and development in animals (Dupont and Holzenberger, 2003). However, evidence that demonstrates whether hepatic gene expression of the IGF system is a useful biomarker for assessing growth performance in nursery pigs is lacking.

In addition, although nutritional parameters are major regulators of the IGF system, previous evidence also indicates that non-nutritional factors, such as immunomodulatory activity, may interfere with transcriptional expression of the IGF

system (Roberts and Almond, 2003; Jenkins et al., 2004; Savage, 2013). Furthermore, some findings suggest that cytokines and acute-phase proteins (APPs) may be useful biomarkers of productivity in pigs (Eurell et al., 1992; Saco et al., 2010). The molecular interaction between the IGF system and immunomodulatory activity, and the usefulness of immunomodulators as biomarkers for productivity, needs further investigation.

1.2 The importance of herd health for swine production

Maximizing productivity is a major priority in swine production. The relationship between swine production and health is complex and consists of a multitude of factors, but understanding the impact of disease on production outcomes is necessary for decision-making and management of a herd. Diseases can commonly impact swine production in multiple ways; reductions in growth performance and feed efficiency, increased rate of culling, extended duration in facilities, change in herd composition and reproductive selections, and increased morbidity and mortality (Regula et al., 2000; Hampson et al., 2006; Olsen et al., 2006; Zimmerman et al., 2006). The negative impacts resulting from poor herd health have cost the industry millions of dollars in lost productivity (Duhamel and Joens, 1994; McOrist et al., 1997; Neumann et al., 2005). Therapeutic interventions, enhancing biosecurity, herd depopulation, and disinfection of facilities are common methods of eliminating disease-causing agents, but such strategies can create a short-term economic burden for producers (Holtkamp et al., 2012). Recent control strategies emphasize the importance and cost efficiency of prevention through enhanced biosecurity measures and vaccination (Corrégé et al., 2012; Alacron et al., 2013). The following section will outline some common viral and bacterial pathogens in swine and the impacts of these pathogens on swine production.

1.2.1 Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is a host restricted positive-strand RNA virus that proliferates primarily in porcine macrophages in various tissues (Pol et al., 1991; Zimmerman et al., 2006). It is a leading cause of reproductive failure in sows, including stillbirths, abortions, and premature farrowing, and it causes significant mortality in suckling piglets (Zimmerman et al., 2006). PRRSV can persist in infected pigs for several months and it is highly transmissible; two factors which have contributed to PRRSV becoming established in many regions throughout the world (Zimmerman et al., 2006). PRRSV significantly reduces ADG in clinically and subclinically affected animals (Regula et al., 2000), and it is rated as the top health-related cause of lost productivity in US breeding herds (Holtkamp et al., 2007). It is predicted that production losses due to PRRSV cost the US swine industry $664 million USD annually (Holtkamp et al., 2013).

1.2.2 Swine Influenza Virus

Traditionally, the most common and important swine influenza virus (SIV) in pig production in North America is H1N1, a type A influenza virus. Genetic reassortment of the negative-sense RNA in this virus produces different genetic and antigenic variants (Olsen et al., 2006). The virus causes fever, anorexia, lethargy, coughing, and sneezing, although the rate of mortality is usually minimal (Olsen et al., 2006). SIV is highly transmissible and naïve herds are particularly susceptible to outbreaks of this virus which can result in significant morbidity (Olsen et al., 2006). In the US, the prevalence of anti-SIV antibodies in swine sera was 22.8%, and of these positive sera samples 66.3% and 33.7% contained antibodies to H1N1 and H3N2 subtypes, respectively (Choi et al.,

2002). In Canada, a seroprevalence of 61.1% and 24.3% for H1N1 has been reported among sows and grower pigs, respectively (Poljak et al., 2008). Previous research has determined that even subclinical infections with SIV can considerably reduce ADG in pigs (Regula et al., 2000). SIV is ranked as the second highest health-related cause of lost productivity in US breeding herds, just behind PRRSV (Holtkamp et al., 2007).

1.2.3 Spirochetes

Brachyspira hyodysenteriae is an anaerobic, intestinal spirochete that causes swine dysentery. It colonizes the mucosa of the large intestine resulting in inflammation, lesions, and haemorrhaging (Hampson et al., 2006). Other haemolytic intestinal spirochetes, including *Brachyspira pilosicoli* and *Brachyspira murdochii*, have been reported to be pathogenic in pigs and may act synergistically with other infectious agents to cause disease (Hampson et al., 2006; Jensen et al., 2010). Swine dysentery can cause significant financial losses for a swine production system due to reduced growth performance and feed efficiency, therapeutic costs, and mortalities (Hampson et al., 2006). Previous research has determined that endemic swine dysentery can reduce the profit margin of production by $5.77 USD per 100 kg of carcass (Polson et al., 1992). In 1994, it was estimated that swine dysentery cost the US swine industry $115 million USD (Duhamel and Joens, 1994).

1.2.4 *Lawsonia intracellularis*

Lawsonia intracellularis is a Gram-negative bacterium that causes proliferative enteropathy in swine by invading the cytoplasm of intestinal epithelial cells (McOrist and Gebhart, 2006). Infections often result in diarrhea, anorexia, and reductions in ADG and

feed in-take; however, almost all pigs with uncomplicated infections recover (McOrist and Gebhart, 2006). Colonization of swine herds with *L. intracellularis* is reported to be 20-100% in major pig-producing regions including the US, Canada, Denmark, Brazil, and Korea (Chiriboga et al., 1999, Stege et al., 2000; Marsteller et al., 2003; Suh and Song, 2005; Paradis et al., 2007). The reduction in growth performance caused by infection can prolong the time it takes for a pig to reach market weight. This costs the swine industry millions of dollars in financial losses each year (McOrist et al., 1997). It is predicted that lost productivity due to proliferative enteropathy costs producers approximately $3-10 USD per infected pig as a result of decreased feed efficiency and longer housing (McOrist et al., 1997).

1.2.5 Enterotoxigenic *Escherichia coli*

Under certain conditions, the colonization of enterotoxigenic *Escherichia coli* (ETEC) in the gut can cause diarrhea in pigs, particularly in young pigs who are the most susceptible (Nagy and Fekete, 1999). Enterotoxins produced by ETEC alter the flux of water and electrolytes in the intestinal mucosa leading to diarrhea (Nagy and Fekete, 1999). In Ontario, enterotoxins were detected in hemolytic *E. coli* strains from 82% of herds with clinical signs of post-weaning *E. coli* diarrhea (Amezcua et al., 2002). Neonatal and post-weaning diarrhea caused by ETEC has economic consequences for swine production systems as this disease can result in significant mortalities in clinically affected herds (Nagy and Fekete, 1999; Amezcua et al., 2002). However, nursery herds afflicted by problematic *E. coli* diarrhea show no difference in growth performance compared to non-afflicted nursery herds (Amezcua et al., 2002).

1.2.6 *Salmonella*

Broad host-range serovars of *Salmonella* can cause salmonellosis in pigs and are a potential cause of salmonellosis in humans due to pork contamination. Newly weaned pigs are the most susceptible to salmonellosis, although clinical disease in swine is uncommon and the clinical outcome is highly variable (Griffith et al., 2006). The most common types that cause salmonellosis in swine are *Salmonella* Typhimurium and *Salmonella* Choleraesuis (Griffith et al., 2006). Although salmonellosis in pigs can cause minor reductions in ADG and feed efficiency (Tubbs and Deen, 1997; Farzan et al., 2010), there is no reliable evidence to indicate that eradication of *Salmonella* within a swine production system will improve productivity (Fraser et al., 2009). However, public perception of pork as a source of *Salmonella* and pressure from government assemblies can compel swine producers to implement different management strategies for reducing *Salmonella* in the production chain (Fraser et al., 2009). Overall, salmonellosis in swine does not appear to be a major source of lost productivity but adopting strategies for reducing the risk of pork contamination because of the public health risk can incur additional expenses for producers.

1.2.7 Methicillin-resistant *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that is a significant cause of infections in humans. Resistance to methicillin is conferred by the *mecA* gene which encodes penicillin binding protein 2a (PBP2a), an altered PBP with a lower affinity for β-lactam antimicrobials (Ubukata et al., 1985). MRSA colonization is common in pigs and people who frequently handle pigs (Khanna et al., 2008; Smith et al., 2009), although the reason for its persistence in swine production is still unclear.

However, genetic profiling does suggest that the porcine MRSA clone sequence type (ST) 398 originated from humans where it moved to pigs and became methicillin-resistant, perhaps as a result of tetracycline exposure (Price et al., 2012).

While pathogenic to humans and many other animal species, MRSA only causes infections in pigs in rare circumstances, although some evidence suggests that it may be a causative agent of ear necrosis (Park, 2011). As it is presently understood, MRSA of porcine origin does not cause any significant loss for swine production systems, but its potential public health risk warrants further investigation.

1.3 Role of the insulin-like growth factor system

The insulin-like growth factor (IGF) system is essential for development, growth, and metabolism in vertebrate species. The IGF system consists of ligands, receptors, binding proteins, and proteases which act in concert to control cellular proliferation and differentiation in bone, muscle, cartilage, brain, and other tissues during early development and throughout life (Dupont and Holzenberger, 2003). IGF-1 and IGF-2 are the two signalling ligands of the IGF system. In humans, IGF-2 is essential for embryonic and fetal development. In post-natal development and adulthood, the role of IGF-2 is largely replaced by IGF-1 (Dupont and Holzenberger, 2003). Production of IGF-1 occurs primarily in the liver and is regulated by growth hormone (GH), a peptide hormone secreted by the pituitary gland (Bichell et al., 1992). Binding of GH to GH receptors (GHR) located on the liver stimulates production of IGF-1 (Bichell et al., 1992). When IGF-1 is secreted into the bloodstream it binds to IGF binding proteins (IGFBPs) which facilitate with transportation and bioavailabity (Mohan and Baylink, 2002). Most IGF-1 is bound to IGFBP-3 which is the most abundant IGFBP found in serum (Dupont and

Holzenberger, 2003). At the target tissue, IGFBP-3 controls the release of IGF-1 to prevent excessive cellular proliferation or apoptosis (Rajah et al., 1999). IGF-1 and IGF-2 bind to the cell surface receptors IGF1R and IGF2R, respectively, and cause a cascade of signal-transducing reactions (Dupont and Holzenberger, 2003). Despite the importance of the IGF system for growth and development, there is a poor understanding of the relationship between the IGF system and growth performance in livestock species, and the role of the immunomodulatory factors that may influence this relationship.

1.3.1 Insulin-like growth factor-1

IGF-1 is an anabolic peptide hormone that is primarily synthesized by the liver but can be produced by most tissues in the body (Rosen, 1999). It is responsible for regulating post-natal growth and development in vertebrate species, and it has been demonstrated to act locally and systemically on muscle, bone, cartilage, brain, ovary, pancreas, and mammary glands (Dupont and Holzenberger, 2003). Production of IGF-1 is primarily regulated by GH, but additional regulatory mechanisms involving leptin and thyroid stimulating hormone (TSH) have been identified (Ajuwon et al., 2003; Ramajayam et al., 2012). IGF-1 acts on target tissue by binding to IGF1R on the surface of target cells which induces a signal transduction cascade (Dupont and Holzenberger, 2003).

In newly born pigs, IGF-1 serum concentrations are relatively high but appear to decrease and stabilize by adulthood (Li et al., 2013). Excessive expression of IGF-1 in neonates likely supports rapid growth during this phase of development (Li et al., 2013). The decrease in serum IGF-1 with age may be a contributor of lameness in pigs as IGF-1 is a critical component of cartilage and joint maintenance (Frisbie et al., 2000; Lejeune et al., 2007). Further research has demonstrated that serum IGF-1 varies between breeds of

pigs during early development (Li et al., 2013), but there is no difference in IGF-1 serum levels between barrows and gilts during the grower stage (Owens et al., 1999).

IGF-1 is thought to be a critical regulator of lean tissue, and it may be an indicator of growth performance and carcass quality in swine production (Owens et al., 1999; Clutter et al., 1995; Li et al., 2013). Research by Li et al. (2013) has demonstrated that liver and serum IGF-1 levels are strongly correlated with body height, body length, and loin muscle of pig carcasses. Further research has determined that gilts from genetic lineages with a high ADG have significantly increased serum IGF-1 levels compared to gilts from genetic lineages with low ADG (Clutter et al., 1995). IGF-1 serum levels are also positively correlated with ADG and voluntary feed in-take in grower pigs (Owens et al., 1999). Despite these findings, hepatic IGF-1 expression has never been investigated for use as a biomarker of growth performance in commercial swine production systems.

Differential levels of IGF-1 are significantly associated with some infectious diseases in swine (Roberts and Almond, 2003; Jenkins et al., 2004). Roberts and Almond (2003) demonstrated that pigs co-infected with PRRSV and *Mycoplasma hyopneumoniae* had significantly reduced serum concentrations of IGF-1 compared to a control group, despite similar feed in-take. Furthermore, pigs infected with *Salmonella* Typhimurium have considerably reduced levels of serum IGF-1 and IGFBP-3 (Jenkins et al., 2004). Both nutritional and non-nutritional factors are suspected to contribute to differential levels of IGF-1 in diseased animals, but it is speculated that increased immunomodulatory activity may independently inhibit the IGF system (Roberts and Almond, 2003; Savage, 2013).

1.3.2 Insulin-like growth factor binding protein-3

The most abundant IGFBP in sera of postnatal pigs is IGFBP-3 (Owens et al., 1991), which forms a ternary complex with IGF-1/IGF-2 and the acid liable α-subunit (ALS), a complex-stabilizing glycoprotein produced by the liver (Baxter, 1988; Baxter, 1994). IGFBP-3 is found in many fluids including serum, milk, urine, cerebrospinal fluid, interstitial fluid, synovial fluid, and amniotic fluid (Rajaram et al., 1997). IGFBP-3 facilitates the transportation of IGFs through fluids and across capillary barriers to tissues where it then controls the release of IGFs to prevent excessive stimulation or apoptosis (Rajah et al., 1999). Depending on the conditions, IGFBP-3 can also potentiate or inhibit the effects of IGF-1 by interacting with surface receptors on target cells (De Mellow et al., 1988; Oh et al., 1993).

IGFBP-3 and IGF-1 levels are concomitantly regulated and an appropriate balance of these proteins is necessary for proper development (Ramajayan et al., 2012; Vottero et al., 2013). Transgenic experiments in mice have revealed that a 4.9 to 7.7-fold increase in IGFBP-3 expression results in a 10% reduction in birth weight, moderate post-natal growth retardation, and reduced organ weight (Modric et al., 2001). It is likely that excessive levels of IGFBP-3 inhibit growth by sequestering all available IGFs, thereby reducing the bioavailability of IGFs for cellular stimulation (Modric et al., 2001). In pigs, it has been demonstrated that increased IGFBP-3 levels are associated with reduced growth performance; gilts from genetic lineages with a low ADG have increased IGFBP-3 in sera (Clutter et al., 1995), and slow-growing breeds of pigs have increased hepatic expression of IGFBP-3 compared to fast-growing breeds (Li et al., 2013). It has

also been demonstrated that serum concentrations of IGFBP-3 are not significantly different between gilts and barrows (Owens et al., 1999).

1.3.3 Growth hormone receptor

Found abundantly in the liver, GHR is a cell surface protein of the class I cytokine receptor superfamily that mediates the growth-promoting effects of GH (Lucy, 2008). GHRs homodimerize upon binding of GH to form a ternary complex of two identical receptors and one ligand (Cunningham et al., 1991). Coupling of GH to GHRs in liver tissue stimulates production and secretion of IGF-1 which in turn controls GH production via negative feedback (Le Roith et al., 2001). During nutritional restriction this feedback loop may be interrupted whereby the actions of GHR are blocked and synthesis of IGF-1 in the liver is decreased (Thissen et al., 1999). *In vivo* experiments in pigs and *in vitro* experiment with porcine hepatocytes have demonstrated that protein and carbohydrate content can significantly affect the activity and expression of GHR (Brameld et al., 1996; Brameld et al., 1999). However, it is unknown whether any non-nutritional mechanisms regulate GHR in pigs.

Previous studies indicate that expression of GHR is positively associated with body weight in animal species (Coschigano et al., 2003). Research with transgenic mice has demonstrated that deletion of the GHR results in severely reduced body weight, and significant reductions in serum IGF-1 and IGFBP-3 (Coschigano et al., 2003). Studies have also identified genetic polymorphisms in the avian and porcine *GHR* gene that is associated with body weight (Feng et al., 1998; Ibeagha-Awemu et al., 2008). However, it is unknown whether hepatic expression of GHR may be used as a biomarker for evaluating growth performance in pigs.

1.4 The acute phase response

The acute phase response (APR) is an innate immune defense mechanism in animals that is induced by infection, inflammation, trauma, and stress (Eckersall, 2000; Petersen et al., 2004; Cray et al., 2009). The primary members of the APR are the acute-phase proteins (APPs) which have vast immunomodulatory effects (Cray et al., 2009). Proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α), travel from the area of inflammation to the liver via the bloodstream to stimulate the synthesis of APPs in hepatocytes (Eckersall, 2000; Cray et al., 2009). It has also been demonstrated that insulin and other growth factors are important modulators of the APR through transcriptional regulation of the APP genes (Campos and Baumann, 1992). The major APPs in pigs are C-reactive protein (CRP), haptoglobin (Hp), and serum amyloid A (SAA) (Petersen et al., 2004). APPs generally function as proinflammatory agents and upregulate the immune response, but they also exhibit anti-inflammatory activity under certain conditions (Du Clos and Mold, 2004; Cray et al., 2009). The 10 to 100-fold increase in APPs as part of the innate immune response has led to their use as valuable biomarkers in human and veterinary medicine (Petersen et al., 2004).

1.4.1 C-reactive protein

The highly conserved immunomodulator CRP is found in vertebrate species and homologous variants have even been identified in invertebrates such as the 200 million-year-old horse shoe crab (*Limulus polyphemus*) (Robey and Liu, 1981; Du Clos and Mold, 2004). CRP is considered a first-line APP as it is synthesized rapidly and early by hepatocytes when stimulated by IL-1 and IL-6 (Hurlimann et al., 1966; Ganapathi et al.,

1991; Du Clos and Mold, 2004; Petersen et al., 2004). At the beginning of the inflammatory response (0-24 h post-infection), CRP is synthesized in moderate amounts, and its cardinal role is to activate the classical complement cascade which mediates the clearance of pathogenic organisms from the body (Weiser et al., 1998; Du Clos and Mold, 2004). However, at the height of the inflammatory response (24-48 hours post-infection) CRP is synthesized very rapidly and in tremendous quantities (600-1000-fold) which results in anti-inflammatory activity (Du Clos and Mold, 2004). The difference between the effects of CRP at low and high levels may be explained by different receptors having different affinities for CRP (Du Clos and Mold, 2004). Due to its gross increase in synthesis during an immune response, CRP has become an effective biomarker for monitoring health and welfare in pigs (Heegaard et al., 1998; Piñeiro et al., 2007a; Gómez-Laguna et al., 2010), but its usefulness for monitoring productive performance in pigs is unknown.

1.4.2 Haptoglobin

The production of Hp is considered the second-line of defense in the APR because the cytokine-stimulated increase in circulating Hp is slower and is sustained for weeks (Petersen et al., 2004). The most important role of Hp is to stabilize free haemoglobin in the blood which prevents the loss of iron (Keene and Jandl, 1965). This has a bacteriostatic effect as it restricts access to free iron – a necessary mineral for bacterial growth (Bullen, 1981; Eaton et al., 1982). Hp is a useful biomarker for diagnosing disease in veterinary medicine (Petersen et al., 2004). Circulating levels of Hp in pigs is influenced by infections, including porcine reproductive and respiratory syndrome virus (PRRS) and ear necrosis (Eckersall et al., 1996; Petersen et al., 2002), and by chronic

conditions such as lameness (Petersen et al., 2002). Additional research suggests that Hp is involved in lipid metabolism (Katoh and Nakagawa, 1999) and that it may be a useful biomarker for assessing productive performance on commercial pig farms (Eurell et al., 1992; Saco et al., 2010). However, there is a deficient understanding of the association between swine herd health, Hp, and growth performance.

1.4.3 Serum amyloid A

The homeostatic effects of SAA functions to inhibit the inflammatory response and restore balance (Petersen et al., 2004; Cray et al., 2009). Circulating levels of SAA usually peak around 72 hours after the onset of the APR and return to baseline levels within a week (Malle and De Beer, 1996). SAA is a useful indicator of illness in pigs as it dramatically increases following natural infection with different porcine viruses and bacteria, including PRRSV, porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae*, and *Actinobacillus pleuropneumoniae* (Parra et al., 2006; Skovgaard et al., 2009). Although it is unclear whether SAA is an indicator of growth performance in livestock production, research suggests that changes in the GH-IGF axis may result in altered levels of SAA (Sesmilo et al., 2000).

1.5 The immune response and the role of cytokines

Much of our knowledge about the cytokine-mediated immune response is conceptualized as the T_H1-T_H2 paradigm (Figure 1). The paradigm represents a structured model of cytokine patterns that results in a mounted immune response against infectious agents (Mosmann and Coffman, 1989). The defense against infectious agents in animals consists of innate protection and long-lasting, adaptive immunity which is orchestrated by a

network of immunomodulatory agents, namely the proinflammatory cytokines (Murtaugh and Foss, 2002). The proinflammatory cytokines are primarily produced by macrophages which are located in tissues throughout the body (Murtaugh and Foss, 2002). IL-1, IL-6, IL-8, and TNF secreted by macrophages are responsible for mediating the innate immune response, whereas IL-10, IL-12, and IL-18 signal adaptive immunity through T cells (Murtaugh and Foss, 2002). T cells also communicate with macrophages via IFN-γ signalling (Foss and Murtaugh, 2000). Macrophages located in the liver (Kupffer cells) are frequently exposed to soluble bacterial and particulate matter originating from the gastrointestinal tract and bloodstream such that it induces cytokine production (Kmieć, 2001). However, little is known about how these cytokines interact with the hepatic expression of the IGF system. The following section will describe the roles and characteristics of some of these cytokines in the immune response.

1.5.1 Interleukin-1

Production of IL-1 is dramatically stimulated by specific components of infectious agents such as cholera toxin (CT) or lipopolysaccharide (LPS) (Foss et al., 1999). IL-1 is a multifunctional regulator; it induces the APR in animals (Dinarello, 1984), is necessary for IFN-γ synthesis (Hunter et al., 1995), increases antibody production (Reed et al., 1989), mediates tissue repair (Zitta et al., 2010), and is involved in modulating the GH-IGF axis (Thissen and Verniers, 1997). In pigs it is recognized as IL-1β and it has an additional role in the regulation of endometrial estrogen synthesis during pregnancy (Franczak et al., 2013).

1.5.2 Interleukin-6

The actions of IL-6 are important for regulating the cytokine-mediated immune response and the APR (Petersen et al., 2004). Specifically, it has been demonstrated that IL-6 regulates production of IL-1 and TNF-α (Schindler et al., 1990), induces differentiation of cytotoxic T lymphocytes (Okada et al., 1988), and significantly contributes to anti-inflammatory pathways (Tilg et al., 1997). It has also been demonstrated that IL-6 weakly induces IGF-1 synthesis in hepatocytes which is likely the result of shared transduction factors and a cross-reaction due to similar receptors (Thissen and Verniers, 1997). Expression of IL-6 in pigs is cell-type specific and peaks around 4-14 hours after stimulation with LPS (Lee et al., 2004).

1.5.3 Interleukin-10

One of the most important anti-inflammatory cytokines is IL-10 (Sabat et al., 2010). It has a multitude of biological effects including down-regulation of pro-inflammatory activity of macrophages (Fiorentino, 1991), inhibition of antigen presenting cells and T cells (D'Andrea et al., 1993), and modulation of macrophage phagocytosis (Buchwald et al., 1999). Current research suggests that IL-10 is an important regulator of PRRS immunology; both American and European strains of PRRSV have been shown to induce a strong IL-10 response in pigs (Suradhat and Thanawongnuwech, 2003), and this may contribute to impaired development of PRRSV-specific IFN-γ-secreting cells which have important antiviral properties (Diaz et al., 2006). It is unknown whether IL-10 can be used as a biomarker for PRRS or whether it interacts with the IGF system.

1.5.4 Interleukin-18

The pro-inflammatory cytokine IL-18, in combination with IL-12, is a potent stimulator of IFN-γ production (Okamura et al., 1995), and an important polarizing-stimulant of both T_H1 and T_H2 immunity (Nakanishi et al., 2001; Reddy, 2004). IL-18 may contribute to lameness in animals as previous research has demonstrated that IL-18 stimulates joint inflammation and cartilage degradation in murine models (Joosten et al., 2004). There is lacking knowledge of any species-specific role of IL-18 in swine immunology.

1.5.5 Interferon-α

The growth-inhibiting cytokine IFN-α belongs to the type I IFN family and is well known for its anti-tumour activity; being the longest used cytokine in oncology to treat over 14 types of cancer (Rizza et al., 2010). IFN-α also has immunomodulatory properties, including inducing differentiation of monocytes (Santini et al., 2000), enhancing antibody production (Le Bon et al., 2001), and playing a role in linking the innate and adaptive immune response (Ferrantini and Belardelli, 2002). Further research has demonstrated that administration of high doses of IFN-α to mice elicits severe growth retardation (Gresser et al., 1975). The IFN-α-inducible protein 6 gene is also reported to be a potential marker for meat and carcass quality in pigs (Kayan et al., 2011).

1.5.6 Interferon-γ

The macrophage-activating cytokine IFN- γ, belonging to the type II IFN family, is a critical immunomodulatory and defense cytokine that has antiviral and antimicrobial properties (Saha et al., 2010); it enhances the ability of macrophages to eliminate bacteria, fungi, and parasites (Gwinn and Vallyathan, 2006), and it inhibits viral

replication in most cells (Horisberger, 1992). IFN-γ enhances macrophage activity and proinflammatory cytokine production (IL-1 and TNF-α) by interrupting the inhibitory effects of IL-10 (Hu et al., 2006). In addition, IFN-γ is a significant suppressor of cellular growth (Chawla-Sarkar et al., 2003), and has important anti-tumor activity (Street et al., 2002). In pigs, IFN-γ may play an important role in remodelling uterine tissue and activating uterine immune cells in preparation for conceptus implantation and subsequent gestation (Cencic and La Bonnardière, 2002).

1.5.7 Tumour necrosis factor-α

The potent proinflammatory cytokine TNF-α is secreted by activated macrophages to stimulate dormant macrophages in the presence of IFN-γ (Parameswaran and Patial, 2010). Macrophage longevity and differentiation is completely dependent on TNF-α signalling (Witsell and Schook, 1992; Lombardo et al., 2007). TNF-α is also considered to be a "master regulator" because of its important role in stimulating proinflammatory cytokine synthesis and mediating inflammation (Maini et al., 1995). Additionally, previous research has determined that TNF-α can significantly reduce insulin-stimulated lipogenesis in neonatal pigs which may result in reduced fat accumulation (Ramsay et al., 2013).

1.6 The use of biomarkers in swine production

Biomarkers are measurable changes in the biochemical, cellular, or molecular components of tissue, cells, or fluids (Hulka et al., 1990). They are used for risk assessment, determining susceptibility to disease, diagnosis and prognosis of disease, evaluating the effectiveness of interventions, and for comparing the physiological state of

individuals (Mayeux, 2004). Biomarkers are advantageous because they provide an objective and quantitative assessment, they are reliable and less bias than other methods, and they are scientifically valid. However, they are limited by the timing of sampling, expense, laboratory error, and lack of previously established baseline values (Mayeux, 2004).

Health-dependent changes in cytokines and APPs have allowed these immunomodulators to become useful biomarkers of illness in livestock species. The most useful biomarkers for assessing herd health are the APPs which usually increase by 100-fold or greater during an infection (Petersen et al., 2004). APPs may also serve as indicators of animal welfare because sustained increases in APPs are stimulated by stressful events (Piñeiro et al., 2007a; Piñeiro et al., 2007b). Furthermore, given the importance of herd health and welfare for production efficiency, cytokines and APPs may be useful biomarkers for evaluating production parameters on commercial farms (Saco et al., 2010). The IGF system, which is critical for growth and development, is also a source of potential biomarkers that may be valuable for predicting or assessing production performance and carcass quality at slaughter (Owens et al., 1999; Li et al., 2013).

1.6.1 Health and Disease

Expression patterns of cytokines and acute-phase proteins at specific time points can be useful for diagnosing and monitoring disease in swine herds (Petersen et al., 2004). It has been demonstrated that certain swine diseases can be accurately diagnosed prior to the appearance of clinical signs by interpreting changes in cytokine and APP profiles (Harding et al., 1997). Many biomarker profiles have been proposed or validated for swine medicine including Hp/Pig-MAP for post-weaning multisystemic wasting

syndrome (Segalés et al., 2004), IFN-α/TNF-α/IL-1 for PRRSV (Van Reeth et al., 1999), and IL-6/IL-8 /TNF-α for experimentally induced staphylococcal pneumonia (Martínez-Olondris et al., 2010). Previous research has also demonstrated that proteomic analysis of serum protein profiles can allow for highly sensitive differentiation of healthy pigs and pigs with porcine circovirus-associated disease (PCVAD) before the appearance of clinical signs (Koene et al., 2012). Furthermore, decreases in serum IGF-1 have also been observed in pigs co-infected with *M. hyopneumoniae* and PRRSV (Roberts and Almond, 2003), and in pigs infected with *S.* Typhimurium (Jenkins et al., 2004).

1.6.2 Animal Welfare

Inconsistencies between animal welfare assessment protocols and differences in the disposition of personnel results in considerable subjectivity for the appraisal of pig welfare (Roberts et al., 2013). Biomarkers have become an attractive alternative because they provide unbiased, scientifically objective results (Mayeux, 2004). Previous research has demonstrated that stressful events can trigger dramatic changes in specific biological compounds in pigs (Piñeiro et al., 2007a; Piñeiro et al., 2007b; Razzuoli et al., 2011). Stress caused by disorderly feeding for several weeks resulted in significantly elevated serum Hp and CRP, but no change was observed for SAA (Piñeiro et al., 2007a). Further research has demonstrated that stress induced by transportation resulted in increased serum Hp, CRP, and SAA at slaughter (Piñeiro et al., 2007b). It has also been suggested that increased serum TNF-α after weaning may be an indicator of stress in pigs (Razzuoli et al., 2011). Further studies are needed to validate these biomarkers as tools for assessing pig welfare.

1.6.3 Production Performance

Genetic markers for reproductive selection have been exhaustively studied in pigs but there is much less research that has explored the use of biomarkers for assessing herd performance (Ibeagha-Awemu et al., 2008). Previous studies with pigs have identified associations between productivity and polymorphisms in the genes that encode growth hormone (GH), growth hormone-releasing hormone (GHRH), leptin (LEP), and ryanodine receptor (RYR1) (Ibeagha-Awemu et al., 2008). Selection of pigs with desirable genetic markers has resulted in larger litter size (Distl, 2007), increased fertility (Dyck et al., 2011), and better pork quality (Davoli and Braglia, 2007). Further research suggests that Hp may be a useful indicator of productivity in swine production systems (Eurell et al., 1992; Saco et al., 2010). However, there is a deficient understanding of how non-genetic markers and transcriptional diversity is associated with production performance in pigs. Particularly, it is unknown whether differential hepatic gene expression of the members of the IGF system, cytokines and APPs is indicative of growth performance in commercial swine production.

1.7 Measuring gene expression using reverse transcription quantitative polymerase chain reaction

The reverse transcription quantitative polymerase chain reaction (RT-qPCR) method is used to quantify the amount of target RNA in a sample. Relative quantification of target RNA is a valuable indicator of transcriptional expression of a target gene and is often recognized as an experimental “gold standard” in molecular biology (Bustin et al., 2009). The typical RT-qPCR protocol involves extraction of RNA, DNase digestion of genomic

DNA, testing of RNA quality and quantity, reverse transcription of RNA into cDNA, and amplification of cDNA with specific primers and indicators (Bustin et al., 2005; Fleige and Pfaffl, 2006). The process is often long and labour-intensive, generally involving multiple quality assurance steps throughout the procedure. Although some disagreement exists regarding the methodology of RT-qPCR experiments, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines assists researchers with reporting adequate methodological information so that readers can critically evaluate the quality of each individual RT-qPCR protocol (Bustin et al., 2009). In general, to ensure accurate and precise results several critical requirements must be fulfilled:

(1) A stable and validated reference gene that is constitutively expressed in the target cells must be used for normalization of gene expression. Typically two references genes are used but a single reference gene can be used if it has proven reliability (Bustin et al., 2005).

(2) High primer efficiency and specificity is necessary for accurate results. Maximal primer efficiency can be achieved by ensuring there is no formation of primer dimers, determining the optimal melting temperature, and by assessing a melting curve of RT-qPCR reactions with differing primer concentrations. The primers should also exclusively amplify the target cDNA and ideally the primers should span an exon-exon junction as a precaution to avoid amplification of DNA contaminants. The optimization of primer performance is a critical determinant of the overall efficiency of the RT-qPCR (Bustin et al., 2009).

(3) The quantity and quality of extracted RNA significantly affects the accuracy of the gene expression results. RNA samples should be free of proteins, genomic DNA, nucleases, and other enzymes which may interfere with the RT-qPCR assay. Ultrasensitive modern spectrophotometers methods are used to quantify and qualify the amount of total RNA in a sample based on optical density ratios. Additionally, gel electrophoresis or modern lab-on-a-chip technologies are also used to assess RNA integrity (Fleige and Pfaffl, 2006).

(4) Biological and technical replicates are useful for assessing sources of variation that arise during the RT-qPCR experiment. Biological replicates are individual samples from two or more organisms that were raised under the same experimental conditions. Technical replicates are when the same sample is analyzed multiple times. Biological replicates are useful for comparing the natural variability between organisms and technical replicates are useful for assessing the variability of the analytical technique (Bustin et al., 2009).

There are also additional parameters that are important for RT-qPCR quantification of RNA which are addressed in the MIQE guidelines (Bustin et al., 2009). The analysis of RT-qPCR output data can be done in many different ways including simple univariable statistical analysis between groups (Livak and Schmittgen, 2001) or multivariable regression analysis (Page and Stromberg, 2011). The use of multivariable analysis allows for the researcher to control variables that may have a confounding effect or to address clustering discrepancies at hierarchal levels. Gene expression is quantified by the number of cycles of the RT-qPCR reaction needed to reach the cycle threshold. This is described graphically in Figure 2; the number of cycles is on the x-axis and the strength of the

fluorescent signal is on the y-axis. The cycle quantification value (C_q) is the number of RT-qPCR cycles needed to amplify the cDNA to give off a fluorescent signal equal to the set cycle threshold value. The reference gene is also amplified alongside the target gene. Since there are differences in the concentration of total RNA between each sample, gene expression must be normalized before C_q values can be analyzed. The difference between the C_q values of the reference gene and target gene is the normalized ΔC_q value. Post-hoc analysis of the RT-qPCR output data can also reveal discrepancies between biological or technical replicates.

There are several limitations of RT-qPCR which can weaken the interpretation of the results. First, the quantification of mRNA is not indicative of translational expression or functional protein content in a cell. The amount of protein and the amount of mRNA does not always correlate, especially for a multiplex protein that is the product of several genes (Bustin et al., 2009). Secondly, the quantification of RNA is relative to the reference gene and the RT-qPCR conditions. This usually means that results from different experiments can only be compared qualitatively and not quantitatively (Bustin et al., 2009). Nevertheless, the use of RT-qPCR for gene expression analysis has greatly advanced research in the era of molecular biology.

1.8 Summary

Current research indicates that IGF-1, IGFBP-3, and GHR are potential biomarkers of growth performance and carcass traits in grower pigs (Owens et al., 1999; Coschigano et al., 2003; Li et al., 2013), but it is unknown whether hepatic gene expression of these growth factors is associated with growth in nursery pigs. There is also evidence which suggests that the IGF system is regulated by immunomodulatory factors (Thissen and

Verniers, 1997; Savage, 2013), and that this may affect growth performance independent of nutritional parameters (Roberts and Almond, 2003; Ramsay et al., 2013). An interaction between the IGF system and immunomodulatory activity may explain the considerable changes in growth factor concentrations in pigs infected with certain bacterial and viral agents (Roberts and Almond, 2003; Jenkins et al., 2004). However, our understanding of the interaction between the IGF system and the immune response is lacking and further research needs to explore this association *in vivo*, particularly under commercial farm conditions where such an interaction may have repercussions for productivity. To address these gaps in the knowledge, the primary objectives of the following research project was:

(1) To determine whether growth performance is associated with the hepatic gene expression of IGF-1, IGFBP-3, GHR, CRP, Hp, SAA, IL-1β, IL-6, IL-10, IL-18, IFN-α, IFN-γ, and TNF-α in nursery pigs.

(2) To determine whether the hepatic gene expression of IGF-1, IGFBP-3, and GHR is associated with any immunomodulating agents, including CRP, Hp, SAA, IL-1β, IL-6, IL-10, IL-18, IFN-α, IFN-γ, and TNF-α, or certain bacterial and viral agents, including *Salmonella* spp., *L. intracellularis*, *Brachyspira* spp., ETEC, MRSA, SIV, and PRRSV, in nursery pigs.

In addition, certain pig and herd characteristics, including sex, weaning age, litter size, sow parity, and herd size, will also be investigated as such factors likely have a pivotal and confounding role in determining the associations described in the primary objectives.

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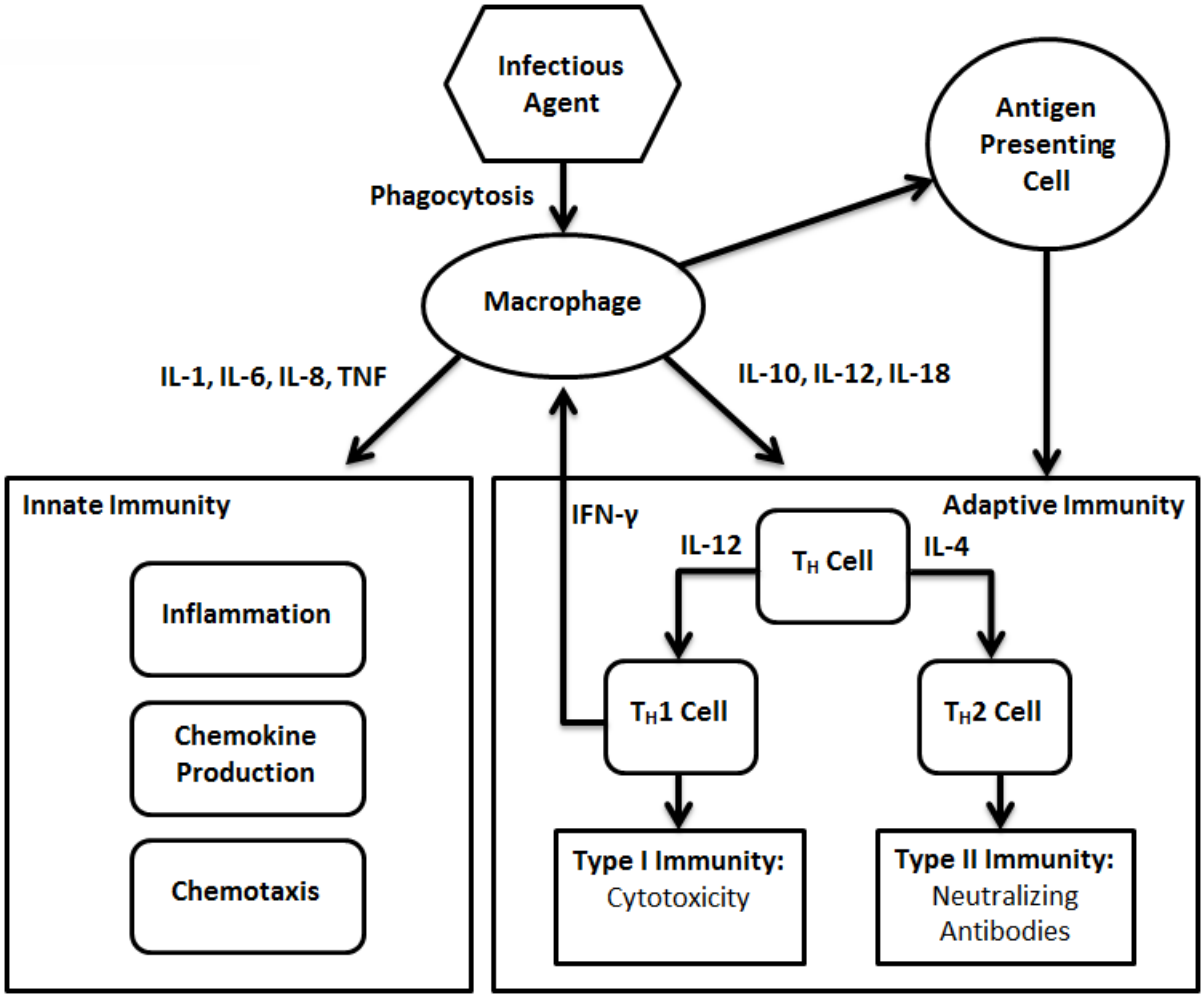

Figure 1.1: The T_H1-T_H2 paradigm and the macrophage-mediated immune response.

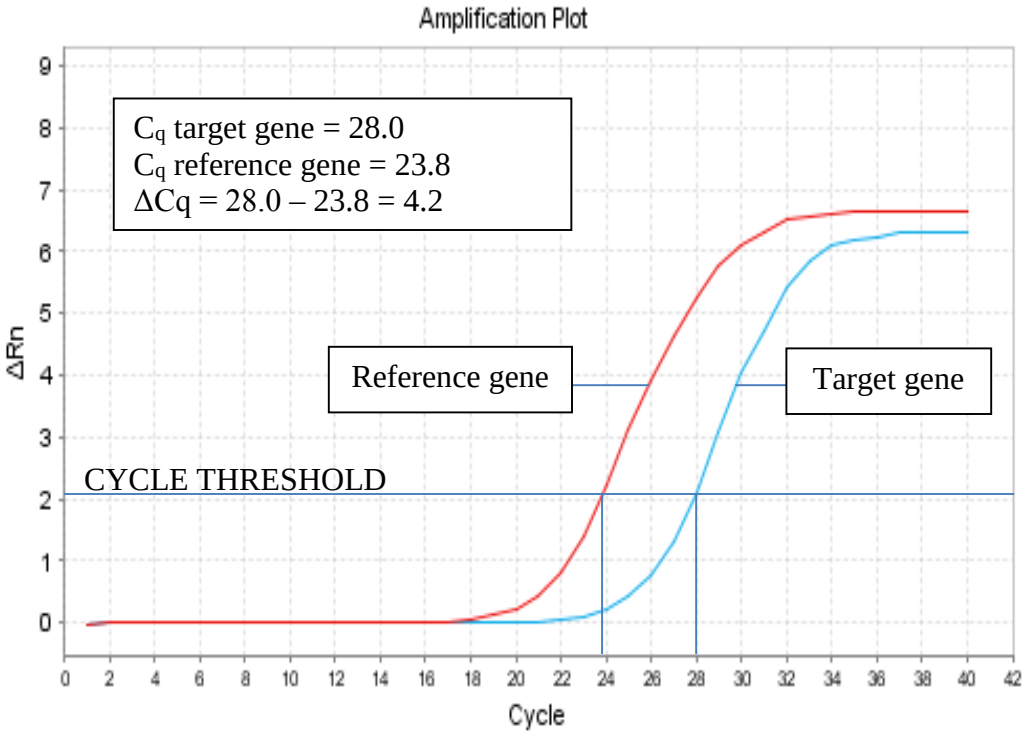

Figure 1.2: RT-qPCR amplification curve of a target and reference gene.

CHAPTER 2: AN EPIDEMIOLOGICAL INVESTIGATION INTO THE ASSOCIATION BETWEEN BIOMARKERS AND GROWTH PERFORMANCE IN NURSERY PIGS.

2.1 Abstract

Biomarkers are useful tools in research and clinical practice where they are often used to detect and monitor differences in the physiological state of an animal. The objective of this study was to determine whether hepatic gene expression of the biomarkers insulin-like growth factor-1 (IGF-1), IGF binding protein-3 (IGFBP-3), growth hormone receptor (GHR), C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin (Hp), interferon-α (IFN-α), interferon- γ (IFN-γ), tumor necrosis factor- α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, and IL-18 are associated with growth performance in nursery pigs. Herd information and growth parameters were collected for 168 piglets from 8 commercial farms in southern Ontario. From these pigs, a subset of liver tissue samples (n=74) was used for real time quantitative-PCR (RT-qPCR) to quantify relative hepatic expression of the proposed biomarkers. Multivariable linear regression methods were used to determine whether genetic expression of the proposed biomarkers was associated with growth performance. Average daily gain (ADG) was significantly associated with IGFBP-3 and GHR expression in the liver ($P<0.05$), and tended to be associated with hepatic IGF-1 expression ($P=0.071$). Similarly, 9-week-old body weight was significantly associated with hepatic expression of IGFBP-3 and GHR expression ($P<0.05$), and tended to be associated with hepatic expression of IGF-1 ($P=0.055$). The age and weight at which pigs are weaned was also an important determinant for nursery performance. Overall, the

hepatic gene expression of IGF-1, IGFBP-3, and GHR are useful biomarkers for monitoring growth performance in nursery pigs.

2.2 Background

The insulin-like growth factor (IGF) system is important for development, growth, and metabolism in livestock species. Insulin-like growth factor-1 (IGF-1) is crucial for stimulating cellular proliferation and differentiation in brain, muscle, and bone tissue (Dupont and Holzenberger, 2003). The expression of IGF-1 is mediated by growth hormone (GH), a peptide hormone released by the anterior pituitary gland (Florini et al., 1996). The binding of GH to growth hormone receptors (GHR) in hepatic tissue induces expression of IGF-1 (Bichell et al., 1992). The expression of IGF-1 is associated with increased body weight as demonstrated by over-expression of IGF-1 in transgenic mice (Mathews et al., 1988). The transportation and bioavailability of IGF-1 is facilitated by IGF binding proteins (IGFBPs) which circulate in the blood (Mohan et al., 2002). The most abundant binding protein is IGFBP-3 which binds most circulating IGF-1 (Rajaram et al., 1997). Experiments using a transgenic mouse model have demonstrated that over-expression of IGFBP-3 is associated with growth impediment (Modric et al., 2001). In pigs, previous research suggests that serum IGF-1 is positively associated with average daily gain (ADG) and serum IGFBP-3 is negatively associated with ADG (Clutter et al., 2001). Additional studies have demonstrated that the IGF system is important for postnatal maturation and the development of desirable carcass traits (Wang et al., 2011; Li et al., 2012; Pierchala et al., 2012). Although these findings demonstrate that the IGF system plays an important role in development, it is not clear to what extent differences

in gene expression affect growth performance in pigs, and whether these genes can be used as biomarkers to monitor growth performance across different commercial farms. There are previous studies which also suggest that growth performance in pigs may be associated with changes in serum concentrations of acute-phase proteins (APP), including C-reactive protein (CRP), haptoglobin (Hp), and serum amyloid A (SAA) (Eurell et al., 1992; Stevenson et al., 2006; Pineiro et al., 2007; Saco et al., 2010). It has been proposed that Hp may be a suitable biomarker for monitoring production performance on commercial swine farms (Saco et al., 2010). Serum levels of certain cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), and tumor necrosis factor-α (TNF-α), have also been associated with body weight in horses and in humans (Bastard et al., 2000; Coppack 2001; Eposito et al., 2002; Bruun et al., 2003; Vick et al., 2007). However, the usefulness of these APPs and cytokines as biomarkers for monitoring the growth performance of pigs on commercial swine farms has not been established. The objective of this study was to investigate the association between the hepatic gene expression of potential biomarkers, including IGF-1, IGFBP-3, GHR, CRP, SAA, Hp, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-18, and growth performance in nursery pigs across diverse commercial farm conditions. A secondary objective of this study was to determine extraneous factors which may influence growth performance in the nursery as such factors are critical for assessing the association between potential biomarkers and nursery performance.

2.3 Materials and Methods

Experimental design and sampling

Eight swine farms in southern Ontario were recruited to participate in a collaborative field study. On each farm, 7 sows were randomly enrolled in the study and 3 piglets (small, medium, and large size) were selected from each sow at weaning and ear tagged. In total 168 piglets (21 piglets per farm) were included. All male piglets recruited in this study had been castrated. A questionnaire (Appendix I) was administered and information about the enrolled sows (farrowing date, parity, litter size, numbers of live born, stillborn), farm management (pig flow, number of gilts, sows, nursery pigs, and grower-finishers, and type of farrowing room floor), health status (recent diseases, mortality in different stage of production), vaccination, and in-feed drug use was collected. Piglets were weighed at weaning and once again at 5 weeks post-weaning. Fecal and blood samples were collected at weaning, 2 weeks post-weaning, and 5 weeks post-weaning.

At 5 weeks post-weaning the piglets were transported to the University of Guelph where they were euthanized. Tissue samples collected from the liver, intestinal lymph node, ileum, and spleen were placed immediately into RNAlater and allowed to sit at 4°C for 24 h before being stored at −80°C. Samples were stored at −80°C for no longer than 4 weeks before being processed for gene expression analysis. Separate portions of the ileum and colon were collected and nasal swabs, blood, and fecal samples were collected from each pig prior to euthanasia.

Calculating growth performance

Growth performance was determined by assessing the post-weaning ADG and the 9-week-old body weight (9-wk BW) of each nursery piglet. The ADG was calculated as the difference between the weaning weight and the 5-wk post-weaning weight, divided by the number of days between each weighing. Body weights were standardized to 9 weeks of age as there was variation in piglet age within each farm and between farms due to differences in the age at which the pigs were weaned.

RNA Extraction

Seventy-four pigs from 4 representative farms with different health status and production parameters were included for gene expression analysis. RNA was extracted from liver tissue using a Qiagen RNeasy Mini Kit (Qiagen). Approximately 30 mg of liver tissue was added to 600 µl of RLT buffer in a RNase-Free tube and homogenized for 30-40 sec using a tissue homogenizer (PowerGen 125, Fisher Scientific). RNA was then extracted from the homogenate as outlined by the manufacturer's protocol without any modifications (Qiagen). The concentration of the eluted RNA was determined using the Thermo Scientific NanoDrop 8000 Spectrophotometer (Fisher Scientific), and RNA was re-extracted from samples with an RNA concentration ≤150 ng/µl.

All RNA samples were treated with an Amplification Grade DNase I kit (Sigma-Aldrich) to remove any genomic DNA. Nucleic acid-free water (24 µl), reaction buffer (3 µl), Amplification Grade DNase I (3 µl), and 3 µg of total RNA (1 unit/µl) were added together and centrifuged for 10 seconds to collect the reaction at the bottom of the tube. The mixtures were incubated at room temperature for 15 min. Then, 3 µl of the Stop

Solution (50 mM EDTA) was added to the mixtures and incubated at 70°C for 10 min. The mixtures were then chilled on ice before being stored at −80°C.

The purity and concentration of RNA was reassessed after DNase digestion using the Thermo Scientific NanoDrop 8000 Spectrophotometer (Fisher Scientific). The integrity of all the RNA samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) before and after DNase treatment to ensure there was no indication of degradation. The mean RNA Integrity Number (RIN) was 8.5, and any samples with RIN<8.0 were repeated as recommended by Fleige and Pfaffl (2006).

Primer Design and Efficiency

Primer designs and efficiencies are listed in Table 1. Primers for IL-1β, IL-10, IFN-α, and IFN-γ were previously designed by Collado-Romero et al. (2010). All other primer sequences were designed and screened for homology using the NCBI Primer-BLAST program. The cDNA sequences used to design the primers were obtained from the GenBank database. Primers were designed to span known exon-exon junctions. Primer efficiency and the coefficient of determination (R^2) were determined using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Analysis of gene expression with RT-qPCR

Relative gene expression was assessed using a real time quantitative polymerase chain reaction (RT-qPCR) assay. The synthesis of cDNA and the RT-qPCR assay were completed by the Advanced Analysis Centre's Genomic Facility at the University of Guelph. For synthesis of cDNA, 1000ng of total RNA was reverse transcribed into cDNA

using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A total reaction volume of 20 µl was used for reverse transcription; 2 µl of reverse transcript buffer, 0.8 µl (100mM) dNTP, 2 µl random priming oligonucleotides , 1 µl of MultiScribe™ Reverse Transcriptase (50 U/µl) (Applied Biosystems), 4.2 µl of nucleic acid-free water, and 10 µl (1000ng) of total RNA. The reverse transcription assay was run at 25°C for 5 min, 37°C for 120 min, 85°C for 5 min, and then cooled at 4°C.

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and receptor-like protein-4 (*RLP4*) were evaluated as potential reference genes for the present study based on validation and reliable performance in previous research (Skovgaard et al., 2007; Svobodova et al., 2008; Li et al., 2009). The stability of these reference genes in the liver tissue samples was measured using RT-qPCR and analyzed using BestKeeper software (Pfaffl et al., 2004). The RT-qPCR cycle quantification (C_q) values of GAPDH and RLP4 had standard deviations of 0.34 and 0.49, respectively. GAPDH was chosen as the only endogenous reference gene to be used for internal normalization of gene expression data in this study due to its very low variability (C_q=18.17±0.34) for the present samples and its reliable performance as a sole reference gene in previous studies (Li et al., 2009; Li et al., 2013).

A single StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used for all RT-qPCR assays in this study. Two technical were completed for each sample. Each 20 µl reaction consisted of 7.5 µl of 2X PerfeCta SYBR Green FastMix ROX (Quanta BioScience), 5 µl of template cDNA, 1.9 µl of molecular grade water, and 0.6 µl of primer. The final primer concentration in each reaction was 200 nM. The RT-qPCR assay conditions were 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s and 60 °C

for 30 s. A melting curve was performed to confirm specificity of the amplicon. The methodology is reported in accordance with the minimum information for publication of RT-qPCR experiments (MIQE) guidelines (Bustin et al., 2009).

Statistical analysis

Linear regression methods were used to model production parameters and gene expression (ΔC_q) in relation to growth performance. First, the C_q values of each biomarker were normalized using the C_q values of the endogenous reference gene GAPDH as described by the following equation:

$$\Delta C_q = [C_q \text{ of target gene}] - [C_q \text{ of reference gene}] \quad (1)$$

The normalized ΔC_q values already exist as the log transformed product of the expression ratios that are conventionally calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), thereby increasing linearity. The linearity of the ΔC_q data was confirmed by assessing a smoothed locally weighted scatterplot. A histogram was used to assess normality of the distribution of values. The ΔC_q values were modelled as the magnitude of gene expression for each target gene.

One multivariable random-effect generalized least squares (GLS) model and 2 multivariable fixed-effect least squares dummy variable (LSDV) models were created in STATA 10.0 (Stata Corporation, College Station, TX) (Appendix II). The GLS model was designed to evaluate the association of production characteristics with 9-wk BW across the 8 farms. The 2 LSDV models were designed to determine the association of

biomarker expression with ADG and 9-wk BW across 4 farms. Extraneous variables that were used for model building include the farm, parity of the sow, stillbirths per litter, live births per litter, sex of the piglet, age at weaning, weight at weaning, and disease status. Each model was manually designed using a parsimonious approach. All variables were first screened individually with univariable linear regression. Variables with a liberal *P*-value of ≤0.20 were included in the initial model. All variables regardless of inclusion were evaluated for confounding. A variable was determined to be a confounder if its inclusion in the model changed the coefficient of any predictor by ≥20%. Two-way interactions were generated for all variables in the initial model and interactions were retained in the model if they were statistically significant ($P<0.05$). Any variable from the initial model was only retained in the final model if it was statistically significant ($P<0.05$), if the variable behaved as a confounder, or if the variable was a component of a statistically significant interaction term. If 2 independent variables presented colinearity ($R^2>0.8$) then the less significant variable was excluded. Clustering at the farm level was accounted for by modelling each farm as a random-effect in the GLS model and as a fixed-effect in the LSDV models. Homoskedasticity and normal distribution of standardized residuals were assessed graphically for the GLS model. The assumptions of constant variance and normal distribution of standardized residuals was evaluated for each LSDV model using the Cook-Weisberg test and the Shapiro-Wilk test, respectively. Diagnostic analysis revealed no outliers or extremely influential observations. Data for 2 pigs were excluded due to missing information. Effects with a $P<0.05$ were considered statistically significant and non-significant effects with a $P<0.10$ were considered to be

suggestive of an association. All descriptive statistics are reported as the mean ± standard deviation.

2.4 Results

The mean growth performance values for each farm are presented in Table 2. The average age and weight at 5-wk post-weaning was 59.5 ± 5.5 d and 19.6 ± 4.3 kg, respectively. From weaning to 5-wk post-weaning, there were 4 mortalities (2.6%) and each mortality occurred on a different farm. Table 3 summarizes the sow characteristics of each farm.

In the first multivariable GLS model, sow parity, live births per litter, stillbirths per litter, sex, age at weaning, and the weight at weaning were used as the primary independent variables to determine an association with 9-wk BW. There were no confounding effects or significant interaction terms detected. The final model revealed that only the weight at weaning and the age at weaning were significantly associated with 9-wk BW (Table 4). This model explained 55.8% of the variation in body weight at 9 weeks of age within each herd. The intraclass correlation coefficient (ICC) was 0.668 based on the herd-level and animal-level variance for the 8 farms.

Multivariable LSDV analysis revealed ADG was not significantly associated with hepatic gene expression of CRP, SAA, Hp, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-18 in the liver (P>0.05), and none of these variables were retained in the final model. However, the effects of IGFBP-3 expression (P=0.016) and GHR expression (P=0.009) on ADG were statistically significant and the effect of IGF-1 expression (P=0.071) was suggestive (Table 5). The model was able to explain 52.9% of the variation in post-weaning ADG. The weight at weaning was retained in the model because it had a

confounding effect. All two-way interactions were not significant and thus excluded from the final model.

The second multivariable LSDV model revealed that hepatic expression of CRP, SAA, Hp, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-18 did not affect 9-wk BW (P>0.05), and all of these variables were excluded from the final model. However, IGFBP-3 expression (P=0.012) and GHR expression (0.016) were significantly associated with 9-wk BW. The hepatic expression of IGF-1 was suggestive (P=0.055) (Table 6). The model was able to explain 69.4% of the variation in the 9-wk BW. The weaning age and the weight at weaning were retained in the model because both variables had a confounding effect. It was also observed that removal of IGF-1 from the model resulted in a major change (>30%) in the IGFBP-3 coefficient indicating substantial confounding. All two-way interactions were excluded from the model as none were statistically significant. Based on the predictions of the LSDV model for 9-week body weight, a 2-fold increase in IGF-1, IGFBP-3, and GHR in a nursery pig of average body weight would result in a 2.6%, 6.8%, and 7.6% change in body weight, respectively.

2.5 Discussion

The weight at weaning and the age at weaning both contributed significantly to growth performance in nursery pigs. The weight at weaning was positively associated with body weight during the nursery period which is consistent with results from previous studies (Wolter and Ellis, 2001; de Grau et al., 2005; Paredes et al., 2012). Additional research has demonstrated that pigs with higher weaning weights reach market weight 9-15 days earlier (Mahan and Lepine, 1991; Wolter and Ellis, 2001). These results support the

importance of pre-weaning interventions that improve weight at weaning and subsequent performance in the nursery.

Growth performance was negatively associated with age at weaning which implies that pigs weaned at an earlier age will reach a greater body weight during the nursery stage. This finding is in agreement with previous research which indicates that late weaning is disadvantageous for post-weaning growth performance (de Grau et al., 2005). Research by Main et al. (2004) determined that weaning at 21.5 d is optimal for nursery performance in multisite production systems. In the present study, 80.4% of the piglets were >21.5 d old at weaning which is indicative of the negative association observed between weaning age and post-weaning growth performance.

The sex of the piglet was not associated with growth performance in the nursery. These findings consistently agree with previous research (Wolter and Ellis, 2001; de Grau et al., 2005). Growth performance of gilts and barrows appears to be significantly different only during the grower-finisher phase of production (Clutter et al., 1995; Wolter and Ellis, 2001). Paredes et al. (2012) demonstrated that sex can be significantly associated with nursery performance in different data sets although the authors attributed this consequence to differences in the proportion of intact males. In the present study, all male piglets had been castrated prior to weaning.

Litter characteristics were not associated with nursery growth performance. The parity of the sow, stillbirths per litter, and live births per litter showed no effect on 9-wk BW. There are conflicting reports of the effects of sow parity on post-weaning growth performance. A study by Smith et al. (2007) suggests that piglets born to primiparous sows have a growth disadvantage compared to higher parity sows. However, the authors

noted that the mean post-weaning weights varied greatly among parties and there was no discernible pattern of growth performance. Other researchers have concluded that sow parity does not influence growth performance at the nursery stage (Paredes et al., 2012). In addition, previous findings have demonstrated that the number of live births per litter is not associated with post-weaning growth performance (Paredes et al., 2012), although the number of live births does appear to be associated with birth weight (Beaulieu et al., 2010). One reason for lack of association between sow characteristics and growth performance in this study may have resulted from selecting piglets of small, medium, and large size from each sow.

Increased expression of IGF-1 in the liver showed a tendency to be positively associated with ADG and 9-wk BW. This is consistent with other studies which have determined that serum IGF-1 concentration is positively associated with growth in pigs and other animals (Clutter et al., 1995; Owens et al., 1999). Furthermore, over-expression of IGF-1 at 1.5-fold the normal levels in transgenic mice resulted in a 30% increase in body weight (Matthews et al., 1988). The statistical significance of the association between IGF-1 and growth may have been undermined by the sample size in this study. The variation in hepatic IGF-1 expression is likely due to the natural variability observed during this stage of development (Owens et al., 1999; Dupont and Holzenberger, 2003). Additionally, differences in production management, feed composition, and antimicrobial usage may have contributed to the large variation in hepatic IGF-1 expression (Hathaway et al., 1996). It is unlikely that differences in breed and sex were a factor in variation as previous studies have demonstrated that IGF-1 levels are not significantly different between breeds (Brameld et al., 1996; Li et al., 2012) or between barrows and gilts

(Owens et al., 1999). These findings suggest that IGF-1 is more useful as a growth-associated biomarker for comparing pigs that are raised in the same herd under the same conditions.

IGFBP-3 expression in the liver was negatively associated with ADG and 9-wk BW. Contrary to the results of our findings, previous research by Owens et al. (1999) showed a weak positive correlation between serum IGFBP-3 concentration and ADG. However, other research has demonstrated that IGFBP-3 is expressed significantly less in gilts bred for enhanced growth performance compared to gilts with diminished growth performance (Clutter et al., 1995), and that hepatic expression of IGFBP-3 is higher in slow-growing breeds than in fast-growing breeds of pigs (Li et al., 2013). Further research using transgenic mice found that a 4.9 to 7.7-fold increase in IGFBP-3 expression resulted in a 10% reduction in birth weight, moderate post-natal growth retardation, and reduced organ weight (Modric et al., 2001). Over-expression of IGFBP-3 in transgenic mice also resulted in a 1.9-2.8-fold increase in circulating IGF-1 levels (Modric et al., 2001), a finding that can be explained by the concomitant mechanisms that regulate both IGF-1 and IGFBP-3 expression (Ramajayam et al., 2012; Vottero et al., 2013). As determined in the present investigation, IGF-1 has a major confounding effect on IGFBP-3 and body weight. This confounding effect may have led Owens et al. (1999) to report a weak positive association between IGFBP-3 and growth performance. Overall, the significant negative association between IGFBP-3 and growth performance found in the present study is consistent with findings in other studies (Clutter er al., 1995; Modric et al., 2001; Li et al., 2013), and with understanding of the basic roles of IGFBP-3 and

IGF-1. These findings indicate that IGFBP-3 can be a useful and reliable biomarker of growth performance in population-based studies of swine herds.

Expression of GHR in the liver was positively associated with growth performance in nursery pigs. A 2-fold increase in GHR expression appeared to have the greatest effect on the magnitude of growth compared to the same increase in IGF-1 and IGFBP-3 expression. A transgenic experiment with mice demonstrated that deletion of the GHR gene resulted in decreased circulating IGF-1 and a substantial reduction in body weight (Coschigano et al., 2003). These findings are consistent with the results presented in this paper. Further studies have demonstrated that *in vitro* GHR expression of swine hepatocytes is significantly influenced by glucose and amino acid concentrations (Brameld et al., 1999), and *in vivo* experiments with pigs indicate that dietary protein concentration is positively associated with GHR expression in the liver, skeletal muscle, and adipose tissue (Brameld et al., 1996). In light of the results from the present study, assessing expression of GHR may be useful for evaluating the effects of different dietary regiments on growth performance at the molecular level. These results also suggest that GHR might be a useful and reliable growth-associated biomarker for population-based studies of swine herds.

Post-weaning ADG and 9-wk BW were not associated with hepatic expression of the APPs assessed in this study. A previous experiment by Saco et al. (2010) demonstrated that Hp is a suitable biomarker for assessing the effects of an immunomodulating feed additive on production parameters. However, Saco et al. (2010) were not able to exclusively identify an association between Hp and growth performance. Additional research by Pineiro et al. (2007) demonstrated differential serum

concentrations of CRP and Hp between 2 groups of pigs subjected to different feeding regiments. Although the ADG was significantly different between groups, Pineiro et al. (2007) attributed the differences in APP levels to stress caused by disorderly feeding. In the present study, it is likely that no significant association was found between APP expression and growth performance because APP levels are influenced considerably by pathogen colonization and stress (Pineiro et al., 2007; Gomez-Laguna et al., 2010); two factors which are not necessarily indicative of growth performance. The results of the present study indicate that CRP, Hp, and SAA are not suitable biomarkers for evaluating the production performance of nursery pigs on commercial farms.

The expression of IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-18 in the liver was not significantly associated with growth performance in nursery pigs. Earlier studies have demonstrated that body weight is significantly associated with circulating levels of IL-6, IL-8, IL-18, and TNF-α in humans (Eposito et al., 2002; Bruun et al., 2003) and differential expression of IL-1, IL-6, and TNF-α in horses (Vick et al., 2007). The lack of association between body weight and hepatic cytokine expression observed in this study is likely explained by previous findings which indicate that excess adipose tissue is the primary source of increased levels of circulating cytokines (Bastard et al., 2000; Coppack, 2001; Fain, 2006). Hence, cytokine expression in the liver is unsatisfactory for monitoring growth parameters in nursery pigs, although serum levels of these cytokines may still prove to be useful biomarkers to evaluate nursery performance.

Measuring the genetic expression or serological levels of IGF-1, IGFBP-3, and GHR may be useful biomarkers for evaluating the growth performance of nursery pigs. It is likely that serological levels of IGF-1, IGFBP-3, and GHR are representative of the

changes in liver expression levels, however further research is needed to confirm this assumption. These biomarkers can be beneficial tools in research and experimental settings where they may be used, in addition to conventional methods, to assess interventions, treatments, or clinical pathologies in relation to growth performance of nursery pigs. However, further studies are needed to determine if the serological levels of cytokines are suitable to monitor growth in pigs as it appears that excessive adipose tissue is the primary source for differential serum concentrations of cytokines in humans (Bastard et al., 2000; Coppack, 2001; Fain, 2006). Future research should also focus on whether RNA and serum levels of IGF-1, IGFBP-3, and GHR are indicative of growth performance at other phases of production and whether biomarker expression during the nursery phase can predict performance in later phases of production. Additionally, there is an opportunity to explore whether any genetic markers in IGF-1, IGFBP-3, or GHR are indicative of growth performance in pigs. Previous research has identified a genetic marker in the GHR gene that is associated with body weight in chickens (Feng et al., 1997). Genetic markers may also be investigated in accessory genes such as the IGFBP proteases which cleave IGFBPs into different forms. Lassarre and Binoux (1994) observed that a different form of IGFBP-3 exists during pregnancy with a lower affinity for IGF-1; a modification that influences growth and development.

2.6 Conclusion

Overall, IGFBP-3 and GHR expression in the liver was significantly associated with growth performance across multiple swine production systems despite variation in operating procedures. IGF-1 expression had a tendency to be associated with the measured growth parameters but the large variation reduced its statistical significance in

this study. Nevertheless, IGF-1 is likely a useful biomarker of growth performance for comparing pigs from the same herd. IGFBP-3 and GHR are suitable biomarkers for evaluating growth performance in nursery pigs, either in a controlled experimental setting or in a population-based study involving multiple swine herds with varying management practices. Hepatic expression of CRP, Hp, SAA, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-18 was not associated with the growth parameters in this study and appear to be inadequate for monitoring growth in pigs.

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Table 2.1: Primers used for RT-qPCR of hepatic biomarkers.

Gene Symbol	Gene Name	Accession Number[a]	Primers (5' – 3')	T_m (°C)	Amplicon Length (bp)	R^2	Primer Efficiency (%)
IL1B	Interleukin-1β	NM_214055	F: GGCCGCCAAGATATAACTGA[b] R: GGACCTCTGGGTATGGCTTTC[b]	59	70	0.991	100
IL6	Interleukin-6	NM_214399	F: CCCTGAGGCAAAAGGGAAAGA R: CGTGGACGGCATCAATCTCA	60	212	0.998	95.2
IL10	Interleukin-10	L20001	F: CAGATGGGCGACTTGTTG[b] R: ACAGGGCAGAAATTGATGAC[b]	57	219	0.993	91.7
IL18	Interleukin-18	NM_213997	F: GCTGCTGAACCGGAAGACAA R: AAACACGGCTTGATGTCCCT	60	192	0.996	95.3
IFNA	Interferon-α	AB257591	F: GACCTGCCTCAGATCCACAG R: ATGGCTTGAGCCTTCTGGAC	60	158	0.986	82.3
IFNG	Interferon-γ	X53085	F: CAAAGCCATCAGTGAACTCATGA[b] R: TCTCTGGCCTTGGAACATAGTCT[b]	60	100	0.985	98.7
TNFA	Tumor necrosis factor-α	NM_214022	F: CCTCTTCTCCTTCCTCCTG[b] R: CCTCGGCTTTGACATTGG[b]	57	194	0.997	100
CRP	C-reactive protein	NM_213844	F: TGCCCAGACAGACATGATCG R: GGTCGGTATAGACACGCAGG	60	131	0.999	100
HP	Haptoglobin	NM_214000	F: TGAATGTGAAGCAGTGTGCG R: CGAGGTGAGGTTATGGTGGG	59	133	0.996	96
SAA	Serum amyloid A	EF362780	F: TGATCAGCGATGCCAGAGAG R: CTTGAGTCCTCCACTCCGTG	60	85	0.998	87.8
IGF1	Insulin-like growth factor-1	JX827417	F: TCTTCTACTTGGCCCTGTGCTT R: CCAGCTCAGCCCCACAGA	61	81	0.998	97.7
IGFBP3	Insulin-like growth factor binding protein-3	NM_001005156	F: GGCATCCACATCCCCAACT R: CCCCGCTTCCTGCCTTT	60	80	0.990	97.3
GHR	Growth hormone receptor	JF276446	F: CTCCACAGGGCCTCGTACTC R: GCTCACATAGCCACACGATGA	60	80	0.999	89.2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001206359	F: ACACACCGAGCATCTCCTGACT R: CGAGGCAGGTCTCCCTAAGC	61	80	0.998	100

[a] Accessed via GenBank.

[b] Designed by Collado-Romero et al., 2010.

Table 2.2: Description of piglet growth performance from 8 commercial swine herds in southern Ontario.

Farm	Age at weaning (days)	Weight at weaning (kg)	9-wk BW (kg)	ADG (g/day)
1[a]	26.7 ± 1.19	6.19 ± 1.54	21.9 ± 3.24	399 ± 62.4
2[a]	22.7 ± 3.36	6.41 ± 1.70	17.4 ± 4.31	267 ± 77.0
3[a]	34.4 ± 7.16	8.93 ± 2.00	21.0 ± 3.47	334 ± 72.4
4	28.3 ± 1.06	6.71 ± 1.88	22.2 ± 4.52	306 ± 65.0
5	30.9 ± 2.92	7.95 ± 1.81	20.2 ± 4.32	360 ± 97.1
6	18.7 ± 0.49	7.04 ± 1.51	22.6 ± 3.01	340 ± 47.5
7	26.1 ± 0.74	8.19 ± 1.82	23.0 ± 4.70	387 ± 143.9
8[a]	22.4 ± 1.96	5.81 ± 1.30	17.9 ± 3.20	296 ± 55.5
Mean	**26.4 ± 5.59**	**7.16 ± 1.96**	**20.7 ± 4.31**	**336 ± 83.2**

BW – body weight; ADG – post-weaning average daily gain

[a] Gene expression analysis was performed on liver tissue from the piglets originating from these farms.

Table 2.3: Description of sow and litter characteristics from 8 commercial swine herds in southern Ontario.

Farm	Number of sows	Sow Parity	Live births per litter	Still births per litter
1	650	2.3 ± 1.9	12.6 ± 2.8	0.9 ± 0.6
2	500	3.6 ± 2.3	11.4 ± 2.8	1.1 ± 1.0
3	1000	2.3 ± 3.2	13.9 ± 1.3	0.4 ± 1.4
4	240	2.3 ± 1.2	11.0 ± 0.5	0.6 ± 0.5
5	123	4.9 ± 2.2	12.3 ± 2.0	1.4 ± 1.0
6	650	3.1 ± 1.9	13.4 ± 1.7	1.0 ± 1.1
7	850	3.6 ± 2.2	11.3 ± 2.7	1.0 ± 0.8
8	670	4.1 ± 2.9	12.9 ± 3.2	0.1 ± 0.4
Mean	**585**	**3.3 ± 2.43**	**12.3 ± 2.46**	**0.8 ± 1.0**

Table 2.4: Characteristics associated with the weight of pigs at 9 weeks of age (Model 1: multivariable random-effect generalized least squares analysis).

Variable	Coefficient[a]	SE	95% CI	*P* value
Weight at weaning (kg)	1.670	0.128	1.419, 1.920	<0.001
Age at weaning (d)	−0.226	0.069	−0.361, −0.090	0.001
Sex	0.524	0.424	−0.307, 1.356	0.217
Parity of sow	0.072	0.097	−0.117, 0.262	0.455
Stillbirths per litter	0.290	0.248	−0.197, 0.777	0.243
Live births per litter	−0.102	0.093	−0.285, 0.081	0.274

SE – standard error; CI – confidence interval

[a] The predicted change in body weight (kg) at 9 weeks of age if the corresponding variable is increased by one unit (ie. the model predicts a decrease of 0.226 kg in body weight at 9 weeks of age if a pig is weaned one day later).

Table 2.5: Change in ADG given a 2-fold increase in gene expression (Model 2: multivariable fixed-effect least squares dummy variable analysis).

Gene	**Change in ADG (g)**	**SE**	**95% CI**	***P* value**
IGF1	12.85	6.990	–1.11, 26.82	0.071
IGFBP3	−33.75	13.65	–61.03, –6.48	0.016
GHR	41.76	15.57	10.65, 72.87	0.009

ADG – post-weaning average daily gain; SE – standard error; CI – confidence interval

Table 2.6: Change in 9-wk BW given a 2-fold increase in gene expression (Model 3: multivariable fixed-effect least squares dummy variable analysis).

Gene	**Change in 9-wk BW (kg)**	**SE**	**95% CI**	***P* value**
IGF1	0.547	0.280	–0.012, 1.105	0.055
IGFBP3	−1.409	0.545	–2.497, –0.320	0.012
GHR	1.580	0.638	0.305, 2.854	0.016

BW – body weight; SE – standard error; CI – confidence interval

CHAPTER 3: IMMUNOMODULATORY FACTORS ASSOCIATED WITH THE HEPATIC GENE EXPRESSION OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1), INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3), AND GROWTH HORMONE RECEPTOR (GHR) IN NURSERY PIGS.

3.1 Abstract

Recent findings suggest there is a complex interaction between the IGF system and the inflammatory immune response. The objective of this study was to determine whether the hepatic gene expression of IGF-1, IGFBP-3, and GHR is associated with the hepatic gene expression of C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin (Hp), interferon- α (IFN-α), interferon- γ (IFN-γ), tumor necrosis factor- α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, and IL-18 as well as with the presence of *Salmonella* spp., *Lawsonia intracellularis*, *Brachyspira* spp., enterotoxigenic *Escherichia coli* (ETEC), methicillin-resistant *Staphylococcus aureus* (MRSA), swine influenza virus (SIV), and porcine reproductive and respiratory syndrome virus (PRRSV) in nursery pigs (n=74) from commercial farms (n=4). Gene expression was quantified using reverse transcription quantitative-PCR (RT-qPCR) and the data was modelled using logistic regression methods. Pigs with elevated IGF-1 expression were less likely to have increased expression of TNF-α (OR=0.14, $P<0.01$) and IL-18 (OR=0.19, $P<0.05$), and less likely to be colonized with PRRSV (OR=0.03, $P<0.01$). Pigs with increased expression of IGFBP-3 were more likely to have elevated IL-6 expression (OR=8.5, $P<0.05$). It was also observed that IGFBP-3 and IGF-1 were significantly associated when Hp expression was low (OR=30; $P<0.05$), but this association was not significant when Hp expression was high ($P=0.54$). Pigs with increased expression of GHR were less likely to have elevated expression of SAA (OR=0.01, $P<0.05$) and IL-1β (OR=0.03, $P<0.05$), but more likely to

have increased expression of CRP (OR=290, $P<0.01$). Overall, there appears to be an inverse association between the hepatic expression of the IGF system (IGF-1, IGFBP-3, GHR) and certain cytokines (IL-1β, IL-18, TNF-α) and acute-phase proteins (SAA, Hp).

3.2 Background

Insulin-like growth factor-1 (IGF-1) is an important anabolic hormone that mediates growth and development in animals. Increases in serum IGF-1 are associated with enhanced growth performance and better carcass traits in livestock species (Clutter et al., 1995; Owens et al., 1999; Li et al., 2013). Circulating IGF-1 is primarily produced by hepatocytes and secreted into the bloodstream where it travels to brain, muscle, bone, and cartilage to stimulate cellular proliferation and differentiation (Dupont and Holzenberger 2003). The most abundant IGF binding protein (IGFBP) in the blood is IGFBP-3 which sequesters IGF-1 to prolong its bioavailability and stability during transportation throughout the bloodstream (Mohan et al., 2002). Hepatic expression of IGF-1 is predominantly regulated by growth hormone (GH) which binds to the growth hormone receptor (GHR) on hepatic tissue and activates a cascade of reactions leading to increased IGF-1 expression (Bichell et al., 1992).

Previous research has indicated that serum concentrations of IGF-1 are associated with certain infectious diseases and health ailments in animals and humans. Decreases in IGF-1 levels are frequently observed in individuals with severe illness even in the presence of normal levels of GH (Donaghy et al., 1995; Wolf et al., 1996; Papastathi et al., 2013). In pigs, it has been observed that reduced concentrations of serum IGF-1 are associated with infection of *Mycoplasma hyopneumoniae*, porcine reproductive and respiratory syndrome virus (PRRSV) (Roberts and Almond, 2003), and *Salmonella*

Typhimurium (Jenkins et al., 2004). Additionally, IGF-1 is important for maintaining healthy cartilage in limb joints, and a cytokine-mediated reduction in circulating IGF-1 may be a contributing factor for lameness in animals (Frisbie et al., 2000; Lejeune et al., 2007). Although such diseases are associated with differential levels of IGF-1, the non-nutritional mechanisms responsible for these changes are not well understood.

Recent findings indicate there is a complex interaction between the IGF system and the cytokine immune response (Thissen and Verniers, 1997; de Martino et al., 2000; Kim et al., 2012; Gude et al., 2012; Suh et al., 2013; Savage, 2013). Previous studies have demonstrated that production of inflammatory cytokines and acute-phase proteins (APPs) are inversely correlated with IGF-1, IGFBP-3, and GHR measurements in various tissues (Thissen and Verniers, 1997; de Martino et al., 2000; Kim et al., 2012; Gude et al., 2012). Further research has demonstrated that TNF-α and IL-1β can inhibit *in vitro* expression of IGF-1 and GHR in hepatocytes even in the presence of GH (Wolf et al., 1996; Thissen and Verniers, 1997), and that such non-nutritional factors may play a critical role in regulating the IGF system in diseased pigs (Roberts and Almond, 2003). It is predicted that elevated levels of inflammatory cytokines and APPs, as a result of disease or stress, may compromise piglet development due to an unfavourable interaction with the IGF system. However, there is still very limited knowledge of the interaction between cytokines, APPs, and the IGF system in pigs with clinical and subclinical diseases. The objective of this study will be to determine whether the hepatic gene expression of IGF-1, IGFBP-3, and GHR is associated with the hepatic gene expression of CRP, SAA, Hp, IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-18 as well as with the presence of *Salmonella* spp., *Lawsonia intracellularis*, *Brachyspira* spp.,

enterotoxigenic *Escherichia coli* (ETEC), methicillin-resistant *Staphylococcus aureus* (MRSA), swine influenza virus (SIV), and PRRSV in nursery pigs from commercial farms.

3.3 Materials and Methods

Experimental design

Eight swine farms in southern Ontario were recruited to participate in a collaborative field study. On each farm, 7 sows were randomly enrolled in the study and 3 piglets (small, medium, and large size) were selected from each sow at weaning and ear tagged. In total 168 piglets (21 piglets per farm) were included. All male piglets recruited in this study had been castrated. A questionnaire (Appendix I) was administered and information about the enrolled sows (farrowing date, parity, litter size, numbers of live born, stillborn), farm management (pig flow, number of gilts, sows, nursery pigs, and grower-finishers, and type of farrowing room floor), health status (recent diseases, mortality in different stage of production), type of feed, vaccination, and in-feed drug use was collected. Piglets were weighed at weaning and once again at 5 weeks post-weaning. Fecal and blood samples were collected at weaning, 2 weeks post-weaning, and 5 weeks post-weaning.

At 5 weeks post-weaning, the piglets were transported to the University of Guelph where they were euthanized. Tissue samples collected from the liver, intestinal lymph node, ileum, and spleen were placed immediately into RNAlater (Ambion, USA) and allowed to sit at 4°C for 24 h before being stored at −80°C. Samples were stored at −80°C for no longer than 4 weeks before being processed for gene expression analysis. Separate portions of the ileum and colon were also collected to test for the presence of *L.*

intracellularis and *Brachyspira* spp., respectively. In addition, nasal swabs were collected prior to euthanasia to be tested for SIV and MRSA. Samples were tested for the presence of *Salmonella* sp., *L. intracellularis*, *Brachyspira* spp., ETEC, and PRRSV at the Animal Health Laboratory at the University of Guelph using standardized protocols.

Growth performance was determined by assessing the post-weaning average daily gain (ADG) of each nursery piglet. ADG was calculated as the difference between the weaning weight and the 5-wk post-weaning weight, divided by the number of days between each weighing.

Detection of viral and bacterial agents

For the isolation of SIV, nasal swab samples were filtered with 0.45 μm pore size filter (Millipore, MA, USA) and 100 μl of the swab preparation was inoculated onto 2 wells of a tissue culture of Madin-Darby canine kidney (MDCK) continuous cell line (10^5 cells) in 24 well plates containing Dulbecco Modified Eagle Medium (DMEM/F12), 10% of fetal bovine serum (FBS), Penicillin 100U/ml, and 100 μg/ml Streptomycin. The inoculum was allowed to absorb at 37°C for 60 min and fresh DMEM/F12 containing 2 μg/ml of TPCK-trypsin, Bovine serum albumin 0.2%, and HEPES 25mM were added. Tissue cultures were harvested upon observation of cytopathic effect (CPE). SIV was purified from cell culture by centrifugation and ultracentrifugation in a Beckman 16.250 rotor at 6000 rpm for 20 min and at 26,000 rpm for 90 min, respectively. Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen). The cDNA was amplified using random primers and 200 U Superscript III reverse transcriptase (Invitrogen), following manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was

performed with 2µl of cDNA in 50µl reaction mixture. The 600bp long fragment of HA gene was amplified by PCR with primer set: H3For (5'-GCTGGTTCAAAGTTCCTCAA-3') and H3Rev (5'-TGAGCTTTTCCCACTTTG-3'). The first cycle of the amplification program consisted of a 2 min period at 95°C and was followed by 35 cycles of 95°C for 20 sec, 52°C for 10 sec and 72°C for 30 sec. The coded protein was identified using BLAST analysis with the GenBank database and degree of identities and similarity was identified using FASTA3 (EMBL-EBI).

Detection of PRRSV in serum samples using RT-PCR was completed using Tetracore real time next generation PCR primers and FAM/MGB probes according to kit instructions (Tetracore Inc., Gaithersburg, MD) and Applied Biosystems 7500 real time thermocycler (Applied Biosystems). The test cannot distinguish between wildtype strains and modified vaccine strains.

For culturing of *Salmonella* spp., fecal samples were enriched in tetrathionate broth and incubated overnight at 41°C. Then a loopful of the enrichment was streaked onto billiants green sulfa (BGS) agar plates and incubated at 35°C overnight. *Salmonella*-like colonies were further identified using matrix-assissted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) and then sent for serotyping to the *Salmonella* OIE reference laboratory at the Public Health Agency of Canada, Guelph, Ontario.

Brachyspira spp. were cultured on selective trypticase soy agar plates containing containing 5% sheep blood, 400 µg/ml spectinomycin, 25 µg/ml vancomycin and 25 µg/ml colistin. Plates were incubated at 41°C in GENbox anaerobic jars (BioMérieux Canada, St-Laurent, Quebec) and monitored for growth of *Brachyspira* spp. every 2 days

for 10 days. Bacterial growth was then washed off the plates with phosphate-buffer saline, and DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) and QIAcube extraction robot following manufacturer's instructions. Presence of *Brachyspira* spp. was determined with PCR using genus specific *nox* gene primers and a probe; BrachysppF284, [5'-AAAGCTACAGATCCTAATTGTCA-3'], BrachysppR723 [5'-CAGCACCAACTACCATAACT-3'] and FAM-5'-TTGCTACTGGTTCTTGGCCT-3'-BBQ. The species of positive *Brachyspira* spp. PCR samples were then determined using RT-PCR, following standard operating procedures used at the Animal Health Laboratory.

For *L. intracellularis* detection, DNA was extracted from ileum samples using FastDNA®kit (MP Biomedicals) and detected using PCR, following manufacturer's instructions. Isolation and detection of ETEC was completed following standard operating procedure used at the Animal Health Laboratory.

The isolation and detection of MRSA from nasal swabs was performed as previously described by Weese et al. (2011). Briefly, nasal swabs were enriched in broth (10 g tryptone/L, 75 g sodium chloride/L, 10 g mannitol/L and 2.5 g of yeast extract/L) and incubated for 24 h at 35°C. Then the broth was inoculated onto MRSA Chromogenic agar, incubated at 35°C for 24-48 h, and screened for *S. aureus* colony morphology. MRSA was confirmed by Gram straining, catalase reaction, coagulase reaction, *S. aureus* latex agglutination, and PBP2a latex agglutination.

RNA extraction from liver tissue

Seventy-four pigs from 4 representative farrow-to-finish farms with different health statuses and production parameters were included for gene expression analysis. RNA was extracted from liver tissue using a Qiagen RNeasy Mini Kit (Qiagen). Approximately 30 mg of liver tissue was added to 600 µl of RLT buffer in a RNase-Free tube and homogenized for 30-40 sec using a tissue homogenizer (PowerGen 125, Fisher Scientific). RNA was then extracted from the homogenate as outlined by the manufacturer's protocol without any modifications (Qiagen). The purity and concentration of the eluted RNA was determined using the Thermo Scientific NanoDrop 8000 Spectrophotometer (Fisher Scientific), and RNA was re-extracted from samples with an RNA concentration ≤150 ng/ul.

All RNA samples were treated with an Amplification Grade DNase I kit (Sigma-Aldrich) to remove any genomic DNA. Nucleic acid-free water (24 µl), reaction buffer (3 µl), Amplification Grade DNase I (3 µl), and 3 µg of total RNA (1 unit/µl) were added together and centrifuged for 10 seconds to collect the reaction at the bottom of the tube. The mixtures were incubated at room temperature for 15 min. Then, 3 µl of the Stop Solution (50 mM EDTA) was added and the mixtures were incubated at 70°C for 10 min. The mixtures were then chilled on ice before being stored at −80°C.

The purity and concentration of RNA was reassessed after DNase digestion using the Thermo Scientific NanoDrop 8000 Spectrophotometer (Fisher Scientific). Samples with low RNA concentrations (<150 ng/µl) were repeated. The integrity of all the RNA samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) before and after DNase treatment to ensure there was no indication of degradation. The mean

RNA Integrity Number (RIN) was 8.5, and any samples with RIN<8.0 were repeated as recommended by Fleige and Pfaffl (2006).

Primer design and efficiency for RT-qPCR

Primer designs and efficiencies are listed in Table 1. Primers for IL-1β, IL-10, IFN-α, and IFN-γ were previously designed by Collado-Romero et al. (2010). All other primer sequences were designed and screened for homology using the NCBI Primer-BLAST program. The cDNA sequences used to design the primers were obtained from the GenBank database. Primers were designed to span known exon-exon junctions. Primer efficiency and the coefficient of determination (R^2) were determined using the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Analysis of gene expression with RT-qPCR

Relative gene expression was assessed using a quantitative polymerase chain reaction (RT-qPCR) assay. The synthesis of cDNA and the RT-qPCR assay were completed by the Advanced Analysis Centre's Genomic Facility at the University of Guelph. For synthesis of cDNA, 1000ng of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A total reaction volume of 20 µl was used for reverse transcription; 2 µl of reverse transcription buffer, 0.8 µl (100mM) dNTP, 2 µl random priming oligonucleotides , 1 µl of MultiScribe™ Reverse Transcriptase (50 U/µl) (Applied Biosystems), 4.2 µl of nucleic acid-free water, and 10 µl (1000ng) of total RNA. The reverse transcription assay was run at 25°C for 5 min, 37°C for 120 min, 85°C for 5 min, and then cooled at 4°C.

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and receptor-like protein-4 (*RLP4*) were analyzed as potential reference genes based on validation and reliable performance in previous studies (Skovgaard et al., 2007; Svobodova et al., 2008; Li et al., 2009). The stability of these reference genes in the liver tissue samples was measured using RT-qPCR and analyzed using BestKeeper software (Pfaffl et al., 2004). The RT-qPCR cycle quantification (C_q) values of GAPDH and RLP4 had standard deviations of 0.34 and 0.49, respectively. GAPDH was chosen as the only endogenous reference gene to be used for internal normalization of gene expression data in this study due to its very low variability (C_q=18.17±0.34) for the present samples and its reliable performance as a sole reference gene in previous studies (Li et al., 2009; Li et al., 2013).

A single StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used for all RT-qPCR assays in this study. Two technical replicates were completed for each sample. Each 20 µl reaction consisted of 7.5 µl of 2X PerfeCta SYBR Green FastMix ROX (Quanta BioScience), 5 µl of template cDNA, 1.9 µl of molecular grade water, and 0.6 µl of primer. The final primer concentration in each reaction was 200 nM. The RT-qPCR assay conditions were 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A melting curve was performed to confirm specificity of the amplicon. The methodology is reported in accordance with the minimum information for publication of RT-qPCR experiments (MIQE) guidelines (Bustin et al., 2009).

Statistical analysis

The crude cycle threshold (C_q) values generated from the RT-qPCR assay were normalized using the C_q values of GAPDH to produce ΔC_q values. Gene expression was

then dichotomized as either high or low using the median ΔC_q value of each gene. Dichotomization was performed to ensure coherent and interpretable coefficients could be generated upon modelling of the data.

The hepatic gene expression of IGF-1, IGFBP-3, and GHR were modelled as dependent variables in three individual logistic regression models (Appendix III). The hepatic gene expression of CRP, Hp, SAA, IFN-a, IFN-y, TNF-a, IL-1b, IL-6, IL-10, and IL-18 were used as independent variables to build the model. Additional extraneous variables used include post-weaning ADG, weight at weaning, age at weaning, number of live births per litter, number of stillbirths per litter, weight at 9-weeks of age, piglet sex, as well as the presence of *Salmonella* spp., PRRSV, and MRSA. However, *L. intracellularis*, *Brachyspira* spp., ETEC, and SIV were not included in the model due to an absence or inadequate number of pigs carrying these organisms.

The linearity of independent variables was assessed using a smoothed locally weighted scatterplot. None of the variables were transformed apart from dichotomization of the gene expression values (ΔC_q) as previously discussed. The initial models were developed through automated stepwise screening. Due to the large number of variables, stringent conditions were used to avoid identifying false associations; variables were first included in the model if $P<0.05$ and then excluded from the model if $P>0.10$. These initial models were then assessed and further developed manually. Farm-level clustering was controlled for by modelling farm as a random-effect unless the intraclass correlation coefficient (ICC) was not significantly different from zero ($P>0.05$). All variables that were excluded during stepwise selection were manually added to the model to determine if they were statistically significant or had a confounding effect on the other variables.

Two-way interaction terms were created between each variable in the model and tested for statistical significance. Variables were retained in the model if they were found to be statistically significant, had a confounding effect of >30%, or were part of a statistically significant interaction term. If a variable was removed from the model, a likelihood-ratio test was performed to determine whether removal of the variable significantly changed the model.

It was determined that all models had binary covariate patterns. If the farm variable was not modelled as a random effect the goodness of fit was determined by the Hosmer-Lemeshow test, and if the farm variable was modelled as a random effect the normality of the best linear unbiased predictions of the random effect and affiliated residuals were evaluated. Furthermore, diagnostic analysis revealed no outliers or extremely influential observations. One pig was excluded due to missing data. In addition, univariable analysis of continuous farm-level parameters (herd size and pathogen prevalence) was assessed by Pearson's correlation coefficient as all variables showed a normal distribution and linear relationship. All statistical analysis was completed using STATA 10.0 (Stata Corporation, College Station, TX). Variables with a $P<0.05$ were considered statistically significant and non-significant variables with a $P<0.10$ were considered to be suggestive of an association.

3.4 Results

Descriptive statistics

Variation in management practices, herd size, and therapeutic interventions on the studied farms is described in Table 2. PRRSV, MRSA, SIV, *Salmonella* spp., and *B. murdochii* were present on 4 (50%), 6 (75%), 1 (12.5%), 2 (25%), and 3 (37.5%) farms,

respectively. *L. intracellularis* and ETEC were not detected on any of the farms. The prevalence of these pathogens among individual farms and the mean prevalence among pigs are displayed in Table 3. None of the pigs in this study exhibited clinical signs of infection. Serotyping of *Salmonella* isolates revealed that all isolates from farm 2 were *S.* Infantis and all isolates from farm 5 were *S.* Worthington. Serum titres of PRRSV on farms 1, 3, 5, and 6 were 3.4×10^6 $TCID_{50}$/mL, 4.5×10^5 $TCID_{50}$/mL, 1.4×10^3 $TCID_{50}$/mL, and 2.6×10^7 $TCID_{50}$/mL, respectively. In addition, it was observed that farms 1, 3, and 6, which had the highest viral titres and prevalence of PRRSV, were also the farms that had the highest prevalence of MRSA. The positive correlation between farm-level prevalence of PRRSV and MRSA was significant (R^2=0.635; *P*=0.018). Farm-level prevalence of PRRSV was also positively correlated with nursery herd size (R^2=0.520; *P*=0.044). Interestingly, antibiotic usage was not associated with MRSA colonization of swine herds (*P*=0.98).

Factors associated with IGF-1 expression in the liver

Multivariable analysis for factors associated with hepatic expression of IGF-1 is shown in Table 4. Pigs with a higher post-weaning ADG had significantly increased odds of elevated IGF-1 expression in the liver. The pig with the highest ADG (556 g/day) was 1,073 (95% CI: 16.02 – 71,892) times more likely to have higher IGF-1 expression than the pig with the lowest ADG (140 g/day) (*P*<0.01). Pigs that had higher expression of IGFBP-3 were more likely to have increased IGF-1 expression, and pigs that had higher expression of the cytokines TNF-α or IL-18 were less likely to have increased hepatic expression of IGF-1. Further, pigs colonized with PRRSV were less likely to have

increased IGF-1 expression in the liver. None of the extraneous variables behaved as a confounder and all two-way interaction terms were not statistically significant. The intraclass correlation coefficient (ICC) was not significantly different from zero (ICC=$1.0 \cdot 10^{-7}$, $P>0.05$).

Factors associated with IGFBP-3 expression in the liver

The multivariable analysis for factors associated with hepatic expression of IGFBP-1 is described in Table 5. Pigs with a higher weight at weaning were more likely to have increased hepatic expression of IGFBP-3 ($P<0.05$). The pig with the highest weight at weaning (14.0 kg) was 1,027 (95% CI: 3.52 – 299,908) times more likely to have increased IGFBP-3 expression than the pig with the lowest weight at weaning (3.1 kg) ($P<0.05$). Pigs with increased hepatic expression of IGF-1, GHR, or IL-6 were more likely to have increased hepatic expression of IGFBP-3 ($P<0.05$). The extraneous variables showed no confounding effect on the model. However, 2 statistically significant interactions were detected and retained in the model; an interaction between Hp expression and IGF-1 expression, and an interaction between Hp expression and the number of live births per litter. At low Hp expression, increased IGF-1 mRNA expression was associated with increased IGFBP-3 expression (OR=29.65; $P<0.05$). However, IGF-1 expression was not associated with IGFBP-3 expression ($P=0.54$) when Hp expression was high. Live births per litter was not significantly associated with IGFBP-3 expression ($P=0.60$) when Hp expression was low, but it was significantly associated with IGFBP-3 expression (OR=2.57; $P<0.01$) when Hp expression increased. The ICC was not significantly different from zero (ICC=$6.5 \cdot 10^{-8}$, $P>0.05$).

Factors associated with GHR expression in the liver

Multivariable analysis for factors associated with hepatic expression of GHR is shown in Table 6. Pigs with increased expression of IGFBP-3 or CRP were more likely to have increased expression of GHR ($P<0.05$). Barrows, pigs from litters with more live births, and pigs with increased expression of SAA or IL-1β were less likely to have increased GHR expression ($P<0.05$). It was also observed that older pigs and increased TNF-α expression had a tendency to be associated with increased expression of GHR ($P<0.10$). The odds ratio for these variables are listed in Table 6. None of the extraneous variables had a confounding effect and there were no statistically significant two-way interaction terms. Data clustering at the farm level was controlled for in the model as the ICC was significantly different from zero (ICC=0.57, $P<0.01$).

3.5 Discussion

The results of this study demonstrate an overall inverse relationship between the hepatic expression of the IGF system and the inflammatory immune response. The IGF system appeared to be inhibited or disrupted by several proinflammatory cytokines and APPs, including IL-1β, IL-18, TNF-α, SAA, and Hp. These findings support the speculation that elevated levels of proinflammatory cytokines and APPs, as a result of disease or stress, may hinder growth and development due to an unfavourable interaction with the IGF system or affiliated pathways (Savage, 2013).

The association between IGF-1 and post-weaning ADG, and between IGFBP-3 and weaning weight was expected and is consistent with previous research which has demonstrated that these proteins are indicative of growth performance and carcass traits

of pigs (Clutter et al., 1995; Owens et al., 1999; Li et al., 2013). Increased IGFBP-3 expression was positively associated with increased IGF-1 and GHR expression which was anticipated as these proteins are concomitantly regulated (Ramajayam et al., 2012; Vottero et al., 2013).

Increased IGF-1 expression was inversely associated with increased expression of 2 potent proinflammatory cytokines; TNF-α and IL-18. The inverse relationship with TNF-α is consistent with studies which demonstrate that TNF-α can inhibit IGF-1 production of *in vitro* hepatocytes even in the presence of GH stimulation (Wolf et al., 1996; Thissen and Vernier, 1997). Although limited research exists that examines the association between IL-18 and the IGF system, previous findings do indicate that IL-18 is negatively associated with IGF-1 and growth (Skopiński et al., 2005; Netea et al., 2006). Future research should explore the mechanisms by which TNF-α and IL-18 may interact with hepatic expression of IGF-1.

Hepatic expression of IGF-1 and IGFBP-3 were associated when Hp expression was low, but this association was not significant when Hp expression increased. This finding is consistent with experiments where administration of recombinant IGF-1 and IGFBP-3 to rats was associated with decreased plasma concentrations of Hp (Jeschke et al., 2000). It appears that increased levels of IGF-1 and IGFBP-3 may inhibit Hp production in the liver. The reason for the observed interaction between Hp and live births per litter is unclear. This association is questionable as sow characteristics (live births per litter and still births per litter) were not the primary targets in this investigation and therefore sow-level clustering of data was not controlled for in the analysis. However, larger litter size is a contributor of compromised pig welfare (Rutherford et al.,

2013) and previous studies have demonstrated that high Hp levels are a strong indicator of decreased welfare on pig farms (Pineiro et al., 2007a; Pineiro et al., 2007b).

Elevated IGFBP-3 expression was associated with increased expression of IL-6, which plays an important role in the anti-inflammatory response (Tilg et al., 1997). It has previously been observed that IL-6 does stimulate production of IGF-1 in hepatocytes, which is likely caused by a cross-reaction with a known receptor of the same superfamily and through stimulation of a transduction pathway factor shared between IL-6 and the IGF system (Thissen and Verniers, 1997). This may explain the positive association between IL-6 and IGFBP-3 that was observed in this study.

Expression of GHR in the liver was inversely associated with expression of SAA and IL-1β. These associations may be explained by other studies which have demonstrated that GH reduces production of inflammatory agents including IL-1β and SAA (Jeschke et al., 2000; Lu et al., 2010). It is likely GH concurrently stimulates GHR expression and inhibits inflammatory cytokine expression in the liver leading to this observed association between GHR and decreased expression of IL-1β and SAA. It was also observed that male pigs were less likely to have increased GHR expression than female pigs. Similar results were reported with rats wherein the males had significantly reduced hepatic expression of GHR when compared to females, and this difference appears unrelated to castration and sex hormones (Ahlgren et al., 1995; Baumbach and Bingham, 1995). The reason for this differential hepatic expression of GHR between the sexes is unknown and further research is needed to address this discrepancy. Pigs from larger litters were also less likely to have elevated GHR expression which may be a result of restricted access to maternal nutrients due to increased litter size (Brameld et al.,

2000). However, clustering of sow-level data (live births per litter and stillbirths per litter) was not controlled for during analysis and therefore any observed association with these sow-level traits is questionable in this study.

It is unclear whether pigs that tested seropositive for PRRSV in the current study had been infected with PRRSV, vaccinated with an attenuated strain, or both. However, research has demonstrated that the change in circulating GH is very similar in vaccinated and unvaccinated pigs exposed to PRRSV (Borghetti et al., 2011). In this investigation, pigs seropositive for PRRSV were less likely to have increased IGF-1 expression compared to pigs that were seronegative for PRRSV. Previous research has demonstrated that pigs co-infected with PRRSV and *Mycoplasma hyopneumoniae* have significantly reduced serum concentrations of IGF-1 despite similar feed in-take when compared to a control group (Roberts and Almond, 2003). This finding suggests that non-nutritional factors, such as the inflammatory response, may contribute to the regulation of the IGF system in pigs (Robert and Almond, 2003). It is possible that early retardation of the IGF system due to nutritional and non-nutritional factors is responsible for the reduced body weight observed in pigs that are seropositive for PRRSV (Regula et al., 2000). Furthermore, the prevalence of PRRSV in nursery pigs was positively correlated with nursery herd size which may indicate that larger swine operations have a higher risk of contracting PRRSV.

The current investigation also suggests that MRSA colonization does not elicit a demonstrable immune response or interact with the IGF system in pigs. This was expected as many swine herds carry this bacterium without incident and it appears to behave as a commensal bacterium (Khanna et al., 2008). However, further research

should explore whether certain types of porcine MRSA are associated with a subclinical immune response. It was also observed that farm-level prevalence of MRSA is positively correlated with PRRSV. This may be the result of poor farm biosecurity or an indication of co-colonization as it has previously been shown that PRRSV reduces the bactericidal activity of porcine macrophages (Thanawongnuwech et al., 1997). In addition, MRSA was present on farms regardless of antibiotic usage which suggests that other factors are contributing to its persistence on pig farms.

Salmonella Infantis was not associated with hepatic expression of the IGF system despite previous studies that identified reduced serum IGF-1 and IGFBP-3 in pigs colonized with *Salmonella* (Jenkins et al., 2004). The lack of association observed in the present investigation may be due to the limited number of liver samples used for RT-qPCR from pigs colonized with *Salmonella* (n=6). RT-qPCR was not performed on liver samples from pigs colonized with *Salmonella* Worthington. Both *S.* Infantis and *S.* Worthington recovered from pigs in this study are rarely reported from clinical salmonellosis in pigs, and it is possible that the effect of *Salmonella* on the IGF system in pigs is serotype-specific.

One of the limitations of this study is that it was not a comprehensive analysis of all swine pathogens that may have been present on each farm. Although none of the pigs showed clinical signs of infection and although the survey inquired about previous illness, these pigs may be carrying pathogens that were not tested for in this study. Any carriage or subclinical infection of undetected swine pathogens may have unknowingly influenced the hepatic expression of the growth factors or immunomodulators.

3.6 Conclusion

Overall, hepatic expression of the IGF system was inversely associated with hepatic expression of several cytokines and APPs which suggests that an inflammatory response, perhaps as a result of disease, vaccination, or stress, can disrupt the balance of the IGF system. Determining the inverse association between inflammatory markers and the IGF system in pigs reinforces the importance of swine health and its potential impact on production. However, future investigations need to assess whether prolonged dysregulation of the IGF system due sustained increases in cytokines and APPs causes significant changes in production outcomes. Differential expression of the IGF system may result in changes to growth performance, carcass quality, or increase the risk of health ailments such as lameness. Fully understanding the consequences of disease and stress is necessary for determining the value of an intervention and for deciding how to balance herd health and production. Lastly, the genetic composition of pigs may partly contribute to the transcriptional expressivity of the IGF system and immune response which was not investigated in the current study, and should be investigated thoroughly.

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Table 3.1: Primers used for RT-qPCR of growth factors and immunomodulators.

Gene Symbol	Gene Name	Accession Number[a]	Primers (5' – 3')	T_m (°C)	Amplicon Length (bp)	R^2	Primer Efficiency (%)
IL1B	Interleukin-1β	NM_214055	F: GGCCGCCAAGATATAACTGA[b] R: GGACCTCTGGGTATGGCTTTC[b]	59	70	0.991	100
IL6	Interleukin-6	NM_214399	F: CCCTGAGGCAAAAGGGAAAGA R: CGTGGACGGCATCAATCTCA	60	212	0.998	95.2
IL10	Interleukin-10	L20001	F: CAGATGGGCGACTTGTTG[b] R: ACAGGGCAGAAATTGATGAC[b]	57	219	0.993	91.7
IL18	Interleukin-18	NM_213997	F: GCTGCTGAACCGGAAGACAA R: AAACACGGCTTGATGTCCCT	60	192	0.996	95.3
IFNA	Interferon-α	AB257591	F: GACCTGCCTCAGATCCACAG R: ATGGCTTGAGCCTTCTGGAC	60	158	0.986	82.3
IFNG	Interferon-γ	X53085	F: CAAAGCCATCAGTGAACTCATGA[b] R: TCTCTGGCCTTGGAACATAGTCT[b]	60	100	0.985	98.7
TNFA	Tumor necrosis factor-α	NM_214022	F: CCTCTTCTCCTTCCTCCTG[b] R: CCTCGGCTTTGACATTGG[b]	57	194	0.997	100
CRP	C-reactive protein	NM_213844	F: TGCCCAGACAGACATGATCG R: GGTCGGTATAGACACGCAGG	60	131	0.999	100
HP	Haptoglobin	NM_214000	F: TGAATGTGAAGCAGTGTGCG R: CGAGGTGAGGTTATGGTGGG	59	133	0.996	96
SAA	Serum amyloid A	EF362780	F: TGATCAGCGATGCCAGAGAG R: CTTGAGTCCTCCACTCCGTG	60	85	0.998	87.8
IGF1	Insulin-like growth factor-1	JX827417	F: TCTTCTACTTGGCCCTGTGCTT R: CCAGCTCAGCCCCACAGA	61	81	0.998	97.7
IGFBP3	Insulin-like growth factor binding protein-3	NM_001005156	F: GGCATCCACATCCCCAACT R: CCCCGCTTCCTGCCTTT	60	80	0.990	97.3
GHR	Growth hormone receptor	JF276446	F: CTCCACAGGGCCTCGTACTC R: GCTCACATAGCCACACGATGA	60	80	0.999	89.2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_001206359	F: ACACACCGAGCATCTCCTGACT R: CGAGGCAGGTCTCCCTAAGC	61	80	0.998	100

[a] Accessed via GenBank.
[b] Designed by Collado-Romero et al., 2010.

Table 3.2: Descriptive characteristics of swine operations and management practices on eight farms.

Farm	Site	Breeding Herd Size	Nursery Herd Size	Nursery Pig Flow	Vaccinations	Antibiotic usage
1[a]	S	650	4,900	AI/AO	MH, AP, PRRSV	Penicillin
2[a]	M	500	2,100	CON	PCV2	None
3[a]	M	1,000	3,000	AI/AO	PCV2, MH	Penicillin, tiamulin, ceftiofur
4	S	240	700	AI/AO	PCV2, MH, LI	None
5	S	123	450	CON	PCV2	None
6	M	650	1,500	CON	PCV2, MH	None
7	M	850	2,200	AI/AO	PCV2, MH	Penicillin, chlortetracycline, sulfamethazine, trimethoprim, sulfadoxine
8[a]	S	670	2,600	CON	PCV2	Penicillin

M – multiple sites; S – single site; AI/AO – all in, all out; CON – continuous; PCV2 – porcine circovirus 2; MH – *Mycoplasma hyopneumoniae*; AP – *Actinobacillus pleuropneumoniae*; LI – *Lawsonia intracellularis*.

[a] Gene expression analysis was completed on liver tissue samples from piglets originating from these farms.

Table 3.3: Prevalence of viral and bacterial pathogen shedding in nursery pigs.

	Number of positive pigs (%)				
Farm	**PRRSV**	**MRSA**	**SIV**	***Salmonella* spp.**	***Brachyspira* spp.**
1[a]	21 (100)	17 (81)	0	0	0
2[a]	0	12 (60)	0	6 (30)	0
3[a]	17 (85)	16 (80)	0	0	0
4	0	7 (33)	3 (14)	0	0
5	1 (5)	0	0	9 (45)	0
6	10 (48)	15 (71)	0	0	3 (14.3)
7	0	1 (5)	0	0	2 (9.5)
8[a]	0	0	0	0	2 (9.5)
Mean	**49 (30)**	**68 (41)**	**3 (1.8)**	**15 (9.3)**	**7 (4.2)**

PRRSV – porcine reproductive and respiratory syndrome virus; MRSA – methicillin-resistant *Staphylococcus aureus*; SIV – swine influenza virus.

[a] Gene expression analysis was completed on liver tissue samples from piglets originating from these farms.

Table 3.4: Analysis of gene expression of IGF-1 in liver tissue from 73 pigs from 4 farms (Model 1: multivariable logistic regression).

Variable	**OR**	**SE**	**95% CI**	***P* value**
Post-weaning ADG (g)	1.017	0.005	1.007 – 1.027	0.001
Colonized with PRRSV	0.034	0.031	0.005 – 0.203	<0.001
High IGFBP-3 Expression	7.161	5.006	1.819 – 28.182	0.005
High TNF-α Expression	0.141	0.104	0.033 – 0.603	0.008
High Il-18 Expression	0.191	0.132	0.049 – 0.741	0.017

PRRSV – porcine reproductive and respiratory syndrome virus.
OR – Odds Ratio; SE – Standard Error; CI – Confidence Interval

Table 3.5: Analysis of gene expression of IGFBP-3 in liver tissue from 73 pigs from 4 farms (Model 2: multivariable logistic regression).

Variable	**OR**	**SE**	**95% CI**	***P* value**
Weight at weaning (kg)	1.889	0.502	1.122 – 3.180	0.017
High IGF-1 Expression	29.65	46.48	1.373 – 640.2	0.031
High GHR Expression	53.38	68.65	4.294 – 663.7	0.002
High Hp Expression	0.008	0.040	4.8×10^{-7} – 133.4	0.330
High Il-6 Expression	8.521	8.343	1.251 – 58.06	0.029
IGF-1 x Hp	0.016	0.034	2.9×10^{-4} – 0.908	0.045
Hp x Live births	2.262	0.888	1.048 – 4.884	0.038

OR – Odds Ratio; SE – Standard Error; CI – Confidence Interval

Table 3.6: Analysis of gene expression of GHR in liver tissue from 73 pigs from 4 farms (Model 3: multivariable random-effect logistic regression).

Variable	**OR**	**SE**	**95% CI**	***P* value**
Male	0.014	0.023	0.001 – 0.369	0.011
Live births per litter	0.593	0.150	0.361 – 0.974	0.039
High IGFBP-3 Expression	98.47	164.1	3.753 – 2583.5	0.006
High CRP Expression	290.0	614.9	4.549 – 18,494	0.007
High SAA Expression	0.005	0.011	1.0×10^{-4} – 0.287	0.010
High Il-1β Expression	0.029	0.048	0.001 – 0.782	0.035

OR – Odds Ratio; SE – Standard Error; CI – Confidence Interval

CHAPTER 4: CONCLUSIONS

4.1 Research Summary and Conclusions

The results of this research have revealed the usefulness of the IGF system as a marker for growth performance and its association with immunomodulatory activity in nursery pigs. In the first component of the research it was demonstrated that hepatic expression of IGFBP-3 and GHR was significantly associated with ADG and 9-week body weight in nursery pigs. Growth and body weight at 9 weeks of age tended to be associated with IGF-1 expression in the first component of the study, but growth was significantly associated with IGF-1 expression in the second component of the study. Nevertheless, IGF-1 is likely to be a useful marker of growth performance if used for comparing nursery pigs on a single farm. Based on these results IGFBP-3 and GHR can be useful growth-associated biomarkers for population based studies that contrast management practices or disease occurrence at the farm level. The hepatic expression of the cytokines (IL-1β, IL-6, IL-10, IL-18, IFN-α, IFN-γ, TNF-α) and APPs (CRP, Hp, SAA) in this study were not significantly associated with growth performance in nursery pigs. Despite previous reports of their growth-related roles (Thissen and Verniers, 1997; Saco et al., 2010; Ramsay et al., 2013), these immunomodulatory agents are not adequate for monitoring growth performance in nursery pigs as their expression can be considerably altered by additional parameters including disease and stress. Additionally, this work demonstrated that production parameters, including weight at weaning and age at weaning, were significant determinants of subsequent growth performance in the nursery phase and this is consistent with previous findings (de Grau et al., 2005; Paredes et al.,

2012). Overall, hepatic expression of IGF-1, IGFBP-3, and GHR are useful markers for monitoring growth performance in nursery pigs. Future research should assess whether IGF-1, IGFBP-3, and GHR levels are biomarkers of growth performance at other stages of production and whether desirable levels of these biomarkers in the nursery phase are indicative of subsequent performance in the grower and finisher stages of pig production.

In the second component of the research it was demonstrated that the hepatic expression of the IGF system is inversely associated with expression of several proinflammatory agents including Hp, SAA, IL-1β, IL-18, and TNF-α. The anti-inflammatory cytokines were not associated with the IGF system except for IL-6 which was positively associated with IGFBP-3 expression. This relationship between inflammatory agents and the IGF system supports the claim that immunomodulatory activity regulates the IGF system independent of nutritional parameters (Roberts and Almond, 2003; Savage, 2013). These findings warrant further research into the long-term effects of immunomodulating agents on growth performance in pigs. For example, do sustained levels of immunomodulating agents cause such dysregulation of the IGF system that it compromises productivity? The molecular mechanisms involved in this nutritional-independent interaction between the IGF system and the immune system are also unknown and need further investigation.

It was also determined that pigs positive for PRRSV (as a result of natural infection or vaccination) were considerably less likely to have increased IGF-1 expression, which is consistent with previous reports (Roberts and Almond, 2003). Future research should explore the usefulness of IGF-1 as a biomarker for PRRSV in pigs. Furthermore, MRSA was detected on pig farms even in the absence of antibiotic usage,

and MRSA colonization was not associated with any immunomodulatory agent which supports speculation that it behaves as a commensal bacterium in swine. The prevalence of PRRSV was also positively correlated with both MRSA prevalence and nursery herd size. In addition, IGF-1 expression was significantly associated with ADG in this component of the research which reinforces the claim that IGF-1 is a valuable biomarker for monitoring growth performance in nursery pigs.

Overall, this study determined that the hepatic expression of the IGF system provides useful biomarkers for monitoring the growth performance of nursery pigs and it appears that increased immunomodulatory activity can dysregulate the IGF system independent of nutritional factors. Furthering our understanding of the factors that influence growth and pioneering methods for monitoring these changes is important for achieving maximal and efficient productivity in pig farming, especially in a rapidly advancing industry. These findings and subsequent research will hopefully lead to improved productivity and the development of advanced methods for evaluating growth performance in swine production.

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Appendix I

Survey

Please note that the information you provide us on this survey will be maintained as confidential.

Visit Date: ……
Farm Number: ……..
Farm owner: …….
Farm Veterinarian ……

1) Type of operation:

- ☐ Farrow to Finish
- ☐ Farrow to feeder
- ☐ Other (……)

2) Herd size:

	Number of animals
Sows	
Nursery pigs	
Grower-Finisher pigs	
Gilts	

3) Number of farrowing rooms: ……..

4) Pig flow in nurseries

- ☐ Continuous
- ☐ AIAO by pen
- ☐ AIAO by room
- ☐ AIAO by barn

5) Pig flow in finishers

- ☐ Continuous
- ☐ AIAO by pen
- ☐ AIAO by room
- ☐ AIAO by barn

6) Do the pigs at the suckling, post-weaning, and finishing stages receive routine vaccination? If yes, please name any vaccines.

7) Do the pigs at the suckling, post-weaning, and finishing stages receive and in-feed, in-water, or injection of antibiotics? If yes, please provide name and root of administration for each group of pigs.

8) In the last year, did this operation have a specific health problem such as diarrhea problem and respiratory diseases in suckling pigs, weaners, and finishers? If yes, please describe (symptoms, mortality, diagnosis, and treatment):

Suckling pigs:
……

……

……

……

Nursery pigs: ……

……

……

……

Finishers:
……

……

……

……

9) What is your feed company?

10) Rations:

11) Type of feed:

- ☐ Dry
- ☐ Dry floor
- ☐ Wet-dry
- ☐ Liquid
- ☐ Mesh
- ☐ Pallets

12) Do we have your permission to contact your veterinarian or feed company for further information?

Comments:
……..
……
……..

Appendix II

```
xtreg weight_9wk wean_weight wean_age sex parity stillborn bornalive, i(farm) re

Random-effects GLS regression                   Number of obs      =       161
Group variable: farm                            Number of groups   =         8

R-sq:  within  = 0.5575                         Obs per group: min =        18
       between = 0.1682                                        avg =      20.1
       overall = 0.4544                                        max =        21

Random effects u_i ~ Gaussian                   Wald chi2(6)       =    191.56
corr(u_i, X)       = 0 (assumed)                Prob > chi2        =    0.0000

------------------------------------------------------------------------------
  weight_9wk |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 wean_weight |   1.669568   .1276296    13.08   0.000     1.419418    1.919717
    wean_age |  -.2255806   .0692366    -3.26   0.001    -.3612818   -.0898793
         sex |   .5240952   .4242166     1.24   0.217    -.3073541    1.355544
      parity |   .0723967     .09687     0.75   0.455     -.117465    .2622584
   stillborn |   .2899609   .2483382     1.17   0.243    -.1967731    .7766949
   bornalive |  -.1019756   .0932212    -1.09   0.274    -.2846857    .0807346
       _cons |   15.19746   2.479592     6.13   0.000     10.33755    20.05737
-------------+----------------------------------------------------------------
     sigma_u |  3.7624153
     sigma_e |  2.6498578
         rho |  .66843389   (fraction of variance due to u_i)
------------------------------------------------------------------------------
```

```
reg adg igf1 igfbp3 ghr wean_weight i.farm

i.farm            _Ifarm_1-8          (naturally coded; _Ifarm_1 omitted)

      Source |       SS       df       MS              Number of obs =      72
-------------+------------------------------           F(  7,    64) =   10.27
       Model |  .247072152     7  .035296022           Prob > F      =  0.0000
    Residual |  .220062538    64  .003438477           R-squared     =  0.5289
-------------+------------------------------           Adj R-squared =  0.4774
       Total |  .467134691    71  .006579362           Root MSE      =  .05864

------------------------------------------------------------------------------
         adg |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        igf1 |  -.0128547   .0069895    -1.84   0.071    -.0268179    .0011085
      igfbp3 |   .0337513   .0136535     2.47   0.016     .0064754    .0610272
         ghr |  -.0417612    .015571    -2.68   0.009    -.0728679   -.0106546
 wean_weight |   .0094545   .0043551     2.17   0.034     .0007542    .0181548
    _Ifarm_2 |  -.1222659    .022279    -5.49   0.000    -.1667733   -.0777586
    _Ifarm_3 |  -.0799087   .0240509    -3.32   0.001    -.1279558   -.0318616
    _Ifarm_4 |  (dropped)
    _Ifarm_5 |  (dropped)
    _Ifarm_6 |  (dropped)
    _Ifarm_7 |  (dropped)
    _Ifarm_8 |  -.1204404   .0211086    -5.71   0.000    -.1626097   -.0782712
       _cons |    .555518   .0742692     7.48   0.000     .4071483    .7038877
------------------------------------------------------------------------------
```

```
xi: reg   weight_9wk igf1 igfbp3 ghr  wean_weight wean_age i.farm

i.farm            _Ifarm_1-8          (naturally coded; _Ifarm_1 omitted)

      Source |       SS       df       MS              Number of obs =      72
-------------+------------------------------           F(  8,    63) =   17.88
       Model |  782.931186     8  97.8663983           Prob > F      =  0.0000
    Residual |   344.82262    63  5.47337493           R-squared     =  0.6942
-------------+------------------------------           Adj R-squared =  0.6554
       Total |  1127.75381    71  15.8838564           Root MSE      =  2.3395

------------------------------------------------------------------------------
  weight_9wk |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        igf1 |  -.5466997   .2797375    -1.95   0.055     -1.10571    .0123111
      igfbp3 |   1.408585   .5448217     2.59   0.012     .3198453    2.497324
         ghr |  -1.579592   .6377337    -2.48   0.016    -2.854001   -.3051825
 wean_weight |   1.403339   .1791896     7.83   0.000     1.045257    1.761421
    wean_age |  -.3128832   .0751784    -4.16   0.000    -.4631153   -.1626511
    _Ifarm_2 |  -5.692979   .9099497    -6.26   0.000    -7.511368    -3.87459
    _Ifarm_3 |  -1.816797   1.045265    -1.74   0.087    -3.905591    .2719984
    _Ifarm_4 |  (dropped)
    _Ifarm_5 |  (dropped)
    _Ifarm_6 |  (dropped)
    _Ifarm_7 |  (dropped)
    _Ifarm_8 |  -5.606505   .9104714    -6.16   0.000    -7.425937   -3.787073
       _cons |   29.96336   3.650611     8.21   0.000     22.66819    37.25852
------------------------------------------------------------------------------
```

Appendix III

```
logit igf1_cat prrsv adg_g il18_cat tnfa_cat igfbp3_cat, or

Logistic regression                                     Number of obs   =         73
                                                        LR chi2(5)      =      31.55
                                                        Prob > chi2     =     0.0000
Log likelihood = -34.819455                             Pseudo R2       =     0.3118

------------------------------------------------------------------------------
    igf1_cat | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       prrsv |   .0341189   .0310229    -3.72   0.000     .0057416    .2027483
      adg_g  |   1.016917   .0052439     3.25   0.001     1.006691    1.027247
    il18_cat |   .1913278   .1322463    -2.39   0.017     .0493657    .7415337
    tnfa_cat |   .1408895   .1044629    -2.64   0.008     .0329422    .6025658
  igfbp3_cat |   7.160774   5.005656     2.82   0.005     1.819439    28.18269
------------------------------------------------------------------------------
```

```
logit igfbp3_cat   igf1_hp  hp_born igf1_cat hp_cat wean_weight bornalive ghr_cat
il6_cat, or

Logistic regression                                     Number of obs   =         73
                                                        LR chi2(8)      =      56.43
                                                        Prob > chi2     =     0.0000
Log likelihood =  -22.32289                             Pseudo R2       =     0.5583

------------------------------------------------------------------------------
  igfbp3_cat | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     igf1_hp |    .016381   .0335548    -2.01   0.045     .0002956    .9076874
     hp_born |   2.262708   .8883577     2.08   0.038     1.048189    4.884467
    igf1_cat |   29.64997   46.47817     2.16   0.031     1.373135    640.2292
      hp_cat |    .008008   .0397161    -0.97   0.330     4.81e-07    133.3881
 wean_weight |   1.88941    .501984     2.39   0.017     1.122475    3.180357
   bornalive |   1.136575   .2777153     0.52   0.600     .7040642    1.834781
     ghr_cat |   53.38417    68.6481     3.09   0.002     4.293684    663.7352
     il6_cat |   8.521369   8.342817     2.19   0.029     1.250672    58.05978
------------------------------------------------------------------------------
```

```
xtlogit ghr_cat bornalive il18_cat tnfa_cat crp_cat sex wean_age igfbp3_cat
saa_cat il1b_cat, i(farm) re or

Random-effects logistic regression              Number of obs      =        73
Group variable: farm                            Number of groups   =         4

Random effects u_i ~ Gaussian                   Obs per group: min =        17
                                                               avg =      18.3
                                                               max =        19

                                                Wald chi2(9)       =     10.66
Log likelihood  = -21.021149                    Prob > chi2        =    0.2996

------------------------------------------------------------------------------
     ghr_cat |         OR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   bornalive |   .5926662    .150111    -2.07   0.039      .360759    .9736507
    il18_cat |   7.320282   9.103139     1.60   0.109     .6397583    83.76059
    tnfa_cat |   7.119641   7.346245     1.90   0.057     .9422464    53.79621
     crp_cat |   290.0397   614.8969     2.67   0.007     4.548545    18494.49
         sex |   .0137588   .0230918    -2.55   0.011     .0005128    .3691254
    wean_age |   1.354151   .2206746     1.86   0.063     .9839067    1.863718
  igfbp3_cat |   98.47365   164.1473     2.75   0.006     3.753518    2583.459
     saa_cat |   .0054566     .01103    -2.58   0.010     .0001038    .2867773
    il1b_cat |   .0285292   .0481929    -2.11   0.035     .0010409    .7819553
-------------+----------------------------------------------------------------
    /lnsig2u |   1.479875   1.128883                     -.7326945    3.692444
-------------+----------------------------------------------------------------
     sigma_u |   2.095804   1.182959                      .693262    6.335839
         rho |    .571758   .2764078                     .1274671    .9242537
------------------------------------------------------------------------------
Likelihood-ratio test of rho=0: chibar2(01) =     6.77 Prob >= chibar2 = 0.005
```