EFFECTS OF LOW LITTER BIRTH WEIGHT ON THE PATHOGENESIS OF INFLUENZA A VIRUS FOLLOWING EXPERIMENTAL INFECTION

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Large Animal Clinical Sciences

University of Saskatchewan

Saskatoon

By

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ABSTRACT

A fetus' *in utero* environment has a profound effect on the individual's development in postnatal life. Research has suggested that intrauterine growth restricted children have a less robust response to vaccination. Studies have confirmed similar results in animal models; however, the effect of low birth weight on clinical disease expression is unclear. This research aims to determine if pigs from low birth weight litters have increased severity of disease after experimental infection with influenza A virus (IAV) when compared to their counterparts from high birth weight litters, thus clarifying the effect of litter birth weight on disease expression.

Pilot trials were conducted to determine the appropriate dose of virus to use and the optimal days post inoculation for necropsy to use for the main trial. The results indicated that the main trial should use an inoculation dose of 1×10^7 plaque forming units of IAV and the time of necropsy should be 48 hours post inoculation. In the main trial, male piglets (n=68) from parity one or two sows were identified at farrowing as coming from high or low birth weight litters. At four weeks of age, intratracheal IAV inoculation was performed (day 0) and pigs were euthanized at 48 hours post inoculation. Clinical signs were assessed prior to euthanasia. After euthanasia macroscopic and microscopic lesion severity were assessed, along with immunohistochemical staining intensity of IAV in lung tissue. SearchLight Chemiluminescent Array Technology was used to measure the concentration of the inflammatory cytokines interleukin 1 beta, interleukin 6, and interleukin 8 in bronchoalveolar lavage fluid. Interferon alpha was measured using fluorescent microsphere immunoassay. Fifty Percent Tissue Culture

Infective Dose was used to measure influenza viral titers in lung tissue. The study found no differences in clinical scores or cytokine concentration between pigs from high and low birth weight litters. Gross, histopathological and immunohistochemical scores were significantly higher in piglets from high birth weight litters and viral titers trended higher in these piglets. These findings indicate that pathologic disease scores in piglets experimentally inoculated with IAV are more severe in piglets from high birth weight litters.

ACKNOWLEDGEMENTS

Thank you to my co-supervisors, John Harding and Susan Detmer; my committee members, Yan Zhou, George Foxcroft and Cheryl Waldner; the Large Animal Clinical Sciences graduate chairs, Joe Stookey and Tasha Epp. I am grateful to the University of Saskatchewan Inter-provincial Graduate Research fund and the Alberta Meat and Livestock Agency for their financial support. I would like to sincerely thank the source herd manager, veterinarian and staff for allowing the research team access to their barns, animals and records. I sincerely appreciate all the assistance received from Natalie Berube, Betty Chow-Lockerbie, Crissie Auckland, and the Animal Care Unit team, Prairie Diagnostic Services, Inc., Drs. Andrea Ladinig, Ted Clark, and Shermila Kulanayake. Thank you to my coworkers at the Saskatoon District Office for all your encouragement. I especially need to thank family for their love and support over the years: Dad, Lawana and Rick, Chet and Camilla, Eric.

Above all, Susan, I could not and would not have done this without you. Thank you for your ability to give it to me straight without making me feel small, listening to my frustrations, and going above and beyond to encourage me. You have no idea how much those things have meant to me during a very arduous period in my life.

DEDICATION

To my Dad:

Everything that is good about me is because of you.

xo

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degrees Celsius
11β-HSD2	11 beta-hydroxysteroid dehydrogenase 2
1M	1 Molar
AB	Alberta
ABC	Avidin Biotin Complex
APC	Antigen Presenting Cells
AUC	Area Under the Curve
BALF	Bronchoalveolar Lavage Fluid
BCG	Bacillus Calmette-Guérin
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CFIA	Canadian Food Inspection Agency
cm	centimeter
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
Ct	Cycle threshold
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic Acid

DPI	Days Post Inoculation
EDEC	Edema Disease Escherichia coli
ELISA	Enzyme Linked Immunosorbent Assay
FAT	Fluorescent Antibody Test
FMIA	fluorescent microsphere immunoassay
GIT	Gastrointestinal tract
H & E	Hematoxylin and Eosin
HA	Hemagglutinin
HBW	High Birth Weight
HI	Hemagglutination Inhibition
HPA	Hypothalamic-Pituitary-Adrenal
HPI	Hours Post-Infection
IAV	Influenza A virus
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-12	Interleukin 12
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta

IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IM	Intramuscular
IUGR	Intrauterine Growth Restriction
KHz	kilohertz
LAMP	Loop-mediated isothermal amplification
LB	Lysogeny Broth
LBW	Low Birth Weight
M	Matrix
MDCK	Madin-Darby Canine Kidney
MEM	Minimum Essential Media
mg/kg	milligrams per kilogram
MHC	Major Histocompatibility Complex
Mhyo	*Mycoplasma hyopneumoniae*
mL	Milliliter
moi	multiplicity of infection
NA	Neuraminidase
NAD	Nicotinamide Adenine Dinucleotide
NAEU	North American and European
NP	Nucleoprotein
ON	Ontario
ORF	Open Reading Frame

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBS+	Phosphate Buffered Saline with Potassium
PBSA+	Phosphate Buffered Saline with potassium, magnesium and calcium
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus
PCV1	Porcine Circovirus type 1
PCV2	Porcine Circovirus type 2
PDS	Prairie Diagnostic Services, Inc.
Pdx1	Pancreatic and duodenal homeobox 1
PFU	Plaque Forming Units
PI	Post Inoculation
PPAR-γ-C1-α	Peroxisome proliferator activated receptor γ coactivator-1 α
PRDC	Porcine Respiratory Disease Complex
PRRSv	Porcine Respiratory and Reproductive Syndrome virus
QC	Quebec
qPCR	quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
sec	seconds

SGA	Small for Gestational Age
SK	Saskatchewan
SN	Serum Neutralization
TCID50	Median Tissue Culture Infective Dose
TNF-α	Tumor Necrosis Factor alpha
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TSE	Tris/Sucrose/EDTA
TTV	Torque-Teno Virus
TX98	Influenza A/swine/Texas/4299-2/1998 H3N2
USA	United States of America
VIDO	Vaccine and Infectious Disease Organization
w/v	weight per volume
μL	Microliter
μm	Micrometer

1. INTRODUCTION

The thrifty phenotype hypothesis proposed by Hales and Barker in 1992 (Hales and Barker, 1992) opened the field of research into prenatal programming. They showed that the intrauterine environment has a profound effect on the postnatal function of the offspring into adulthood (Hales and Barker, 1992). Birth weight and growth characteristics of the fetus have been shown in the literature to be proxies for the intrauterine milieu in several species such as humans and rodents (Hales and Barker, 1992; Simmons et al., 2001; Stoffers et al., 2003; Landgraf et al., 2007; Meaney et al., 2007; Coe et al., 2008; Mastorci et al., 2009; Liguori et al., 2010; Shen et al., 2010; Zamudio et al., 2010; Parraguez et al., 2013; Pinney, 2013; Xie et al., 2013) and overwhelmingly, low birth weight or what in human medicine is described as "small for gestational age" (SGA) is a signal that the fetus has been exposed to an abnormal process during gestation. Several studies use only birth weight below the 10^{th} percentile for gestational age to determine SGA (Stoknes et al., 2012; Goodman et al., 2013; Kramer et al., 2013; Lohaugen et al., 2013), although other deviations from the mean such as less than the 5^{th} or 2.5^{th} percentile are also used (Chamberlain, 1991). Other definitions include body measurements to determine if an infant is SGA. An abdominal circumference measurement below the 10^{th} percentile is a broad indicator of SGA that could have resulted from myriad factors including normal variations, congenital malformations, and placental bed disease (Chamberlain, 1991). Babies born SGA that have normal head to abdominal circumference ratios are considered to be at the lower end of biological variation, whereas those born SGA due to placental insufficiency will shunt blood flow to the brain resulting in conserved head growth at the expense of the body

(Chamberlain, 1991). SGA is considered a proxy for intrauterine growth restriction (IUGR) (Wu et al., 2006; Stoknes et al, 2012; Lohaugen et al., 2013), which is defined by asymmetrical growth with the occurrence of relative brain sparing (Ladinig et al., 2014a). It is important to highlight that intrauterine growth restriction encompasses more than just the number on a scale; rather it is a result of processes that prevent the fetus from reaching its genetic growth potential. The prenatal programming hypothesis suggests that the fetus will adjust to the predicted postnatal environment by changing its metabolic or stress response phenotype based on hormonal or nutritional signals received transplacentally from the mother (Pluess and Belsky, 2011; Sandman et al., 2011.) A large proportion of the literature has focused on the effect of birth weight on postnatal metabolism, though there has been work investigating the effect of birth weight on the immune system. The investigation into the effect on the immune system has delved into the function of individual parts of the innate and adaptive systems in both humans and other animals and has also uncovered variations in response to vaccination between low and normal birth weight humans (Neumann et al., 1998; McDade et al., 2001; Moore et al., 2004; Landgraf et al., 2007). The transgenerational effect of low birth weight has also been investigated. SGA females are more likely to give birth to low birth weight offspring in the subsequent generation in both rat (Jimenez-Chillaron et al., 2009) and human (Klebanoff et al., 1987; Liguori et al., 2010; Pinney, 2013) studies. In the research presented herein, piglets of average weight within their litter were chosen from high and low birth weight litters to ensure that the overall intrauterine milieu was represented, rather than choosing the smallest or largest pigs overall, which may have represented a unique local variation in that individual. Individual and litter birth weight can affect

postnatal performance. Studies have shown that even average birth weight pigs selected from low birth weight litters carry the same negative phenotypic traits associated with IUGR as their lower birth weight litter mates (Pardo et al., 2013). By selecting litters at the extremes of litter average birth weight, this study adopted the strategy used in previous studies in which these differences in average litter birth weight has been associated with clear “brain sparing” effects (Smit et al., 2013).

This work is the first to investigate the effect of low litter birth weight on the clinical disease expression of Influenza A virus (IAV) in pigs. The goal of this work was to better elucidate how the intrauterine environment provided by the sow could affect the clinical disease expression of the individual piglet following experimental disease challenge. Based on the evidence provided by the literature (Neumann et al., 1998; McDade et al., 2001; Moore et al., 2004; Landgraf et al., 2007), the expectation was that average birth weight pigs from low birth weight litters would have more severe clinical disease expression compared to average pigs from high birth weight litters. It was then proposed that the results of this investigation could be used to provide recommendations for more rigorous record keeping to assess birth weight when choosing replacement breeding stock to help improve herd health if the hypothesis was confirmed.

IAV was chosen as the exemplifier disease for several reasons. Firstly, IAV is a common cause of respiratory disease in swine. It can manifest on its own or as part of the Porcine Respiratory Disease Complex (PRDC). In either case, IAV has the potential to result in financial loss to swine producers. Secondly, IAV can be obtained, propagated and utilized with relative ease since it is ubiquitous and many strains can be manipulated within a biosafety level 2 (BSL2) laboratory setting. Thirdly, the clinical course of

disease with IAV is relatively short. This allows for a rapid turnaround time and reduced need for simultaneous use of multiple animal care rooms.

This thesis contains seven main chapters. The literature review first focuses on the relevant literature regarding the effects of low birth weight in humans and animals and possible mechanisms of these effects; and then provides an overview of diagnostic methods for IAV; adaptive immunity and the importance of cytokines in IAV infection. Since IAV often occurs concurrently with other pathogens, it is important to be able to rule out other pathogens or at least understand the status of concurrent infections in order to have a more complete picture of concurrent challenges to the immune system. This chapter, therefore, also includes brief summaries of diagnostic methods for *Mycoplasma hyopneumoniae* (Mhyo), Porcine Respiratory and Reproductive Syndrome virus (PRRSV), Porcine Circovirus Type 2 (PCV2), and Torque Teno virus (TTV). Chapter Three outlines the hypothesis and objectives of the thesis. Chapter Four presents the pilot trials that were undertaken to determine the best experimental design for the main trial. The full manuscript of the main trial is presented in Chapter Five. Chapter Six describes the challenges encountered in performing the research and finally, Chapter Seven is the general discussion, which outlines the overall conclusions reached.

2. LITERATURE REVIEW

2.1 General Introduction

Prenatal programming implies that the *in utero* environment can alter the structure and function of a mammal. Intrauterine growth restriction (IUGR) refers to a condition in which a neonate does not meet its growth potential at birth, usually due to abnormal processes during gestation. This growth restriction has been found, in humans and animals, to affect various physiological functions into adulthood (Hales and Barker, 1992; Wu et al., 2006). The effects of IUGR on carbohydrate metabolism have been investigated and many effects have been elucidated. The potential effect on other physiological processes is an area of increasing research interest. Various studies have delved into the effect of IUGR on immune system function; however, this research is the first to investigate the effect of IUGR on the clinical disease expression of Influenza A virus (IAV) in pigs. Most research has focused on and found that IUGR results in a detriment to the function of an animal later in life and the scope of this review is from that perspective.

IAV is a genus within the family of Orthomyxoviridae viruses that can cause disease in birds and mammals. In pigs, IAV is a common contributor to porcine respiratory disease complex (PRDC) (Opriessnig et al., 2011), which can cause production and financial losses for the swine industry every year. In addition, IAV poses a zoonotic risk as the transmission between pigs and people has been well documented (Wentworth et al., 1997; Alavanja et al., 2007; Ma et al., 2009; Terebuh et al., 2010). Multiple methods of diagnosis exist for IAV, each with various strengths and weaknesses. However, since influenza can exist simultaneously with other respiratory

pathogens, the diagnosis of IAV should also include diagnostic tests for other common respiratory pathogens of pigs. The pathogenesis of IAV has been well characterized and is intimately involved with the immune response.

In this review, the relevant literature on IUGR is reviewed for both human and animal models and includes evidence showing the effect of IUGR on carbohydrate metabolism and disease. Possible mechanisms of epigenetic modifications are discussed. An investigation of the literature for clinical and pathological methods of IAV diagnosis is presented, and cytokine function in the immune response is reviewed, especially the proinflammatory cytokines. Adaptive immunity to IAV is discussed to give insight into how the immune system affects pathogenesis and finally a brief review of diagnostics for other porcine respiratory pathogens is discussed.

2.2 Intrauterine Growth Restriction (IUGR)

2.2.1 Introduction

In humans, low birth weight can be used to describe babies that are either full or pre-term. The phrase “small for gestational age” (SGA) was used to differentiate those that were born full term but in the bottom tenth percentile of birth weights. IUGR refers to a fetus that has not met its genetic growth potential, indicated by asymmetric growth, with brain mass conserved at the expense of other fetal organs, and is considered a result of an abnormal process, such as placental insufficiency. An IUGR fetus is usually born SGA but this is not necessarily so (Lim and Ferguson-Smith, 2010). In livestock production swine, ovine, equine, and bovine neonates born small due to IUGR are at higher risk of intestinal, circulatory, neurological, and respiratory dysfunction (Wu, et al., 2006). The majority of the relevant literature on IUGR is centered on the causes and

effects on humans and in rodent models, although work has also been done using livestock models.

2.2.2 Human studies

The “thrifty phenotype” hypothesis initially described by Hales and Barker (1992) proposed that if the human fetus is exposed to a nutritionally restrictive intrauterine environment, this programs the fetus to thrive in the same type of postnatal environment. The subsequent metabolic adaptations actually prove maladaptive if the neonate encounters a less restrictive postnatal environment (Seckl et al., 2000; Singhal and Lucas, 2004; Gicquel et al., 2007; Gluckman et al., 2011; Harris et al., 2011; Pluess and Belsky, 2011). This less nutrient restrictive environment can result in compensatory growth, which may lead to the growth restricted infant reaching a similar or higher body weight than a non-restricted infant at a specific time point.

2.2.2.1 Metabolic consequences

Human infants born SGA as a result of IUGR have a higher risk for developing metabolic syndrome, a cluster of hypertension, insulin resistance, and hypercholesterolemia. These can lead to type II diabetes and other cardiovascular and metabolic disorders later in life which is independent of external factors such as smoking or obesity (Hales and Barker, 2001; Dunger and Ong, 2005; Fowden et al., 2005; Meaney et al., 2007; Waterland and Michels, 2007; Harris et al., 2011; Woroniecki et al., 2011) and remains valid over the spectrum of birth weights (Dunger and Ong, 2005; Gicquel et al., 2007; Meaney et al., 2007; Gluckman et al., 2011; Harris et al., 2011). Beltrand et al., (2009) showed that fat mass restoration was related to compensatory growth in humans. This study also showed that infants with fetal growth restriction had low cord blood

leptin levels at birth, but venous leptin levels were higher than in infants without fetal growth restriction at one year of age, even though the fat mass between the two groups was not different. In fact, the leptin levels at one year of age were positively correlated with change in body mass index (BMI) between birth and 4 months of age and negatively correlated with growth velocity between birth and one year. The compensatory growth in this study was proportional to height and did not result in a large BMI, which indicates that fat mass at birth and throughout compensatory growth, as well as increased leptin levels seen at one year of age, may be more important influencers on later metabolic perturbances (Beltrand et al., 2009).

While IUGR most apparently affects body mass, organ growth and development can also be affected. Humans with IUGR as a result of prenatal exposure to excess glucocorticoids were shown to have decreased numbers of nephrons (Gicquel et al., 2007; Meaney et al., 2007; Woroniecki et al., 2011). IUGR caused by nutrient restriction in humans and non-human primates is associated with down regulation of the insulin-like growth factor (IGF) system, possibly due to increased cortisol, which could lead to future impairment of brain function and growth (Meaney et al., 2007; Xie et al., 2013).

2.2.2.2 Causes of IUGR

Insufficient blood flow to the placenta is one of the most common reasons for IUGR in human pregnancies (Stoffers et al., 2003; Pinney, 2013). IUGR and low birth weight can result from fetal hypoxia due to placental insufficiency. Fetal hypoxia is seen in high altitude pregnancies (>2500m above sea level); however it is difficult to separate the effect of hypoxia in human pregnancies from malnutrition due to the frequently concurrent substandard economic conditions of high altitude regions such as the Andean

plateau of Chile (Parraguez et al., 2013), although others dispute this conclusion (Zamudio et al., 2010). In their study of high altitude human pregnancies, Zamudio et al (2010) provide evidence that the decreased birth weights seen are not due to a lack of fetal oxidative metabolism, but rather to a decline in fetal glucose availability and metabolism due to escalated anaerobic metabolism by the placenta. Ovine models show IUGR as a result of fetal hypoxia and can be replicated at high altitude; the resultant damage due to oxidative stress can be averted by antioxidant administration possibly by increasing placental sex-steroidogenesis (Parraguez et al., 2013).

During pregnancy, the placenta has many important functions to support the healthy growth and development of the fetus. One of these functions is to act as a gateway between the maternal and fetal blood flows to ensure adequate nutrition and prevent harmful substances from crossing into the fetal circulation. One example of this is the enzyme 11 beta-hydroxysteroid dehydrogenase 2 (11β-HSD2) in the placenta that serves to limit fetal exposure to maternal glucocorticoids and their detrimental effects on the fetus. A deficiency of 11β-HSD2 has been shown to impair fetal growth in humans (Meaney et al., 2007).

Prenatal overexposure to maternal glucocorticoids, which can be induced by stress, has the ability to re-program the hypothalamic-pituitary-adrenal (HPA) axis of the fetus (Seckl et al., 2000; Sandman et al., 2011). This can lead to higher glucocorticoid levels in the offspring, which in turn can lead to changes in adiposity, cardio-vascular effects, affective disorders, immunosuppression, hyperglycemia and hypertension, among others (Seckl et al., 2000; Gicquel et al., 2007; Meaney et al., 2007; Pitale and Sahasrabuddhe, 2008; Harris et al., 2011). HPA activity can predict birth weight as

glucocorticoid administration will change fetal gene expression to enhanced reactivity of the cardiovascular system, enhanced storage and production of energy substrates and reduces fetal growth, especially if administered during the high growth seen during late gestation (Meaney et al., 2007). Stressors during pregnancy that result in low birth weight for humans are also implicated in alterations in feeding behavior of the offspring with preferences skewed towards high carbohydrate and/or fat diets (Portella et al., 2012). As well, prenatal stressors that result in lower birth weight humans can also predict infant physiological stress reactions; therefore, lower birth weight can function as a marker for exposure to an adverse prenatal environment (Pluess and Belsky, 2011). Furthermore, prenatal stress will prime the HPA axis to produce an increased response to stress later in life; stressful events in humans are known to contribute to cardiovascular events (Meaney et al., 2007; Pitale and Sahasrabuddhe, 2008; Sandman et al., 2011).

2.2.2.3 Transgenerational effects and Epigenetics

Multiple studies show that epigenetic programming *in utero* can be transmitted to subsequent generations (Waterland and Michels, 2007; Kirchner et al., 2013). Women exposed to the Dutch Hunger Winter during pregnancy had daughters of low birth weight, who then subsequently gave birth to another generation of low birth weight daughters (Liguori et al., 2010; Pinney, 2013). An expansive study in Tennessee showed that women that were born SGA were at higher risk for giving birth to a SGA infant (Klebanoff et al., 1987).

Epigenetic modification is postulated to contribute to premature advancement of the aging process in IUGR human infants and thus influence fetal origin of adult disease based on epigenetic dysregulation seen in specific loci from cord blood sourced stem

cells (Einstein et al., 2010). Indeed, loci have been reported that are linked to both adult disorders and birth weight (Seckl et al., 2000). Lending further credence to the premature aging hypothesis, human placentas from pregnancies resulting in IUGR have been found to have shorter telomeres than controls; telomeres are known to shorten in response to oxidative stress and cellular senescence (Pitale and Sahasrabuddhe, 2008; Biron-Shental et al., 2010). Dysregulation of the epigenome is more likely to occur and be propagated as disruption in stem cell regenerative capacity or cell function during times of rapid cell growth, such as during fetal development and thus increase susceptibility to age-related disease such as type 2 diabetes (Einstein et al., 2010). Evidence of epigenetic modification of the fetal HPA axis has also been shown in humans, in whom maternal depression and anxiety leads to increased cortisol levels in the infants at three months of age and hypermethylation at a specific site in the genome (Pluess and Belsky, 2011).

In humans, mothers exposed to famine around the time of conception had offspring with hypomethylation of the IGF-II gene that was still evident after 60 years; hypomethylation of this maternally imprinted gene could lead to bi-allelic expression, although this is yet to be determined (Liguori et al., 2010; Gluckman et al., 2011; Patti, 2013). Peroxisome proliferator activated receptor γ coactivator-1 α (PPAR-γ-C1-α) is a transcriptional co- activator important in the regulation of mitochondrial genes; mitochondrial function is a determinant of insulin secretion in pancreatic islet cells. Human patients with type II diabetes have increased DNA methylation on this gene's promoter, leading to decreased expression in muscle and pancreatic islet cells (Liguori et al., 2010; Gluckman et al., 2011). Young, healthy men that were born in the bottom tenth percentile for birth weight showed persistent hypermethylation of the PPAR-γ-C1-α gene

when fed both control and high fat diets compared to their normal birth weight counterparts who only when fed the high fat diet (Liguori et al., 2010; Gluckman et al., 2011). The embryonic genome is almost completely demethylated around the time of fertilization and becomes remethylated just after implantation, possibly shaping one critical period in which environmental changes can alter the epigenome (Gicquel et al., 2007; Waterland and Michels, 2007).

2.2.3 Animal studies

2.2.3.1 Animal models of intrauterine growth restriction

Various experimental strategies have been employed to alter the uterine environment in order to study the effects of IUGR. Maternal nutrient deprivation during gestation has been used as a non-surgical model of inducing IUGR in animals. Gilts fed a protein-restricted diet containing 0.5% protein during gestation leads to offspring with lower birth weight and higher relative brain weights than offspring from control or energy restricted gilts (Atinmo et al., 1974). In 4^{th} parity sows whose feed was supplemented with 100mg of L-carnitine from day 1 to 57 of gestation, total litter and individual fetal weights were unchanged at 57 days of gestation, whereas litter sizes were larger compared to unsupplemented sows (Waylan et al., 2005) Fetal crown-to-rump length was also similar between the two groups indicating that increased nutrient availability may help to prevent IUGR in large litters (Waylan et al., 2005). In rats (Desai et al., 2007; Landgraf et al., 2007; Landgraf et al., 2008; Reamon-Buettner et al., 2014) and mice (Jimenez-Chillaron et al., 2009), restricting feed intake to 50% of *ad libitum* during gestation leads to lower birth weight in offspring compared to control dams. Nutritional models of IUGR allow the study of IUGR effects without altering litter size.

Comparing normal pregnancies to those in which the litter size has been artificially reduced can help indirectly elucidate the effects of IUGR due to relative uterine crowding. However, since litter sizes are reduced in this model, it is technically seen as a model of uterine spaciousness unless the control sows have exceptionally high ovulation and fertilization rates, in which case "crowded" and "spacious" uterine environments can be directly compared. In pigs, the ovulation rate available for fertilization can be reduced by unilateral oviduct ligation. Oviduct ligation prevents the oocytes ovulated from the ligated side from entering the uterus where they could be fertilized. Post-implantation, the number of conceptuses is matched to the functional uterine capacity. At 30 days of gestation, this results in lower total numbers of embryos present; higher embryonic survival rate; heavier placentas and lower placental efficiency in ligated sows when compared to control sows. At 90 days of gestation, the numbers of fetuses remains lower; fetal survival rate and placental weight remain higher; fetuses are heavier with lower brain:liver weight ratios and there is no difference in placental efficiency in ligated sows when compared to non-ligated controls (Town et al., 2004). Similar relative uterine crowding results have been achieved with laparoscopic unilateral oviduct cauterization model instead of ligation (Dhakal et al., 2013). In this study, offspring from cauterized sows were heavier at birth and 21 days of age, and had higher average daily gains from birth to 7 weeks of age than offspring from non-cauterized control sows.

In rats, uterine horn crowding can be achieved by hemiovariectomy. In this model, mice are hemiovariectomized prior to breeding, which leads to compensatory ovulation from the remaining ovary. This leads to uterine horn crowding in rats, since

each uterine horn has its own cervix and are therefore independent in this species, and exaggerates the naturally occurring differences in placental blood flow based on the location of the fetus within the uterine horn (Coe et al., 2008). Similarly in pigs, gilts subjected to unilateral ovariectomy-hysterectomy prior to breeding displayed uterine horn crowding due to compensatory ovulation from the contralateral ovary. Prior to 35 days of gestation, conceptus survival and growth were not different between the two groups, however after day 35, the number of live fetuses, percent fetal survival to day 100 and fetal growth were significantly lower in hemiovariectomized-hysterectomized gilts compared to control gilts. Placental length and weight were higher at all stages of gestation in control gilts compared to ovariectomized-hysterectomized and is likely due to the increased surface area of the endometrium available for placentation (Knight et al., 1977). Uteroplacental insufficiency can also be replicated in animal models by uterine artery ligation (Simmons et al., 2001; Stoffers et al., 2003; Puglianiello et al., 2007). Late gestational bilateral uterine artery ligation in rats leads to IUGR by reducing the availability of substrates to the fetus (Simmons et al., 2001). In sheep, placental function and growth can be restricted by surgically removing all but 4 caruncles from each uterine horn prior to mating, which leads to a 20% reduction in birth weight compared to non-surgically altered ewes (Woolridge et al., 2014). Surgical models reduce the need to restrict nutrients or feed intake during animal studies and methods employing laparoscopic techniques can be minimally invasive.

IUGR naturally occurs in litter bearing species where ovulation rates and uterine capacity are limiting factors for litter size. This is evident in swine where runt pigs in a normal weight litter have abnormally low birth weights (usually $\leq$ 700g) and tend to have

slower growth rates than their littermates. This lower growth rate is not explained simply by lower birth weights (Foxcroft et al., 2006). Pigs born per sow per year is an important metric for the evaluation of the financial and reproductive health of contemporary commercial swine production. Selection for litter size in replacement breeding females is commonly used to increase overall productivity. However, depending on the interaction among the component traits (ovulation rate, embryonic and fetal survival, and uterine capacity) that determine litter size born, this selection for increased litter size can disproportionately increase the number of surviving conceptuses in early gestation, leading to uterine crowding and IUGR (Foxcroft et al., 2006). In both an experimental (Town et al., 2004) and commercial context (Smit et al., 2013), the crowding that occurred early in gestation due to increased numbers of conceptuses by day 30 of gestation is not compensated for in the fetuses that survive until term, and each pig born in the litter will display IUGR (Foxcroft et al., 2009).

2.2.3.2 Metabolic consequences

2.2.3.2.1 Glucose metabolism

In the bilateral uterine artery ligation model, birth weights were lower in the ligated group compared to the sham surgical group. The growth rate of IUGR pups exceeded that of controls between seven and ten weeks of age and resulted in obesity by 26 weeks (Simmons et al., 2001). Coe et al., achieved similar results using the hemiovariectomy crowded uterine horn model (2008). The uterine artery ligation model also showed that, IUGR pups had inappropriate responses to both exogenous glucose and insulin administration. As the pups aged, they stayed hyperglycemic after exogenous glucose administration and had minimal to no endogenous insulin secretion in response to

this stimulus at 26 weeks. As early as one week of age in these pups, exogenous insulin administration had little to no effect on glucose concentrations, indicating severe insulin resistance (Simmons et al., 2001; Stoffers et al., 2003). Morphometric analysis of the pancreas showed that β-cell mass present in the pancreas of IUGR pups was one-third that of normal controls (Simmons et al, 2001). These findings are phenotypically very similar to type II diabetes in humans and thus this experimental model is important for predicting the effects of IUGR in both animals and humans (Simmons et al., 2001; Stoffers et al., 2003).

Other rodent models also show that IUGR leads to alterations in carbohydrate metabolism later in life that are worsened by compensatory growth due to postnatal nutrient excess (Seckl et al., 2000; Cottrell and Ozanne, 2008; Patti, 2013). It also appears that the timing of this compensatory growth is important, since if the nutrient restriction continues through lactation and compensatory growth is not seen until later in life, the offspring appear metabolically normal; this is also seen when nutrient restriction is due to large litter size (Desai et al., 2007; Cottrell and Ozanne, 2008). As well, the type of nutrient supplementation may contribute to whether or not compensatory growth is exhibited. Evidence from a pig model showed that piglets with a birth weight 30 percent lower than the average birth weight of the herd exhibited a higher relative growth rate than average birth weight piglets through the lactational period when fed either a high or adequate protein milk replacer; this effect on growth rate did not extend into the post-weaning phase (Morise et al., 2011). As well, lipid and protein composition of the carcasses were not different at 70 days of age. This shows that protein supplementation in

the suckling period may not contribute extensively to compensatory growth or body composition later in life.

2.2.3.2.2 Lipid metabolism

Uteroplacental insufficiency models have been used to assess the effect of IUGR on hypothalamic lipid sensing in rats. Puglianiello et al., (2007) showed that in a late gestation bilateral uterine artery ligation model, the level of the brain isoform of carnitine palmitoyletransferase 1 was much lower at birth in IUGR neonates. This enzyme is a regulator for long chain fatty acid entry into mitochondria where they undergo β-oxidation. This rate of oxidation acts as a signal of nutrient availability to the hypothalamus, thus IUGR rats at birth have impaired lipid-sensing capability (Puglianiello et al., 2007).

The interaction between fetal exposure to maternal nutrient restriction and leptin treatment can program transcriptional responses in the liver for many genes, including those involved in steroid synthesis, carbohydrate metabolism, complement cascade, innate immunity (up regulated), major histocompatibility complex (MHC) antigens and lymphocyte activation (down regulated), and are at least as important as post weaning diet in rats (Ellis et al., 2014). The endogenous hormone leptin in adults acts as a satiety signal to indicate that the body has adequate energy stores. Low blood leptin can induce hunger and fat storage, whereas high leptin levels can help suppress hunger and increase energy expenditure (Cottrell and Ozanne, 2008). In pigs, average and small birth weight piglets fed either a high protein or adequate protein milk replacer exclusively up to 28 days of age, plasma leptin levels at 70 days of age were higher in both piglets that were fed the high protein milk replacer and in small birth weight piglets (Morise et al., 2011).

Both total body fat and plasma leptin concentration are increased by a high fat post weaning diet, by fetal nutrient restriction and by postnatal leptin treatment individually: however, when rat fetuses that were exposed to maternal nutrient restriction were treated with leptin, both total body fat and plasma leptin concentration decreased. It was, therefore, hypothesized that any of these factors induce a phenotypic switch to the "thrifty liver phenotype" on their own, but not when fetal exposure to maternal nutrient restriction and postnatal leptin treatment are seen together (Meaney et al., 2007; Gluckman et al., 2011; Ellis et al., 2014). This thrifty liver phenotype seems to favor carbohydrate metabolism at the expense of immunity and may be due to growth restriction seen prenatally when the dam is nutrient restricted, or postnatally with leptin administration (Ellis et al., 2014). Circulating leptin levels normally surge in early development and then drops in accord with satiety and fat stores (Galjaard et al., 2013; Ellis et al., 2014). Early postnatal nutrient restriction will prevent this leptin surge, which will rebound if nutritional levels are restored. Ellis et al. (2014) hypothesized that this surge and drop determines the metabolic set point: Therefore, if the surge occurs at a normal or high body weight, the homeostatic set point will be relatively normal, whereas a low body weight at the time of the surge will set a lower calibration. Low body weight neonates that exhibit compensatory growth could preferentially deposit fat and thus the body composition at the time of leptin surge could be much higher, which could lead to metabolic syndrome.

2.2.3.3 Maternal Stress and Glucocorticoids

IUGR has been shown to have an effect on the growth and function of fetal organs. Various mechanisms are likely at work, since proteomic studies show differences

in the proteins expressed amongst different organs of IUGR rats. Rats exposed to a 6% isocaloric diet compared to control diets containing 22% protein during all of gestation had IUGR progeny whose kidneys showed thinner cortices and thicker areas of nephrogenesis. There was decreased expression of proteins involved in cell signaling, cell proliferation, apoptosis and metabolism in these rats, which indicates these processes may be involved in the abnormal nephrogenesis seen in IUGR (Shen et al., 2010). Studies in rats show that IUGR fetal kidneys are smaller and contain fewer glomeruli and are more likely to have proteinuria and decreased glomerular filtration long term (Shen et al., 2010). IUGR rodent fetuses, and often those exposed to prenatal glucocorticoid excess, also show decreased numbers of nephrons (Meaney et al., 2007). The placental enzyme 11β-HSD2 inactivates a large portion of maternal glucocorticoids, ensuring that fetal levels are mainly sourced from the fetal adrenal, and maternal protein malnutrition in the rat model blunts these effects of 11β-HSD2 (Seckl et al., 2000; Fowden et al., 2005; Gicquel et al., 2007; Meaney et al., 2007). A deficiency of 11β-HSD2 will impair fetal growth in rats, and these rats also show increased anxiety responses later in life (Meaney et al., 2007). Stressors during pregnancy that result in low birth weight in rat models are also implicated in alterations in feeding behavior of the offspring, with preferences skewed towards high carbohydrate and/or fat diets (Portella et al., 2012). Early life stress has also been shown to cause epigenetic changes in gene expression that can lead to persistent hyperactivity of the HPA axis (Meaney et al., 2007; Harris et al., 2011; Patti, 2013). Furthermore, this effect is transgenerational in rats up to the third generation past the insult (Harris, et al., 2011).

2.2.3.4 Transgenerational effects and epigenetics

Epigenetic regulation results in changes in gene expression without changing DNA sequences (Gicquel et al., 2007; Waterland and Michels, 2007; Gluckman et al., 2011; Woroniecki et al., 2011; Galjaard et al., 2013; Kirchner et al., 2013; Reamon-Buettner et al., 2014). This locally regulated gene silencing or transcription can occur throughout the genome or as tissue specific phenotypic adaptations (Waterland and Michels, 2007; Kirchner et al., 2013). Gene expression is altered in instances of IUGR, which leads to changes in structure and function of organs such as the pancreas or liver (Stoffers et al., 2003). The effect the paternal environment has on his progeny highlights the epigenetic transmission of traits across generations. Nutrient restriction in the male mouse before breeding can lead to glucose and body weight alterations of the offspring, even if the dam maintains a normal nutritional status (Patti, 2013). These alterations in metabolism can be seen if the male was exposed to nutrient restriction during their intrauterine development, even if nutrition around the time of breeding is normal (Patti, 2013). Multiple rodent models show that epigenetic programming *in utero* is transmitted to subsequent generations (Waterland and Michels, 2007; Kirchner et al., 2013).

In rats, alterations in glucose homeostasis were seen in first, second or third generation progeny of animals exposed to restricted diets during pregnancy (Gicquel et al., 2007; Liguori et al., 2010; Patti, 2013). Interestingly, glucose intolerance, obesity and reduced birth weight were found in first and second generation offspring in a mouse model of under nutrition, even when nutrition was not restricted during the subsequent pregnancies and aspects of these phenotypes were transmitted maternally, paternally or both (Jimenez-Chillaron et al., 2009). Epigenetic mechanisms are heritable by mitosis

and likely also by meiosis (Waterland and Michels, 2007; Woroniecki et al., 2011). The transgenerational effects of various maternal exposures is likely due to direct effects on the developing germ line of her progeny, which will then exert effects on the F2 progeny, even in the absence of further environmental exposures (Pitale and Sahasrabuddhe, 2008; Patti, 2013).

A rodent model assessed epigenetic modifications of pancreatic and duodenal homeobox 1 (Pdx1), which is a transcription factor necessary for beta cell function and development, and found that mRNA expression of Pdx decreased in IUGR neonatal and adult rats, likely due to decreased transcription from oxidative stress induced by uteroplacental insufficiency (Stoffers et al., 2003; Park et al., 2008; Schwitzgebel et al., 2009; Gluckman et al., 2011). Mitochondrial function and production of nicotinamide adenine dinucleotide hydride (NADH) is disabled by even a moderate decrease in Pdx expression, leading to impaired glucose stimulated insulin release; furthermore, IUGR rodents have been shown to have abnormal mitochondrial function in pancreatic islet cells (Stoffers et al., 2003).

One mechanism of epigenetic regulation is DNA methylation, specifically of cytosine at the 5-carbon position (Woroniecki et al., 2011; Kirchner et al., 2013; Reamon-Buettner et al., 2014). This effectively silences DNA sequences that are not to be transcribed (Gicquel et al., 2007; Kirchner et al., 2013), which is seen in normal silencing of imprinted genes, such as those maternally or paternally inherited or in X chromosome inactivation (Waterland and Michels, 2007; Woroniecki et al., 2011). In pigs, sows that were fed restricted amounts of feed in the last week of lactation had less viable female embryos at 28 days of pregnancy than their un-restricted counterparts

(Vinsky et al., 2006) and a follow-up study determined that in similarly restricted sows, Insulin-like growth factor receptor expression variance was lower in female embryos and X-chromosome specific transcript expression variance decreased in the male embryos of these sows (Vinsky et al., 2007). This suggested that female embryonic loss before day 30 of gestation may be due to maternally inherited epigenetic effects (Vinsky et al., 2007).

Various rodent studies show that IUGR induced by mechanisms such as maternal protein restriction has an effect on methylation patterns and subsequent gene expression in a multitude of genes in both the placenta and the fetus, including those that help regulate fetal growth (Pinney, 2013; Reamon-Buettner et al., 2014). There appear to be tissue specific differences in the timing of methylation; in rats, methylation of the glucocorticoid receptor promoter regions in the hippocampus is not completed until day 6 of postnatal life (Waterland and Michels, 2007).

2.2.3.5 Embryonic Development of Swine

After fertilization the zygote undergoes a series of divisions before entering the uterus at the morula stage (Marrable, 1971). By 12 days post-fertilization, the embryo is a flat disc but has undergone lamination and the plan of the three germ layers has already begun to outline the future pig (Marrable, 1971). Implantation of the embryo begins at 13 to 14 days and is complete by 40 days (Althouse, 2005). Fourteen to sixteen days after fertilization, the primitive brain has already begun to form from an enlargement of the neural tube and the heart is beating by 15-16 days (Marrable, 1971). By 36 days, the structural differentiation of the main body systems is complete and skeletal mineralization has begun (Marrable, 1971; Althouse 2005). After this time, the

developing organs continue to grow, differentiate and mature in preparation for birth at 115 days of gestation.

The weight of the gastrointestinal tract (GIT) increases linearly with fetal weight but accelerates after day 56 of gestation. The GIT is not necessary to process nutrients during fetal life, however the growth acceleration may be in anticipation of this need postnatally (McPherson-McCassidy 2003). Similarly, liver growth decelerates after day 63 (McPherson-McCassidy, 2003) perhaps since the erythropoeitic activity of the liver is important specifically in early gestation, beginning at 28 days (Kruml et al., 1970; Dyce, 2002). The spleen begins to form at 22 days of gestation (Rothkotter et al., 2002) and extramedullary hematopoeisis becomes evident in the spleen by 51 days (Kruml et al., 1970). Primary and secondary muscle fibers form at different times during gestation in the pig. Primary fibers are formed by day 38 and serve as scaffolds for secondary fibers, which form around day 54 and are complete by day 90 (Wigmore and Stickland, 1983). Increased feed intake in sows during early gestation before secondary fiber hyperplasia has been shown to cause increased numbers of secondary, but not primary fibres compared to sows with increased feed intake in mid or late gestation (Dwyer et al., 1994).

Genesis of the developing immune system, including the spleen and thymus, occurs in early gestation. The thymus begins forming at day 21 of gestation (Rothkotter et al., 2002) and pro-T lymphocytes are appreciable in the thymic rudiment by 38 days (Sinkora et al., 2000). The thymus is fully differentiated by day 77 (Kruml et al., 1970). Different immune cell populations become evident during different days of gestation as well. The liver contains evidence of cells able to secrete interferon-alpha (IFN-α) by day 26 (Splichal et al., 1994). Lymphocytes are seldom observed peripherally until

hematopoeisis begins in the bone marrow around 45 days of gestation (Sinkora et al., 2000; Sinkora and Butler, 2009), however CD3+ lymphocytes are detectable by day 30 (Rothkotter et al., 2002). B-lymphocytes are appreciable in the periphery by day 40 (Sinkora et al, 1998; Rothkotter et al., 2002), and cells that can secrete immunoglobulin are present in spleen and liver by day 50 (Rothkotter et al., 2002). The natural killer cell population stabilizes at 70 days of gestation (Sinkora and Butler, 2009) and the fetus is considered immune competent by this time (Althouse, 2005). Due to these described differences in the ontogenetic timing of various organs and systems it follows that if the timing of prenatal insults that lead to IUGR occur before or during these critical windows of development, there could be negative effects on the developing organ system.

2.2.3.6 IUGR, Immune and Inflammatory Responses, and Infectious Disease

The research linking IUGR with infectious disease is scarce when compared to the literature concerning IUGR and metabolic consequences. This section describes the human and animal studies investigating this link, and includes discussions on asthma and allergic responses, as these conditions are relevant to respiratory disease in humans.

A longitudinal study of human infants in Kenya showed that smaller IUGR infants (birth weight less than or equal to 2.5 kg) had significantly lower white blood cell counts, which persisted in a small percentage of these children until 1 year of age (Neumann et al., 1998). Mean T lymphocyte counts were also lower in IUGR infants in this study, especially among those with birth weights between 1.5 and 2.5 kg. These differences were exacerbated by the duration of IUGR, with the lowest T lymphocyte counts observed in those offspring exposed to long term under nutrition *in utero* compared to those exposed later in pregnancy. In this study, IUGR infants were split into

three groups with defined levels of IUGR severity. Severe IUGR was defined as birth weight less than or equal to 2500 g (less than 3rd percentile) and moderate IUGR was defined as birth weight between 2501 and 2799 g (3rd to 10th percentile). All infants had no clinical abnormalities otherwise. A group of full-term control infants with birth weight greater than or equal to 2800 g was also included. Low T lymphocyte counts at 6 or 12 months of age was predicted better by degree of IUGR than whether or not catch-up growth was exhibited. When these children were intradermally administered a tuberculin purified protein derivative 6 months after the Bacillus Calmette-Guérin (BCG) vaccination at birth, IUGR infants of 2.5 kg birth weight or less showed the lowest intensity of delayed-type hypersensitivity reaction and highest percentage of negative reactors compared to infants of higher birth weight. Additionally, infants with birth weight less than or equal to 2.5 kg had the highest illness rates in the 0-3 month and 7-10 month age range, with male infants having higher rates than females (Neumann et al., 1998). These results mirrored the decreased T lymphocyte counts found in this group of infants.

Studies in Pakistan assessed the response to vaccination in adults that were born SGA or normal for gestational age. These studies found that low birth weight was associated with decreased response to vaccination in adulthood or adolescence, independent of other variables that could affect response, when vaccinated with a *Salmonella typhi* vaccine, but not when vaccinated for rabies suggesting that non-T lymphocyte dependent mechanisms of serum antibody generation are affected in the immune systems of adults born SGA (McDade et al., 2001; Moore et al., 2004). Further evidence shows that low birth weight due to malnutrition of the mother during pregnancy

is associated with altered lung function and chronic lung disease in the offspring (Landgraf et al., 2008).

Studies using animal models of birth weight have shown similar results on the number and function of immune cells. Rats fed 50% of the normal *ad libitum* feed intake during pregnancy were shown to have progeny with lower birth weights than their normally nourished counterparts. The male pups from this study were shown to have lower total leukocyte and bone marrow cell counts as well as reduced leukocyte migration at 8-9 weeks of age. The mechanisms for this could be related to the observed decreased expression of L-selectin, decreased type IV collagen in the basal membrane and decreased leukotriene B4 after an inflammatory stimulus in these IUGR rats (Landgraf et al., 2007). A rat model of low birth weight due to maternal malnutrition showed a reduction in allergic lung inflammation, as evidenced by decreased mucus secretion, decreased leukotriene and immunoglobulin E (IgE) production and infiltration of inflammatory cells into the airways that was highly dependent on the age at which the low birth weight offspring were challenged (Landgraf et al., 2008). In snatch-farrowed pigs, IUGR piglets had decreased circulating cytokine concentrations and lymphocyte proliferation at 7 days of age compared to normal birth weight piglets indicating impaired immune function in IUGR piglets (Zhong et al., 2012).

Several studies have found correlations between low birth weight and/or catch-up growth and asthma or decreased lung function (Tedner et al., 2012; Mu et al., 2014). Low birth weight children were also more likely to have respiratory illnesses in adulthood that required hospitalization, as compared to their normal birth weight counterparts (Tedner et al., 2012). These results have been disputed by studies that have found a decreased risk of

asthma in children whose full term birth weights were less than 2500g (Koshy et al., 2013).

Surgical restriction of placental function and implantation in sheep induces IUGR, which was associated with fewer positive cutaneous hypersensitivity reactions when challenged post sensitization, suggesting that neonates born SGA are less susceptible to allergy (Woolridge et al., 2014). Antibody responses in these sheep after Clostridial vaccine were not different between placentally restricted offspring and controls, suggesting that all aspects of the immune response are not negatively impacted by IUGR (Woolridge et al., 2014).

2.3 Influenza A virus of pigs

IAV is a respiratory pathogen of pigs that can cause important production losses and may pose a risk to public health. Influenza virus was first isolated from swine in 1930 (Shope, 1931) but clinical signs in pigs had been observed in the United States coincidentally with the human influenza pandemic during 1918 (Koen, 1919). The virus isolated in 1931 was the precursor virus that gave rise to the classical H1N1 swine influenza that predominated swine infections worldwide until 1979, at which time they were replaced by avian-like H1N1 strains in Europe (Yu et al., 2009). An H3N2 strain that contained a triple reassortment internal gene (TRIG) complex was identified in North American swine in 1998 and subsequently the TRIG gene became established in H3N2, H1N1 and H1N2 strains that are still endemic in North American swine herds (Webby et al., 2000; Karasin et al., 2002; Vincent et al., 2010; Lorusso et al., 2013).

The process of reassortment occurs when a cell is infected by more than one IAV and any of the 8 genes of the segmented IAV genome are picked up by the other virus to

form a new reassortant strain. One of the best examples of this reassortment was the 2009 pandemic H1N1 (pH1N1) strain. In 2009, a virus containing genes from Eurasian and North American swine-origin IAVs was first detected in humans and gave rise to the first influenza pandemic of the 21st century (Arias et al., 2009). The virus was subsequently transmitted to pigs around the world and was well established in the North American pig population shortly thereafter (Ducatez et al., 2011).

IAV infection in pigs is characteristically a disease of high morbidity and low mortality, with a sudden onset and rapid recovery from respiratory signs including coughing, nasal discharge and dyspnea accompanied by fever and anorexia. IAV is a component of porcine respiratory disease complex (PRDC), which also includes *Mycoplasma hyopneumoniae* (Mhyo), porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) (Opriessnig et al., 2011). PRDC can complicate IAV infection and the immune system's response. Various diagnostic methods, including immunologic, molecular and bacteriological assays, can be used to rule out these pathogens and confirm IAV infection.

2.3.1 Methods of Diagnosis

2.3.1.1 Clinical signs

Studies using experimental inoculation of IAV in pigs have used the assessment of clinical signs such as anorexia, respiratory stridor, coughing, conjunctivitis, dyspnea, erythema, loss of interest and elevated rectal temperature to indicate successful experimental infection with IAV (Van Reeth et al., 1999; Masic et al., 2010; Barbé et al., 2011; Xu et al., 2012; Kowalczyk et al., 2014). The presence, severity and duration of clinical signs, including fever, can vary with influenza subtype, time of sampling (days

post infection), and individual variation (Masic et al., 2009). One study using H1N1 experimental inoculation in juvenile pigs found that five out of nine inoculated pigs displayed a transient elevation in rectal temperature over 40ºC between days 1 and 3 post inoculation (Kowalczyk et al., 2014). In this same study, no evidence of coughing was noted in IAV only inoculated pigs. Clinical signs of tachypnea and loss of interest after experimental infection of piglets with an H1N1 strain have been shown to peak at 24-30 hours post-inoculation, and viral lung titers were also shown to peak during this time (Barbé et al., 2011).

2.3.1.2 Gross Pathology

Gross pathological lesions due to IAV infection in pigs are classically deep plum colored, sharply demarcated areas in the cranioventral portions of the lung (Masic et al., 2009; Sreta et al., 2009; Babiuk et al., 2011; Vander Veen et al., 2012; Charoenvisal et al., 2013; Janke, 2014) and can be reproduced experimentally by intratracheal inoculation (Vleeschauwer et al., 2009). The classical distribution, however, may be masked by interlobular edema in severe cases (Janke, 2014). The presence and severity of gross lesions can depend on the influenza subtype and the duration of infection (Masic et al., 2009). Pigs infected with a Danish strain of H1N1 IAV showed less than 10% consolidation of the middle and left cranial lobes, whereas those infected with a Danish H1N2 strain showed 80% consolidation of the left cranial lobes, 40% consolidation of the middle lobe and 20% consolidation of the accessory lobe with minimal amounts of right lobes affected four days after intranasal inoculation (Trebbien et al., 2011). Gross lesions tend to appear between 48 and 72 hours post-inoculation (PI) (Van Reeth et al., 1998) and were found to be most severe, in one study, at 48 hours (Sreta et al., 2009). In studies

where animals are necropsied after two days PI, only a portion of pigs displayed gross lesions with less severity than those necropsied at nine days PI (Kowalczyk et al., 2014).

2.3.1.3 Histopathology

While histopathological assessment of the lung will not detect IAV per se, the lesions induced are almost pathognomonic in nature (Janke, 2014). Histopathological evaluation consists of staining sections with hematoxylin and eosin (H&E) and assessing the degree of peribronchiolar inflammation and bronchiolar epithelial changes (Masic et al., 2009; Khatri et al., 2010; Trebbien et al., 2011). Typical lesions seen with IAV infection are necrotizing bronchiolitis that may be accompanied by bronchointerstitial pneumonia (Sreta et al., 2009; Masic et al., 2010; Vander Veen et al., 2012; Charoenvisal, 2013; Janke, 2014). Neutrophil infiltration and bronchiolar epithelial degeneration is seen within 24 hours of inoculation (Van Reeth et al., 1999; Vander Veen et al., 2012). Pulmonary atelectasis, and bronchiolar epithelial cell necrosis with hyperplasia are also seen (Babiuk et al., 2011; Trebbien et al., 2011). Inflammatory changes observed microscopically are known to mirror gross lesions (Khatri et al., 2010). Infiltration with neutrophils, macrophages and lymphoplasmacytic cells are seen in bronchioles and alveoli (Sreta et al., 2009; Babiuk et al., 2011; Trebbien et al., 2011).

2.3.1.4 Fluorescent Antibody Test and Immunohistochemistry

Direct and indirect fluorescent antibody tests (FAT or IFAT) using polyclonal antibodies were often employed for diagnosis using both tissue samples and cell cultures. One potential downfall of this method is the requirement for frozen samples, which could preclude its use in certain field samples (Detmer et al., 2013; Janke, 2014). For immunohistochemistry (IHC), the application of monoclonal antibody to nucleoprotein

reveals viral antigen in sections of formalin fixed tissues and can detect all subtypes of IAV since nucleoprotein is conserved across IAVs. (Detmer et al., 2013; Janke, 2014). Different subtypes can be detected by using subtype specific antibodies to hemagglutinin (HA) (Detmer et al., 2013).

Immunostaining methods can be direct or indirect. Direct methods utilize an antibody labeled with detector substances such as enzymes, fluorophore, colloidal gold or biotin. Indirect methods require two antibodies. The first is unlabeled and binds to the antigen in the sample and the second is anti-Immunoglobulin G (IgG) and so binds to the antibody-antigen complex if formed. The second antibody is labeled and since multiple secondary antibodies can bind to the primary antibody, this will multiply the amount of labeled antibody detected in the sample (Detmer et al., 2013). In particular, the Avidin Biotin Complex (ABC) method has commonly been used as a sensitive test for IAV, although background staining of endogenous biotin can be problematic (Detmer et al., 2013). Alveolar and bronchiolar epithelial cells show evidence of nuclear and cytoplasmic staining in pigs challenged with pandemic H1N1 (Babiuk et al., 2011). The immunoreactivity for the anti-influenza A nucleoprotein antibody is most concentrated within areas containing histologic lesions (Sreta et al., 2009).

2.3.1.5 Virus Isolation

Virus propagation in embryonated chicken eggs is the gold standard for avian influenza virus isolation, although antigenic variation has been noted due to amino acid changes in the HA gene when propagating mammalian viruses in this manner. Isolation in certain mammalian cell lines, especially Madin-Darby Canine Kidney (MDCK) cells, does not display this problem (Detmer et al., 2013). Virus propagation in cell culture can

be used to identify and quantify the virus present. Propagation in MDCK cells is used in studies to produce virus of known amount for use as intranasal or intratracheal inoculation (Masic et al., 2009; Khatri et al., 2010; Ducatez et al., 2011; Kowalczyk et al., 2014). Propagation of the virus in mammalian cell lines causes cell lysis, known as cytopathic effect (CPE). Culture supernatant can be tested to determine the viral subtype; viral titration to quantify the amount of virus present can be performed by inoculating serial dilutions into cell cultures (Detmer et al., 2013). Peak viral titers PI are seen between 18 and 24 hours (Van Reeth et al., 1999).

2.3.1.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is more rapid, sensitive and specific than antigen detection or virus culture and, to a great extent, has replaced these methods (Wenzel et al., 2010). Advantages of PCR are that it can be easily adapted for mass screening and can be done on samples that are in less than perfect condition. Influenza subtype can be differentiated using PCR either by performing simplex tests in series or by using multiplex assays (Malanoski and Lin, 2013; Janke, 2014). It is important to remember when using PCR as a diagnostic test is that it only detects the presence of the targeted nucleic acid sequence and cannot determine if viable virus is present. IAV is a negative sense RNA virus and as such, reverse transcriptase PCR (RT-PCR) must be used. RNA must first be extracted from samples, either directly, in the case of lavage fluids or swabs, or after processing in the case of tissue samples. These are considered routine diagnostic samples for farm surveillance and disease investigations (Culhane and Detmer, 2014).

Nasal swabs, tissues and bronchoalveolar lavage (BALF) are used for IAV detection in experimental IAV inoculation studies (Kowalczyk et al., 2014). Processing of lung tissue usually consists of creating a 10% weight per volume (w/v) homogenate in viral culture media (Detmer et al., 2013). Commercial kits for RNA extraction are available based on either solid phase adsorption or magnetic bead extraction, the latter being more successful for fluid samples containing PCR inhibitors or with low virus concentration (Detmer et al., 2013). This can be an important consideration for inoculation studies of differing duration. In an experimental study, viral RNA levels from nasal swabs peaked at four days PI, were low on day 1 and undetectable by day 9, whereas lung levels were not different on days 2 and 4 (Kowalczyk et al., 2014). PCR primers are designed to detect conserved proteins, matrix (M) or nucleoprotein (NP), to ensure unexpected subtypes are not overlooked (Malanoski and Lin, 2013). When determining subtype, the relatively rapid evolution of the virus may necessitate the use of wobble primers which utilize a degenerate site within the HA or Neuraminidase (NA) primer to account for the variability within the sequence without cross-reacting with other subtypes (Detmer et al., 2013). Commonly used chemistries for quantitative PCR (qPCR) are TaqMan® and SYBR green. TaqMan® uses taq polymerase to add a fluorescent-labeled probe to a specific sequence, whereas SYBR green intercalates fluorescent dye in the minor grooves of the amplified DNA (Kumar et al., 2012).

2.3.1.7 Serology

Serologic assays are performed on serum to detect antibody (predominantly IgG) to IAV. These assays can be broad if they are detecting a well-conserved protein or more specific if they target a protein with genetic variability.

2.3.1.7.1 Hemagglutination Inhibition

Red blood cells (RBCs), especially chicken and turkey RBCs, will naturally agglutinate in the presence of IAV. Serial dilutions of IAV can then be used to measure the HA titer of the virus. This reaction is inhibited by the presence of antibody to influenza and is therefore the basis for the hemagglutination inhibition (HI) test (Malanoski and Lin, 2013). As described by Detmer et al., (2013), serial dilutions of the sample in question are plated in a 96-well microtiter plate. A standardized amount of virus is added to each well, followed by RBCs after a period of incubation. The presence of antibodies to influenza in the test serum will inhibit the agglutination of the red blood cells. The titer is then read by taking the reciprocal of the highest dilution of serum that inhibits the agglutination reaction. This standard method has been used in experimental inoculation studies with multiple subtypes to determine the immune status of pigs prior to inoculation (Kowalczyk et al., 2014) and to determine the level of antigen specific immunoglobulins during the course of vaccination studies (Masic et al., 2010). There is known cross-reactivity between phylogenetic clusters of HA that makes it difficult to correctly define the virus subtype using this test; this is somewhat ameliorated by the fact that heterologous reactions are of less intensity than homologous reactions (Detmer et al., 2013).

2.3.1.7.2 Serum Neutralization

Serum neutralization (SN) detects the presence of virus neutralizing antibody in serum. As described by Detmer et al., (2013), the SN test begins with the same two steps as the HI assay. MDCK cells are then inoculated with the virus and serum mixture and the titer is determined to be the reciprocal of the highest dilution that prevents CPE in the

cell culture, i.e., the dilution that can neutralize the virus. This shows not only if antibodies are present, but also if they are biologically active. As with the HI, the interpretation of the SN test is subjective and can vary within and between observers.

2.3.1.7.3 Serum ELISA

Detection of antibodies to influenza in serum by enzyme linked immunosorbent assay (ELISA) follows the same principles as other immuno-detection methods. For example in a blocking ELISA, 96-well plates are coated with influenza viral antigen and incubated with the serum in question. The plate is washed to remove unbound antibody. Enzyme conjugated anti-influenza monoclonal antibody is added and the plate is washed again to remove unbound material. The enzyme substrate, which changes color in the presence of the enzyme, is added and the amount of color produced is quantified by analysis by a photospectrometer. The more color produced, the less the amount of antibody present in the sample (Detmer et al., 2013; Malanoski and Lin, 2013). As with any test, cross reactivity amongst influenza subtypes may lead to unexpected positive or negative results and early infections may go undetected if insufficient time has passed to allow sufficient antibody production to occur.

2.3.2 Cytokines

Cytokines are cell-signaling proteins that have various effects on the mammalian physiology (Table 2.1). Interleukin-6 (IL-6) can stimulate the production of both acute phase proteins and antibodies; interleukin-1 beta (IL-1β) can promote leukocyte migration from the blood stream; tumor necrosis factor alpha (TNF-α) can induce a febrile response and bronchoconstriction; and interferon gamma (IFN-γ) is immunomodulatory and antiviral (Zhang et al., 2013; Janke, 2014). Infected respiratory

epithelial cells produce interferon-α (IFN-α) to inhibit viral replication and induce a proinflammatory response (Janke, 2014). Inflammatory cytokines, such as TNF-α, IFN-α, IL-1β, and IL-6, are associated with the modulation of fever and inflammation in IAV pathogenesis (Masic et al., 2009; Van Reeth et al., 2002; Janke, 2014; Kowalczyk et al., 2014). Clinical signs are highly correlated with levels of IFN-α, IL-6 and TNF-α in BALF in experimentally IAV infected pigs, whereas only weak correlations were seen with IL-1 and interleukin 8 (IL-8) (Van Reeth et al., 1999; Barbé et al., 2011).

Different cytokines have peak levels at different times in the course of disease associated with IAV. In one study, IL-8 was shown to peak later in the course of IAV infection, whereas IL-1, interleukin 4 (IL-4), IL-6 and TNF-α were stable or undetectable through the course of a nine-day study and IFN-α peaked at day 1 (Kowalczyk et al., 2014). Other studies have shown that TNF-α, IFN-α, IL-1, and 6 peaked between 18 and 30 hours post intra-tracheal inoculation (Van Reeth et al., 1999; Van Reeth et al., 2002; Barbé et al., 2011; Janke, 2014) and IFN-α, IL-1β and IL-6 are still detectable at five days PI (Masic et al., 2010). Kowalczyk et al., also showed that the site of pulmonary lesions corresponded to high viral replication and selective expression of IL-8 and IFN-α thus indicating the role of these cytokines in IAV pathogenesis. IFN-α has also been shown to have a role in the induction of IAV symptoms. In a study by Barbé et al., in 2010, pigs that were treated with IFN-α neutralizing antibodies and inoculated with IAV had delayed onset of clinical signs when compared with pigs not given IFN-α neutralizing antibodies and inoculated with IAV. Barbé et al., (2011) also showed that clinical signs are correlated with lung viral titers and cytokine concentrations in BALF after experimental intratracheal IAV inoculation. Van Reeth et al., (1998) provided

corroborating evidence by showing that TNF-α, IL-1 and IFN-α production coincides with lung pathology.

The cytokines IL-1, 6 and TNF-α are known to induce acute phase proteins, which are involved in the pathogenesis of respiratory disease (Petersen et al., 2004; Barbé et al., 2011). Interestingly, blood samples showed that IL-6 and TNF-α producing cells were at a high level early in the course of the disease in Severe Acute Respiratory Syndrome (SARS) patients who had a negative outcome (Openshaw, 2004). Similarly for influenza, IL-6 and IFN-α peaked at day two post infection and TNF and IL-8 increased between days three to seven in humans voluntarily infected (Hayden et al., 1998). Other cytokines have been proposed to be involved in pathogenesis, such as IFN-γ, which has antiviral activity and interleukin 12 (IL-12) and interleukin 18 (IL-18), which help to modulate IFN-γ (Barbé et al., 2011). It has been suggested that this body of evidence shows that the pro-inflammatory cytokines produced in the early acute phase of IAV infection are the cause of clinical signs and microscopic pathology associated with this disease (Van Reeth et al., 1998; Van Reeth et al., 2002; Masic et al., 2009).

2.3.2.1 Cytokine Measurement

Cytokine levels in biological samples can be measured by conventional ELISA, which needs separate samples for each analyte, or by multiplex protein assays, which can detect multiple analytes within the same sample (Lash et al., 2006). The two main types of multiplex assays are those that use standard sandwich ELISA technology, or those using bead techniques (Lash et al., 2006). In the bead assays, such as fluorescent microsphere immunoassay (FMIA), a flow cytometer is used to detect fluorescent dye combinations on the beads, which have capture antibodies conjugated to their surfaces

(Lash et al., 2006). Since the different detection and capture antibodies as well as the cytokines are all present in the mixture, cross reactions can be problematic for FMIA (Martins et al., 2002). The precision of this type of assay is high since each microsphere acts as an individual assay, meaning there are hundreds of replicates of each assay in one mixture (Martins et al., 2002).

SearchLight technology is a sandwich ELISA based test that uses a 96-well flat bottom plate with each well containing spots of different capture antibodies (Lash et al., 2006; Bastarache et al., 2011). SearchLight has been shown to have a better lower end detection limit when compared to other sandwich ELISA or bead assays for tissue samples (Lash et al., 2006). Other advantages to this technology include increased sensitivity due to the chemiluminescent detecting agent and low sample size required (Bastarache et al., 2011). Serious concerns have been raised, however, with respect to reproducibility and reliability of the SearchLight assay due to intra-well spot irregularities, incorrect capture antibody spotting, and high plate-to-plate variability (Bastarache et al., 2011).

It is important to consider that for techniques relying on antibody capture, naturally produced antibody in the sample could interfere with the tests (Bartels and Ribel-Madsen, 2013). Sample acquisition and handling can also affect these tests. The samples may contain the cells that produce the cytokines in question and simply taking the sample could stimulate cytokine production. This can be prevented by collecting samples in ethylenediaminetetraacetic acid (EDTA) tubes and by removing the cellular portion of the sample as soon as possible (Bartels and Ribel-Madsen, 2013). Soluble

cytokine receptors may also be present in the sample, which could then artificially decrease detected concentrations (Bartels and Ribel-Madsen, 2013).

2.3.3 Influenza Pathogenesis

Influenza viruses in mammals have an affinity for the respiratory tract, especially the bronchial and alveolar epithelial cells of the lung (de Vleeschauwer et al., 2009; Khatri et al., 2010; Janke, 2014). The majority of viral replication occurs in these locations; however, other tissues such as tracheobronchial lymph nodes, tonsil, trachea and nasal mucosa can also be affected (de Vleeschauwer et al., 2009; Barbé et al., 2011; Janke, 2014). The resultant distribution of bronchopneumonia is due to the virus entering via the airways and infecting cells within the cranial bronchial tree due to gravitational diffusion within the lung (Janke, 2014). Once the influenza virus invades the host cells it effectively hijacks protein synthesis, which stops the cell from producing its own proteins and expressing its own genes, and switches the cell to viral protein synthesis. This causes necrosis of the cell, which in turn recruits an inflammatory response due to cytokine production (Janke, 2014). During acute infection, viral replication in the lungs will immediately cause the production of several cytokines, such as IL-1, IL-6, IL-12, IFN-γ, IFN-α, and TNF-α (de Vleeschauwer et al., 2009; Khatri et al., 2010). Infected respiratory epithelial cells produce primarily IFN-α, which functions to induce the proinflammatory response through cytokines and inhibits both viral replication and infection of neighboring cells (Janke, 2014). TNF-α and IL-1 and -6 up regulate leukocyte adhesion factors on endothelial cells (Janke, 2014), which is the first step to leukocyte migration into the tissues. IFN-α and Il-6, in addition to these local effects,

also function in systemic effects such as fever and acute phase protein production (Janke, 2014).

Cytokine levels in BALF are positively correlated with clinical signs, viral lung titers and infiltration of inflammatory cells in the lung (Khatri et al., 2010; Barbé et al., 2011; Van Reeth et al., 2012; Janke, 2014). Neutrophil infiltration and necrotizing bronchiolitis and bronchitis are commonly seen microscopically in tissue lesions (Barbé et al., 2011; Janke, 2014). Surface proteins expressed on infected cells recruit neutrophils to the site of damage (Janke, 2014). Neutrophil infiltration can both obstruct the airways and directly damage the lung due to enzyme release (Van Reeth et al., 2012). Subsequently, lymphocytes and macrophages are recruited to the damaged sites (Janke, 2014). Ultimately, while the infection of and replication within host cells of influenza virus causes direct damage to those cells, the subsequent activation and response of the immune system through cytokine production and leukocyte recruitment leads to the most severe damage to the respiratory tract.

2.3.4 Influenza Adaptive Immunity

The surface proteins of influenza virus, such as HA, are strongly immunogenic but the error prone RNA polymerase essential to influenza's replication makes it susceptible to antigenic changes that can foil the adaptive immune system (Stanekova and Vareckova, 2010; Kreijtz et al., 2011). Humoral immunity helps to prevent IAV infection through anti-influenza virus antibodies found in the serum or mucosa (Burlington et al., 1983; Masic et al., 2010), whereas cell mediated immunity promotes recovery from infection by helping to promote viral clearance in the respiratory tract (Thomas et al., 2006; Masic et al., 2010). Antibodies to the surface proteins HA and NA confer

protective immunity (Kreijtz et al., 2011). Humoral immunity is more effective against viral strains that are homologous to the anti-influenza antibody present, because conserved viral coat proteins are targeted by these antibodies to block viral entry to cells and these coat proteins can quickly mutate thus leading to reduced protection (Thomas et al., 2006). Interestingly, studies have shown that in certain cases challenge after vaccination with a heterologous virus lead to immune-mediated enhancement of disease, which may be related to an overzealous cytokine response (Janke, 2014).

B-lymphocytes can recognize unaltered antigen and produce an antibody response mediated by immunoglobulin A (IgA), which acts as an initial barrier to influenza in the upper respiratory tract, and IgG, which helps protect the lower respiratory tract (Stanekova and Vareckova, 2010). Since IgA is polymeric, it can act more broadly on heterologous virus but the monomeric IgG antibody produced in response to hemagglutinin can only block homologous virus attachment to susceptible cells (Stanekova and Vareckova, 2010). IgA is a rapidly produced, neutralizing antibody that confers local protection and its presence indicates recent exposure to influenza (Kreijtz et al., 2011). Anti-neuraminidase antibodies prevent virion release from infected cells (Stanekova and Vareckova, 2010; Kreijtz et al., 2011). Other mechanisms by which antibody can mediate viral clearance are antibody dependent cell-mediated cytotoxicity, complement mediated cytotoxicity and FcR-dependent phagocytosis (Stanekova and Vareckova, 2010; Kreijtz et al., 2011). Antibody is induced to more conserved proteins such as nucleoprotein or matrix, but does not appear to be protective: Rather, these internal proteins are more important in cell-mediated immunity (Stanekova and Vareckova, 2010).

Cell mediated immunity targets internal viral proteins that can be expressed by antigen presenting cells. Cluster of differentiation (CD)4 or CD8 receptor bearing T cells can secrete cytokines or induce apoptosis of infected cells in response to foreign antigens (Thomas et al., 2006; Stanekova and Vareckova, 2010). Influenza specific CD8+ cells proliferate in bronchiolar lavage fluid, lungs, tracheobronchiolar lymph nodes and tonsils of experimentally infected pigs. These CD8+ cells are important for cross immunity with different IAV subtypes and for effecting viral clearance (Khatri et al., 2010). CD4+ cells increase later in the course of the disease after exposure to peptides presented by class II MHC on antigen presenting cells (APC) (Kreijtz et al., 2011). These are important for generating neutralizing antibodies to the virus and, in keeping with their helper cell nomenclature, assists in proliferation of cytotoxic T lymphocytes (CTL) specific to the offending virus, which also contributes to viral clearance (Khatri et al., 2010; Stanekova and Vareckova, 2010). CD4+ cells have been shown to markedly decrease in tracheobronchal lymph node, lung, tonsil, and peripheral blood mononuclear cell (PBMC) populations at three days post infection, but markedly increase in BALF and PBMC by day 6 (Khatri et al., 2010). CD8 bearing cells mature into CTL in the lymphoid tissue and are recruited to the site of infection to destroy virus-infected cells (Stanekova and Vareckova, 2010; Kreijtz et al., 2011). Secretion of IFN-γ by CTL on encountering viral peptides presented by MHC I on infected cells mediates the cellular destruction (Stanekova and Vareckova, 2010). A number of CTL become memory CTL after the course of infection and retain broad cross-reactivity against conserved proteins such as nucleoprotein and matrix upon subsequent infection (Khatri et al., 2010; Kreijtz et al., 2011).

2.4 Other Respiratory Pathogens of Pigs

PRRSV, PCV2 and Mhyo are common pathogens that, along with IAV, are associated with PRDC and must be ruled out, or at least the status of these pathogens within the pig must be understood, when evaluating the effect of influenza clinically or pathologically. A brief discussion of the diagnostics available for these pathogens is presented.

2.4.1 Mycoplasma hyopneumoniae

Moorkamp et al. (2008) suggested that lung tissue is the sample of choice for detection of Mhyo in diseased pigs, whereas BALF is more appropriate in the early stages of infection. BALF was also shown to be more reliable since targeted lung sampling of small areas may not be large enough to select regions containing Mhyo and Mhyo has a predilection for ciliated epithelium, which is not present in the small terminal bronchioles of the lung (Kurth et al., 2002). The gold standard for diagnosis is culture: however, since this method is time consuming and can require specialized techniques, alternative methods such as PCR are more often utilized (Thacker, 2004). Various types of PCR tests are available for Mhyo detection; including nested, qPCR (Strait et al., 2008; Woolley et al., 2012) and single stage conventional PCR (Mattson et al., 1995). Nested PCRs are very sensitive, which can lead to false positive results due to environmental contamination (Kurth et al., 2002).

2.4.2 Porcine Circovirus Type 2

There are two types of PCV, type 1 and type 2. Both types of PCV are commonly detected in both healthy and diseased pigs in North America, but PCV2 is more often associated with clinical symptoms than is PCV1 (Hamel et al., 2000). PCV2 is associated

with a variety of clinical signs, such as wasting and respiratory signs. Necropsy findings often show non-collapsed lungs with interstitial edema and enlarged lymph nodes grossly and lymphohistiocytic interstitial pneumonia, depletion of lymphoid tissue and intracytoplasmic basophilic inclusion bodies in macrophage-like cells microscopically (Ouardani et al., 1999). Methods for detecting PCV include in situ hybridization, viral isolation, immunohistochemical techniques, electron microscopy and serology (Ouardani et al., 1999). Additionally, PCR is a sensitive and expeditious method for the detection of viral nucleic acid. Both conventional and real-time methods are commonly used for many swine viruses. Conventional PCRs are sensitive, however they require the additional step of gel electrophoresis, which often uses the carcinogen ethidium bromide, to visualize the PCR products. Conventional PCR for PCV can be done in simplex, to detect the presence of PCV, or multiplex, to detect the presence of both PCV genomes (Ouardani et al., 1999). Real-time quantitative PCR abolishes the need for gel electrophoresis and has the added benefit of the ability to determine the amount of viral particles in the sample. The accuracy of this method depends in part on the sample tissue, the extraction process and the presence of PCR inhibitors in the sample. In the SYBR green qPCR developed by McIntosh et al. (2009), the accuracy of the test for spiked serum samples was high, however, as the number of copies approached the lower detection limit of the PCR, the accuracy diminished by up to 2 log between the expected and observed copies. The high accuracy indicates either that few PCR inhibitors are present in serum, or the extraction process does not affect accuracy as compared to lung tissue or feces. This PCR could successfully detect and quantify PCV2 DNA in a wide variety of tissues and was tested in both naturally infected and spiked tissues. Molecular beacon PCRs are another method of

real-time quantitative PCR that use fluorophores and quenchers to detect the targeted DNA sequence rather than intercalation within the DNA sequence, like SYBR green technology, and have similar to improved sensitivity and specificity to conventional PCR (McKillen et al., 2007). Loop-mediated isothermal amplification (LAMP) is a rapid method of isothermal amplification of DNA sequences. Chen et al. (2008) developed a LAMP technique for PCV2 with improved sensitivity over conventional PCR, especially for liver, blood, spleen and lymph node.

2.4.3 Porcine Respiratory and Reproductive Syndrome Virus

PRRSV detection has been accomplished through various methods including in situ hybridization in formalin fixed tissues, indirect immunofluorescence in frozen lung sections (Cheon et al., 1997; Spagnuolo-Weaver et al., 2000), viral isolation, serological methods (Spagnuolo-Weaver et al., 2000; Xiao et al., 2008) and PCR. Both conventional and real-time reverse transcriptase (RT)-PCRs have proven to be sensitive and specific methods of detection, especially when using probe based methods (Mardassi et al., 1994; Spagnuolo-Weaver et al., 2000; Egli et al., 2001; Martinez et al., 2008; Xiao et al., 2008; Drigo, Franzo, Belfonti et al., 2014; Drigo, Franzo, Gigli et al., 2014). The real-time assay developed by Egli et al. (2001) showed sensitivity up to 100 times greater than that achieved with conventional PCR when tissue or serum was tested. The SYBR Green real time RT-PCR developed by Martinez et al. (2008) was able to detect PRRSV in challenged pigs before seroconversion. Both North American and European strains of PRRSV exist, both able to cause varying degrees of respiratory disease, and multiple primer and probe sets can be designed to distinguish between them and their respective strains for both conventional and real time RT-PCR assays (Mardassi et al., 1994;

Spagnuolo-Weaver et al., 2000; Egli et al., 2001; Martinez et al., 2008; Drigo, Franzo, Belfonti et al., 2014). As the PRRSV is subject to variations in its genome, sensitivity and specificity of published PCR protocols can degrade over time. A study by Drigo et al. (2014) showed that in-house, probe based PCRs underperformed commercial probe based kits, SYBR Green and RT-PCRs based on primer-genome interaction. Detection of PRRSV in infected lungs by in situ hybridization is more likely in the anterior and middle lobes than the accessory or caudal lobes (Cheon et al., 1997). PRRSV replicates primarily within alveolar macrophages, type II pneumocytes and interstitial macrophages, and is not detected in bronchiolar epithelium (Cheon et al., 1997). The preference of PRRSV for pulmonary alveolar macrophages has posed problems for virus isolation in the past (Mardassi et al., 1994).

2.5 Summary

IUGR is the term used to describe a fetus that has failed to meet its genetic growth potential due to a usually abnormal process *in utero*. In both humans and animals, IUGR is associated with later life metabolic disorders, independent of persisting environmental factors. Compensatory growth, where the IUGR neonate exhibits rapid growth acceleration resulting in similar weights at a given time point to a non-IUGR cohort, has been shown to worsen these metabolic effects. IUGR offspring also have alterations in both humoral and cell mediated immunity, which could lead to higher incidence of disease or vaccine failure. Maternal malnutrition or stress during pregnancy can also lead to IUGR fetuses. The transgenerational effect of IUGR through various epigenetic mechanisms has been noted in humans, with women that were born small for gestational

age being more likely to give birth to SGA infants. Metabolic disturbances were also shown to be transgenerational in rodent models.

Clinical signs of IAV in pigs can vary in duration and intensity and not all symptoms that can be attributed to IAV will be present in the individual animal or group. Clinical signs usually appear approximately 24 hours after infection and are short lived, often lasting to a maximum of 72 hours post infection. Gross pathological lesions associated with IAV are also variable in severity and begin to appear approximately 24 hours after clinical signs appear. The right lung lobes are more affected than the left in pigs, especially after intratracheal inoculation. Histopathological signs may be used in conjunction with gross pathology and clinical signs to support the diagnosis of IAV but does not provide definitive diagnosis. Definitive diagnosis requires the detection of the antigen, agent or antibodies to the agent. Immunostaining and ELISA techniques detect antigen in samples, and virus isolation and PCR detect the agent itself. Serological techniques such as HI or SN detect antibodies to the agent and as such, are more retrospective diagnostics rather than detecting the immediate presence of the disease-causing agent. If IAV is suspected in pigs, it is important to rule out other respiratory pathogens or search for those that could potentiate the effects of IAV. Cytokines are cell-signaling molecules that can have myriad physiological effects (Table 2.1). The proinflammatory cytokines are important for influenza pathogenesis.

Table 2.1 Proinflammatory cytokines important in IAV pathogenesis, physiological effect and time of peak production PI (Adapted from: Hayden et al., 1998; Van Reeth et al., 2002; Petersen et al., 2004; Masic et al., 2010; Barbe et al., 2011; Zhang et al., 2013; Janke 2014; Kowalczyk et al., 2014)

Cytokine	Physiological effect	Time PI of peak
IL-6	Induces acute phase proteins Induces antibodies Modulates febrile response Modulates inflammation	24-48 hours
IL-1β	Promotes leukocyte migration Modulates febrile response Modulates inflammation Production coincides with lung pathology Induces acute phase proteins	24-30 hours
TNF-α	Induces/modulates febrile response Bronchoconstriction Production coincides with lung pathology Induces acute phase proteins	24 hours-7 days
IFN-γ	Immunomodulatory Antiviral	
IFN-α	Inhibits viral replication Proinflammatory Modulates febrile response Modulates inflammation Production coincides with lung pathology	24-48 hours

3. HYPOTHESIS AND OBJECTIVES

This research aims to determine if pigs from low birth weight litters which are expected to show characteristics of intrauterine growth restriction (IUGR), have increased severity of disease after experimental infection with influenza A virus (IAV) when compared to their counterparts from high birth weight litters, thus clarifying the effect of litter birth weight on disease expression. The goal of this work was to better elucidate how the intrauterine environment provided by the sow could affect the clinical disease expression of the individual piglet following experimental disease challenge. Based on the evidence provided by the literature (Neumann et al., 1998; McDade et al., 2001; Moore et al., 2004; Landgraf et al., 2007), the expectation was that average birth weight pigs from low birth weight litters would have more severe clinical disease expression compared to average pigs from high birth weight litters. This work is the first to investigate the effect of low litter birth weight on the clinical disease expression of IAV in pigs.

In chapter 4, pilot trials were conducted in order to determine the appropriate dose of virus to use and the optimal days post inoculation for necropsy to use for the main trial. Chapter 5 describes the main trial that tested the null hypothesis that there would be no difference in clinical disease expression between average birth weight pigs from low birth weight litters and average pigs from high birth weight litters. In the research presented herein, piglets of average weight within their litter were chosen from high and low birth weight litters to ensure that the overall intrauterine milieu was represented rather than choosing the smallest or largest pigs overall, which may have represented a unique local variation in that individual.

The following chapter describes the pilot project conducted in order to determine the appropriate dose of virus to use and the optimal days post inoculation for necropsy to use in the main trial. In order to determine the effect of low litter birth weight on clinical disease expression using IAV it was important to establish an inoculum dosage that ensured infection and disease symptoms, therefore the first part of this project discusses the trials done to achieve this objective. It was also important to determine the optimal time of necropsy post inoculation so that the most relevant post-mortem data could be recorded. As the literature review has shown, the salient and measurable aspects of disease associated with IAV occur at different intervals of time post-inoculation. The second part of this chapter describes an inoculation trial where pairs of pigs from one inoculated batch were necropsied at specific time points post inoculation to determine this optimal time for maximal data collection.

4. PILOT TRIALS TO DETERMINE OPTIMAL VIRAL DOSAGE AND TIMING OF NECROPSY

Original Contribution: My responsibilities included performing all laboratory work associated with virus production, ensuring the welfare of the animals under my care, performing the viral inoculation and necropsies and plaque assays for viral titration, and sample preparation for third party analyses. Prairie Diagnostic Services, Inc. (PDS) performed pre-inoculation screening for influenza A virus, histologic tissue preparations and immunohistochemistry for influenza A virus. Aushon Biosystems performed the cytokine analysis. Dr. Ted Clark performed the microscopic tissue examinations.

This chapter is not intended for publication apart from in this thesis.

4.1 Abstract

Pilot projects were conducted to determine the appropriate virus dose and the optimal days post inoculation (PI) for necropsy in the main trial. The virus used was Influenza A/swine/Texas/4299-2/1998 H3N2 (TX98). Piglets were selected from parity one or two dams only. Litters were deemed high or low birth weight based on total born weight being ±0.7 standard deviations from the historical average litter birth weight of the dam's parity group. Two average birth weight male piglets from each selected litter were chosen for the trial. In pilot trial #1 two, 4-week old pigs were intratracheally inoculated on experimental day 0 with 1 x 10^6 plaque forming units (PFU) of TX98. Necropsies were performed after 96 hours. Increased respiratory rate and serous nasal discharge were observed PI. Gross pathological lesions and positive RT-PCR results from lung and nasal swab were obtained from one piglet. This trial was repeated using a higher inoculation dose of 1 x 10^7 PFU (Pilot trial #2). Necropsies were performed at 72 hours PI and both had multiple lobular areas of consolidation mostly in the cranial lobes bilaterally. Pilot trial #3 was conducted to determine the optimal time PI for necropsy in the main trial. Four high/low birth weight pairs were inoculated as before, with 1 x 10^7 PFU of TX98. Clinical signs were evaluated for all pigs, and one high/low pair was euthanized and necropsied every 24 hours. The results of the pilot trials indicated that clinical signs would peak at 24 hours PI; cytokine concentration and immunohistochemistry score would peak at 48 hours; and pathological lesions would peak between 72 and 96 hours. Therefore the main trial was conducted using an inoculation dose of 1 x 10^7 PFU of TX98 and pigs were necropsied at 48 hours PI.

4.2 Introduction

A series of pilot experiments were conducted in order to determine the appropriate dose of virus to use and the optimal days post inoculation (PI) for necropsy to use for the main trial. In order to determine the effect of low litter birth weight on clinical disease expression using Influenza A virus (IAV) it was important to establish an inoculum dosage that was sufficient to ensure infection and disease symptoms; therefore the first part of this project discusses the trials done to achieve this objective. It was also important to determine the optimal time of necropsy PI so that the most relevant post-mortem data could be recorded. The second part of this project describes an inoculation trial where pairs of pigs from one inoculated batch were necropsied at specific time points PI to determine the optimal time for data collection. This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use University committee on animal care and supply (UCACS) Protocol Number 20090157.

4.3 Materials and Methods

4.3.1 Virus

The virus used for the pilot project was Influenza A/swine/Texas/4299-2/1998 H3N2 (TX98). This virus was originally isolated from an outbreak of respiratory disease in pigs in Texas in 1998 and was determined to be a triple reassortant between genes from avian influenza, classical H1N1 swine influenza and a human H3N2 that was circulating in 1995 (Zhou et al., 1999). Pigs infected with TX98 exhibit clinical signs of sneezing, rough hair coat, cough, moderate fever, and occasionally sow mortality has been noted (Zhou et al., 1999). The virus was grown from stock obtained from Dr. Yan

Zhou, Vaccine and Infectious Disease Organization-Intervac (VIDO-Intervac) at the University of Saskatchewan. TX98 was propagated in Madin-Darby Canine Kidney (MDCK) cells with Eagle's Minimum Essential Medium (Life Technologies, Grand Island, New York, USA), containing 10% heat inactivated fetal calf serum, 1M Hepes and non-essential amino acids (MEM+). Using a multiplicity of infection (moi), or ratio of virus to cells, of 0.001, the inoculated culture was incubated at 37°C and 5% CO_2 for at least 48 hours until full cytopathic effect (CPE) was observed (Meguro et al. 1979). Once flasks were at full CPE, virus was harvested and purified as described below.

4.3.2 Virus propagation

MDCK cells were obtained and propagated at the Vaccine and Infectious Disease Organization (VIDO) at the University of Saskatchewan. Cells are grown using MDCK complete media made using MEM+ (Internal document, VIDO). Once confluent, MDCK cells were washed with versene (Life Technologies) and incubated with a 0.25% trypsin solution (Lonza, Walkersville, Maryland, USA) at 37°C to loosen the attached cells. The cell suspension was transferred to 50 ml centrifuge tubes and centrifuged at 2000×*g* for 10 minutes. After this mixture was centrifuged, the remaining pellet was resuspended in MEM+ and the required amount of suspended cells was added to fresh MEM+ in flasks. The amount required depended on the interval of time before the next passage. For example, if cells were required to be confluent in 48 hours, 2 mL of resuspended cells would be added to the 13 mL of fresh media in the flask.

IAV was propagated in MDCK cells using MEM+. Prior to propagation, the seed stock virus titer and the number of cells present in the tissue culture flask must be determined. In this case, the seed stock virus was known to be 1.7×10^7 plaque forming

units/milliliter (PFU/mL) and the number of cells was 1 x 10^7 as determined by hemocytometer count. The moi needed for this infection was 0.001 and was calculated from the aforementioned numbers. To perform the infection, media was removed from a T75 flask with fully confluent MDCK cells. The cells were washed with 1 molar (1M) phosphate buffered saline at pH 7.3 with potassium, magnesium and calcium (PBSA+) taking care not to disturb the cells. PBSA+ was removed and 3 mL of inoculum was added and the flask was incubated at 37°C for 1 hour, rocking every 20 minutes. All of the media was removed and MEM+ and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (Thermo Fisher Scientific, Rockford, Illinois, USA) was added to the flask and incubated at 37°C for 48 hours. Virus was harvested and then purified, as described below.

4.3.3 Virus purification

The harvested virus was spun at 2000×*g* for 10 minutes. The supernatant was collected then ultra-centrifuged at 53,406×*g* for 2.5 hours. The resulting pellet was resuspended in Tris/Sucrose/EDTA (TSE) and allowed to soften overnight at 4°C. All resuspended pellets were pooled and layered on a sucrose overlay: 2.5 mL of 60% sucrose in TSE with 5 mL of 30% sucrose in TSE carefully added so the layers do not mix. 3.5 mL of pooled pellets were added carefully so they do not mix with the lower layers. This overlay was ultra-centrifuged at 53,406×*g* for 2.5 hours. The virus was harvested from the resulting opalescent band that appears at the 60/30 sucrose boundary. The propagated virus had a titer of 1.4 x 10^9 PFU/mL determined by plaque assay (as described below). This virus was then divided into 10 microliter (μL) aliquots of purified

virus so that it contained 1 x 10^7 PFU of IAV when 1990 μL of PBS was added to comprise the 2 ml inoculum for each pig.

4.3.4 Plaque assay

MDCK cells were seeded in a 6-well plate at 5.5 x 10^5 cells/well 24 hours before infection. For infection, virus dilutions were prepared in MEM+ on ice. MDCK cells were washed with 1M phosphate buffered saline with potassium (PBS+) at pH 7.3 and 400μL of virus inoculum was added to each well. The plates were inoculated at 37°C for 1 hour, rocking the plate every 15 minutes. The inoculum was then removed and 2 mL of an agarose/media overlay was added and allowed to solidify for 30 minutes. Plates were then incubated at 37°C until full CPE. To stain the plaque assay, the media overlay was removed gently and coomassie blue stain was added to each well. Wells were then rinsed gently with tap water and allowed to dry overnight. The number of plaques in each well was counted to determine the titer by using the following calculation:

$$\lambda = n(di)(1/V)$$

where λ = virus titer

n = number of plaques produced

di = dilution of virus

V = volume of inoculum in mL

This method was used to determine the titer of the purified virus inoculum and the virus titers for lung tissue from all pigs used in the pilot trial (Reed and Muench, 1938).

4.3.5 Inoculation trials

Twelve, 4-week-old, purebred Large White male pigs were purchased from a farm free of *Mycoplasma hyopneumoniae* (Mhyo) and porcine reproductive and

respiratory syndrome virus (PRRSV) in Saskatchewan, Canada and housed in an animal isolation facility at the University of Saskatchewan that was equipped with negative pressure air flow and individual room air filtration (Saskatoon, SK, Canada). The pigs were given free access to water and were fed an antibiotic-free, age appropriate diet (Whole Earth Pig Starter, Federated Co-operatives Ltd., Saskatoon, SK, Canada) *ad libitum* that met or exceeded recommended nutrient requirements. The animal phase of this study was conducted in accordance with the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (UCACS Protocol Number 20090157).

Piglets were selected from parity one or two dams only because older sows, third parity or higher, had serum antibodies to IAV indicative of a past infection. Nasal swabs taken from 20 nursery pigs ranging in age from 4 to 9 weeks of age tested IAV negative by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) tested at PDS using the Canadian Food Inspection Agency (CFIA)-approved protocol (Spackman et al., 2002; Detection of Type A Influenza Viruses and Avian H5 and H7 Hemagglutinin Subtypes by Real-Time RT-PCR Assay, CFIA protocol), indicating that no active circulation of virus was present. Therefore, piglets from first and second parity sows were deemed acceptable and appropriate for the experiment. Piglets were determined to originate from high or low birth weight litters based on being ±0.7 standard deviations (Z-score) from the historical average litter birth weight of the dam's parity group from the barn after controlling for total born litter size, where: $Z_{Litter\ i}$ = (litter weight$_i$ – mean litter weight$_{cohorts}$)/SD$_{cohort\ mean\ litter\ weight}$, and where *cohorts* includes the population of sows of similar parity and litter size over the previous 2 years.

These extreme low birth weight litters were expected to show the phenotypic characteristics of intrauterine growth restriction (IUGR). Litters were selected at birth based on weight, including stillbirths, and two average birth weight male piglets from each selected litter were chosen at weaning for the trial. Trial animals were subjected to regular processing as per barn protocol, such as castration and iron treatment. Upon arrival at the isolation facility, serum samples from all piglets were sent to Prairie Diagnostic Services, Inc. (PDS) and confirmed negative for antibodies to IAV by H1N1 and H3N2 ELISA (Idexx Laboratories, Westbrook, Maine, USA). As well, nasal swabs of all pigs were collected and tested by a matrix (RT-PCR) procedure at PDS using the Canadian Food Inspection Agency (CFIA)-approved protocol (Spackman et al., 2002; Detection of Type A Influenza Viruses and Avian H5 and H7 Hemagglutinin Subtypes by Real-Time RT-PCR Assay, CFIA protocol). The pigs were acclimated for 5 days and room temperature was maintained at approximately 27°C.

4.3.5.1 Pilot Trial 1: Determining response to viral inoculum dose of 1 x 10^6 PFU TX98 at 96 hours post inoculation

Immediately prior to inoculation on experimental day 0, one pair of pigs, consisting of one pig from each group of high and low birth weight litters, were transferred from a biosafety level 1 (BSL1) to a BSL2 room and intratracheally inoculated in sternal recumbency with 1 x 10^6 PFU TX98 in 3 mL of MEM while anesthetized with a single intramuscular dose (up to 20 mg/kg) of ketamine (Ketalean®, Bimeda-MTC, Cambridge, ON, Canada) and 2 mg/kg xylazine (Rompun®, Bayer HealthCare, Toronto, Ontario, Canada). Clinical signs were assessed every 6 hours (Table 4.1) and by a qualified veterinarian at least every 12 hours. After 96 hours, both

pigs were euthanized with a lethal dose of pentobarbital (Euthanyl Forte®, Bimeda-MTC) and a necropsy performed. Any lung lobes that showed gross pathological evidence consistent with IAV were collected *en bloc*. If no lesions were evident, the right cranial lobe was collected. Lung samples and nasal swabs from both pigs were collected and tested by a matrix real-time RT-PCR procedure at PDS using the CFIA approved protocol.

4.3.5.2 Pilot trial 2: Determining response to viral inoculum dose of 1 x 10^7 PFU TX98 at 96 hours post inoculation

One high/low pair of piglets were anesthetized and inoculated as described previously, except the inoculation dose was increased to 1 x 10^7 PFU. Clinical signs were assessed every 8 hours. Pigs were euthanized at 72 hours post inoculation (PI).

4.3.5.3 Pilot Trial 3: Determining optimum time post inoculation for necropsy

The remaining eight pigs were divided into high/low pairs (one pig was from a high birth weight litter, the other from a low birth weight litter) and inoculated as previously described with 1 x 10^7 PFU TX98. Clinical signs were evaluated for all pigs, and one high/low pair was euthanized and necropsied every 24 hours in order to assess which time point had the most severe lesions. Serum samples were obtained just prior to performing the necropsy and samples were stored at -80°C. At necropsy, bronchoalveolar lavage fluid (BALF) samples were collected as previously described (Detmer et al., 2013) using PBS. After gently massaging the lungs, the PBS was aspirated and 5 ml aliquots of the aspirated fluid were stored at -80°C. Each lung lobe was collected *en bloc* and 2cm^3 sections of each lung lobe that exhibited lesions consistent with IAV were collected in 10% neutral buffered formalin, trimmed and embedded in paraffin within 24 hours of

collection. If no lesions were present, a $2cm^3$ section was collected from the centre of the lobe in 10% neutral buffered formalin and prepared as previously described. The remaining lung lobes were stored individually at -80°C. In addition to the lung sections, $2cm^3$ sections of nasal turbinate, thymus, tracheobronchial lymph node and spleen as well as a 2cm full circumference, linear section of trachea, were also collected in formalin and processed for immunohistochemistry (IHC) as described previously.

4.3.6 Cytokine analysis

One aliquot of BALF from each piglet was sent to Aushon BioSystems Inc. (Billerica, Massachusetts, USA) to determine the cytokine concentrations of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α) using their SearchLight Array Technology. Four cytokines were chosen specifically due to their proinflammatory effects during IAV infection and because the literature search indicated that a discrete concentration peak would be evident in bronchoalveolar fluid after IAV inoculation. Interferon gamma (IFN-γ) is produced by activated Th1 lymphocytes in pigs and high levels are not produced until approximately 6 days PI (Khatri et al., 2010).

4.3.7 Histopathology

PDS performed the micro section staining with hematoxylin and eosin (H&E) for histopathologic examination and IHC with anti-influenza A ribonucleoprotein antibody (National Institute of Allergy and Infectious Disease, Bethesda, Maryland, USA) at 1:5,000 dilution as previously described with 3,3-diaminobenzadine (IMMpact™ DAB; Vector Laboratories, Burlingame, California, USA) chromagen (Haines, 1993). Dr. Ted Clark (consulting, Calgary, AB) conducted the microscopic examination of the lung

samples while blinded to the group and time of necropsy of each pig. The lesions were examined for bronchiolar epithelial attenuation and inflammation within the bronchioles and interstitium (0 = no lesion, 1 = mild, 2 = moderate). For IHC, the amount of immunoreactivity was scored 0 to 3 (0 = none, 1 = minimal, 2 = moderate, and 3 = abundant).

4.3.8. Polymerase Chain Reaction

DNA and RNA were extracted from day 0 serum samples and lung tissue using a commercial kit (AllPrep DNA/RNA Mini Kit, Qiagen, Toronto, ON, Canada) and frozen at -20°C. Tissue for extraction was chosen from lung tissue exhibiting lesions or from random lobes if no lesions were apparent. Conventional nested PCR for Torque Teno Virus (TTV) was performed on extracted DNA from serum as previously described (Kekarainen et al., 2006); SYBR Green Real Time porcine circovirus type 2 (PCV2) PCR was performed on day 0 serum DNA extractions as previously described (McIntosh et al. 2009); and a gel based conventional PCR was performed for PRRSV Open Reading Frame (ORF)7 from serum RNA and Mhyo from lung DNA (Mattson et al., 1995).

4.3.9 Lung titers

Lung tissue homogenates (10% w/v) in MEM+ were mixed with RN-ase free disposable pellet pestles (Thermo Fisher Scientific, Rockford, Illinois, USA) and sonicated at 20 kHz for 60 seconds. Viral titer of the resultant homogenate was determined by plaque assay, as described earlier. All samples collected were frozen at -80°C until the end of the trial and tested at the same time.

4.4 Results

4.4.1 Pilot trial 1

Respiratory rate was compared to the pre-inoculation baseline score and an increased respiratory rate was seen in pig 244 at 12, 18, 24 and 36 hours post inoculation (PI) and at 18, 66, 72, 78 and 90 hours PI in pig 325. Serous nasal discharge was not observed in either pig during the pre-inoculation period, however serous discharge was observed at 48 and 84 hours PI in pig 325, but not in pig 244 (Table 4.2). Pig 244 had a peak body temperature of 41.8°C at 12 hours PI. Pig 325 exhibited a peak body temperature of 39.8°C at 12, 42, and 54 hours PI (Figure 4.1). An esophageal abscess and no lung lesions consistent with IAV were found at necropsy in Pig 244 and RT-PCR on lung and nasal swab were negative for IAV in this animal as well. Pig 325 had one small (<1 cm) area of plum colored consolidation present in the right middle lobe. RT-PCR on lung and nasal swab from this animal were positive.

4.4.2 Pilot trial 2

The respiratory rate was increased compared to pre-inoculation baseline scores and an increased respiratory rate was observed beginning at 8 hours PI in both pigs and persisted until euthanasia. Sneezing was noted periodically, at 16, 40 and 64 hours PI in pig 248 and at 32, 48 and 64 hours PI in pig 312. Pig 248 exhibited its peak body temperature of 40.4°C at 2 days prior to inoculation. This pig's highest body temperature after inoculation was 40.0°C and this was observed at 40 and 48 hours PI. Pig 312 exhibited a peak body temperature of 40.8°C at 24 hours PI (Figure 4.2). The two pigs inoculated at this dosage were necropsied 72 hours PI and both had multiple lobular areas of consolidation mostly in the cranial lobes bilaterally (Table 4.2).

4.4.3 Pilot trial 3

Peak body temperatures ranged from 39.8°C to 41.4°C and 6 of 8 pigs exhibited their highest temperature at 24 hours PI (Figure 4.3). PI respiratory rate, when compared to pre-inoculation rate, did not increase. No dyspnea or sneezing was noticed at any time point. The pair euthanized at 24 hours PI showed no gross lesions indicative of IAV (Table 4.2). One pig in this pair was found dead the morning of the scheduled necropsy, therefore not all data was able to be collected from this pig. In the pair euthanized at 48 hours PI, lung lesions consistent with IAV were only found in one pig and no lesions were found in both pigs euthanized at 72 hours (Table 4.2).

Peak concentrations of IL-1β were found in the pigs necropsied at 48 hours PI; no consistent peak was evident for the other cytokines tested. Interstitial inflammation and epithelial attenuation peaked at 72 hours, whereas IHC intensity peaked at 48 hours. The highest titer was measured in one of the pigs necropsied at 24 hours PI and remained detectable through to 96 hours. Nasal swab titers were not detectable until 96 hours PI.

No pigs were positive for TTV genotype 1, however, 8 pigs were positive for genotype 2 (Table 4.2). All samples tested for PCV2 were classified as detectable but not quantifiable. RT-PCR results for PRRSV (Tetracore PRRS real-time PCR kit, Tetracore, Inc., Rockville, US) and Mhyo (Mattson et al., 1995) were both negative.

4.5 Discussion

In Pilot trial 1, one of the pigs in this pair had a body temperature of 42°C at 12 hours PI, however, an esophageal abscess was noted on necropsy likely due to trauma from intubation. The gross pathology and RT-PCR results from this trial indicated that the inoculation was only successful in one of the two pigs and that the dosage of IAV was

possibly not sufficient to result in significant lesions. Thus, it was decided to repeat the inoculation experiment using 1 x 10^7 PFU in Pilot 2.

In Pilot trial 2, both pigs had multiple lobular areas of consolidation mostly in the cranial lobes bilaterally, which is suggestive of IAV infection. Based on the increased severity of gross lesions found in the pigs at this dosage, it was decided to use this dose going forward. The next step was to determine the optimum days post infection to perform the necropsies.

The results of both pilots 2 and 3 indicated that clinical signs, specifically elevations in body temperature, should peak at approximately 24 hours PI. Cytokines, based on the concentration of IL-1β, should peak at 48 hours PI. The results also indicated that pathological lesions should peak later, between 72 and 96 hours. Not all pigs in pilot 3 had quantifiable lung titers. This was likely due to inadvertent intraesophageal inoculation rather than intratracheal, and was rectified prior to the main trial. Positive IAV titers on nasal swabs were not detectable until 96 hours PI, indicating that nasal shedding post intratracheal inoculation takes approximately that long to develop or that the plaque assay technique used to quantify the titer was not sensitive enough to detect lower titers. We decided to perform necropsies in the main trial at 48 hours PI in order to optimize the amount of data available to test our hypothesis.

Table 4.1 Clinical signs of respiratory disease caused by IAV infection assessed for each pig.

Score	Clinical Signs
Attitude and responsiveness	
0	Alert and active
1	Alert, but slower than pen mates
2	Reluctant to move, but gets up by stimulation
3	Down, doesn't respond with stimulation or demonstrates seizures (leg paddling, recumbency, opisthotonous)
Respiratory rate & effort	
0	Normal
1	Increased respiratory rate
2	Increased respiratory rate, slight abdominal breathing (dyspnea)
3	Increased respiratory rate and marked abdominal breathing (dyspnea)
Appetite and body condition	
0	Normal appetite and body condition
1	Reduced feed intake, normal body condition
2	Anorexic, slight loss of body condition and weight (>10% loss of body weight)
3	Anorexic, moderate loss of body condition and weight (>15% loss of body weight)
Coughing and sneezing	
0	No coughing or sneezing
1	Observed coughing/sneezing once while in the room
2	Observed coughing/sneezing 2-3 different times while in the room
3	Observed continual coughing/sneezing while in the room

Table 4.2 Preinoculation TTV2 status, clinical pathological signs (present or absent), and lung titers measured in PFU/ml for pigs inoculated with IAV TX98 at necropsy with variable numbers of hours post-infection (HPI).

Pig ID	Pilot #	HPI	IAV Lung Titer (PFU)	Fever	Nasal Discharge	TTV2 status	Gross lesions	IHC +ve
244	1	96	0	Y	N	-	N	N
325	1	96	$1.25x10^5$	N	N	+	Y	Y
248	1.2	72	$1.06x10^6$	Y	N	-	Y	Y
312	1.2	72	$5.31x10^6$	Y	N	+	Y	Y
302	2	24	$1.33x10^7$	N	N	+	N	Y
313	2	24	0	N	N	-	N	N
317	2	48	$7.88x10^4$	Y	N	+	Y	Y
318	2	48	0	Y	Y	+	N	N
237	2	72	0	N	N	+	N	N
309	2	72	0	Y	N	+	N	N
307	2	96	0	Y	N	-	N	N
320	2	96	$9.38x10^5$	Y	N	+	Y	Y

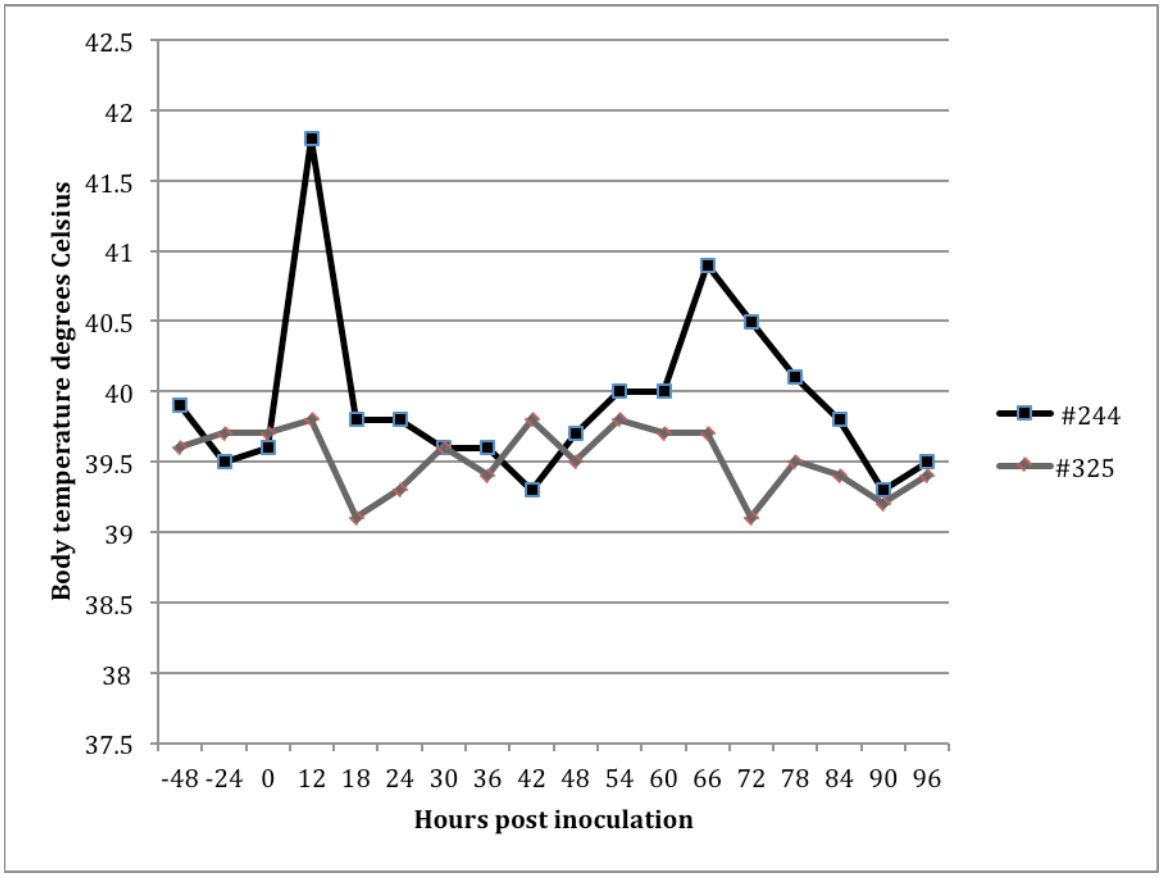

Figure 4.1 Body temperatures of pig 244 and pig 325 during the pilot study 1.Body temperature was measured rectally every 24 hours starting at 48 hours before inoculation up to the time of inoculation and then every 6 hours until 96 hours PI.

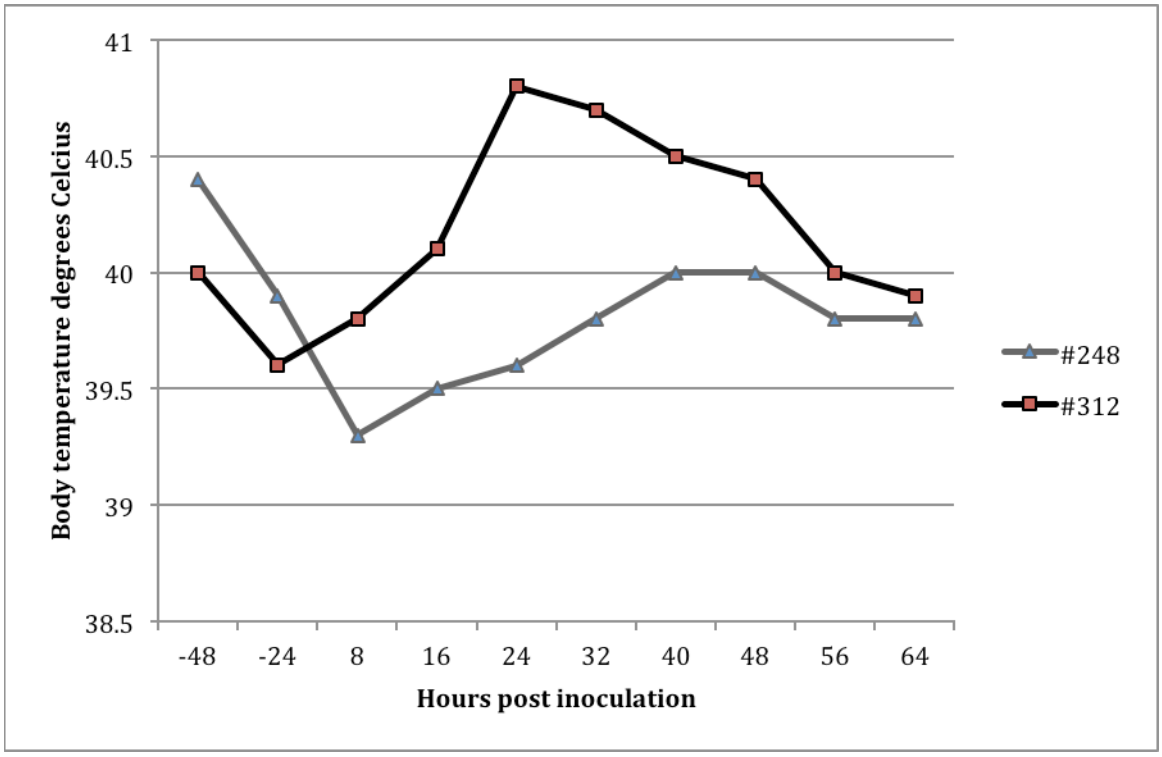

Figure 4.2 Body temperature of pig 248 and pig 312 during pilot study 2. Body temperature was measured rectally every 24 hours starting at 48 hours before inoculation up to the time of inoculation then every 8 hours until 64 hours PI**.**

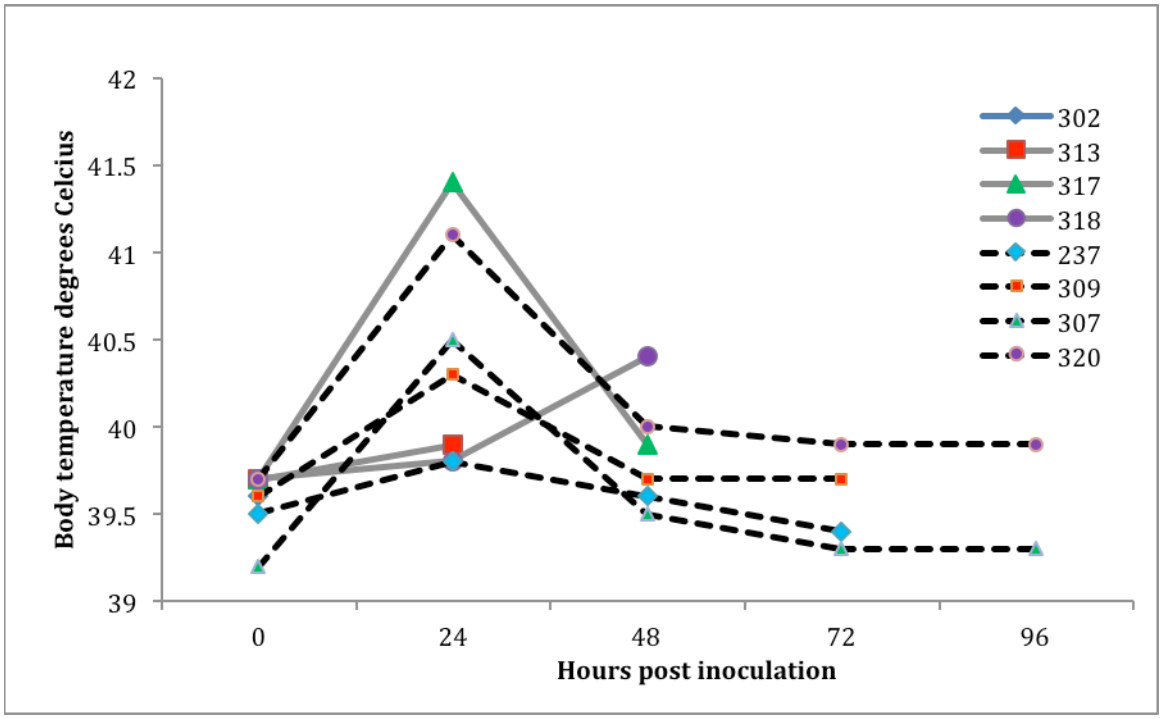

Figure 4.3 Body temperature of the pigs during pilot study 3. Starting at the time of inoculation until 96 hours PI, body temperatures were measured rectally every 24 hours until termination.

5. COMPARISON OF INFLUENZA A VIRUS INFECTION IN AVERAGE PIGS OF HIGH- AND LOW-BIRTH-WEIGHT LITTERS.

The final, definitive version of this paper has been published as:

Detmer SE, Gunvaldsen RE, Harding JC. Comparison of influenza A virus infection in high- and low-birth-weight pigs using morphometric analysis. Influenza and Other Respiratory Viruses. 2013;7 Suppl 4:2-9.

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Original contributions: Co-author, RE Gunvaldsen, was the principal person who conducted the animal experiments, sample collection, and certain laboratory analyses for this study, which represents over one year of dedicated work. The version published in this thesis contains the full data analysis and interpretations of the results conducted by said co-author, who is the principal writer of this chapter.

The following chapter describes the main trial that tested the null hypothesis that there would be no difference in clinical disease expression between average birth weight pigs from low birth weight litters and average pigs from high birth weight litters. The development of the experimental protocol detailed herein relied on the information gleaned from the available literature on intrauterine growth restriction and influenza A virus as well as the results of the pilot experiments designed to determine the appropriate inoculum dose and termination point for the study.

Prairie Diagnostic Services, Inc. performed pre-inoculation screening for influenza A virus, histologic tissue preparations and immunohistochemistry for influenza A virus. University of Minnesota Veterinary Diagnostic Laboratory performed the hemagglutination inhibition assays. Aushon Biosystems Inc. performed the SearchLight cytokine analysis and Dr. Andrea Ladinig performed the interferon alpha fluorescent microsphere immunoassay test. Dr. Susan Detmer performed the microscopic tissue examinations. Betty Chow-Lockerbie and Dr. Shermila Kulanayake performed the virus titration assays for the study.

5.1 Abstract

Human and animal studies have shown a relationship between low birth weight and susceptibility to later onset of metabolic disease; however the relationship between birth weight and infectious disease is not clear. The objective of this study was to determine if average birth weight pigs from low birth weight litters, which are expected to show the phenotypic characteristics of intrauterine growth restriction, would have more severe clinical disease expression compared to average pigs from high birth weight litters following experimental inoculation with influenza A virus. At birth, litters were selected as high- or low- birth weight; two average birth weight male piglets were selected at weaning and after the serologic results for influenza A virus were confirmed negative from each of 17 high- or 17 low-birth weight litters originating from a Porcine Respiratory and Reproductive Syndrome Virus and *Mycoplasma hyopneumoniae* free farm. The piglets were intratracheally inoculated with the challenge virus influenza A/swine/Texas/4199-2/1998 H3N2 in a biosafety level 2 animal care unit room. Clinical scores and rectal temperatures were recorded twice daily for each pig until euthanasia 48 hours post inoculation. At necropsy, lung samples were collected to determine gross and histopathological lesion severity, influenza A virus staining intensity score and influenza viral titers. Bronchoalveolar lavage samples were collected for inflammatory cytokine quantification. No differences in clinical scores between pigs from high- and low-birth weight litters were observed. Gross, histopathological and immunochemical scores were significantly higher in piglets from high-birth weight litters. Viral titers trended higher in piglets from high-birth weight litters. These findings indicate that pathological lesion

severity in piglets experimentally inoculated with influenza A virus is more severe in piglets from high compared to low birth weight litters.

5.2 Introduction

Low birth weight in humans is associated with increased risk of developing metabolic syndrome, which is a cluster of hypertension, insulin resistance and hypercholesterolemia, type II diabetes and other cardiovascular and metabolic disorders later in life, which is independent of external factors such as smoking or obesity (Hales and Barker, 2001; Dunger and Ong, 2005; Fowden et al., 2005; Meaney et al., 2007; Waterland and Michels, 2007; Harris et al., 2011; Woroniecki et al., 2011). Animal models have shown a relationship between birth weight and risk of intestinal, circulatory, neurological, and respiratory dysfunction (Wu et al., 2006). There have also been studies showing that low birth weight can contribute to decreased response to vaccination in adolescence in certain human subsets (McDade et al., 2001; Moore et al., 2004). From this, the question arose whether low litter birth weight in pigs could be associated with increased severity of disease. Average birth weight pigs from high- or low-birth weight litters were chosen in order to ensure that the birth weights from each group did not overlap, which would make the differences between the groups unclear. Using an influenza A virus (IAV) as an exemplifier disease, the objective of this study was to determine if average birth weight pigs from low birth weight litters, which are expected to show the characteristics of intrauterine growth restriction (IUGR), would have more severe clinical disease expression compared to average pigs from high birth weight litters following experimental inoculation with IAV.

5.3 Materials and Methods

5.3.1 Virus

The challenge virus, influenza A/swine/Texas/4199-2/1998 H3N2 (TX98) was grown from stock obtained from Dr. Yan Zhou, Vaccine and Infectious Disease Organization-Intervac (VIDO-Intervac) at the University of Saskatchewan. TX98 was propagated in Madin-Darby Canine Kidney (MDCK) cells with Eagle's Minimum Essential Medium (Life Technologies, Grand Island, New York, USA) containing 4% bovine serum albumin (Life Technologies, Grand Island, New York, USA) and 0.5% gentamycin (Lonza, Walkersville, Maryland, USA) (MEM+). Using a multiplicity of infection (moi) of 0.001, the inoculated culture was incubated at 37°C and 5% CO_2 for at least 48 hours until full cytopathic effect (CPE) was observed (Meguro et al. 1979). Once flasks were at full CPE, virus was harvested and purified (unpublished document, VIDO). Briefly, harvested virus was spun at 2000×*g* for 10 minutes. The supernatant was collected then ultra-centrifuged at 53,406×*g* for 2.5 hours. The resulting pellet was resuspended in Tris/Sucrose/EDTA (TSE) and allowed to soften overnight at 4°C. All resuspended pellets were pooled and layered on a sucrose overlay: 2.5 mL of 60% sucrose in TSE with 5 mL of 30% sucrose in TSE carefully added so the layers do not mix. 3.5 mL of pooled pellets were added carefully so they do not mix with the lower layers. This overlay was ultra-centrifuged at 53,406×*g* for 2.5 hours. The virus was harvested from the resulting opalescent band that appears at the 60/30 sucrose boundary. Aliquots of virus each having a titer of $1 \times 10^{6.3}$ median tissue culture infective dose ($TCID_{50}$)/ml as calculated by the Spearman-Kärber method (Villegas and Alvarado 2008) were prepared, and stored at -80°C until used.

5.3.2 Animals

Sixty-eight 4-week-old pigs were purchased from a farm free of *Mycoplasma hyopneumoniae* (Mhyo) and porcine reproductive and respiratory syndrome virus (PRRSV) in Saskatchewan, Canada and housed in an animal isolation facility at the University of Saskatchewan that was equipped with negative pressure air flow and individual room air filtration (Saskatoon, SK, Canada). The pigs were given free access to water and were fed an antibiotic-free, age appropriate diet (Whole Earth Pig Starter, Federated Co-operatives Ltd., Saskatoon, SK, Canada) *ad libitum* that met or exceeded recommended nutrient requirements. The animal phase of this study was conducted in accordance with the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use and University Committee on Animal Care and Supply (UCACS) Protocol Number 20090157.

Piglets were selected from parity one or two dams only, and were determined to be coming from high or low birth weight litters based the litters on being ±0.7 standard deviations (Z-score) from the historical average litter birth weight of the dam's parity group from the barn. Litters were selected at birth based on weight, including stillbirths, and two average birth weight male piglets, as compared to their littermates, were selected at weaning from each selected litter for inclusion in the trial. One-week prior to weaning, serum samples from selected piglets were sent to Prairie Diagnostic Services, Inc. (PDS) and confirmed negative for antibodies to IAV by H1N1 and H3N2 ELISA (Idexx Laboratories, Westbrook, Maine, USA). Additionally, serum samples were also confirmed to be negative for antibodies to TX98 by hemagglutination inhibition (HI)

assay at the University of Minnesota Veterinary Diagnostic Laboratory. Trial animals were subjected to regular processing as per barn protocol, such as castration and iron treatment. Due to the size of the barn and availability of high- (HBW) and low- (LBW) birth weight litters on a given week, it was not possible to obtain the required number of pigs on a single day; therefore, pigs were selected in weekly batches of 4 to 12 piglets, depending on farrowing rates and the availability of litters and pigs that fell within selection criteria. Within batches, at least two piglets were selected from each of high and low litter to allow the effect of batch to be controlled for in the statistical analyses.

5.3.3 Experimental procedures

Upon arrival at the animal care unit (ACU), nasal swabs of all pigs were collected and tested by a matrix real-time reverse transcriptase-polymerase chain reaction (RT-PCR) procedure at PDS using the Canadian Food Inspection Agency (CFIA)-approved protocol (Spackman et al., 2002; Detection of Type A Influenza Viruses and Avian H5 and H7 Hemagglutinin Subtypes by Real-Time RT-PCR Assay, CFIA protocol). Animals were housed in a biosafety level 1 (BSL1) room within the ACU and acclimated for 5 days; room temperature was maintained at approximately 27°C. The inoculum was prepared the morning of day 0 for each batch. On experimental day 0, all pigs in the batch were transferred to a BSL2 room and intratracheally inoculated in sternal recumbency with TX98 while anesthetized with a single intramuscular dose of up to 20 mg/kg ketamine (Ketalean®, Bimeda-MTC, Cambridge, ON, Canada) and 2 mg/kg xylazine (Rompun®, Bayer HealthCare, Toronto, Ontario, Canada) after sedation with a single intramuscular dose (0.3 ml) of azaperone (Stresnil®, Merial, Baie D'urfé, QC, Canada). After inoculation, piglets were monitored until all could stand on their own. *Ad*

libitum feed and water, identical to that provided during acclimation, were provided. Four pigs were removed from the study, leaving 32 pigs per treatment group. Two pigs were removed due to death from anesthetic complications; one pig died during the acclimation period before inoculation and one pig was removed due to an unrelated lung abscess.

5.3.4 Clinical observations, sampling and pathological examination

The pigs were checked twice daily for any evidence of injury or illness from the time of arrival until termination of the study. Clinical scores and rectal temperatures were recorded for each pig. Clinical signs specific to IAV were also scored twice a day following inoculation and recorded (Table 5.1). Observers were blinded to treatment group at all times.

Two days post-inoculation (DPI), all pigs were euthanized with a lethal dose of pentobarbital (Euthanyl Forte®, Bimeda-MTC) and a necropsy examination specific to the respiratory tract performed. A nasal swab and serum was collected from all pigs immediately after euthanasia and both samples were stored at -80°C. All necropsies were performed following blood collection. The lungs were removed with the trachea attached and evaluated for the percentage of the lung affected with purple, lobular consolidation typical of IAV infection in swine. The percentage of consolidation for each lobe was calculated using weighted proportions of the total lung volume (Halbur et al., 1996). The proportions were 10% each for the right and left cranial lobes, right and left middle lobes and the accessory lobe, and 25% each for the right and left caudal lobes for a total of 100%. The bronchoalveolar lavage fluid (BALF) sample was collected as previously described (Detmer et al., 2013) using 50mL of phosphate buffered saline (PBS). After

gently massaging the lungs, the PBS was aspirated and 5 ml aliquots of the aspirated fluid were stored at -80°C.

Lung samples were collected 2.5 cm from the tip of the left cranial (A), left middle (B), right cranial (C) and right middle (D) lung lobes and labeled with colored beads and safety pins (Figure 5.1). An additional 1 cubic cm sample of each of A, B, C and D were collected, individually labeled and stored at -80°C until used for further testing. The tissue from the tip of the lobes was fixed in 10% formalin for 24 h, trimmed and paraffin embedded so that the histological sample examined came from approximately 2 cm from the tip of the lobe. The first 4 μm serial section was used for the hematoxylin and eosin (H&E) staining and the second section was used for immunohistochemistry (IHC) with anti-influenza A ribonucleoprotein antibody (National Institute of Allergy and Infectious Disease, Bethesda, Maryland, USA) at 1:5,000 dilution as previously described with 3,3-diaminobenzadine (IMMpact™ DAB; Vector Laboratories, Burlingame, California, USA) chromagen (Haines, 1993).

Dr. Susan Detmer, Assistant Professor in the department of veterinary pathology at the Western College of Veterinary Medicine (WCVM), performed all microscopic pathological assessments in a blinded manner. The H&E stained lung sections were examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation in large, medium and small bronchioles. The following scoring stratagem was used: 0 = no airways affected, 0.5 = only a few isolated airways affected, 1 = localized cluster of affected airways (in 1 or 2 lobules), 1.5 = several airways affected throughout section plus minimal interstitial infiltrates, 2 = several airways affected throughout section plus mild to moderate interstitial infiltrates, 2.5 = several airways

affected, often severely plus moderate interstitial and alveolar infiltrates, and 3 = many airways affected, often severely plus moderate interstitial and alveolar infiltrates (Detmer et al., 2013) (Figure 5.2).

The immunostained lung sections were examined microscopically for the amount of staining intensity present in both airways and alveoli. The following scoring stratagem was used: 0 = no immunoreactivity, 1 = rare to occasional immunoreactivity, 2 = scattered immunoreactivity (<25% of area), 3 = moderate immunoreactivity (25-50% of area), and 4 = abundant immunoreactivity (>50% of area). The separate scores for immunoreactivity in the airways and alveoli were added together for the final score (Gauger et al., 2013) (Figure 5.2).

5.3.5 Virus titration

The nasal swabs collected at necropsy were placed in a vial containing 1 ml Eagle's Minimum Essential Medium (Life Technologies, Grand Island, New York, USA), containing 10% heat inactivated fetal calf serum, 1M Hepes and non-essential amino acids (MEM+) and vortexed for 30s, followed by centrifugation for 15 min at $2272\times g$ within 24 hours of collection and stored at -80°C. The 10% weight per volume (w/v) lung tissue homogenates were made in MEM+ using a Retsch MM400 homogenizer (Retch GmbH, Haan, DE) with 5/32" S/S Grinding balls 97007-688 (VWR International, Edmonton, Alberta) for 2 cycles of 2 minutes at 30Hz followed by centrifugation for 15 min at $2200\times g$. The homogenates for lobes A, B, C and D were pooled, aliquoted, and stored at -80°C.

Nasal swab supernatant and lung homogenate samples were titered using 10-fold serial dilutions and 4 wells per dilution that were inoculated in 48-well plates containing

monolayers of Madin-Darby Canine Kidney (MDCK) cells and using MEM+. All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for five days under an inverted light microscope to observe cytopathic effect (CPE). Virus titers calculated by the Spearman-Kärber method (Villegas and Alvarado, 2008). All negative titers were confirmed negative by matrix real-time RT-PCR procedure (Spackman et al. 2002; Huang et al. 2012; Detection of Type A Influenza Viruses and Avian H5 and H7 Hemagglutinin Subtypes by Real-Time RT-PCR Assay, CFIA protocol) using RNA extraction kit and a real-time RT-PCR kit (Qiagen Inc., Valencia, California, USA) performed at PDS.

5.3.6 Cytokines and additional diagnostic testing

One aliquot of BALF from each piglet was submitted to Aushon BioSystems Inc. (Billerica, Massachusetts, USA) to determine the cytokine concentrations of Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), and Interleukin 8 (IL-8) using their SearchLight Array Technology. The lung collected from the healthy pig had no significant macroscopic or microscopic lesions (same A, B, C and D samples), and was RT-PCR negative for influenza A virus (as described in the previous section), PRRSV, porcine circovirus type 2 (PCV2), and Mhyo (all tested by PDS).

Additional diagnostic testing was performed to determine the status of all inoculated pigs for North American and European (NAEU) variants of PRRSV, PCV2 and Mhyo. DNA and RNA were extracted from 30 mg of the right middle lung lobe using a commercial kit (AllPrep DNA/RNA Mini Kit, Qiagen, Toronto, ON, Canada). Extracted product was stored at -20°C. RNA was tested for PRRSV using a commercial kit as per manufacturer instructions (Tetracore RTqPCR, Gaithersburg, MD). DNA from

lung samples was tested for PCV2 by SYBR green quantitative, real time polymerase chain reaction (qPCR) (McIntosh et al., 2009) and Mhyo by conventional PCR (Mattsson et al., 1995).

5.3.7 Statistical Analysis

Statistical analysis was performed using Stata Statistical Software, Release 13 **(**StataCorp. 2013, College Station, TX: StataCorp LP**).**

Raw data was assessed for normality by evaluating the skewness and kurtosis values. Non-normal variables were log base 10 transformed. Normality of the log-transformed variables was assessed. Multilevel mixed-effects linear models (XTMIXED for continuous outcomes and XTMELOGIT for dichotomous outcomes) accounting for batch and sow ID were developed to assess the relationships between outcome variables and biologically plausible fixed effects. Fixed effects included: treatment group, parity, whether or not the sow was induced to farrow, season of farrowing (season 0 = wean dates between November 3 and December 7; season 1 = wean dates between January18 and March 7), Z-score of litter birth weight from historical average, wean age and lactational average daily gain. Random effects were litter of origin (sow ID) and weekly batch. Dichotomous outcomes included: presence or absence of nasal discharge. Continuous outcome variables included: area under the curve (AUC)[1] of body temperatures throughout the trial period, percent lung lobe affected, percent total lung affected, cytokine concentration in BALF (IL1β, IL6, IL8), histological score of bronchiolar epithelium and peribronchiolar inflammation in medium and small bronchioles, IHC scores (amount of immunoreactivity present), and IAV titers (TCID50s)

[1] AUC=(24-0)*(temp24hPI + tempD0)/2 + (30-24)*(temp24hPI + temp30hPI)/2 + (48-30)*(temp30hPI + temp48hPI)/2

in the 10% w/v lung homogenates and nasal swabs. Certain variables were dichotomized, where 0 indicated absence and 1 indicated presence at any time point during the study, based on their natural distribution in the dataset, including: responsiveness (level of alertness), respiratory rate and effort, physical appearance of hair coat, respiratory signs (coughing or sneezing), histological inflammation scores of trachea and turbinate.

Three-level models accounting for piglet, batch and litter of origin were initially run. If the variance at the batch-level was or approached zero, this random effect was removed and a 2-level model re-run. If the model fit improved, the simplified model was retained. If the random effect controlled a substantial portion of the total variation, it was left in the model. Fixed effects with $P<0.2$ in univariate analyses were considered in the full model if biologically plausible and not correlated or intervening. A backwards-stepwise removal of fixed effects was performed, and fixed effects $P<0.05$ were retained in the final model. Final models were evaluated for assumptions of normality and homoscedasticity of residuals at all levels of random effects. Outliers were identified and removed and model diagnostics repeated where appropriate.

5.4 Results

5.4.1 Animals

In total, 68 piglets were selected for the study. One died during the acclimation phase, two died during recovery from sedation, and one piglet was diagnosed with bacterial pneumonia on post mortem examination. All of these were excluded from the analyses. All analyses included 64 piglets from a total of 14 weekly batches.

The average birth weights of HBW and LBW pigs selected were 1.61 ± 0.07 and 1.29 ± 0.17 kg, respectively, and the average Z-scores of the high and low birth weight

litters were 1.0 and -1.9 standard deviations respectively ($P < 0.05$ for both). The Z-score was calculated as: $Z_{Litter\ i}$ = (litter weight$_i$ – mean litter weight$_{cohorts}$)/SD$_{cohort\ mean\ litter\ weight}$, where *cohorts* includes the population of sows of similar parity and litter size over the previous 2 years (see Table 5.3). The average weaning age of HBW and LBW pigs selected were 21.5 ± 1.6 and 21.5 ± 1.7 days, respectively. Across both groups, the mean growth rate during lactation was 0.214 ± 0.043 kg/d, which did not differ by group. Average wean weights, 6.2 ± 1.1 for HBW and 5.9 ± 1.0 for LBW, did not differ by group but the numerical difference (0.3 kg) was similar to the numerical difference in birth weight.

5.4.2 Clinical Scores

No significant differences were found in either qualitative or quantitative measures of clinical disease expression.

5.4.3 Gross Pathology

The percentage of total lung affected by lesions consistent with IAV infection was higher ($P \leq 0.05$) in piglets from HBW compared to LBW (Table 5.2). When the extent of pathological involvement of the accessory lobes, cranial lobes and middle lobes were compared across groups, the same differences were noted with the percentage of lung affected in these lung lobes being higher in HBW when compared to LBW pigs. During model diagnostics for pathological involvement of cranial lobes, one piglet (528) was identified as an outlier in that its score fell outside of 3 standard deviations from the mean. This animal was not included in the final model.

5.4.4 Histopathology

Histological scores for the left cranial lobe, right middle lobe and total lung were all higher ($P \leq 0.02$, 0.02, 0.01, respectively) in the HBW compared to LBW group. There were no significant differences between histological scores for left middle and right cranial lung lobes between HBW and LBW pigs (Table 5.2). There were no significant differences between the presence of histological scores in trachea or turbinates between the two groups.

5.4.5 Immunohistochemistry

Immunohistochemistry (IHC) scores were higher in the middle lobes in the HBW versus LBW pigs (Table 5.2). The average overall IHC lung score decreased ($P \leq 0.001$) in the winter batches compared to the autumn batches. Mean IHC values were as follows: lung average = 1.9 ± 1.0 vs 1.25 ± 0.56 (p=0.001); right middle lobe = 2.0 ± 1.1 vs 1.3 ± 0.71 (p=0.002); left middle lobe 2.0 ± 1.2 vs 1.3 ± 0.86 (p=0.007) for autumn and winter batches, respectively. As well, IHC score in the left middle lobe increased (coefficient=0.15; standard error = 0.08; confidence interval -0.02 to 0.29) with increasing weaning age (p=0.05). No significant differences in IHC scores in the cranial lobes were found between the two groups (mean IHC score left cranial lobe = 1.4 ± 0.9; mean IHC score right cranial lobe = 1.4 ± 0.87).

5.4.6 Virus titers

The log10 mean virus titer from nasal swabs was lower in progeny from parity 2 sows than from parity 1 sows (mean parity 1 = 2.4 ± 1.5; mean parity 2 = 1.4 ± 1.4; p=0.01). The mean virus titer from lung tissue trended lower in LBW versus HBW pigs, although the difference was not significant (Table 5.2).

5.4.7 Cytokines

Mean log10 values of cytokines were as follows: ILβ 2.3 ± 0.52; IL6 1.5 ± 0.57; and IL8 1.6 ± 0.37. No significant differences in cytokine concentrations in BALF samples between HBW and LBW pigs were noted.

5.5 Discussion

The statistically significant findings in this study were that HBW pigs had a higher percentage of total lung, accessory, cranial and middle lobe involvement, higher histological scores for the left cranial lobe, right middle lobe and total lung, and higher IHC scores in the middle lobes. The average overall IHC lung score decreased in the winter batches compared to the autumn batches.

IUGR is specifically defined as asymmetrical growth with relative brain sparing (Ladinig et al., 2014a). Body and organ weight measurements are required to completely ascertain whether or not a neonate is IUGR; however, small for gestational age (SGA) is considered a proxy for IUGR (Stoknes et al, 2012; Lohaugen et al., 2013; Wu et al., 2006). Obtaining on farm birth weights relative to gestational age is relatively easy and is thus commonly used to detect IUGR (Wu et al., 2006). Studies have shown that even average birth weight pigs selected from low birth weight litters carry the same negative phenotypic traits associated with IUGR as their lower birth weight litter mates (Pardo et al., 2013). For these reasons, a finding of low litter birth weight was used as a marker for the occurrence of IUGR in piglets born in these litters. Choosing average birth weight piglets from each litter rather than selecting a random sample of low, medium, and high birth weight pigs, reduces the potential sources of variation for the model by only assessing the effect of between litter variation and not within litter variation. Ensuring

that the average litter birth weights, represented by Z-scores, were significantly different between groups and did not overlap confirmed that the pigs within each group differed in terms of their developmental programming.

The average birth weights of HBW pigs selected ranged from 1.54 to 1.68kg and the average birth weights of LBW pigs ranged from 1.12 to 1.46kg. Neither the mean growth rate during lactation nor the average wean weights differed by group. This indicates that the LBW piglets grew just as quickly as the HBW piglets during lactation but remained 0.3kg lighter at weaning. Smit et al. found that pigs from low birth weight litters tended to have lower average daily gains during lactation than piglets from high birth weight litters and this difference became significant in the nursery and grow-finisher phases of production (Smit et al., 2013). It is possible that similar results could have been achieved in the present study if the piglets had not been sacrificed before reaching the nursery. Christenson et al. found that pigs born to gilts subjected to unilateral hysterectomy-ovariectomy had a higher lactational growth rate despite having lower birth weights than pigs born to non-altered gilts. This was attributed to more nutrient availability during lactation since the litters were smaller in the surgically altered gilts and no cross fostering was performed (Christenson et al., 1987). Fix et al. found that lactational average daily gain and weaning weight increased at a decreasing rate with increasing birth weight indicating that the differences were greater for lighter birthweight than heavier birthweight pigs. As well, there was a clear interaction between cross fostering and birth weight with cross fostering exacerbating the differences in lactational average daily gain and weaning weight (Fix et al., 2010). In the present study, cross

fostering was permitted but not tracked so any differences in average daily gain that could have been due to differences in competition were not assessed.

These findings were unexpected based on the original hypothesis that LBW pigs would have more severe disease with IAV infection than HBW pigs although these results are similar to those found by Ladinig et al., (2014a) when investigating birth weight, IUGR and susceptibility to PRRSV. Previous research has shown that, in humans, low birth weight is associated with a predisposition to the development of metabolic and cardiovascular disorders such as type II diabetes and metabolic syndrome later in life (Hales and Barker, 2001; Dunger and Ong, 2005; Fowden et al., 2005; Gicquel et al., 2007; Meaney et al., 2007; Waterland and Michels, 2007; Gluckman et al., 2011; Harris et al., 2011; Woroniecki et al., 2011), altered lung function and chronic lung disease (Landgraf et al., 2008) and significantly lower white cell counts, especially T-cells, in the first year of life (Neumann et al., 1998).

Similarly, in food animal production, low birth weight neonates are at higher risk of intestinal, circulatory, neurological, and respiratory dysfunction (Wu et al., 2006) and have higher preweaning mortality (Smit et al., 2013). In rats, low birth weight male pups have lower total leukocyte and bone marrow cell counts as well as reduced leukocyte migration at 8-9 weeks of age (Landgraf, et al., 2007). The lack of significant differences between HBW and LBW groups in qualitative or quantitative measures of clinical disease expression was, therefore, unexpected. This is consistent with the results achieved by Ladinig et al., (2014a), in which no difference in clinical signs were detected between high and low birth weight gilts in a PRRSV infection model. This lack could indicate that high or low birth weight group has no effect on clinical disease expression. Alternatively,

the strain of virus chosen for this study has previously demonstrated relatively low virulence (Richt et al., 2006; Masic et al., 2009; Masic et al., 2013) and may not have been potent enough to cause clinical signs in the majority of piglets with reasonably robust immune systems.

The finding that the microscopic lesion scores and IHC scores were significantly higher in HBW pigs than in LBW pigs was unexpected especially given the lack of differences in cytokine concentrations in BALF between these two groups. One possible explanation is that the HBW pigs may have a more robust immune system which would lead to an enhanced proinflammatory cytokine response and leukocyte recruitment, in turn leading to more cellular damage, however this was not supported by the cytokine data in the present experiment.

In both groups, the microscopic lesion scores were highest in the right cranial lobe than the other 3 lobes examined (Table 5.2). This may be due to the anatomy of the pig lung. The pig has a tracheal bronchus, which originates cranial to the carina and this supplies the right cranial lobe. The right bronchus supplies the right middle lobe. The bronchial supply to the left lung differs in that the left cranial and middle lobes are supplied by bronchioles that arise from the ventrolateral surface of the left bronchus (Nakakuki, 1994). Histological scores for the left cranial lobe, right middle lobe and total lung were all higher in the HBW group. This is consistent with the gross pathological results. There were no significant differences in the amount of caudal lung lobes affected between the two groups. This would be consistent with intratracheal influenza inoculation and the normal cranioventral distribution of IAV in pigs.

The average overall IHC lung score decreased in the winter batches vs. the autumn batches. Autumn can be a challenging time for ventilation in commercial hog barns. Days are often still very warm while the overnight temperatures can drop rapidly to quite low temperatures. Stage 2 fans in the farrowing rooms are not yet closed up during this season and ventilation rates can be high to combat the high daytime temperatures. When the evening temperatures drop, the ventilation systems often cannot adjust as rapidly as the temperatures fall and this can lead to chilling of the piglets in these rooms. During the winter, usually only the first stage fans run to combat humidity and the temperature in the rooms tend to stay more constant. The stress due to ventilation challenges and potential chilling of the piglets classically leads to increases in respiratory disease in young pigs in commercial barns (Dennis, 1986; Sanchez-Vazquez et al., 2012).

The autumn weaned batches of pigs in this study could have been exposed to higher levels of stress and chilling due to ventilation challenges during the 4 weeks spent in the commercial barn before being moved to the ACU. This higher level of stress can increase glucocorticoid secretion, which in turn has an immunosuppressive effect (Davis, 1998). These ventilation challenges could also have an effect on innate barriers to infection. The transition from the high ventilation rates of summer to lower nighttime ventilation rates in the fall could increase the amount of dust, endotoxin and bacterial contaminants within the farrowing rooms. This higher exposure could lead to compromise of the mucosal epithelium of the respiratory tract thereby allowing more influenza virus access to the lung.

In the statistical model, the weaning age had an effect on the IHC scores for the left middle lobe. Overall the score increased as weaning age increased; however, there is no apparent biological explanation for this finding.

The mean virus titer from lung tissue trended lower in LBW pigs, although the difference was not significant. This is also consistent with the results of Ladinig et al (2014a), who found no significant differences in serum and tissue PRRS viral load between high and low birth weight pregnant gilts even though their IUGR fetuses had lower PRRS viral loads in the thymus and at the maternal-fetal interface than non-IUGR fetuses. The titer results in the IAV study are appropriate in light of the gross, histological and immunopathological data collected. The implication is that LBW pigs have lower levels of replicating virus in the lower respiratory tract 48 hours post inoculation. No differences in nasal swab titers were noted between the two groups. This is to be expected as the intratracheal route of inoculation results in low nasal titers and therefore any differences, if they exist, may not be detected by the sample size used in this study (Vleeschauwer et al., 2009). The concentration of virus isolated from nasal swabs was significantly lower in progeny from parity 2 sows indicating that these piglets were shedding fewer viruses. Decreased nasal shedding could be due to parity 2 progeny having reduced viral replication in the lung. Alternatively, there may have been factors present in parity 2 progeny preventing nasal shedding. Parity 2 progeny may have experienced peak nasal shedding earlier and, therefore, the concentration would be lower than that of parity 1 progeny by the time of sampling.

Cytokines were not significantly different between groups. This was not surprising given that there were no differences in clinical signs between the groups and

the levels of the cytokines IL-1, IL-6, IL-8 and IFN-γ correspond with clinical signs (Van Reeth et al., 2002; Kowalczyk et al., 2010; Barbé et al., 2011). This is also consistent with the results published by Ladinig et al., (2014a) who found no differences in the levels of investigated cytokines were found between high and low birth weight groups.

Table 5.1 Clinical signs of respiratory disease caused by IAV infection that were assessed for each pig.

Score	Clinical Signs
Attitude and responsiveness	
0	Alert and active
1	Alert, but slower than pen mates
2	Reluctant to move, but gets up by stimulation
3	Down, doesn't respond with stimulation or demonstrates seizures (leg paddling, recumbency, opisthotonous)
Respiratory rate & effort	
0	Normal
1	Increased respiratory rate
2	Increased respiratory rate, slight abdominal breathing (dyspnea)
3	Increased respiratory rate and marked abdominal breathing (dyspnea)
Appetite and body condition	
0	Normal appetite and body condition
1	Reduced feed intake, normal body condition
2	Anorexic, slight loss of body condition and weight (>10% loss of body weight)
3	Anorexic, moderate loss of body condition and weight (>15% loss of body weight)
Coughing and sneezing	
0	No coughing or sneezing
1	Observed coughing/sneezing once while in the room
2	Observed coughing/sneezing 2-3 different times while in the room
3	Observed continual coughing/sneezing while in the room

Table 5.2 Pathologic lesion assessments in LBW compared to HBW 48h PI.

	LBW (n=32)		HBW (n=32)		*P* value
	Mean	SD	Mean	SD	H vs. L)
Gross lung lesion percentages					
Cranial lobes	3.2	3.0	4.2	2.3	**0.01**
Middle lobes	3.8	3.0	5.7	3.2	**0.01**
Accessory lobe	1.3	1.7	1.8	1.3	**0.05**
Caudal lobes	1.8	1.8	2.0	1.8	0.71
Total lung	10.1	8.3	13.7	7.6	**0.05**
Microscopic lesion severity (scored 0 to 3)					
Left cranial	1.6	0.6	1.8	0.6	**0.02**
Left middle	1.5	0.7	1.7	0.7	0.09
Right cranial	1.9	0.5	2.0	0.5	0.99
Right middle	1.4	0.7	1.8	0.6	**0.02**
Total lung	1.6	0.4	1.9	0.3	< **0.01**
Influenza antigen staining intensity in lung (scored 0 to 4)					
Left cranial	1.4	0.9	1.4	0.88	0.83
Left middle	1.3	1.1	1.8	0.9	**0.02**
Right cranial	1.4	0.9	1.4	0.9	0.99
Right middle	1.3	1.0	1.8	0.9	**0.01**
Total Lung	1.3	0.8	1.6	0.8	0.13
Viral concentration isolated from lung and nasal cavities					
Lung (log 10)	3.0	1.5	3.5	1.4	0.06
Nasal cavity (log 10)	2.0	1.6	1.9	1.5	0.58

Bolded *P* values are significant (*P* < 0.05) based on multilevel regression (STATA; xtmixed)

Table 5.3 Individual litter characteristics showing high/low birthweight group, batch, sow parity, z score, total born and total born weight.

Z-score	Sow ID	Group	Batch	Parity	Total born	Average birth weight (kg)
-4.36	G2633	L	13	1	M	0.85
-3.33	G2480	L	4	1	15	1.13
-3.20	G2205	L	4	2	17	1.13
-2.32	G2640	L	12	1	13	1.26
-2.31	G2618	L	10	1	15	1.24
-2.26	G2383	L	11	2	12	1.26
-1.92	G2608	L	9	1	11	1.36
-1.76	G2662	L	12	1	16	1.17
-1.63	G2564	L	5	1	11	1.39
-1.59	G2147	L	4	2	10	1.50
-1.55	G2540	L	7	1	12	1.26
-1.37	G2117	L	1	2	13	1.40
-1.01	G2590	L	8	1	10	1.42
-0.99	G2399	L	14	2	12	1.41
-0.90	G2271	L	6	2	14	1.43
-0.75	G2216	L	3	2	10	1.57
-0.98	G2132	H	4	2	16	1.58
0.70	G2403	H	12	2	17	1.52
0.72	G2304	H	7	2	11	1.65
0.72	G2537	H	6	1	13	1.59
0.75	G2583	H	10	1	14	1.57
0.80	G2465	H	4	1	12	1.58
0.83	G2588	H	9	1	15	1.57
0.87	G2339	H	8	2	11	1.68
0.87	G2574	H	5	1	12	1.59
0.97	G2387	H	11	2	17	1.55
0.97	G2668	H	14	1	17	1.53
1.09	G2149	H	2	2	15	1.63
1.16	G2579	H	12	1	12	1.63
1.28	G2126	H	1	2	16	1.61
1.86	G2649	H	13	1	M	1.7
2.01	G2213	H	4	2	15	1.74

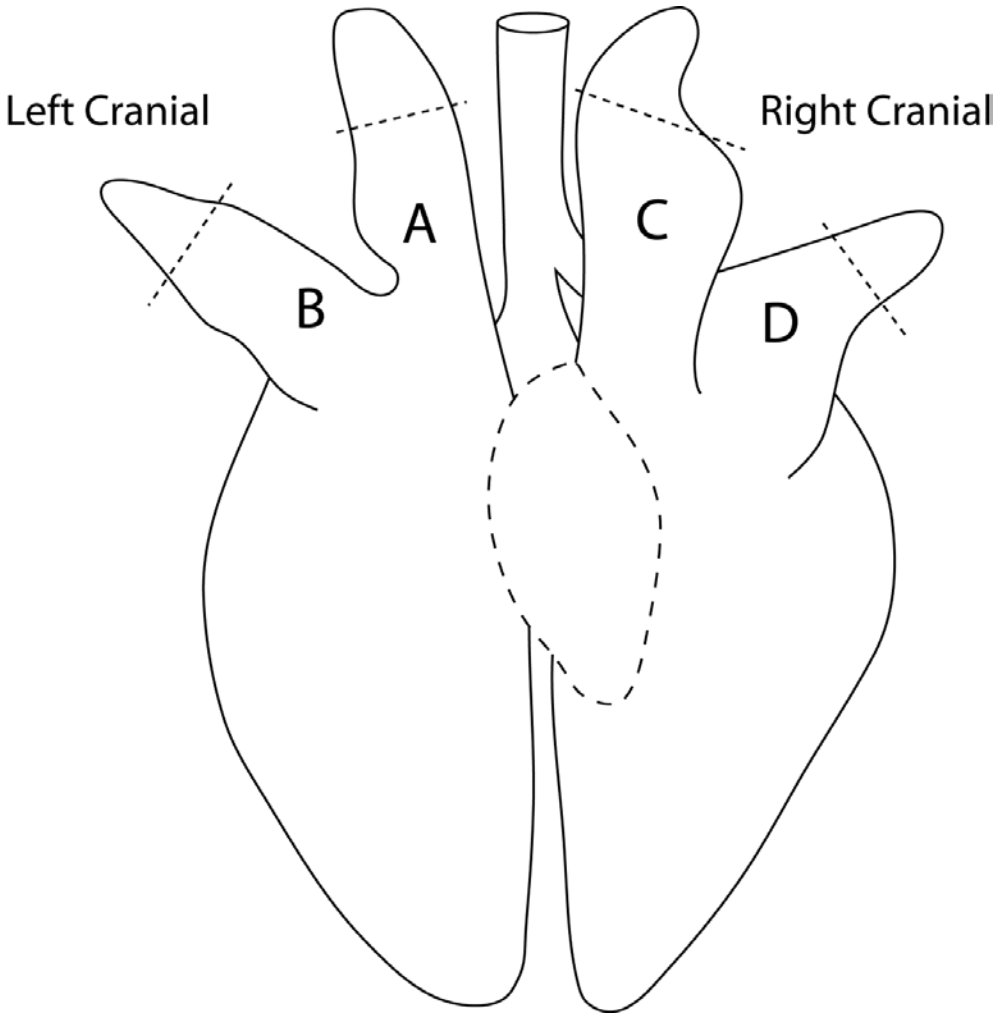

Figure 5.1 Diagram of the dorsoventral aspect of the lungs showing lung lobe designations (A, B, C, and D) and the approximate location of sampling.

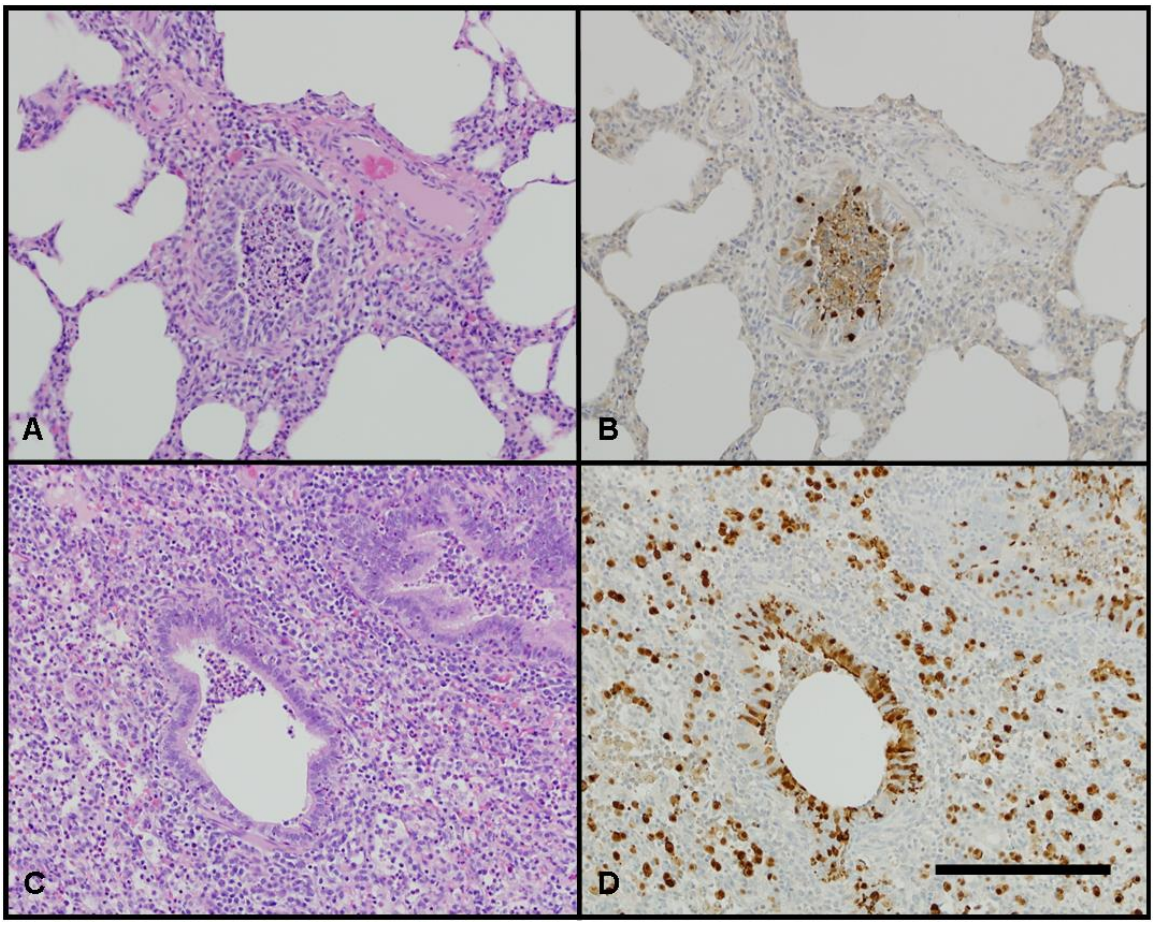

Figure 5.2 Examples of histopathological and immunohistochemical scoring system used in piglets of high and low birth weight litters experimentally inoculated with influenza A virus. (A) The bronchiolar epithelial changes are in only a few isolated airways (score 0.5) to a localized cluster within one or two lobules (score 1). (B) The immunoreactivity is mild and affecting < 25% of the bronchiolar epithelial cells. (C) An example of severe inflammation affected bronchioles, alveolar walls and alveolar spaces (Score 3). (D) The immunoreactivity is abundant within the bronchiolar epithelial cells and alveoli. Note: actual scores are based on scan of the entire tissue section. Bar = 200μm.

6. STUDY LIMITATIONS

The research presented in this thesis represents the first study to directly investigate the effect of intrauterine growth restriction (IUGR) on the clinical disease expression of influenza A virus (IAV) in pigs. The majority of the research has used retrospective human studies to evaluate the effect of IUGR on metabolic disease or immune response to vaccine challenge; and rodent models have been mostly used to determine the mechanisms of inducing IUGR and its effect on indirect measures of immune function such as immune cell number and function. This research is the first to evaluate the effect of naturally induced IUGR, which is common in pigs, on infectious disease severity using animals experimentally infected with influenza. This chapter details the limitations encountered during development of the experimental protocols and while conducting the experiments, and Chapter 7 considers future directions that should be pursued as a result of the findings, and the general conclusions of the research presented herein.

6.1 Protocol development issues

6.1.1 Animal Sourcing

Finding an appropriate barn from which to source piglets for this trial proved to be difficult. The first batch of 6 piglets was nasal swabbed for IAV and blood sampled for Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), Torque Teno Virus (TTV) and Porcine Circovirus type 2 (PCV2) testing at arrival. Two of the nasal swabs were positive for IAV when tested by a matrix real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) procedure at Prairie Diagnostic Services, Inc. (PDS) using the Canadian Food Inspection Agency (CFIA)-approved protocol

(Spackman et al., 2002; Detection of Type A Influenza Viruses and Avian H5 and H7 Hemagglutinin Subtypes by Real-Time RT-PCR Assay, CFIA protocol). PDS also tested the serum samples for antibodies to IAV H1N1 and H3N2 by Enzyme Linked ImmunoAssay (ELISA) (Idexx Laboratories, Westbrook, Maine, USA). Nasal swab samples were repeated two days after the initial swabs were taken. This time, 5 of 6 swabs and 2 of 6 serum samples from these same pigs were positive. The positive RT-PCR results on the nasal swabs indicated an active infection in this group, with nasal shedding. The positive serology could have been indicative of a mounting antibody response within the individual pig, or could have been due to maternal antibodies. No follow up serum samples were taken to determine if the initial result was due to acute, convalescent or lactational antibodies. Due to these results, animals from this farm were not used and a second farm was sought as an animal source. An alternative farm to supply pigs for the experiment was identified. Both sows of varying parities and nursery piglets were bled and nasal swabbed from the second selected barn, and antibodies to IAV were found in sows of all parities. Again, this farm was ruled out as a source of pigs and a third barn was sought. The third barn, from which pigs were ultimately sourced, showed antibodies to IAV but only in sows of third parity or higher, which indicated a past infection. Nasal swabs were taken from 20 pigs in the nursery and all were negative by RT-PCR tested at PDS, indicating that no active circulation of virus was present. Therefore, piglets from first and second parity sows were deemed acceptable and appropriate for the experiment.

6.1.2 Pilot 3

The goal of this trial was to euthanize one pair of piglets consisting of one high-birth weight litter (HBW) and one low-birth weight litter (LBW) piglet every 24 hours post inoculation (PI) to assess which time point had the most severe lesions. One piglet was found dead at 24 hours post inoculation (HPI) and was necropsied immediately. On gross examination, the only abnormal finding was that the lungs failed to collapse and were heavy and moist, likely indicative of pulmonary edema. Due to the unexpected death, not all of the planned data could be collected for this pig.

6.1.3 PCV2 PCR

Development issues occurred when testing DNA extracted from lung collected at necropsy for PCV2 by in-house SYBR green PCR (MacIntosh et al., 2009). The established melting curves for the PCR product contained peaks that did not correspond to the melting temperatures for PCV2. There was a possibility that excess primers, which resulted in primer dimers, were affecting the melting curves. A second run was completed using half the original primer concentration and with a new aliquot of ROX dye. No change was observed with this alteration in the protocol. The PCR products were run on a conventional gel with no bands visualized, indicating a lack of DNA amplification. The unexpected peak was noted at 72°C, whereas the expected PCV2 peak is at 78°C. If the unexpected peak at 72°C was due to primer-dimers, re-running the PCR with a read at 75°C should eliminate the 72°C peak and still capture any PCV2 peaks. No difference was observed with this protocol. The protocol was tried with a different master mix and also in the PDS lab with no effect on Ct. Small peaks were noted in the melting curves for 6 of 7 samples. Samples were re-extracted, run at 50% primer concentration

and the cycles were cut off at 40 and a full standard curve was run. All samples produced the same results and were classified as detectable but not quantifiable.

6.1.4 Tissue homogenates

Tissue homogenates (10% w/v) were created from the two piglets from Pilot #2 since both pigs had lung lesions consistent with IAV at necropsy. This exercise elucidated the problems with creating lung homogenates without the proper tools. Initially, 10 mL of homogenate was created. The exact amount of tissue was weighed to determine the corresponding amount of growth medium to make a 10% solution. Lung samples were cut up in a large tissue culture dish with a #20 scalpel blade. This scored the bottom of the dish and made it difficult to remove all of the lung tissue by flushing, thus impacting the exact weight of lung in the homogenate. No hand held homogenizers nor stomachers were available, so the homogenate was sonicated at 20 kHz at 60 second intervals to try to produce a smooth homogenate. This proved to be impossible; as the time taken to produce a somewhat smooth homogenate by this method was impractical and heated the solution to an extent that killed the virus, as was evidenced by subsequent negative plaque assays. Disposable pellet pestles and one cycle of sonication were used for subsequent homogenates; however, this too did not produce a smooth homogenate thus calling into question the accuracy of the plaque assay titers. The plaque assay results from this method of homogenizing were 2.5×10^5 and 1.25×10^6 plaque forming units (PFU)/mL, respectively, for the two pigs sampled for this initial test compared to the pure virus titer of 3×10^8 PFU/mL. For the main trial, a Retsch MM400 homogenizer (Retch GmbH, Haan, DE) with 5/32” S/S Grinding balls 97007-688 (VWR International,

Edmonton, Alberta) was used to provide a consistent protocol for creating the homogenate.

6.1.5 IAV multiplex PCR

Validation of a real-time reverse transcriptase multiplex PCR for Matrix, H3 and N2 genes of IAV (Nagarajan, et al., 2010) was attempted for use in our lab. The protocol was used as described for these three genes without success. No fluorescence was observed on any channel. Another attempt was made using a different Mastermix on the same samples. Two of the channels (Matrix and N2) amplified, where the third (H3) did not. Since these early attempts were done on clinical samples, another attempt was done using RNA extracted from a dilution of pure virus. This produced the same result, with H3 not amplified. The primer and probe sequences were double checked against the sequences provided in the paper and were deemed to be correct. Since the H3 probe came from a different manufacturer than the other two probes, the protocol was run using only the H3 probe to determine if there was interference between the other primers and probes. No amplification was observed. The protocol was repeated without the probe (as a conventional RT-PCR) and the product was run out on a gel. No band was observed indicating that no RNA was amplified. Another attempt was made using the same Mastermix as described in the conventional PRRSV RT-PCR protocol and used the following program to conduct the amplification:

RT step: 50°C for 30 min

Denature: 95°C for 15 min

Amplification: 94°C for 30 sec; 57°C for 30 sec; 72°C for 30 sec

The amplification step was repeated for 35 cycles. This protocol produced a band at the appropriate weight when the RT-PCR product was run on a gel. This indicated that the primers were successful. The RT-PCR product from this run was purified and frozen at -20°C. For the next attempt, N2 and M were run together, but separate Master mixes were used for each. RT-PCR products from this protocol were run on a gel, where M was amplified but N2 was not. Finally, N2 was amplified on its own with the same protocol and this time a band was produced. This RT-PCR product was purified and stored at -20°C.

Since running the RT-PCR protocol for each gene individually in a conventional thermocycler had more success than multiplexing in real time, we tested the two master mixes for H3 both with the probe and without to see if the inclusion of the probe was causing problems on the real time thermocycler. We decided to run the CFIA protocol on the real time thermocycler at PDS to also determine if there was any issue with the real time thermocycler in our lab, as one specific machine was used in all real time runs. Fluorescence was detected in all samples tested on this run and the RT-PCR products generated were run out on a gel to visualize. All samples produced a band, although the OneStep Mastermix compared to the Quantitect produced the brightest bands. The multiplex protocol was attempted in parallel on both machines; neither was successful in producing Ct results for all 3 genes. RT-PCR products from this protocol were run out on a gel and after 50 minutes, 3 separate bands were resolved showing that the protocol did amplify RNA from each gene, although this was not able to be shown quantitatively on the real time thermocycler. Conventional RT-PCR was performed on RNA extracted from pilot pigs' lung samples and the results correlated exactly with

immunohistochemistry (IHC) results from these samples. This shows that the RT-PCR could be used to determine if the samples were positive or negative, but could not yet be used as a quantitative test. Five, 2-fold serial dilutions were made from virus stock in ultrapure water and the RNA was extracted from this using the QIAamp Viral RNA Mini kit. RT-PCR on the extracted RNA produced amplification of all dilutions in this series within detection limits with a linear standard curve. RT-PCR products were run out on a gel and three bands were observed for each sample at molecular weights corresponding to amplicons for Matrix, H3 and N2.

All 3 purified RT-PCR products were nanodropped to determine the concentration of nucleic acid present and then cloned using TOPO and transformed using One Shot Chemical Transformation Protocol. Patch plates were made colonies formed on the plates and PCR testing was done to ensure the success of transformation. The two colonies that showed the brightest bands on the PCR gel were chosen from the patch plate. These were collected and incubated in Lysogeny Broth (LB) + ampicillin overnight. Each inoculum was frozen in LB + 30% glycerol + ampicillin and stored at -80°C. Plasmid DNA was purified from each inoculum using BIO BASIC Molecular Biology kit – Plasmid DNA kit, nanodropped to determine concentration, and stored at -20°C. Standards were created from purified plasmid DNA with the number of copies/μL calculated from the concentration determined by nanodrop for each plasmid. One master standard mix was created containing 5.76×10^9 copies of nucleic acid from each plasmid/μL. Serial dilutions were created down to 10^0 dilution and stored at -20°C and a real time qPCR program was run on these standard dilutions as previously described in this section. Standard curves had r^2 values between 0.98 and 0.99.

A 20mg sample of lung tissue was excised from presumed IAV negative pigs and a 10-fold dilution of IAV was added to each consecutive sample to create the spiked tissue samples. Samples and viral dilutions were stored at 4°C for 3 hours. These spiked lung samples were then homogenized with a disposable tissue homogenizer. RNA from viral dilutions was extracted using QIAamp Viral RNA Mini kit. RNA from spiked lung samples was extracted using All Prep DNA/RNA Minikit using "Tissues" protocol for unstabilized fresh tissues. When PCR was run on standards, virus dilutions, and spiked lung samples, only the 10^{-2} dilution of virus and spiked lung tissue were considered positive within the detection limits of the standard curve. This is likely an issue with the extraction yield of the kits themselves.

RT-qPCR was performed on RNA extracted from pilot pigs' lung samples and nasal swabs using Qiagen OneStep RT Mastermix and Matrix standards created from purified M3 colony plasmids. The same primer and probe concentrations were used as for the quantiTect kit. Matrix protein was detected, but resulted in high Ct values and the standard curve was not linear. These results indicated that the RT-qPCR protocol used could detect influenza Matrix protein, but only if the sample contained very high amounts of viral RNA and could not be used to calculate a virus titer. Therefore, for the experiments we needed to use the CFIA-approved protocol at PDS.

7. GENERAL DISCUSSION

The viral dosage used in this study is similar to those used in other IAV inoculation studies in pigs (Masic et al., 2009; Xu et al., 2012; Kowalczyk et al., 2014). While the intratracheal route of inoculation used in this study does not exactly mimic the natural infection route, it does have benefits for use in a clinical trial. This method bypasses the upper respiratory tract and its barriers and has been shown to cause infection with increased clinical symptoms and more prominent gross pathological lesions than intranasal inoculation (de Vleeschouer et al., 2009). However, in the present study clinical signs were not consistently noted and so in a future experiment it might be beneficial to use a combination of intranasal and intratracheal administration to increase the severity of clinical signs (de Vleeschouer et al., 2009).

The main conclusions of this study were that pigs born in high birth weight litters had more gross and histopathological evidence of disease when experimentally inoculated with IAV. This is contrary to what was expected based on a review of the available literature review and seems to suggest that pigs from high birth weight litters are more at risk from influenza virus infection. It will be important to further identify the mechanisms that could be at the root of these results before any broad recommendations regarding birth weight are proposed. The first step in the pathogenesis of influenza is the attachment and replication of the virus within the respiratory epithelium. In this study, the mean virus titer from lung tissue trended lower in LBW pigs, but was not statistically significant. If the virus titer were indeed lower in the lung tissue of LBW pigs, this would support and help explain the decreased pathologic evidence of disease in this group compared to HBW pigs. However, Ladinig et al., (2014a) did not find any differences in

PRRS viral load between high and low birth weight pregnant gilts and that their IUGR fetuses had lower PRRS viral loads in the thymus and at the maternal-fetal interface than non-IUGR fetuses.

The finding that the microscopic lesion scores and IHC scores were significantly higher in HBW pigs than in LBW pigs was unexpected especially given the lack of differences in cytokine concentrations in BALF between these two groups. In a related study in which the transcriptome of cranial lung samples was compared between a subset of high and low birth weight pigs from this study, pigs with higher percentage of cranial lung lobe consolidation had higher expression levels of genes associated with the inflammatory response (Wilkinson et al., 2014). Thus, there may have been other inflammatory factors or interactions contributing to the increased severity of pathologic lesions that were not investigated in the present study.

This study did not investigate the effect of litter birth weight on the number or function of immune cells, such as lymphocytes. Ladinig et al., (2014a) did investigate differences in peripheral blood mononuclear cell counts between high and low birth weight gilts. The only significant differences found were that HBW gilts had lower γδ T cell counts than LBW gilts. This is not likely to be a factor in a short duration IAV study since γδ T cells production should not be appreciable at 72 hours post inoculation.

Weaning age had an effect on the IHC scores for the left middle lobe. Overall the score increased as weaning age increased. It was speculated that pigs that were older at weaning might have benefited from extra days on the sow. Also, older pigs would be larger and this could translate into a larger lung tidal volume allowing more virus to reach deeper into the lower respiratory tract. However, since the increased IHC staining

intensity was only evident in one lobe, it is less likely that these reasons could adequately explain these findings.

It will be critical to understand differences in immune function between high and low birth weight litters to understand the implications of the results found in this study. It would be interesting to use this model to investigate total viral shedding over a longer period of time or to investigate recovery from disease. Although piglets from high birth weight litters had more gross and microscopic pathological lesions, this could be protective. While the infection of and replication within host cells of influenza virus causes direct damage to those cells, the subsequent activation and response of the immune system through cytokine production and leukocyte recruitment leads to the most severe damage to the respiratory tract. This might indicate that pigs with more pathological lesions would experience a shorter interval for return to normal lung anatomy. Total viral shedding over the natural course of disease would be important to ascertain for herd health reasons. If piglets from high birth weight litters have a shorter disease course and shed fewer viruses overall than pigs from low birth weight litters, then litter birth weight could be an important consideration for the epidemiology of IAV in commercial barns.

Since cytokines are important in the pathogenesis of IAV, studies focusing on cytokine production will be necessary. An experiment with a shorter interval from inoculation to euthanasia and/or using a more virulent strain of IAV to produce more consistent and measurable clinical signs will elucidate the differences in cytokine production between HBW and LBW litters. Collection of BALF for cytokine analysis should be done at 24 hours post inoculation to coincide with peak production. Repeating

this study using a different disease or by using a co-infection model with IAV and Mhyo may potentiate the clinical signs of IAV, which could further clarify the results. Research using bacterial diseases may also illuminate whether or not these results are unique to viral infection or if similar results are achieved with disparate diseases. Given the difficulty of finding a barn where all parities of sows do not have pre-existing antibodies to IAV, future studies could employ specific pathogen free, caesarian derived-colostrum deprived or snatch-farrowed piglets to ensure a more representative cross section of parities and to avoid gilt litters. The results of the research presented here are surprising given the evidence provided in the literature published on this subject and as such, the most important implication is that the knowledge is incomplete. Most of the literature in humans and animals centers on the effect of birth weight on metabolism, and relatively few focus on how birth weight affects the immune system. The literature that does exist implicates low birth weight as a detriment to immune function, however there are examples of high birth weight also being disadvantageous to future health outcomes of the offspring such as the association between high birth weight and increased risk of schizophrenia (Wegelius et al., 2011), childhood obesity (Oldroyd et al., 2011) and type 2 diabetes in indigenous populations in humans (Klomp 1999); and larger, faster mammary tumor growth in rats (de Assis et al., 2006). This indicates that more research is necessary to provide a clear understanding of the effect of birth weight on clinical disease expression.

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