Dietary Supplementation of Omega-3 Fatty Acids Influences the Equine Maternal Uterine Environment and Embryonic Development

Robert D. Jacobs

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Sciences

Rebecca K. Splan
Alan D. Ealy
Jim Knight
Lori K. Warren

June, 12, 2015
Blacksburg, VA

Keywords: DHA, Pregnancy, Fetal Programming

ABSTRACT

Adverse maternal events around the time of conception influence embryonic development. Thus, aberrations in the uterine environment during early pregnancy, such as those resulting from maternal metabolic or nutritional disruption, can alter gene expression in the developing embryo, leading to variations in its developmental trajectory. Dietary supplementation of long-chain omega-3 polyunsaturated fatty acids (LCPUFA), especially Docosahexaenoic acid (DHA) improves metabolic and reproductive health across species. The objective of this study was to evaluate the effects of peri-conceptual LCPUFA supplementation on endometrial gene expression, uterine health and embryonic gene expression in overweight horses. Thirteen non-lactating light horse mares (mean ± SEM age=13.56±0.11 yr; mean ± SEM BCS=7.07±0.21) were supplemented with concentrate (n=6) or an isocaloric diet containing 0.06 g/kg BW algae-derived omega-3 LCPUFA (n=7) beginning 60 d prior to sample collection. Four consecutive ovulatory cycles were monitored, and uterine endometrial samples were obtained 12 d post-ovulation in cycles 1, 3 and 4. Mares were bred and embryos were flushed 12 d post ovulation 2,3 and 4. Endometrial biopsies obtained from supplemented mares contained increased DHA and omega-3 fatty acids as a percent of total fat ($P< 0.05$). Endometrial biopsy scores were assigned to endometrial tissues and mares receiving the LCPUFA supplementation had improved scores during the first ovulatory period as compared to control animals ($P=0.009$). Candidate genes essential to inflammation, prostaglandin synthesis and embryonic development were evaluated by quantitative reverse transcriptase polymerase chain reaction. Data were log transformed and analyzed using the GLM procedure in SAS

(v9.3). When examining the data independent of breeding and pregnancy status, endometrial obtained samples from LCPUFA supplemented mares contained reduced *IL6* (*P*= 0.04) and TNFa (*P*=0.03) mRNA abundance and tended to have increased transcript abundance for *Uterocalin* (*P*= 0.09)*, SAA* (*P*= 0.06) and *IL10* (*P*= *0.06*). Endometrial samples from mares fed LCPUFA pregnant in cycle 3 contained greater *IL10* (*P*< 0.001), *PTGFS* (P=0.05), *OXTR* (P=0.05) and *PLA2G3* mRNA (*P*= 0.009) and had a tendency for increased *SAA* (*P*= 0.08), *PTGES* (P=0.10) and *SLCO2A1* (P=0.10) mRNA abundance. Supplemented mares bred but not pregnant at day 12 in cycle 3 had reduced expression of *PTGER2* (*P*=0.001) and *PTGS1* (*P*= <0.001) in endometrial samples. In embryos obtained post ovulatory cycle 3 and 4, relative transcript abundance of *GATA4* and *GATA6*, markers of endoderm differentiation, along with *GATA3* and *ELF3*, markers of trophectoderm differentiation were greater (*P*< 0.05) in embryos from LCPUFA supplemented mares (n=5), than controls (n=5). These results indicate that algae-derived LCPUFA supplementation during the peri-conceptual period alters the post-ovulatory uterine environment in the horse by modifying expression of genes related to inflammation and regulating prostaglandin synthesis. Additionally, embryos obtained from supplemented mares displayed differential gene expression related to embryonic lineage specification.

ACKNOWLEDGEMENTS

I would first like to thank my family without whom I would not have gotten to this point. My wife Alana has stood by me unconditionally as I have worked towards my educational and professional goals. Without her support and love, I would not be where I am today. As far back as I can remember my parents have pushed me to pursue goals that I may have thought to be impossible. No matter what I decided I wanted to do in life, I knew that I had the support of my parents and I cannot thank them enough. My brother has always appreciated my educational goals no matter how different they were from his own and no matter what would always take the time out to listen to me. From the bottom of my heart, thank you to all of you.

My advisor and mentor Dr. Rebecca Splan has truly supported me from the beginning. Whether it was learning from her or learning with her, I appreciated the time that she took to make sure that I succeeded. From the beginning Dr. Splan recognized my strengths and my weaknesses and helped me to improve where I needed and grow wherever possible. I realize the time and devotion that she put in to mentoring me and preparing me for a successful future career. I thank her for teaching me, trusting me and helping me to develop as a researcher and as an educator.

My committee members, Dr. Alan Ealy, Dr. Jim Knight and Dr. Lori Warren have all been instrumental in my academic and professional development. Dr. Ealy accepted me into his lab and treated me as one of his own graduate students and I truly valued the experiences working and learning from him. Dr. Knight trusted me with teaching responsibilities from the start that allowed me to truly develop as an educator. I value the time he has spent on my committee as well as the time he has spent observing me

teach and give seminars. Dr. Warren has been present since the start of my graduate career at the University of Florida. She has been an incredible mentor and resource and has supported me through both of my graduate degrees. Thank you to all my committee members for the guidance you have given me, and your devotion to my academic and professional growth.

I would also like to thank the various staff and interns of the Virginia Tech MARE Center, without whom I would not have been able to get to this point. Rita Rollison, Jake Grove and all of the undergraduate and graduate interns were instrumental in helping me with my research and allowing me to be a part of the MARE Center alumni. I would like to thank all of the graduate students, postdocs and lab techs that I have had the pleasure to work with at Virginia Tech. Dr. Jennifer Bradley provided me with unending technical support in the laboratory and without her, none of this work would have been completed. Sarah McCoski, Bradley Reinholt, Meghan Macghee, Jason Smith and all of the other graduate students that I have worked with have truly been a family here at Virginia Tech and I thank you all for your support. Finally, I would like to thank my collaborators from the Smithsonian Conservation Biology Institute. Parker Pennington and Dr. Budhan Pukhazhenthi have been absolutely fantastic to work with and I have enjoyed the time spent with both of them.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AAEP	American Association of Equine Practitioners
AIRg	Acute insulin response to glucose
ALA	Alpha linolenic acid
BCS	Body condition scoring
BMI	Body mass index
CDX2	Caudal-related homeobox 2
CNS	Cresty neck score
COX-2	Cyclooxegenase 2
DHA	Docosahexaenoic acid
DI	Disposition Index
DoHAD	Developmental Origins of Health and Disease
ECD	Equine Cushings Disease
eCG	Equine chorionic gonadotropin
EMS	Equine Metabolic Syndrome
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FOAD	Fetal Origins of Health and Disease
FSH	Follicle stimulating hormone
FSIGTT	Frequently sampled intravenous glucose tolerance test
GLUT4	Glucose transporter 4
GnRH	Gonadrotopin releasing hormone

ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IL	Interleukin
IR	Insulin resistance
IVF	In vitro fertilization
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LH	Luteinizing hormone
MIRG	Modified insulin-to-glucose ratio
n-3	Omega 3
NAHMS	National Animal Health Monitoring System
OCT4	Octamer 4
PE	Parietal endoderm
PGE_2	Prostaglandin E_2
$PGF2_a$	Prostaglandin $F2_a$
PPID	Pituitary Pars Intermedia Dysfunction
PrE	Primitive endoderm
PS	Primitive streak
PUFA	Polyunsaturated fatty acid
QUICKI	Quantitative insulin sensitivity check index
RISQI	Reciprocal inverse square of basal insulin
Sg	Glucose effectiveness
Si	Insulin sensitivity

TE	Trophectoderm
TNF	Tumor Necrosis Factor
USDA	United States Department of Agriculture
VE	Visceral endoderm
VFA	Volatile fatty acid

CHAPTER 1
INTRODUCTION

Female obesity and metabolic dysfunction can impair reproductive function by negatively altering the uterine environment. The subsequent effects on uterine health can alter embryonic development, predisposing the offspring to long-term adverse health conditions. The concept of fetal development affecting later adult disease predisposition represents a recent shift in thinking, forcing researchers to consider how the maternal environment impacts embryonic development.

The negative effects of obesity on reproductive function largely stem from metabolic dysregulation and alterations in inflammatory status. While equine obesity alone is a serious health concern, it is associated with equine metabolic syndrome (EMS), a dangerous condition related to metabolic dysfunction (Geor, 2008). Development of equine metabolic syndrome is associated with various pathologies in the horse, including insulin resistance and chronic low-grade systemic inflammation, which are the key regulators in obesity-related reproductive dysfunction. (Vick et al., 2006; Frank et al., 2010)

The health benefits of omega-3 long chain polyunsaturated fatty acid supplementation have long been recognized and applied across species. The equine industry, recognizing the anti-inflammatory properties associated with omega-3 fatty acids, has routinely incorporated supplementation of these compounds into nutrition practices. Recently, the benefits of omega-3 fatty acid supplementation on the metabolic health of the horse have also been described (Hess et al., 2013). Many of the negative effects of obesity on reproductive health largely stem from metabolic

dysfunction and inflammation dysregulation. That fact coupled with the increasing body of evidence supporting the ability of omega-3 fatty acid supplementation to improve metabolic health and reduce inflammation has lead to investigation into these compounds role in altering reproductive health.

It is important to understand that presently the National Research Council's Nutrient Requirements for Horses does not differentiate between a horse at maintenance and one during early pregnancy with the exception of those that are lactating. With advancements in our understanding of early development and developmental programming, it is worthwhile to consider encouraging feeding strategies to promote offspring health through to adulthood. Increasingly, epidemiological and experimental evidence in a growing number of species supports the hypothesis that early environmental factors experienced by embryos *in utero* may predispose offspring to obesity, metabolic syndrome, and other degenerative diseases common in the current equine population.

This dissertation presents research designed to elucidate potential mechanisms by which alterations of the maternal uterine environment, due to over conditioning and nutritional supplementation during the periconceptual period, may alter embryonic development. It was hypothesized that nutritional supplementation of omega-3 long chain polyunsaturated fatty acids (LCPUFA) from an algae source during the pre and periconceptual period in overweight mares with metabolic dysfunction, would alter endometrial composition and gene expression. Further, it was predicted that these alterations in endometrial gene expression and competency would result in marked differences in gene expression of conceptus tissues relating to embryonic development.

CHAPTER 2
REVIEW OF LITERATURE

Impact of Obesity on Reproductive Function

Modern equine management and nutrition practices combined with reproductive and genetic selection have increased the prevalence of obesity in recent years (Giles et al., 2014). The United States Department of Agriculture (USDA) National Animal Health Monitoring System (NAHMS) in 1998, through an owner-reported analysis, estimated that only 1.4% of the U.S. horse population was overweight or obese. However, a recent study indicated that in a group of 300 mature horses, 19% were reported as overweight while a further 32% were considered obese (Thatcher et al., 2012). Horses in the United States are not alone in this epidemic of increased obesity. Similar increases in the prevalence of obesity are evident in Great Britain as well (Stephenson et al., 2011; Robin et al., 2015). This section will provide a background on equine obesity and its related maladies as well as the effects of obesity on reproduction. Additionally, this section will cover various aspects of equine reproduction and early pregnancy.

Obesity: Determination, effects and pathology

Determination of obesity rates in horses has been difficult. Owner-reported studies, such as the 1998 USDA NAHMS survey, often severely underestimate the prevalence of obesity. Many owners struggle in determining the nutritional status of their horses relying solely on body weight. However, the body weight of an individual horse may fluctuate over a given day resulting in a misrepresentation of the true nutritional status of the horse.

The use of body condition scores to evaluate the nutritional status of horses continues to be adopted by horse owners around the world. Body condition scoring (BCS) as developed by Henneke et al. (1983), is a tool used create a standardized scale to asses a horse's nutritional status (Henneke et al., 1983). Designed to be completed without the use of specialized equipment, BCS measurements are made by visible and physical palpation of six major areas of the horse as displayed in Figure 2-1.

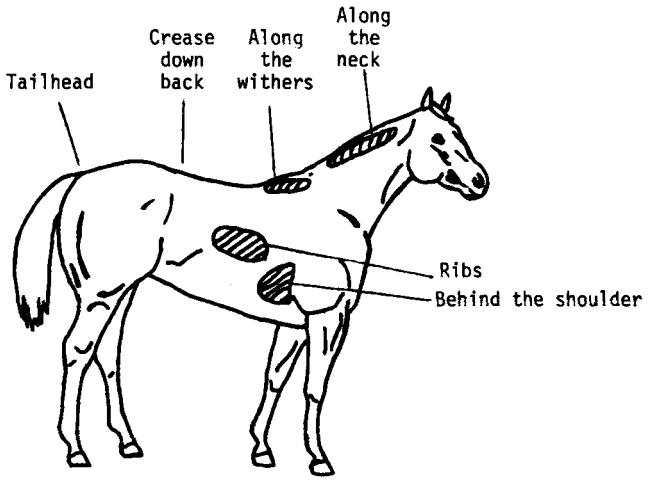


Figure 2-1- Diagram of areas palpated to estimate body fat and condition score (Henneke et al., 1983).

Scores are assessed to individual areas of the horse and overall score is determined as an average across all areas measured and horses are scored on a scale of 1-9. Horses scoring closer to 1 are considered extremely emaciated, while horses scoring closer to 9 are considered morbidly obese. Ideally, the average horse is healthiest with a body condition score falling between 4 and 6; however, an 'ideal' BCS may differ among breeds, disciplines, gender, function and reproductive or lactational

status. Identifying the correct balance of nutrition and exercise is imperative when determining ideal body condition. Body condition score measurements are heavily influenced by an evaluator's ability to assess levels of subcutaneous fat, and while BCS is a better measurement for determining nutritional status of horses than body weight alone, it is still a subjective measurement. Variations on the Henneke system have been adapted to better evaluate specific breeds of horses, but shortcomings and incorrect measurements are still common (Kienzle and Schramme, 2004).

In human medicine, waist to hip ratio and body mass index (BMI) are morphometric measurements commonly used to determine overall adiposity. Researchers have attempted to apply the same BMI measurements to horses, however only a moderate correlation was observed (Donaldson et al., 2004). Most recently associations between regional adiposity and obesity in horses has been investigated with a significant focus on nuchal crest adiposity (Giles et al., 2015). Regional, and more specifically, abdominal fat deposition in humans is associated with various negative health consequences and has been linked to alterations in circulating blood glucose and other metabolic complications (Wagenknecht et al., 2003). Similarly, regional adiposity along the neck and other areas in horses has been associated with various metabolic abnormalities and disorders (Frank et al., 2006). To better classify horses that may be at risk for metabolic dysfunction, a classification known as "Cresty Neck Score" (CNS) was developed by Carter et al., in 2009 (Carter et al., 2009a). This scoring system provides a guideline to assess the deposition of fat along the crest of the neck on a scale of 1 to 5 with increasing fat deposition associated with a higher score. While it is unknown whether neck crest adiposity is the cause of altered metabolic

function, recent evidence suggests that there may be a correlation between higher CNS scores and increased risk of metabolic dysfunction (Carter, 2007; Burns et al., 2010).

Equine Metabolic Syndrome

While obesity alone is a serious health concern that is consistently targeted as a focus area for the American Association of Equine Practitioners (AAEP), it is the constellation of associated maladies that are of the greatest concern to owners, breeders and veterinarians. Excessive weight gain leading to obesity predisposes horses to a condition known as equine metabolic syndrome (EMS). EMS is classified as an endocrinopathy caused by hormone imbalances related to adipose tissue. Adipocytes have been found to be a much more metabolically active tissue than previously thought (Kershaw and Flier, 2004). Adipocytes can release various steroids and cytokines, as well as leptin, adiponectin, resistin and similar compounds now classified as adipokines (Arner, 2005; Li et al., 2015). As adipocyte number and size increase, as is seen in obesity, more steroids and adipokines are released into systemic circulation, and this alters the metabolic status of the individual. Adipocytes also play a significant role in the secretion of various cytokines related to inflammation. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 are highly expressed in obese individuals with increased adiposity (Vozarova et al., 2001; Krogh-Madsen et al., 2006). The increase in circulating and macrophage derived cytokines play a role in the perpetuation of a cycle of increased inflammatory status in obese individuals.

Prior to 2002, maladies now associated with EMS, namely obesity, laminitis and abnormal adipose deposition, were routinely misdiagnosed as being attributed to

hypothyroidism (Johnson, 2002). However, various clinical observations and research trials failed to attribute all the related symptoms to hypothyroidism (Frank et al., 2003; Breuhaus et al., 2006). Clinicians and researchers, striving to better understand the metabolic dysfunction in these horses, began to confuse these cases with equine Cushing's disease (ECD), also known as pituitary pars intermedia dysfunction (PPID), or peripheral Cushing's disease. It was hypothesized that greater adipose deposition resulted in increased synthesis of cortisol like that seen in humans suffering from peripheral Cushing's syndrome (Johnson, 2002). Unfortunately, this hypothesis remains unproven, with little to no evidence that horses develop a disorder similar to peripheral Cushing's disease.

The lack of a clear, pre-existing malady led researchers to categorize this as a new disorder, EMS. In its broadest definition, EMS is an alteration in the metabolic state of the horse. A collection of clinical signs is associated with this pathology, and these include:

1. Increased regional adiposity (Carter et al., 2009a)
2. Insulin dysregulation (Schuver et al., 2014)
3. Predisposition to laminitis (Treiber et al., 2006a)
4. Altered reproductive cycling (Gentry et al., 2002; Vick et al., 2006)
5. Increased systemic inflammation (Vick et al., 2007)
6. Hyperleptinemia (Cartmill et al., 2003)
7. Hypertension (Bailey et al., 2008)

Most commonly, a horse afflicted with EMS will present clinically with obesity, regional adiposity and the development of pasture-induced laminitis (Frank, 2009). As in

humans, horses develop obesity due to increased caloric intake coupled with decreased energy expenditure. This is largely a result of modern management practices, stemming from owner ambivalence and/or ignorance, in which horses are kept in stalls and consume high quantities of grain and high quality forages in multiple daily meals (Thatcher et al., 2012). Certain breeds and types of horses, known as 'easy keepers' are at an especially high risk of excess weight gain. These horses typically maintain excess body condition even with a reduced caloric intake. The genetic component by which horses may be predisposed to developing EMS is not fully understood and further research is required in this area (Frank et al., 2006; Treiber et al., 2006b). However, it has been well established that EMS is observed in a variety of breeds including Arabians, Morgans, Saddlebreds, Quarter Horses and ponies.

Insulin Dysregulation

As a defining characteristic of EMS, insulin resistance is one of the more dangerous pathologies due to its ability to predispose horses to developing laminitis (Kahn, 1978). The mechanism implicating insulin resistance to laminitis has yet to be fully elucidated, but insulin resistance is associated with decreased glucose delivery to the hoof as well as the vasodilatory effects of insulin in conjunction with dysregulation of inflammatory products. Recently, studies involving insulin resistance in horses have evolved to better define the situation as insulin dysregulation (Frank and Tadros, 2014). As a broader definition, insulin dysregulation encompasses insulin resistance, fasting hyperinsulinemia and postprandial hyperinsulinemia (Carter et al., 2009c).

In order to understand insulin dysregulation, it is important to first understand the various approaches utilized to assess insulin sensitivity. The minimal model of glucose

and insulin dynamics can be utilized to determine insulin sensitivity (Hoffman et al., 2003a). This mathematical model accounts for glucose clearance independent of plasma insulin and in response to changes in insulin levels (Bergman et al., 1979; Ward et al., 1991). This model, used to study diabetes in humans has been used in a modified manner to assess insulin sensitivity in horses (Hoffman et al., 2003a). As evaluated by Hoffman et al., (2003a), a frequently sampled intravenous glucose tolerance test (FSIGTT) can be used to collect the data necessary for the minimal model analysis. Briefly, an FSIGTT consists of baseline blood samples collected approximately 30 min prior to the start of the FSIGTT. At 0 min, a glucose bolus is administered intravenously followed by a bolus of insulin at 20 min post glucose infusion. During the 3-h duration of the FSIGTT, 30 venous samples are collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min post glucose administration. (Caumo et al., 2000). Blood samples are analyzed for glucose and insulin concentration and these data are utilized by the minimal model analysis (as depicted in Figure 2-2) to determine glucose effectiveness (Sg), insulin sensitivity (Si), acute insulin response to glucose (AIRg) and disposition index (DI). Definitions are as follows:

Glucose effectiveness- Capacity of glucose to mediate its own disposal independent of a change in plasma insulin

Insulin sensitivity- Capacity of insulin to promote glucose clearance

Acute insulin response to glucose- Endogenous insulin secretion in response to glucose

Disposition index- Describes beta cell responsiveness. Takes into account both AIRg and Si.

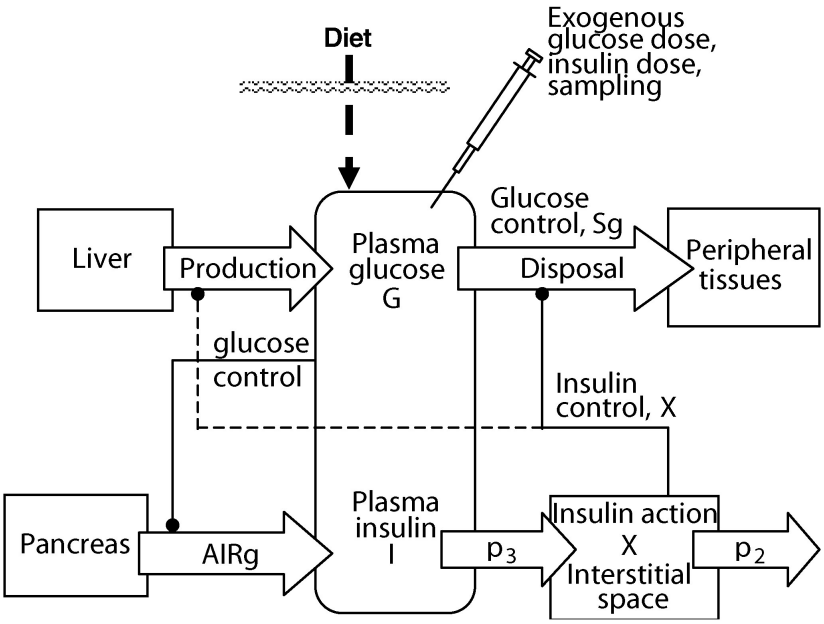


Figure 2-2- Diagram of the minimal model compartments used to interpret glucose-insulin dynamics from a modified FSIGTT. G=plasma [glucose]; I=plasma [insulin]; Sg= glucose effectiveness; X= insulin-mediated glucose response; AIRg= acute insulin response to glucose for the first 10 minutes of secretion post glucose infusion; p_3= contribution of plasma to the remote compartment; p_2= fractional rate of insulin clearance from the remote compartment (Hoffman et al., 2003a).

In addition to extensive investigation as is seen in the FSIGTT, simple single sample predictors of insulin sensitivity have also been used extensively to categorize insulin resistance and glucose responsiveness in human studies (Fukushima et al., 1999; Katz et al., 2000; Laaksonen et al., 2002; Uwaifo et al., 2002; Gungor et al., 2004). These single plasma samples may provide an adequate representation of the

uninterrupted state of the animal. Utilizing parameters of the minimal model, researchers have determined that proxies defined by reference ranges, are adequate in predicting insulin response and sensitivity in horses (Treiber et al., 2005). These proxies, having been evaluated and compared to Si and AIRg all displayed strong correlations (r^2>0.73) (Treiber et al., 2005). Proxies that have been analyzed and adapted for equine use are as follows: reciprocal inverse square of basal insulin (RISQI), modified insulin-to-glucose ratio (MIRG) and quantitative insulin sensitivity check index (QUICKI). Formulas are as follows:

RISQI= $Insulin^{-0.5}$(=1/ √Insulin)

MIRG=(800-0.3 x $[Insulin-50]^2$)/(Glucose-30)

QUICKI= 1/(log[fasting Insulin] + log[fasting Glucose])

These proxies allow researchers and veterinarians to quickly asses the insulin status of the horse, only requiring a single resting plasma sample as opposed to the more intense sampling required with an FSIGTT.

A basic understanding of the methods involved in insulin-glucose dynamics and regulation allows for a better understanding of the pathophysiology of insulin dysregulation. Broadly defined, insulin resistance (IR) represents a failure of the insulin-sensitive tissues, including skeletal muscle, adipose and liver, to respond to insulin (Frank and Tadros, 2014). Due to the inability of these tissues to respond to insulin, horses with IR display hyperglycemia due to increased gluconeogenesis in the liver and increased levels of circulating free fatty acids (FFA) due to lipolysis. Insulin resistance

has continually been linked to the development of laminitis, with dysfunction of glucose transporter 4 (GLUT4) being a key regulator of this physiological phenomenon (Kronfeld et al., 2006). Predisposition of horses with IR to laminitis may be related to the inability of the body to supply adequate amounts of glucose to the hoof, which has a relatively high glucose requirement (Kitamura and Accili, 2004). Horses with EMS may further be predisposed to laminitis due to the direct effects on vascular tone by insulin (Eades et al., 2007). Functioning through a vasodilatory mechanism, hyperinsulinemia would cause a decrease in nutrient flow to the hoof. Horses with EMS demonstrated increased vasoconstriction in the hoof following a carbohydrate challenge, underlying the potential for increased laminitic episodes in EMS horses (Asplin et al., 2007).

Hyperinsulinemia is associated with both increased insulin secretion and delayed insulin clearance in horses. (Frank et al., 2006; Frank and Tadros, 2014). The inability of tissues to recognize or respond to circulating levels of insulin has been implicated as a causative factor in hyperinsulinemia (Treiber et al., 2005). Fasting hyperinsulinemia is a consequence of persistent pancreatic beta cell stimulation (Shanik et al., 2008). Modern management practices in which horses are meal-fed with long fasting periods may play a role in the development of fasting hyperinsulinemia. Of more concern to the equine population is postprandial hyperinsulinemia due to the continual consumption of nutrients in the pasture-grazing horse (Hintz et al., 1978). Even when horses are removed from pasture and fasted, digestion continues in the hindgut, and this produces a long-lasting, hyperinsulinemic response almost constantly. While obesity is a major component of EMS, it is important to note that some obese horses may have a normal insulin response while some non-obese horses may be afflicted with insulin

dysregulation. These cases have forced researchers to consider obesity as a modifying factor, rather than a causative one, which implicates the genetics of the individual for their role in insulin dysregulation. (Johnson, 2002; Carter et al., 2009b; Carter et al., 2010; Borer et al., 2012).

Acting in concert, hyperinsulinemia, obesity and IR may promote poor metabolic health in horses. Hyperinsulinemia stimulates obesity via alterations in lipid metabolism, and through the anabolic effects of insulin on lipid metabolism, may play a role in the development of obesity (Shanik et al., 2008; Mehran et al., 2012). This cycle, correlating aspects of EMS has been proposed and research is ongoing to fully elucidate the mechanisms by which one sign of EMS may be responsible for the development of further signs.

Management of Equine Obesity

Management of obesity in horses can be a complex process requiring alterations in nutritional practices and exercise protocols. Caloric intake can be restricted in obese horses simply by removing all grains from the diet and relying on lesser quality pasture and forages (Frank, 2009). Many horse owners believe that simply moving an obese horse to pasture can reduce BCS, however the unrestricted nature of pasture provides limitless caloric intake for horses (Van Weyenberg et al., 2008). Limiting grazing time, reduction of pasture size and/or the use of grazing muzzles may be advantageous in reducing BCS of the obese horse. The source of calories should also be considered when dealing with obese horses. Forage in the form of moderate quality grass-type hay fed at approximately 2% of body weight should be the sole source of calories for obese horses (Van Weyenberg et al., 2008). Coupled with a reduction in caloric intake, an

increase in energy expenditure may also be beneficial. Horses with an elevated BCS should be exercised to further facilitate weight loss (Powell et al., 2002). Precautions should be taken however, as some exercise protocols may be inappropriate for severely obese horses that may be suffering from laminitis. As with all changes in diet and exercise, the alterations should be made gradually as to not further aggravate problems such as laminitis or joint discomfort. With the interrelationship between obesity and insulin dysregulation, management of obesity may also help to diminish the negative health effects of insulin resistance.

Obesity/Metabolic Disorders and Reproductive Dysfunction

The role of obesity in reproductive dysfunction has been investigated for some time. Perhaps the first known observation came from Hippocrates who wrote: “People of such constitution cannot be prolific… fatness and flabbiness are to blame. The womb is unable to receive the semen and they menstruate infrequently and little” (Brewer and Balen, 2010). Since that time, our understanding of both obesity and reproduction has progressed, however the mechanisms linking obesity to reproductive dysfunction have yet to be fully elucidated (Norman, 2010).

The original mechanisms proposed indicated that obesity caused perturbations in endocrinological pathways, which caused a disruption in the hypothalamic-pituitary-ovarian axis (Hartz et al., 1979). In humans, these disruptions resulted in menstrual cycle disruption leading to reductions in overall fertility associated with altered ovulatory cycles and infertile ovulations. Coupled with fertility decline associated with ovulation disruption, obesity is associated with pregnancy loss among women (Hamilton-Fairley et al., 1992). Demonstrating that the negative effects of obesity on reproduction can be

reduced, multiple studies have observed a rebounding effect on fertility with resumption of normal cyclicity and ovarian function once the individual has lost weight or decreased BMI (Clark et al., 1995; Clark et al., 1998; Tang et al., 2006). These very basic observations implicate obesity for its role in reproductive dysfunction and warrant further investigation.

The reproductive cycle is highly dependent on endocrine signaling through out the body and is governed by a variety of cells and tissues. Adipose tissue is an extremely metabolically active tissue and is the site of significant steroid production and metabolism (Pasquali and Gambineri, 2006). Significant conversion of androgens to estrogens occurs in adipose tissue while the bioavailability of steroid hormones is further impacted by adipose tissue (Gambineri et al., 2002). Overall, obese women display a hyperandrogenaemic state, which is thought to contribute to the reproductive dysfunction and more specifically the abnormal cyclicity seen in these individuals (Leenen et al., 1994). As in humans, obese horses display altered estrous cyclicity with prolonged cycle length and in some cases no anestrous periods commonly associated with the seasonally polyestrous animal (Gentry et al., 2002).

A more direct way that obesity impacts reproduction is through the manipulation of oocyte developmental competency. Perturbations in oocyte development even at early stages may lead to reductions in conception rate and an alteration in the developmental trajectory of the oocyte. The majority of studies involving oocyte quality and competency in relation to obesity have been in relation to artificial reproductive technologies such as intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) in humans (Wittemer et al., 2000; Dokras et al., 2006; Esinler et al., 2008). Results

from multiple studies have demonstrated that oocytes derived from obese women may be delayed in maturation or have impaired maturation (Esinler et al., 2008). When observing oocyte quality as a product of maturation rate, it has further been demonstrated that fertilization rates from oocytes of obese women are reduced during IVF (Rutherford et al., 2001; Matalliotakis et al., 2008). Impairment of oocyte development, competency and maturation may lead to increased pregnancy loss seen in obese individuals; however, these effects have not been investigated in the horse and warrant further exploration (Lashen et al., 2004; Robker, 2008).

Obesity may alter reproductive function through direct alteration in endometrial health or function. While numerous studies have been conducted to investigate the effects of obesity on endometrial function, contradictory results have been reported (Bellver et al., 2003; Wattanakumtornkul et al., 2003; Styne, 2005; Bellver et al., 2007). The uterine environment during conception, implantation and pregnancy is subject to variations in hormone and nutrient composition, thus altering the uterine milieu and potentially compromising endometrial function. Obesity may impact the endometrium by perturbing implantation and embryonic receptivity of the endometrium (Metwally et al., 2007). Alterations in insulin signaling, associated with obesity, may also be responsible for the reduction in fertility associated with the endometrium. Obese women have lower levels of insulin-like growth factor binding protein-1 in endometrial tissue, a molecule that plays an important role in the maternal-fetal interface (Levens and Skarulis, 2008). The development of endometrial insulin resistance has not been fully described; however, it can be assumed that the development of insulin dysregulation in the uterus would affect fertility (Strowitzki et al., 1993). While these perturbations are likely

responsible in part for the reproductive dysfunction seen in obese individuals, the mechanisms by which the endometrium is impacted by obesity are not fully understood.

The final puzzle piece in understanding the role of obesity on reproduction is potential effects on the embryo itself. Because obesity has a negative effect on oocyte quality and development, embryonic development may likewise be impacted by obesity. Women with a high body mass index (BMI) (> 30 kg/m^2) had lower quality embryos than those from women with a lower BMI (20-30 kg/m^2) (Carrell et al., 2001). Obese women also display gonadotropin resistance during IVF, and it has been suggested that the higher doses of gonadotropins may ultimately disturb embryonic development and implantation (Tamer Erel and Senturk, 2009). This phenomenon has not yet been described in horses and as the use of artificial reproductive technologies increases in the equine industry, more research must be done to evaluate the effects of obesity on embryonic development.

To summarize, the prevalence of obesity in horses is rising (Thatcher et al., 2012), and EMS and its related pathologies are of major concern to the equine industry. While the development and management of obesity is generally well understood, the far-reaching effects on reproduction remain largely unresolved. The following section will provide insight into the unique aspects of equine reproduction and explain early pregnancy, embryonic development and early embryonic loss in the mare.

Reproductive Anatomy

The ovary of the mare consists of three layers including the cortex, medulla, and the hilum. Additionally, it is unlike that of other farm animals in that it is oriented with an inner zone known as the cortex, composed of germinal epithelium, and an outer zone

designated as the medulla. This is in contrast to other domestic livestock species in which the germinal epithelium is oriented on the outer surface of the ovary with the connective medullary region inside the ovary. Due to the unique orientation of the ovary, ovulation in the mare occurs at a single point known as the ovulation fossa (Ginther and Pierson, 1984b).

The uterus of the mare is considered a bicornuate uterus with a single body and less-developed horns than that of the sow. It's arrangement relative to the attachment of the broad ligament allows for palpation of the uterus through the rectum (Ginther and Pierson, 1984a). The uterus consists of three distinct layers. As the outermost layer, the serous layer is continuous with the broad ligament. The myometrium consists mainly of smooth muscle, and functions to provide myometrial contractions during parturition and uterine involution. Known as the endometrium, the innermost layer is a complex mucosal membrane containing a rich blood supply that houses and supports the developing fetus during pregnancy. The endometrium of the uterus is glandular and secretory in nature (Ginther and Pierson, 1984a).

Uterine endometrial biopsies can be used to evaluate uterine health and competency based on inflammation and fibrosis present. These biopsies are useful in diagnosing reasons for infertility in mares and create a prognosis as to the mare's future reproductive potential. Biopsies are graded on a scale from Grade I, which is a normal endometrium to a Grade III, which is indicative of severe inflammation or diffuse fibrosis. A grade of IIB results in a 10-50% less chance of conceiving or carrying a foal to term, while a grade of III indicates less than a 10% chance of carrying a foal to full term (Ricketts, 1975).

Reproductive Cycle

Horses are long-day seasonal breeders with an ovulatory estrous cycle occurring between May and October (Aurich, 2011). The average estrous cycle length is 22 d with a behavioral estrus period ranging from 5-7 d. In addition to season, estrous cycle length is impacted by breed, age, reproductive status, pregnancy status and lactation, with significant variability in length between mares (Heidler et al., 2004). As mares transition into cycling periods during early spring and into anestrous in the fall, irregular estrous cycles are common (Ginther et al., 2009).

As in other domestic livestock species, the estrous cycle of the mare is dominated by a follicular phase and a luteal phase. The follicular phase, controlled by a large estrogen secreting follicle, is a time of increased receptiveness to the stallion by the mare (Crowell-Davis, 2007). Estrus behaviors displayed during this time will include clitoral “winking”, behavioral urination and general interest in the stallion. These behaviors are subject to extreme variation in mares and may be influenced by age, individual receptivity and presence of a foal. In contrast to the follicular phase, the luteal phase is governed by increased progesterone secretion by a corpus luteum. Progesterone will inhibit behavioral estrus with mares displaying decreased willingness to be bred by the stallion (Hedberg et al., 2007). Further differentiating horses from other livestock species is a fertile estrous period approximately 10 d (range 7-20 d) following parturition. This facilitates early conception, allowing for the production of a foal each year from an individual mare.

The estrous cycle of the mare is dictated by endocrine signals through the hypothalamic-pituitary-gonadal axis (Irvine and Alexander, 1987). The gonadotropins,

luteinizing hormone (LH) and follicle stimulating hormone (FSH) are synthesized and released by the anterior pituitary in response to stimulation by hypothalamic gonadotropin releasing hormone (GnRH). The pulse frequency of GnRH changes throughout the estrous cycle yielding different patterns of LH and FSH secretion which acts on the ovary of the mare and dictates folliculogenesis (Aurich et al., 1995). Generally mares will exhibit 1-2 follicular waves during a given estrous cycle, however various breed-specific patterns are observed in estrous cyclicity (Ginther, 2000). Mares, in direct contrast to other species, do not exhibit a single preovulatory LH peak; rather, there is a gradual rise in LH resulting in elevated concentrations of LH for several days (Ginther et al., 2010). Follicular development is dictated by FSH concentrations with increasing concentrations of FSH until follicular size reaches approximately 13 mm or around 40% of dominant follicle size (Gastal et al., 1997). Once follicular dominance is established, FSH concentrations decline to levels that would not support growth of subordinate follicles. Follicular deviation is controlled by the dominant follicle synthesis and secretion of estrogen and inhibin (Gastal et al., 1999). In comparison to other species, the dominant follicle in horses is significantly larger at ovulation and grows at an average rate of 3 mm per day until a final diameter of approximately 35 mm (Ginther et al., 2008). Final preovulatory size of the dominant follicle is breed specific and individual mares will tend to ovulate follicles of similar size each year.

Following ovulation, mares will develop a functional corpus luteum in the inner aspect of the ovary (Yagi et al., 2005). Circulating levels of progesterone will begin to rise as luteal tissue formation occurs with maximal levels of progesterone occurring approximately 8 d post ovulation with a subsequent decrease until luteolysis

(Vanniekerk et al., 1975). Functional luteolysis in the mare, occurring around d 15-17 of the cycle is characterized by decreased levels of circulating progesterone as well as morphogenic regression of the corpus luteum (Ginther et al., 2005; Watson et al., 2005). Luteolysis is predicated by increased endometrial secretion of prostaglandin $F2_a$ ($PGF2_a$). In contrast to ruminant species, the mare lacks the counter-current system of circulation between uterine veins and arteries; thus endometrial secretions must enter peripheral circulation. As a result, the ovary of the mare is significantly more responsive to changes in $PGF2_a$ levels with increased receptor affinity. In the mare, $PGF2_a$ synthesis is regulated by cyclooxegenase-2 (COX-2), a key enzyme in the synthesis of the luteolytic hormone (Boerboom et al., 2004). While it is understood that the release of endometrial $PGF2_a$ is stimulated by oxytocin, the signal for increased oxytocin synthesis is not fully understood.

Equine Pregnancy

Maternal endocrine status during the first 14 d of pregnancy is similar to that of the nonpregnant mare. The lifespan of the corpus luteum is dictated by the events of luteolysis, which occur 15-17 d post ovulation. In the pregnant mare, the release of an as of yet undetermined maternal recognition signal inhibits luteolysis thus increasing the lifespan of the corpus luteum. Various conceptus-derived pregnancy recognition signals have been investigated in the mare. Much of the research that has been conducted in this area has been based upon findings in other domestic livestock species including pigs and ruminants. Conceptus-derived estrogens play a major role in pregnancy recognition in pigs (Bazer and Thatcher, 1977) whereas interferons, and more specifically interferon-tau (IFNT) have been identified as the conceptus-derived signal

for maternal recognition of pregnancy in ruminants (Bazer et al., 1997). The process of maternal recognition of pregnancy in the mare remains a potential target for nutritional intervention related to improving embryo development and developmental programming.

Similar to other large domestic livestock species, lysis of the corpus luteum is dependent upon pulsatile release of PGF_{2a}. In the mare, corpus luteum regression, declining progesterone levels and increased pulsatility of PGF_{2a} coincide with each other, indicating the importance of PGF_{2a} in luteolysis (Kindahl et al., 1982). During pregnancy when the conceptus is present in the uterus between days 10 and 14, the pulsatile release of prostaglandin is altered in the mare (Douglas and Ginther, 1976). However after day 18, the levels of prostaglandins in pregnant mares are similar to those of cyclic mares at the same time point, indicating that the conceptus delays rather than inhibits prostaglandin secretion (Berglund et al., 1982; Stout and Allen, 2002). When cultured in combination with a conceptus or conceptus-conditioned medium, the amount of PGF_{2a} secreted by endometrial cells is reduced further underlying the importance of a conceptus or conceptus-derived signal in the prevention of luteolysis (Berglund et al., 1982; Watson and Sertich, 1989; Ealy et al., 2010).

The equine conceptus is unique in many ways relative to other livestock species (Allen and Wilsher, 2009). Beginning on d 7 following ovulation, the conceptus secretes a glycoprotein network of mucin-like molecules originating from the underlying trophectoderm that completely surrounds the embryo forming an acellular and rigid capsule (Oriol et al., 1993). The function of this capsule is unknown, however it has been hypothesized that the capsule provides a rigid structure allowing the embryo to

travel freely in the uterine lumen with little risk of damage. Removal of the capsule during early pregnancy results in early embryonic death, underlining its importance in maintenance of pregnancy. Capsule development is dependent upon the presence of the uterine environment. Embryos produced *in vitro* secrete the capsular glycoproteins, however they fail to produce a typical capsule. The glycoprotein capsule persists until approximately d 23, undergoing compositional changes coinciding with zona degradation beginning on day 9 followed by alterations in sialic acid composition beginning on d 16, coinciding with embryo fixation (Stout et al., 2005).

Constant motility of the equine embryo through the intrauterine space is another distinguishing factor of early equine embryogenesis (Ginther, 1983b). Prior to d 16 of pregnancy the equine conceptus moves frequently through the entire uterine lumen driven by peristaltic contractions of the myometrium (Stout and Allen, 2001). Cessation of this movement is the result of increased uterine tone as well as the increasing size of the conceptus. Conceptus motility is stimulated by the conceptus itself, through the stimulation of contractility in the uterine myometrium (Ginther, 1984). Research into mechanisms regarding conceptus motility has focused on the production of prostaglandins, which are known to cause myometrial contractions. The equine conceptus begins producing prostaglandin E_2 (PGE_2) as early as d 5 of gestation, and continues producing various prostaglandins including $PGF2_a$ throughout the motile stage of early embryogenesis. Restriction of embryo movement causes an early termination of pregnancy (McDowell et al., 1988). It has been hypothesized that the role of conceptus motility in early pregnancy is the delivery of an unknown anti-luteolytic factor throughout the entire surface of the endometrium. Reducing the area of

conceptus-endometrium interaction to less than 80% leads to failure of pregnancy (McDowell et al., 1988).

Beginning around day 30 of pregnancy, unique structures begin to form on the developing conceptus. Specifically forming on the advancing portion of the allantois, the chorionic girdle is characterized by avascular, highly proliferative cells. The chorionic girdle represents an area of intense maternal endometrial invasion during which chorionic girdle cells invade through the maternal endometrium penetrating as deep as the stromal layer locating themselves between uterine glands (Sharp, 2000). Once invasion is complete, the cells undergo hypertrophy until the formation of large, raised, concave endometrial cups protruding above the endometrial surface (Antczak et al., 2013).

Formation of endometrial cups is generally completed by day 38 of pregnancy. Once formed, endometrial cups begin to synthesize and secrete equine chorionic gonadotropin (eCG) (Antczak et al., 2013). The overall function of eCG has been debated, but its secretion corresponds with and is thought to be responsible for the formation of accessory corpora lutea. The formation of these progesterone secreting accessory corpora lutea is essential for pregnancy maintenance in the horse. Additionally, it has been hypothesized that eCG functions to blunt the maternal immune response to the developing placenta (Samuel et al., 1974). Recent research has discovered that chorionic girdle cells exhibited high expression of interleukin (IL)-22, an immunomodulating cytokine (Brosnahan et al., 2012). Equine chorionic gonadotropin has been investigated for its role as a luteotropic agent. It has been demonstrated that eCG, functioning as a luteotropin, stimulates progesterone secretion from luteal tissue

(Albrecht and Daels, 1997; Daels et al., 1998). Functioning as both a luteotropin and an immunomodulator, the requirement of equine chorionic girdle cells and their production of eCG for maintenance of pregnancy has been well established and the supplemental progesterone produced by the accessory corpora lutea is essential for successful pregnancy in horses.

While embryo fixation has occurred by day 16 of pregnancy, implantation of the equine embryo will not occur until approximately 60 days of pregnancy (Sharp, 2000). At this time, structures known as microcotyledons begin to form from the chorioallantoic macrovilli. These microcotyledons form the basis for the major nutrient exchange mechanisms of the placenta. These tissues continue to develop and by day 100 of pregnancy display maximal vascularization and size (Ginther and Pierson, 1984a). In contrast to other species, implantation of the equine embryo occurs significantly later than other domestic livestock species.

Early Equine Embryonic Development

Embryonic genome activation, a process by which the embryo begins to transcribe its newly formed genome occurs during the 6-cell stage in the horse (Brinsko et al., 1995; Grondahl and Hyttel, 1996). Before this period, the fertilized embryo is transcriptionally inactive with maternal mRNA driving the first cell cycles. Following embryonic genome activation, cellular division continues until the development of the blastocyst cavity. Contrary to other species, the equine blastocyst forms in a multicentric manner with a loose association of cells in the inner cell mass. These cells migrate along the inside of the trophoblast and form a continuous layer resulting in a bilaminar blastocyst (Bruyas et al., 1993; Tremoleda et al., 2003; Hinrichs et al., 2007). The first

major differentiation event in embryogenesis occurs when trophectoderm cells (TE), precursors of placental development, and the inner cell mass (ICM), precursors of embryonic formation, are specified (Narasimha and Leptin, 2000). The molecular control of TE formation has been well described in mice and expression of caudal-related homeobox 2 (*CDX2*) in combination with repression of octamer-4 (*OCT4*) is required for TE cell line commitment (Chambers and Smith, 2004). In contrast, differentiation of ICM is controlled by expression of *OCT4* (Chambers, 2004). Once ICM and TE differentiation has occurred, a population of ICM cells will undergo further differentiation forming the primitive endoderm (PrE), cells responsible for eventual formation of the visceral endoderm (VE) and the parietal endoderm (PE) (Prendiville et al., 1994). The formation of the VE and PE is critical as these tissues will develop into tissues that play a role in early embryonic gas exchange, nutrient exchange and fetal-maternal communication prior to placentation (Jollie, 1990; Cross et al., 1994; Bielinska et al., 1999).

Following this important developmental milestone is an important step in embryonic development in horses known as gastrulation. During gastrulation, three distinct germ layers are formed and oriented in an anterior-posterior manner along the embryonic axis. The embryo at this point is dominated by the TE cells and the ICM cells, however during gastrulation the formation of lineage-specific germ layers occurs with the formation of the endoderm, mesoderm and ectoderm (Solnica-Krezel, 2005). The outer ectoderm gives rise to the epidermis and neural tissue, while mesoderm cells will differentiate into connective tissue, muscle, blood cells and specific organs including the heart, kidney and gonads. Endoderm cells will differentiate into the digestive tract

and its associated tissues (Solnica-Krezel, 2005). One of the defining characteristics of gastrulation is the development of the primitive streak (PS) (Hay, 1995). This development establishes bilateral symmetry in the embryo and is characterized by the migration of specific cells, which ultimately are precursors of mesoderm and endoderm formation. The presence of a PS on a developing embryo indicates the onset of gastrulation and begins on day 12 of embryonic development in horses. Completion of gastrulation occurs by day 18 of pregnancy in horses at which point the embryo has developed an embryonic disk, PS and three distinct germ layers (Gaivao et al., 2014).

Early Equine Embryonic Loss

Equine early embryonic loss is generally defined as failure to maintain pregnancy following fertilization prior to day 40 of gestation (Vanderwall, 2008). Pregnancy diagnosis in horses via transrectal ultrasonography can be accomplished as early as 10 days following ovulation. Estimations of early embryo death range from 2.6% to 24.0% with an average of 8.6% (Woods et al., 1987; Hemberg et al., 2004; Allen et al., 2007). The highest rates of embryo mortality were detected in aged mares greater than 18 years of age (Carnevale and Ginther, 1992). Given the increased costs associated with additional breeding of mares and decreased foal production, early embryonic loss represents a significant economic burden to the equine industry.

Various causative factors have been implicated for their role in early embryonic loss in horses. It is easiest to evaluate the causative factors of early embryonic loss in three categories: intrinsic, extrinsic and embryonic (Woods et al., 1987; Ball, 1988). Intrinsic factors are those that are related to the mare itself. These may include endometrial disease (Kenney, 1978; Adams et al., 1987), progesterone insufficiency

(Allen, 2001; Newcombe et al., 2001), lactation (van Niekerk and van Niekerk, 1998; Newcombe and Wilson, 2005), maternal age (Ball et al., 1989; Carnevale et al., 1999) or any factors related to the process of insemination/fertilization (Koskinen et al., 1990; Woods et al., 1990; Barbacini et al., 1999) and fixation/implantation (Ginther, 1983a). Extrinsic factors are those that are generally out of the control of the mare generally involving induced stress (van Niekerk and Morgenthal, 1982; Baucus et al., 1990; Daels et al., 1991). Stressors such as transrectal palpation and ultrasound (Irwin, 1975; Voss et al., 1975; Vogelsang et al., 1989), semen quality (Platt, 1973; Blanchard et al., 1994) and nutrition (Henneke et al., 1984; Brendemuehl et al., 1994; Webb et al., 2004; Youngblood et al., 2004) are often implicated as extrinsic factors linked to early embryonic loss. Finally, embryonic factors are those associated directly with the embryo and its development and include diagnosis of pregnancy via transrectal ultrasound or palpation (Chevalier and Palmer, 1982; Ginther et al., 1985; Bergfelt et al., 1992), mare handling (McKinnon et al., 2000; Vanderwall et al., 2006; Hinrichs et al., 2007) and overall reproductive management (Darenius et al., 1988). It is important to remember however, that embryonic factors are often linked to both extrinsic and intrinsic factors. While some factors such as increasing maternal age have been consistently identified as causative factors in early embryonic loss others such as nutrition are still not fully understood. Proper reproductive and overall equine management may mitigate the effect of individual factors; however, further investigation is necessary to fully understand the mechanisms of early embryonic mortality in horses.

The previous section explored equine obesity and highlighted the effects of equine obesity on the development of a range of health issues. Additionally,

reproductive anatomy and function was explained. Recently, a new area of research contends that the impacts of obesity may be multi-generational, and that in utero exposure to obesity adversely impacts postnatal development and the incidence of obesity-related disorders. This concept is discussed next.

Metabolic Programming

The programming of long-term health and disease was once thought to be a process controlled solely through genetics with alterations only possible through breeding and lifelong nutritional intervention. This idea has evolved to include events occurring at the earliest stages of development that may be responsible for predisposition of offspring to disease and adverse health later in life. This section will discuss the 'Developmental Origins of Health and Disease', the observation of an intergenerational cycle of obesity and specific epigenetic regulation of metabolic programming and development.

Developmental Origins of Health and Disease

Almost 25 years ago a hypothesis emerged from epidemiological studies of birth and death records in humans indicating that birth weight may be associated with later adult death rates (Barker and Osmond, 1986). This hypothesis developed into an area of research known as the 'Developmental Origins of Health and Disease (DOHaD)', the 'Fetal Origins of Health and Disease', or by a more colloquial term, 'Barker's Hypothesis' (Barker et al., 1989a; Barker et al., 1993; Wadhwa et al., 2009). Initially only investigated as a relationship between birth weight and the lifetime risk of coronary

artery disease, this hypothesis has evolved into a highly studied area of research across species (Barker et al., 1989b).

The concept of DoHAD represents a fundamental shift in the way that we think about lifelong health and disease. Events occurring during the earliest stages of embryonic development may play a role in the development of various diseases including type-2 diabetes, asthma, cardiovascular disease, cancer, obesity, osteoporosis and most recently autism (Kwong et al., 2000; Gillman et al., 2007; Shanik et al., 2008; Fleming et al., 2012; Xu et al., 2014). Research into the role that maternal nutrition plays on DoHAD has become an area of intense international research utilizing various animal models including the rat (Lillycrop et al., 2005), mouse (Cooney et al., 2002), sheep (Zhang et al., 2011a) and non-human primate (Aagaard-Tillery et al., 2008). Various tools have been utilized to evaluate the effects of adverse developmental programming including maternal or grand maternal diet modification, in *utero* growth restriction and uterine artery ligation, all stressors of uterine function and subsequent embryonic development (McMillen and Robinson, 2005). The working hypothesis is that various stressors may result in the development of physiology immediately suited to adverse conditions but which may not be appropriate for lifelong health. Few studies have investigated the role of maternal nutrition, specifically over nutrition, on early embryonic development in the horse. Additionally, the intergenerational effect of maternal diet has been relatively unstudied in the horse.

Specifically relating to the broodmare, maternal nutrition during pregnancy and the periconceptual period is highly misunderstood. Several effects are evident. Direct effects of overconditioning include a reduction in reproductive rates (Gentry et al.,

2002), and a lack of an anestrous period during the normal non-breeding season, an unnatural occurrence that can reduce reproductive efficiency of mares (Vick et al., 2006). Longer estrous cycles and less predictable ovulations, both hallmarks of obese mares, result in more difficult and costly breeding practices as compared to mares with a BCS around six. Additionally, mares normally become more insulin resistant as pregnancy progresses (Hoffman et al., 2003b; George et al., 2011), and for mares beginning pregnancy at an insulin resistant and obese state, this can progress to a level of insulin resistance that not only has maternal effects, but can pose health risks for the developing embryo. It has also been hypothesized that increased inflammation associated with obesity may increase early embryonic loss.

In contrast to many other domestic livestock species as well as the human population, there are currently limited recommended feeding strategies for horses during the pre-breeding or early gestational period (National Research Council (U.S.). Committee on Nutrient Requirements of Horses., 2007). Recommendations for mares during early pregnancy mirror those of horses at maintenance. The scarcity in research-based feeding programs has led to common management practices in which broodmares are fed a high calorie diet potentially leading to the development of obesity, IR and EMS.

Intergenerational Cycle of Obesity

The molecular mechanisms involved in the modulation of early embryonic development, specifically relating to the predisposition of offspring to lifelong disease risk, are poorly understood across species, but specifically in the horse. The concept that maternal diet can influence periconceptual events including oocyte maturation and

embryonic development is relatively new, and questions have been raised as to the mechanisms by which this occurs. Increasingly, the idea of maternal-fetal communication through endometrium-derived substrates has given rise to the notion that maternal effects on fetal development begin prior to implantation and placental development (Haig, 1996). The periconceptual period, corresponding to early embryonic development, is characterized by incredible remodeling of the female reproductive tract in preparation for the intense requirements of sustaining a pregnancy (Fleming et al., 2012). During this time, both maternal and fetal cellular phenotype changes dramatically as oocyte maturation progresses through fertilization and into embryonic genome activation. As the embryo develops and cell fate is decided, uterine environmental conditions can play a major role in development that can induce persistent changes in cellular phenotype and dictate embryonic and fetal development (Ulbrich et al., 2013). The uterine environment, reflecting what is observed in the external environment is thought to play a role in fetal adaptation and subsequent survival (Tauson et al., 2006). During development, the conceptus translates nutrients received from the maternal diet as a representation of the external environment. These bioactive nutrients can then act through epigenetic and other mechanisms to alter gene expression. Specifically epigenetic regulation of gene expression involves modifications to the genome that do not specifically alter the DNA sequence. If maternal inputs do not adequately represent the outside environment, or if there are any extremes experienced by the developing embryo or fetus, overall development may be compromised as the fetus adapts to these changes. These adaptations to the extreme uterine environment, though necessary at

the time may be unsuitable for later life leading to development of chronic disease (Steward and Moser, 2004).

Children of obese mothers have been shown to have a higher risk of developing a variety of adult diseases including but not limited to:

1. Cardio-metabolic disorders (Drake and Reynolds, 2010)
2. Cardiovascular disease (Dietz, 1994)
3. Hypertension (Barker, 1994)
4. Non-alcoholic fatty liver disease (Oben et al., 2010)
5. Central nervous system malformations (Galtier et al., 2008)
6. Autism (Krakowiak et al., 2012)

It should not be surprising that maternal nutrition and body condition, dictating the hormonal environment of the developing offspring should influence infant body composition. However, what should be of note is that the nutritional environment experienced *in utero* has long lasting and detrimental effects that endure throughout life (Power and Jefferis, 2002). Another emerging concern is the rise of a probable intergenerational cycle of obesity (Figure 2-3). Various studies indicate that children born to obese mothers were more likely to be obese by a young age, persisting into adulthood (Whitaker, 2004). This intergenerational cycle of obesity contends that women at a heavier weight during gestation will give birth to heavier children that are then predisposed to a life of obesity (Zhang et al., 2011b). Gestational diabetes in humans, similar to mares with insulin resistance during pregnancy results in alterations in fetal blood glucose concentrations that result in fetal hyperinsulinemia, overgrowth and developmental problems (Dorner and Plagemann, 1994).

Figure 2-3- The Intergenerational Cycle of Obesity (Zhang et al., 2011b)

Evidence supporting the hypothesis of DoHAD is strong and has been reinforced by research across species. The link between aberrations in maternal nutrition and the predisposition of offspring to lifelong disease risk should not be undervalued. Equine diets, specifically related to pregnancy and the periconceptual period have been severely under researched. Further evaluation of the effects of maternal over nutrition on developmental programming of offspring is necessary. It is important to understand the underlying mechanisms behind the intergenerational cycle of obesity in order to potentially disrupt the causative factors related to the development of obesity related pathologies.

Metabolic Programming through Nutrition and Epigenetics

The effect of diet on overall health and development occurs through several different mechanisms. Recently, the concept of diet altering phenotype through epigenetics has been advanced and multiple studies have investigated the role of

overall nutrition as well as individual nutritional components on the epigenetic modifications of cells. Epigenetics describes a phenomenon in which gene expression is altered in an organism by mechanisms other than alteration in the genetic code itself (Ledford, 2008). These changes to the genome represent alterations to the transcriptional ability of the cell and may or may not be heritable. In order to understand the role of epigenetics in gene regulation, it is important to remember that even though all somatic cells of an individual contain an identical DNA sequence, not all cells have a similar phenotype. One mechanism through which this occurs is epigenetics. Epigenetic modifications allows different genes to be activated or inactivated depending on various modifications to the gene sequence or structure. Generally, there are three methods by which epigenetics alters gene activity including DNA methylation, histone acetylation and histone methylation (Egger et al., 2004).

DNA methylation is the most widely understood and studied aspect of epigenetic modification. A methyl group added to a specific site on the DNA, known as a CpG site modifies the structure of the DNA and functions to silence a gene (Ozanne, 2015). This modification is under the control of a family of enzymes known as DNA methyltransferases (DNMT) and results in the formation of a molecule thought of as a fifth base known as methyl cytosine.

Histone modifications are a more complicated function of epigenetic regulation. Histones, comprised of a chromatin core on which DNA is wrapped, can be modified in two ways. The addition of an acetyl group to an amino acid residue on chromatin results in histone acetylation while histone methylation involves the addition of a methyl group to the amino acid residues on chromatin (Egger et al., 2004). Histone acetylation,

governed by the enzyme histone acetyltransferase (HAT), coincides with euchromatin, and activation of the gene while deacetylation is associated with the inactive form, heterochromatin. Methylation of histones is more complicated with either repression or activation occurring based on the individual methyl codes associated with the histone modification.

As our understanding of gene regulation progresses, the role of nutrition on epigenetic regulation has become apparent. Nutritional modification of epigenetics is accomplished through bioactive compounds and nutrients that are capable of altering transcription. Nutrients or molecules that contain either DNMT's or HAT's are able to alter the epigenome through DNA methylation or histone acetylation (Kirkland, 2009). Compounds that contain donors of either methyl groups or acetyl groups, essential for acetylation and methylation are also responsible for epigenetic modification. Such compounds include green tea, resveratrol, folate and other B-vitamins, all present in diets (Keyes et al., 2007). Alterations in enzyme activity due to epigenetic modifications as a result of diet impact physiologic processes that may play a role in health and disease.

Modifications to the epigenome have been implicated for their role in disease status across species. A pertinent example is the role of histone acetylation in the regulation of inflammation. The regulation of pro-inflammatory genes and anti-inflammatory genes by HDAC has been observed (Villagra et al., 2010). Enzymes responsible for regulating inflammation can also be modified through diet-induced epigenetic modifications. Resveratrol, a compound commonly found in the skin of red grapes has displayed anti-inflammatory properties (Donnelly et al., 2004; Youn et al.,

2009; Cui et al., 2010). The high-fat and high calorie diets associated with obesity have also been connected to epigenetic modifications through DNA methylation that may lead to insulin resistance and progression of obesity (Widiker et al., 2010). Since many diseases involve epigenetic changes, researchers are attempting to counteract these changes with epigenetic-specific treatments. Specifically, treatments utilizing epigenetic modifications through DNA methylation have been used to treat various cancers (Egger et al., 2004). While this epigenetic-based treatment has proven beneficial, it must still be controlled. Generally these treatments have not been able to target specific cells or cell types and healthy cells in some cases have been converted into cancer cells by inactivation of tumor suppressing genes or activation of genes that had been silenced.

Knowledge of nutritional regulation of epigenetic modifications is still limited. However, the ability for epigenetics to impact phenotype without base pair modifications in addition to the reversible nature of epigenetic modifications, represents an attractive field of research in developmental biology. As our understanding of various bioactive compounds progresses, it may be possible to utilize the epigenetic modifications progressed through nutrition to our advantage (Burdge et al., 2012).

Metabolic programming and metabolic imprinting are terms that describe events occurring at the earliest stages of development that impact later biological outcomes. While both of these terms have been used interchangeably, it is important to distinguish between them. Metabolic programming refers to events that must occur during specific developmental windows during which the embryo or fetus is in a stage of developmental plasticity (Levin, 2006). Metabolic imprinting, which is an example of metabolic programming refers to effects directly on the genome (Waterland and Garza, 1999).

This idea as presented earlier, refers to the effects that are observed in the offspring when maternal conditions are impacted during pregnancy.

There are a variety of factors to consider in regards to metabolic programming including timeframe of exposure, duration of exposure and the concept of developmental plasticity. Single stressors may cause varying effects on individuals based on when the exposure occurs and the duration of the exposure. Nutritional aberrations at any stage of pregnancy lead to a variety of health concerns, but of significance is that changes in nutrition at different times during pregnancy result in very different outcomes (Symonds et al., 2007). This association makes sense when it is understood that organs and systems develop at varying timepoints during pregnancy. The concept of developmental plasticity implies that during times of increased development, negative effects of environment or diet would be amplified leading to increased disease susceptibility later in life (Javaid et al., 2006). Correlations have been found between a wide range of dietary exposures and health endpoints (Hanley et al., 2010).

Our understanding of developmental programming is extremely limited in horses. Few studies have investigated maternal nutrient status during equine gestation, however impaired insulin sensitivity and disrupted endocrine signaling was observed in foals of nutrient restricted dams (Ousey et al., 2008; George et al., 2009). While caloric intake is important in understanding nutritional effects on pregnancy, dietary composition may play a role in the developmental programming of offspring. Mares on pasture during late gestation, supplemented with either high fiber or high starch diets delivered foals with significantly altered glucose and insulin dynamics prior to weaning

(George et al., 2009). Further research is necessary to evaluate the effects of maternal over nutrition on embryonic and fetal development in horses.

Omega-3 Fatty Acids: Background and Health Benefits

As our understanding of developmental programming has progressed, so has the potential to impact later-life growth and development through nutritional supplementation. Researchers have consistently sought new ways to impact reproductive health, embryonic development and systemic health. Omega-3 long chain polyunsaturated fatty acids have been investigated for some time for their role in improving disease status by reducing inflammation and modulating various aspects related to health. This section will provide insight into omega-3 fatty acids as they relate to horses and reproduction.

Background

Omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) are fatty acids with a double bond located at the third carbon from the terminal end of the carbon chain (Scorletti and Byrne, 2013). These fatty acids are considered long-chain fatty acids based on the number of carbons present in the fatty acid chains. Fatty acids containing 16-20 carbon atoms are considered long-chain fatty acids with medium chain and short chain fatty acids falling into categories containing fewer carbon atoms in succession. Short-chain fatty acids, also known as volatile fatty acids (VFAs), are produced solely in the digestive tract as byproducts of microbial fermentation and provide a significant contribution to the horses' energy usage (National Research Council (U.S.). Committee on Nutrient Requirements of Horses., 2007). The three most common omega-3 LCPUFA's are alpha-linolenic acid (ALA), derived from plant oils and eicosapentaenoic

acid (EPA) and docosahexaenoic acid (DHA), both of which are commonly derived from marine sources (Scorletti and Byrne, 2013).

In mammals, linoleic acid (LA) and alpha linolenic acid (ALA) are considered to be essential fatty acids, as mammals lack the necessary enzymes required to introduce double bonds into fatty acid chains (National Research Council (U.S.). Committee on Nutrient Requirements of Horses., 2007). Linoleic acid, an omega-6 fatty acid and ALA an omega-3 fatty acid are both derived from different dietary sources. Linoleic acid, mainly from soy, corn and safflower oil and ALA derived mainly from flax must be supplied in the diet in some way. While generally low in total fat, pasture is contains a large proportion of ALA as total fat. As essential fatty acids are supplied in the diet, they can be converted into polyunsaturated fatty acids (PUFAs) that are highly integrated into cell membranes. The conversion and interconversion of PUFA's in the cell is complex and outlined in Figure 2-4.

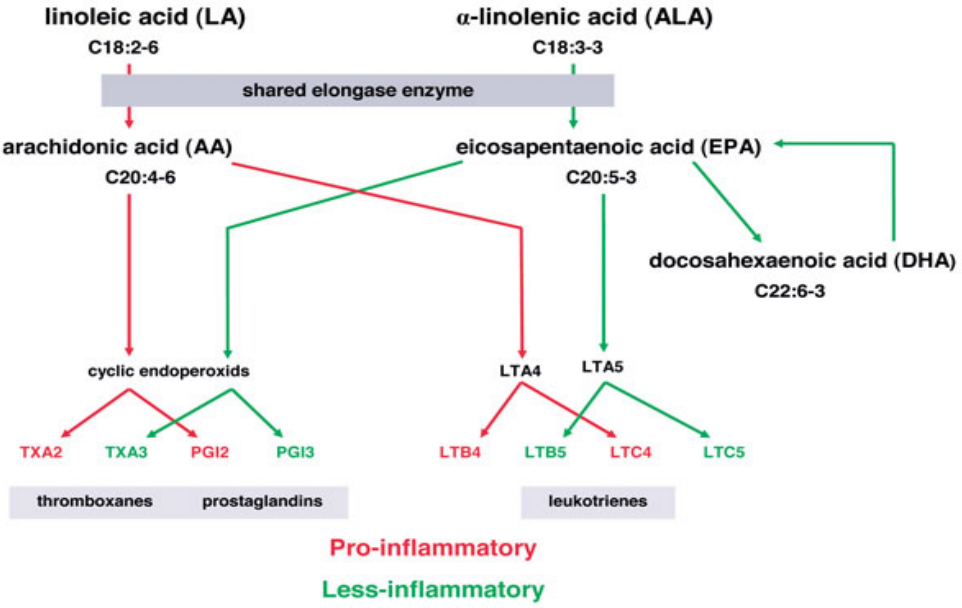


Figure 2-4- Biochemical pathways of omega-3 and omega-6 fatty acids (Yamamoto and Smith, 2002).

One of the major functions of fats in the body is their incorporation into the phospholipids that make up the cell membranes (Simopoulos, 1999). Alteration of the fatty acid composition of the diet alters the composition of cell membranes (Simopoulos, 1999; Hess et al., 2012; Hess et al., 2013). Diets supplemented with EPA and DHA in humans (Hulbert et al., 2005) and horses (Hess et al., 2013) alters the cell membrane by incompletely replacing omega-6 fatty acids, specifically arachidonic acid. Adjustments to the composition of cell membranes change the substrates available for biochemical processes. As seen in Figure 4 arachidonic acid (AA) is utilized as a substrate for various pro-inflammatory prostaglandins, leukotrienes and thromboxanes, molecules known collectively as eicosanoids. Replacement of AA with EPA and DHA lead to the production of less-inflammatory thromboxanes, cytokines, prostaglandins and leukotrienes. Additionally, EPA inhibits specific enzymes necessary for the production of pro-inflammatory eicosanoids (Weber et al., 1986). Further highlighting the protective effects of EPA and DHA is the propensity for them to be converted into protective molecules known as resolvins and protectins (Serhan and Petasis, 2011). These names represent a family of molecules whose functions have not been completely defined. However, it is understood that these molecules play a major role in anti-inflammatory and immunoregulatory processes and are responsible for the return of tissues to normal function after an inflammatory event (Kohli and Levy, 2009). While conversion of ALA to EPA and DHA occurs in the body, this conversion may not be efficient (Gerster, 1998). In order for EPA and DHA to be incorporated into the phospholipid cell membranes in a quick, efficient manner, these compounds must be supplied directly in the diet. Conversion of ALA to DHA is limited in humans (Burdge

and Wootton, 2002; Arterburn et al., 2006), and multiple studies have demonstrated limited conversion of ALA to DHA in horses (Hansen et al., 2002; Vineyard et al., 2010) To achieve the full benefit of omega-3 LCPUFAs, it is recommended that these products be supplemented directly without relying on the conversion of ALA.

Health Benefits of Omega-3 Supplementation

The health benefits of omega-3 supplementation are well documented in several mammals. Arguably, the best-known benefit for omega-3 supplementation in humans is reduction in inflammatory status and resulting prevention of cardiovascular disease (Calder, 2001). Dietary supplementation of omega-3 LCPUFA's also regulates cholesterol triglycerides in humans (Ferramosca et al., 2012) in addition to improvements in child learning and development (Montgomery et al., 2013). In the horse, omega-3 LCPUFA supplemented as DHA and EPA has historically been investigated for its role in reducing inflammation in joints and maintaining joint health (Munsterman et al., 2005) as well as a treatment for other inflammation related maladies such as those associated with airway obstruction (Nogradi et al., 2015).

An increasing body of literature has highlighted the reproductive benefits of oral supplementation of omega-3 LCPUFAs. Research involving cattle (Waters et al., 2012a; Leroy et al., 2013), swine (Smit et al., 2013) and mice (Wakefield et al., 2008) has demonstrated an increase in conception rates, improved oocyte and embryo quality and overall reproductive benefits in animals supplemented with DHA and EPA. Several reasons for this outcome are possible.

It is hypothesized that during the periconceptual period and leading into the early preimplantation period, the majority of influence on the oocyte, follicle and embryo is a

product of the endometrium and its specific secretions. Recent evidence suggests that endometrial gene expression can be altered by dietary omega-3 fatty acid supplementation (Waters et al., 2012a). Genes involved in prostaglandin synthesis, steroidogenesis and other important reproductive processes were impacted by omega-3 supplementation (Waters et al., 2012a). Gene expression in omega-3 supplemented cattle displayed a shift towards the production of luteotropic prostaglandin E_2 (PGE_2) and away from luteolytic prostaglandin $F2_a$ ($PGF2_a$). Furthermore, omega-3 LCPUFA supplementation has also been shown to impact key enzymes involved in fatty acid synthesis and metabolism (Waters et al., 2009). This, along with other evidence of the reproductive benefits of omega-3 LCPUFA supplementation, supports the necessity of investigating the effect of supplementation on equine reproduction and early embryonic development.

Dietary Inclusion of Omega-3 Fatty Acids

Modern diets in both humans and livestock have evolved through time. A striking example of this is evident when comparing the omega-6 to omega-3 fatty acid ratio of our ancestors (1:1) and of modern day humans on a western diet (10:1-25:1) (Simopoulos, 1991; Wathes et al., 2007). Horses are naturally adapted to a diet rich in omega-3 fatty acids, especially ALA as opposed to a diet high in omega-6 fatty acids, specifically LA (National Research Council (U.S.). Committee on Nutrient Requirements of Horses., 2007). This ratio results from the fact that horses, grazing animals, would spend large portions of the day consuming grasses high in ALA. As our management of horses has evolved, many horses are now spending increasing amount of time in stalls consuming rations consisting of high quantities of grains inherently rich in omega-6 fatty

acids. The addition of vegetable oil to equine diets as an energy supplement has further skewed the ratio of omega-6: omega-3 to a level that may be putting the animal at risk of developing various pathologies including gastric ulcers, allergies, joint pain, reproductive inefficiencies and a multitude of other avoidable maladies. As both human diets and equine diets have evolved it is important to understand the role that nutrition plays on overall health and disease. Deviation from the diets that were present during the time that genetic patterns were established may have detrimental effects on disease status and reproductive efficiency. Better understanding the role that omega-3 LCPUFA play on early embryonic development may provide us with the knowledge needed to incorporate different nutritional recommendations to individuals struggling with reproductive inefficiencies. Additionally, dietary supplementation of omega-3 fatty acids may prove beneficial in improving offspring health.

Summary of Current Literature and Experimental Aims

Obesity and its related maladies are a high research priority for owners, breeders, veterinarians, funding agencies and private industry groups. However, the effects of obesity and nutritional disruption during the periconceptual period progressing through early pregnancy are not well understood. Dietary influences play a major role in the predisposition of offspring to lifelong disease risks and negative outcomes in other species. It is imperative that we acknowledge the role of nutrition on early developmental programming in the horse. Omega-3 LCPUFA's have routinely been shown to have beneficial health effects across species and their potential role in mitigating the negative effects of a compromised uterine environment is intriguing.

The research presented in this dissertation was conducted to decipher the physiological mechanisms underlying the role of maternal nutrition on uterine health and embryonic development. Elucidation of the mechanisms dictating endometrial health may lead to the development of further nutritional strategies for the mare during the critical times around conception and early pregnancy. The specific objectives are as follows:

- Determine if DHA supplementation alters endometrial composition.
- Determine if DHA supplementation alters endometrial biopsy score.
- Characterize gene expression in endometrium from mares fed a DHA supplement vs. a control diet.
- Characterize gene expression in 12.5-day embryos from mares fed a DHA supplement vs. a control diet.

CHAPTER 3
DIETARY SUPPLEMENTATION OF ALGAE-DERIVED LCPUFA ALTERS EQUINE ENDOMETRIAL COMPOSITION AND GENE EXPRESSION AND MODIFIES EMBRYONIC GENE EXPRESSION

R.D. Jacobs[1], A.D. Ealy[1], P.M. Pennington[2,3], B. Pukazhenthi[2], L.K. Warren [4], A.L. Wagner[5], A.K. Johnson[6], T.M. Hess[7], R.K. Splan[1,8]

Virginia Polytechnic Institute and State University, Blacksburg, VA, USA[1], Smithsonian Conservation Biology Institute, Front Royal, VA, USA[2], George Mason University, Fairfax, VA, USA[3], University of Florida, Gainesville, FL, USA[4], Cooperative Research Farms, Richmond, VA, USA[5], Auburn University, Auburn, AL, USA[6], Colorado State University, Fort Collins, CO, USA[7], Virginia Tech MARE Center, Middleburg, VA, USA[8]

Corresponding Author:

Dr. Rebecca K. Splan

Virginia Tech MARE Center

5527 Sullivans Mill Road

Middleburg, VA 24073

540-687-3521

Animals housed at Virginia Tech Middleburg Agricultural Research and Extension (MARE) Center, Middleburg, VA. Work completed at Virginia Tech MARE Center and Virginia Polytechnic Institute and State University Department of Animal and Poultry Science, Blacksburg, VA. IACUC Approval Number: 13-031 APSC

Abstract

Emerging evidence in humans and other species suggests that obesity, as well as maternal diet around the time of conception influences gene expression and cellular function in developing offspring. These changes may lead to aberrant embryonic development or predisposition of offspring to lifetime metabolic dysfunction. Dietary supplementation of docosahexaenoic acid (DHA), and other marine-derived long-chain omega-3 polyunsaturated fatty acids has also been shown to improve reproductive, metabolic and inflammatory health across species. The objective of this study was to evaluate effects of peri-conceptual DHA supplementation on endometrial health and gene expression and embryonic gene expression in overweight horses. Non-lactating light horse mares (mean ± SEM age=13.56±0.11 yr; mean ± SEM BCS=7.07±0.21; mean ± SEM BW 655.94±13.21 kg) were supplemented with concentrate (n=6) or an isocaloric diet containing 0.06 g/kg BW algae-derived omega-3 DHA (n=7) 60 d prior to first sample collection. Four consecutive ovulatory cycles were monitored and uterine endometrial samples were obtained 12 d post ovulations 1, 3 and 4. Mares were bred to one stallion on ovulatory cycles 2, 3 and 4, and embryos were flushed 12 d post ovulation. Candidate genes essential to inflammation, prostaglandin synthesis and embryonic development were evaluated by quantitative PCR. Data were log transformed and analyzed using the GLM procedure in SAS (v9.3). When examining the data independent of breeding and pregnancy status, endometrial samples from DHA supplemented mares contained reduced *IL6* (*P*= 0.04) and TNFa (*P*=0.03) mRNA abundance and tended to have increased transcript abundance for *Uterocalin* (*P*= 0.09), *SAA* (*P*= 0.06) and *IL10* (*P*= *0.06*). Endometrial samples obtained from mares fed

LCPUFA pregnant in cycle 3 (n=2) contained greater *IL10* (*P*< 0.001), *PTGFS* (P=0.05), *OXTR* (P=0.05) and *PLA2G3* mRNA (*P*= 0.009) and had a tendency for increased *SAA* (*P*= 0.08), *PTGES* (P=0.10) and *SLCO2A1* (P=0.10) mRNA abundance. Endometrial tissues obtained from supplemented mares bred but not pregnant in cycle 3 (n=5) had reduced expression of *PTGER2* (*P*=0.001) and *PTGS1* (*P*= <0.001). In the conceptus, relative transcript abundance of *GATA4* and *GATA6*, markers of endoderm differentiation, along with *GATA3* and *ELF3*, markers of trophectoderm differentiation were greater (*P*< 0.05) in embryos from LCPUFA supplemented mares (n=5), than controls (n=5). These results indicate that algae-derived DHA supplementation during the peri-conceptual period alters the post-ovulatory uterine environment in the horse by modifying expression of genes related to inflammation, while regulating prostaglandin synthesis. Additionally, embryos obtained from supplemented mares displayed altered gene expression related to lineage differentiation.

Introduction

Environmental factors during early pregnancy alter the developmental trajectory of offspring. This phenomenon describes circumstances in which specific conditions during early embryonic and fetal development give rise to later life outcomes that may be detrimental to lifelong health, predisposing the offspring to potential adverse heath outcomes (Levin, 2006). While this concept has been advanced rapidly in recent years, it is not a recent observation. The idea that occurrences during fetal development could impact health later in life was first proposed by Barker in his pivotal papers (Barker, 1992, 1994, 1995). Since then, this idea of fetal programming has evolved to include alterations in nutrition and growth at specific developmental points and their potential to result in detrimental long-term effects (Kakar et al., 2005; Wadhwa et al., 2009).

Changes to the uterine environment may alter fetal development and result in shifts in developmental trajectory (Steward and Moser, 2004). The uterine environment is dictated by maternal inputs including nutrition, stress, body condition and various other environmental factors. Fetal development allows adaptation to the ever-changing uterine environment as developmental trajectory is established (Rossdale and Silver, 1982). Any misrepresentation of the postpartum environment, as is commonly observed with obese dams, may predicate a maladaptation of offspring for later life (Tauson et al., 2006). The negative effects of obesity on reproduction have been well documented with research focusing on the altered mechanisms involved with conception and implantation (Al-Azemi et al., 2004; Balen et al., 2006; Bellver et al., 2011). Further implication of obesity as a negative effector of reproductive health is the apparent existence of an intergenerational cycle of obesity (Whitaker, 2004). This term explains the observation

that offspring born to obese individuals were more likely to be obese by a young age, persisting into adulthood (Power and Jefferis, 2002). It is still relatively unknown what effects maternal nutrition has on equine embryonic development. The relatively few studies that have been conducted have demonstrated that maternal nutrition during various timepoints in pregnancy is a crucial component to optimum developmental programming (Rossdale and Ousey, 2002; Tauson et al., 2006; Fowden et al., 2013). Alterations in the metabolic health of foals as a result of maternal dietary manipulation has been observed as a result of maternal nutrient restriction (Ousey et al., 2008) and dietary composition modifications (George et al., 2009).

While proper nutrition is necessary for reproductive success, the question remains as to what effect obesity has on the reproductive function in the horse. Much of the research has focused on the growing population of obese horses and those afflicted with the related disease of equine metabolic syndrome. Many of the negative effects of obesity on reproduction stem from the metabolic dysfunction and inflammatory dysregulation associated with the disease (Frank et al., 2010). Specifically relating to reproductive dysfunction, inflammation has been routinely implicated for reductions in fertility and early embryonic loss (Weiss et al., 2009). Recently, mares with equine metabolic syndrome were reported to have an altered follicular environment (Sessions-Bresnahan and Carnevale, 2014). Any alterations of the delicate milieu that is the follicular environment could result in a failure to ovulate or a compromised oocyte derived from the altered follicle. As the prevalence of obesity and metabolic dysfunction rises in horses it is prudent to question what effects this is having on oocyte quality, reproductive function and embryonic development (Thatcher et al., 2012).

Fatty acid supplementation, and more importantly supplementation of omega-3 fatty acids, including DHA has been explored as a potential health benefit across species (Wakefield et al., 2008; Waters et al., 2012b; Leroy et al., 2013; Moallem et al., 2013; Smit et al., 2013). While omega-3 fatty acid supplementation has been integrated into equine diets for some time, the effects of a marine-derived omega-3 fatty acid supplement on uterine health and reproductive function are unknown. In a recent study, cattle supplemented with an omega-3 supplement displayed altered endometrial gene expression related to prostaglandin synthesis, steroidogenesis and other important reproductive processes (Waters et al., 2012b). Specifically relating to prostaglandin synthesis, omega-3 fatty acid supplementation alters the release of PGF_{2a} and PGE_2 (Meier et al., 2009). Further, embryos obtained from omega-3 supplemented cows were of increased number and improved overall quality (Childs et al., 2008). Recently, studies have demonstrated an improvement in insulin sensitivity in horses supplemented with a marine-derived omega-3 fatty acid supplement (Hess et al., 2013). Additionally, omega-3 fatty acid supplementation alters the membrane composition in red blood cells and incorporates into the skeletal muscle of supplemented horses (Hess et al., 2012). Further, the incorporation of omega-3 fats into stallion diets has been investigated for its role in improving semen quality (Brinsko et al., 2005). The potential for omega-3 fatty acid supplementation to improve uterine health in obese, metabolically compromised horses is of increasing interest to both researchers and the equine industry.

The reproductive and metabolic health benefits of omega-3 supplementation have been demonstrated across species. In this study we utilized an overweight horse model to investigate the effects of supplementing an algae-derived omega-3

supplement on uterine health and subsequent embryonic development. It was hypothesized that the omega-3 supplementation would modulate uterine composition resulting in improved uterine health leading to an alteration in embryonic gene expression.

Materials and Methods

Animals and Diets

All animal procedures were approved by the Institutional Animal Care and Usage Committee (IACUC: 13-031 APSC) at Virginia Polytechnic Institute and State University (approval number 13-031). All animals were housed at the Virginia Tech Middleburg Agricultural Research and Extension (MARE) Center in Middleburg, VA. Thirteen non-lactating, light horse mares (mean ± SEM age=13.56±0.11 yr; mean ± SEM BCS=7.07±0.21) were utilized for this study. Mares were matched by age, weight, BCS, parity and insulin sensitivity and randomly assigned to one of two groups. Mares in the omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) supplemented group (n=7) were fed a diet containing 0.06 g/kg BW algae-derived omega-3 LCPUFA (DHA Gold® DSM Nutritional Products Inc. Ames, IA) along with a commercially available concentrate diet (Southern States®, Richmond, VA; mean ± SEM 1282.4 ± 30.4 g). Mares in the control group received an isocaloric concentrate diet (Southern States®, Richmond, VA; mean ± SEM 1560.1 ± 52.8 g). Body weights and body conditions were recorded weekly and diets were adjusted on a weekly basis to account for body weight variations. Diets were fed 60 d prior to tissue and embryo collection following a 20 d dietary acclimation period during which increasing amounts of supplement and concentrate were fed until no refusals were noted. All mares were housed on a single pasture containing mixed fescue and orchardgrass with free choice access to water and a vitamin/mineral mix. Dietary supplementation began in May and proceeded through November. Endometrial tissue and embryo collections began in June and continued through the end of dietary supplementation. Pasture samples were collected once every

two weeks and analyzed for nutrient composition. Pasture samples obtained were representative of available forage types and selected from areas with evidence of grazing. Once collected pasture was stored at -20°C until further analysis. Pasture samples were analyzed individually by collection time point and results were grouped into three categories: mid summer (mean of samples in June and July), late summer (mean of samples in August and September) and Fall (mean of samples in October and November). Concentrate aliquots were collected every two weeks and stored at -20°C until further analysis for nutrient composition. Concentrate was analyzed individually by collection time point and results were pooled due to limited variation in nutrient composition. All nutrient requirements for mares were met by the diets. Nutrient composition of feedstuffs is outlined in Table 3-1.

Experimental Procedure and Schedule

Synchronization of estrous cycles in mares was accomplished using a single intramuscular (IM) dose of cloprostenol (250 mg, Estrumate®, Schering-Plough Animal Health, Kenilworth, NJ) followed by daily oral administration of 0.044 mg/kg BW altrenogest (Regumate®, Intervet Inc., Summit NJ) for 2 wk, and finally a second dose of cloprostenol (250 mg, Estrumate®, Schering-Plough Animal Health, Kenilworth, NJ) injected IM on d 15. Estrous synchronization took place 45 d following the start of dietary supplementation. Following the second injection of cloprostenol (250 mg, Estrumate®, Schering-Plough Animal Health, Kenilworth, NJ), mares were monitored via daily transrectal ultrasound and ovarian follicular activity as well as uterine edema was recorded for four consecutive ovulatory cycles. Ovulation was confirmed via transrectal ultrasonograpy as the presence of a corpus luteum on the ovary in place of

the dominant follicle. Twelve days following ovulations 1, 3 and 4, endometrial biopsies were obtained trans-cervically using an equine uterine biopsy instrument (Kruuse, Denmark) and divided into three aliquots. Two aliquots were snap-frozen in liquid nitrogen and then stored at -80°C until needed for RNA extraction and fatty acid analysis. A final aliquot was fixed in formalin and paraffin embedded. Five micrometer tissue sections were cut and affixed to glass slides. Tissue were stained with H & E (hematoxylin and eosin) under standard procedures and then graded by an experienced theriogenologist blinded to treatment (Ricketts, 1975). Following the biopsy, a single dose of cloprostenol (250 mg, Estrumate®, Schering-Plough Animal Health, Kenilworth, NJ) was administered to induce luteolysis.

Embryo Recovery

Once a follicle of ≥ 35 mm in diameter and appropriate edema was detected mares were artificially inseminated every other day until ovulation using fresh semen from a single stallion of known fertility with at least 500 million motile spermatozoa for ovulatory cycles 2-4**.** Semen was collected using an artificial vagina and extended using Inra 96® (IMV Technologies, Normandy, France). Mares were not bred during the first ovulatory cycle. Twelve days following ovulation, transrectal ultrasound was used to observe the presence or absence of an equine conceptus. Immediately following ultrasound, a modified nasogastric tube was passed transcervically to the opening of the uterus. Approximately 2 liters of Bio Life 'Advantage' Complete embryo flush media (AgTech Inc., Manhattan, KS) was infused into the uterus. Transrectal uterine massage was used to ensure complete uterine filling and media was flushed out of the uterus into a collection vessel. The process was repeated twice or until a conceptus was observed

in the collection vessel. Following embryo recovery all mares received a single dose of 20 IU oxytocin (AgriLabs, St. Joseph, MO) to ensure uterine fluid clearance and a single dose of cloprostenol (250 mg, Estrumate®, Schering-Plough Animal Health, Kenilworth, NJ), to ensure luteolysis, administered IM. Mares were monitored via transrectal ultrasound following embryo recovery to ensure complete uterine fluid clearance.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from endometrial tissues using TRIzol reagent and PureLink RNA isolationcolumns (Life Technologies, Carlsbad, CA) according to manufacturer instructions. Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and then stored at -80°C. Total RNA (50 ng) was treated with DNase I amplification grade and first strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) according to the manufacturers instructions.

Total RNA was isolated from conceptus tissues using Qiagen® AllPrep DNA/RNA Kit (Qiagen®, City, State, Country) according to manufacturer instructions. Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Further conceptus RNA quality was determined using Experion RNA Standard Sensitivity Chips (Bio-Rad, Hercules, CA) per manufacturer recommendations. All RNA and DNA samples were stored at -80°C until needed for analysis. Total RNA (50 ng) was treated with DNase I amplification grade and first strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) according to manufacturer instructions.

Gene Expression Analysis by qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on candidate genes of interest depicted in Table 3-4 for endometrial samples. All qRT-PCR on endometrial samples was conducted using the 7500 Fast Real Time PCR System (Life Technologies, Carlsbad, CA). Each sample was carried out in duplicate, and contained 5 µl of SYBR® Green Master Mix (Life Technologies, Carlsbad, CA), 1 µl of cDNA template, 1 µl each of 100µM forward and reverse primer mix and 2 µl of nuclease-free water. The PCR reaction cycle and conditions were as follows: 1 cycle at 95°C for 20 sec, followed by 40 cycles of 3 sec of denaturation at 95°C, 30 sec of annealing at 60°C. Endometrial samples were normalized using *BACTIN*.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on genes of interest depicted in Table 3-5 for conceptus samples. All qRT-PCR on conceptus samples was conducted using the Eppendorf RealPlex^4 Mastercycler epgradient S (Eppendorf, Germany). Each 20 µl was carried out in duplicate, and contained 10 µl of SYBR® Green Master Mix (Life Technologies, Carlsbad, CA), 2 µl of cDNA template, 1 µl each of 100µM forward and reverse primer mix and 6 µl of nuclease-free water. The PCR reaction cycle and conditions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 15 sec at 55°C and 20 sec at 68°C. Conceptus samples were normalized using *GAPDH.*

For all qRT-PCR analysis, samples were repeated if Ct values were >1 Ct between duplicates. Controls were verified to serve as controls as described previously (Klein et al., 2011). Primer specificities were confirmed by melting curve analysis.

Samples were repeated if Ct values were >1 Ct between duplicates. Relative expression level of target genes was calculated using $2^{-\Delta Ct \text{ gene of interest}}/2^{-\Delta Ct \text{ reference}}$ gene. Statistical analyses were performed on log transformed data.

Fatty Acid Analysis

Endometrial biopsies were obtained from mares approximately 12.5 days post ovulation 1. Mares from the control group (n=6) were on diet for an average of 49 d (range 47-53 d) while mares from the DHA supplemented group (n=7) were on diet for an average of 49.5 d (range 45-51 d). Endometrial samples obtained following the first ovulatory period were stored at -80°C and utilized for fatty acid composition analysis. At least 25 mg of endometrial tissue was utilized for fatty acid extraction and analysis. All fatty acid analysis and extraction was completed by the University of Missouri-Columbia Agricultural Experiment Station and Chemical Laboratories (ESCL; Columbia, MO). Briefly, fatty acids were extracted using the Folch extraction method for total lipids from animal tissues (Folch et al., 1957). Fatty acid analysis was completed using AOAC and AOCS official methods by gas-liquid chromatography.

Statistical Analysis

All statistical analysis was performed and analyzed using Statistical Analysis Software (v9.3, SAS Inst. Inc. Cary, NC). Gene expression data expressed as $2^{-\Delta Ct}$ values were log transformed and analyzed using the GLM procedure. Body condition score and body weight measurements were tested for normality and analyzed using the GLM procedure in SAS. Endometrial biopsy scores were transformed into an ordered scale and analyzed as repeated measures using ANOVA. Endometrial fatty acid composition was analyzed using the GLM procedure in SAS. Gene expression data are

presented as $2^{-\Delta Ct}$ values. Differences were considered significant at $P \leq 0.05$ and a tendency was considered at $P \leq 0.10$.

Results

All horses remained healthy over the course of the supplementation period and experiment. Body weight and body condition remained constant through the course of supplementation with no changes over time or between groups (Table 3-2). Following the adaptation period, horses readily consumed both the control diet as well as the DHA supplement. During the acclimation period some initial refusals were observed, but ceased quickly once the correct feeding procedures were optimized. Mares readily consumed the diet after mixing the textured concentrate with the supplement prior to feeding. Slight refusals were observed from only a single mare during estrus during the monitored ovulatory cycles, however refusals were of an insignificant amount and she resumed consumption for the remainder of the supplementation period.

Endometrial Fatty Acid Composition

Endometrial fatty acid composition is shown in Table 3-3. Mares from the DHA group had increased incorporation of DHA into endometrial tissue as compared to control mares (P=0.0002*).* Additionally, supplemented mares had increased DHA as a percent of total fat (P=0.0155) and as a percent of wet tissue weight (P=0.0027). When total omega 3 fatty acids were calculated as the sum of linolenic acid (18:3n3), clupanodonic acid and docosahexaenoic acid (22:6n3), mares from the supplemented group had increased total omega 3 (P=0.0001), total omega 3 as a percent of total fat (P=0.015) and total omega-3 as a percent of wet tissue weight (P=0.002) as compared to control mares. Total omega-6 fatty acids were calculated as the sum of linoleic acid (18:2n6) and arachidonic acid (20:4n6). Biopsies from mares supplemented with DHA had increased total omega-6 fatty acids (P=0.002) and a tendency for increased total

omega-6 fatty acids as a percent of wet tissue weight (*P*=0.101). The ratio of total omega-3 to total omega-6 fatty acids in biopsies from supplemented mares was higher than those from control mares (*P*=0.017).

Endometrial Biopsy Scores

Endometrial biopsy scores from DHA supplemented mares (n=7) were significantly less following the first ovulation (*P*≤0.05) than those obtained from control mares (n=6). Thereafter, biopsy scores between groups remained similar with no significant differences between them. In order to provide the best representation of the uterine environment prior to manipulation no pre-treatment samples were obtained. Endometrial biopsy score data is depicted in Figure 3-1.

Endometrial Gene Expression

Endometrial gene expression was altered across all time points in DHA supplemented mares as compared to non-treated control animals. A total of 38 endometrial biopsies were obtained across four ovulatory cycles. Endometrial samples were analyzed by qRT-PCR for a wide variety of genes depicted in Table 3-4. Data were analyzed between groups as well as by pregnancy status and ovulatory cycle.

When evaluated independent of pregnancy status and ovulatory cycle, genes related to inflammation and uterine nutrient transport displayed differential transcript abundance. Results of gene expression analysis are depicted in Figure 3-2. Endometrial tissues obtained from DHA supplemented mares displayed decreased mRNA abundance of the pro-inflammatory signaling genes *IL6* and *TNFA* ($P \leq 0.05$). Additionally elevated transcript levels were observed for the anti-inflammatory gene *IL10* ($P \leq 0.05$) in endometrial samples obtained from DHA supplemented mares.

Interestingly, a trend was observed for increased expression of *SAA*, ($P \leq 0.10$), a gene related to systemic inflammatory status in endometrial samples obtained from DHA supplemented mares. Expression levels of *UTEROCALIN*, a gene associated with uterine nutrient transport, tended to be higher in endometrial samples obtained from DHA supplemented mares as compared to control animals ($P \leq 0.10$). No differences in other transcripts were observed.

Endometrial Gene Expression in Cyclic Mares

All endometrial biopsies taken post-ovulatory cycle 1 were obtained from non-bred, cycling animals. When evaluated between DHA supplemented animals and control animals, differential expression of transcripts relating to prostaglandin signaling and inflammation signaling pathways were detected (Fig. 3-3). Endometrial samples obtained from DHA supplemented mares displayed increased transcript abundance of *PTGFS* ($P \leq 0.05$) and *PPARA* mRNA ($P \leq 0.10$) and reduced abundance of *IL6* ($P \leq 0.05$) as compared to those obtained form control animals. No differences in other transcripts were observed.

Post Ovulatory Cycle 3 Endometrial Gene Expression

Endometrial samples obtained from mares approximately 12.5 days following ovulation 3 could be categorized into those from mares that were pregnant (DHA group n=3; control group n=4) and those that were bred, but did not become pregnant (DHA group n=4; control group n=1). Endometrial biopsies obtained from pregnant mares during the third ovulatory cycle displayed differential expression of genes related to prostaglandin signaling and inflammation signaling pathways. Transcript abundance of *PLA2G4A* was increased ($P \leq 0.05$) in DHA supplemented mares, as was mRNA

abundance of *SAA* ($P \leq 0.10$) and *IL10* ($P \leq 0.05$). Endometrial samples obtained from non-supplemented mares that were bred but did not become pregnant displayed increased transcript levels of *PTGS1, PTGER2* and the steroid hormone receptor *PGR* ($P \leq 0.05$) as compared to DHA supplemented animals. Results of gene expression analysis are depicted in Figure 3-4.

Post Ovulatory Cycles 3 and 4 Endometrial Gene Expression

Endometrial samples obtained from mares following ovulation in cycles 3 and 4 were further analyzed together for differential expression of genes relating to prostaglandin synthesis, inflammation signaling, fatty acid metabolism and uterine nutrient transport. These samples came from both pregnant animals (DHA group n=6; control group n=5) and those that were bred but did not become pregnant (DHA group n= 7; control group n= 5). Results of gene expression analysis are depicted in Figure 3-5. Among samples obtained from pregnant animals, differential expression of prostaglandin signaling genes was observed. Samples obtained from DHA supplemented animals contained increased mRNA abundance of *PTGFS* and *OXTR* ($P \leq 0.05$) as well as increased transcript abundance of *PTGES* and *SLCO2A1* ($P \leq 0.10$) as compared to control animals. Among samples obtained from mares that were bred but did not become pregnant, increased transcript abundance of *PTGER2* ($P \leq 0.05$) and PTGS1 ($P \leq 0.10$) was observed in non-supplemented control animals as compared to those obtained from DHA supplemented mares. Additionally, endometrial tissue from non-pregnant DHA supplemented mares contained increased mRNA levels of *UTEROCALIN* ($P \leq 0.10$) as compared to non-supplemented controls.

Embryo Recovery and Conceptus Gene Expression

A total of 13 conceptuses were recovered from mares at 12.5 d post ovulation in cycles 3 and 4. Following RNA extraction and quality evaluation, 10 conceptuses were utilized for gene expression analysis (DHA n=5; Control n=5). The remaining 3 conceptuses did not contain sufficient quantity and quality of RNA for qRT-PCR analysis. Conceptus gene expression was evaluated for a variety of candidate genes depicted in Table 3-5. Differential expression of genes related to trophectoderm (TE) and primitive endoderm (PrE) was observed (Figure 3-6). Embryos from DHA supplemented mares contained increased transcript abundance of *GATA3, TFAP2A* and *ELF3*, markers of trophectoderm differentiation ($P \leq 0.05$). Additionally, embryos from DHA supplemented mares contained increased transcript abundance of the PrE differentiation related genes *GATA4* and *GATA6* ($P \leq 0.05$).

Discussion

The current study, to the authors' knowledge, is the first to evaluate the effects of supplementing an algae-derived omega-3 fatty acid on endometrial and subsequent embryonic gene expression in horses. Additionally, as far as the authors are aware, this is the first study to report the incorporation of supplemental dietary fatty acids into endometrial tissue in the horse. Previous studies have found that supplementation of an EPA and DHA source altered red blood cell membrane composition and skeletal muscle composition (Hess et al., 2012). The current study demonstrated alteration of inflammatory signaling and prostaglandin signaling in the endometrial tissue obtained from DHA supplemented mares. While it is likely that the biological changes mediated by omega-3 fatty acid supplementation are mediated by the coordinated expression of a large number of genes, this seminal study investigated candidate genes representing pathways impacted by omega-3 fatty acid supplementation. The most significant alterations in expression were found in genes responsible for inflammatory signaling and prostaglandin synthesis. Finally, this study is the first to describe changes in gene expression in embryos obtained from mares supplemented with an omega-3 DHA supplement. Notably genes related to lineage differentiation were differentially expressed between supplemented and control mares. Taken together these results indicate that an algae-derived omega-3 fatty acid supplement administered to mares during the pre and peri-conceptual period alters the uterine environment and subsequently embryonic development.

Tissue Incorporation and Uterine Health

In the current study fatty acid composition of endometrial tissue was altered in mares supplemented with an algae-derived DHA supplement. Total DHA was increased in tissues obtained from supplemented mares as well as DHA as a percentage of total fat. When evaluated as levels of omega-3 fatty acids, tissue composition was similarly altered. Previous studies have indicated that conversion of the parent fatty acid, ALA, into the long chain fatty acids EPA and DHA is inefficient in horses (Hess et al., 2012). Results from this study indicate that conversion of DHA into EPA does not occur at appreciable levels, as indicated by the lack of EPA in analyzed endometrial samples. Dietary supplementation in this study was by a relatively pure DHA source with only trace amounts of EPA included in the supplement. Taken together these results suggest the need to supplement DHA directly when desiring an increased incorporation of these omega-3 long chain polyunsaturated fatty acids.

Endometrial biopsy scoring is a tool commonly used in the equine industry during the breeding soundness exam. Generally, these biopsies are graded using the Kenny scale with a lower score indicative of improved uterine health as compared to a higher score (Snider et al., 2011). Scores are based on abnormalities in the endometrium associated with inflammation and various other degenerative qualities (Kenney, 1978). While the effects of obesity on equine endometrial biopsy scores has yet to be determined, an overall decrease in reproductive health has been noted in obese individuals. This study sought to understand the effects of DHA supplementation on overall endometrial health and results indicate a significant decrease in biopsy scores following the first ovulatory period, coinciding with improved uterine health, from

samples obtained from supplemented mares following the first ovulatory cycle. While significance was lost over subsequent estrous cycles, the authors attribute this to continued manipulation of the uterus coinciding with breeding, embryo flushing and ensuing endometrial biopsies. Early embryonic loss in the mare, defined as pregnancy failure between fertilization and day 40-60 of pregnancy, is of significant value to the equine industry with rates estimated to be 10-15% in young mares and approaching 70% in aged mares (Vanderwall, 2008). Endometrial disease has been implicated for its role in early embryonic loss and is generally characterized by endometrial inflammation (Adams et al., 1987; Carnevale and Ginther, 1992). The anti-inflammatory nature of omega-3 fatty acids represents a potentially interesting method of reducing inflammation in the uterus of the mare. These results indicate a potential for the recommendation of supplementation of omega-3 fatty acids to overweight or obese mares during the breeding season to reduce uterine inflammation. Improvements in biopsy scores relate to increased conception and pregnancy maintenance.

Endometrial Gene Expression

Endometrial gene expression was altered in supplemented mares across all experimental timepoints, most notably in genes related to prostaglandin biosynthesis, inflammation and uterine nutrient transport. Prostaglandin signaling and inflammatory signaling are key regulators of reproductive function. The ability of omega-3 fatty acid supplementation to alter gene expression of these pathways is of significance. Obesity is associated with alterations in systemic inflammation and disruption in inflammation-related pathways (Ferrante, 2007). While the effects of obesity on reproductive function of the mare have yet to be fully elucidated epidemiological evidence and understanding

of the role of inflammation on reproductive dysfunction, implicate obesity as a negative modulator of reproductive health (Vick et al., 2006).

Prostaglandins play a critical role in a variety of reproductive functions including fertilization, ovulation and implantation. Additionally, prostaglandins are important in establishment and maintenance of pregnancy as evidenced by their role in degradation of the progesterone producing corpus luteum during luteolysis (Boerboom et al., 2004; Atli et al., 2010). Prostaglandins are synthesized through a series of complex enzymatic reactions stemming from the release of arachidonic acid from the cell membrane by phospholipase A2 (Crofford, 2001). From that point, various metabolic steps are undertaken and interconversion of prostaglandins is controlled by the expression of the cyclooxygenase enzymes, COX-1 and COX-2 (also known as PGHS-1 and PGHS-2) (Dubois et al., 1998). Products of cyclooxygenase metabolism include the luteolytic prostaglandin $F2_a$ (PGF_{2a}) and the more luteoprotective prostaglandin E_2 (PGE2) (McCracken et al., 1999). Specifically relating to the horse, modulation of the COX-2 enzyme is a crucial aspect of pregnancy recognition and maintenance (Boerboom et al., 2004). It is important to understand that the starting source of these metabolic pathways are fatty acids present in the lipid bilayer of the cell membrane. Alterations in the composition of the cellular membrane may alter the output of these complex metabolic pathways. Coinciding with altering the cellular membrane is the modulation of the expression of the key enzymes related to prostaglandin synthesis. One such method of altering the cellular membrane composition and changing the expression patterns of key enzymes is through fat supplementation in the diet.

Omega-3 supplementation alters the expression of genes involved in prostaglandin biosynthesis in cattle (Waters et al., 2012b). This report is the first to indicate that omega-3 supplementation during the pre- and periconceptual period alters expression of genes related to prostaglandin biosynthesis *in utero* in both pregnant and nonpregnant mares. In cattle supplemented with omega-3 fatty acids, there was a shift in expression of genes towards production of the more luteoprotective PGE_2 (Waters et al., 2012b). Prostaglandin synthesis pathways were altered in the current study as well. Across all timepoints, candidate genes related to prostaglandin synthesis displayed differential expression between supplemented and non-supplemented mares. In DHA supplemented mares post ovulatory cycle 1 there was an increase in prostaglandin F synthase *PTGFS* compared to non-supplemented mares. While this may appear to be in contrast to results seen by Waters *et al.,* 2012 from cattle, the samples taken from these mares was from a different point in the estrous cycle. Obesity prolongs estrous cycles through a prolonged diestrus period with little follicular activity (Leenen et al., 1994). Increased expression of the *PTGFS* gene could indicate the more rapid priming of the uterus for subsequent estrous cycles. In mares that were bred but did not become pregnant post-ovulatory cycle 3, there was an increase in transcript abundance of *PTGS1* that again may indicate a quicker return to proestrus and increased follicular activity. Pregnant mares displayed altered expression of genes related to prostaglandin synthesis. Supplemented, pregnant mares displayed increase expression of *PTGES, PTGFS, SLCO2A1* and *OXTR.* Maintenance of pregnancy is not fully understood in the mare, but it has been established that certain levels of prostaglandins are necessary for survival of the corpus luteum. Alterations in expression of genes related to

prostaglandin synthesis demonstrated in the current study possibly implicate omega-3 fatty acids for their potential to produce a more hospitable uterine environment for embryonic development.

Overall, expression patterns of genes related to prostaglandin biosynthesis were altered. While results were in contrast to those observed in previous studies, it is important to remember that the different sampling time point and the species variation may be responsible for the discrepancies. Further research is necessary to elucidate the specific role of prostaglandins in early equine pregnancy. The current study demonstrates a significant effect on the modulation of prostaglandin biosynthesis through supplementation of an omega-3 fatty acid supplement.

Inflammation is usually considered a negative modulator of reproductive function (Vanderwall, 2008). Further, uterine inflammation has been identified as an important modulator of maternal recognition of pregnancy in the mare (Patterson et al., 2012). This study demonstrates an alteration in the inflammatory pathways in the endometrium of mares as a result of DHA supplementation. In supplemented mares, regardless of pregnancy status or ovulatory cycle, an overall anti-inflammatory environment was observed. Decreased expression of pro-inflammatory cytokines *IL6, and* TNF_a coupled with increased expression of the anti-inflammatory cytokine *IL10* in supplemented mares demonstrates the anti-inflammatory effects of DHA supplementation. Supplemented non-bred mares post-ovulatory cycle 1 displayed decreased expression of *IL6,* while supplemented mares pregnant post-ovulatory cycle 3 displayed increased expression of *IL10*. A reduction in the inflammatory status of the endometrium may lead to improvements in overall reproductive health in horses and is likely to contribute to

increased fertility resulting from a more hospitable environment for embryonic development. These results indicate that omega-3 fatty acid supplementation altered endometrial gene expression, potentially modulating the uterine environment of overweight mares through gene-signaling pathways, resulting in a less inflammatory environment. When analyzed in conjunction with the improved biopsy scores observed in supplemented mares following the first ovulatory cycle, these results may provide evidence that omega-3 fatty acid supplementation improves the uterine environment of overweight mares leading to a more hospitable condition for embryonic development.

The equine pregnancy is unique in relation to other domestic livestock species in that implantation occurs approximately 40 d post-ovulation. This prolonged pre-implantation period highlights the need for proper uterine-derived support for embryonic development. In this study, the expression of *UTEROCALIN*, a gene responsible for nutrient transport to the developing embryo showed a tendency to be increased in DHA supplemented mares. This may indicate the presence of a more hospitable uterine environment further highlighting the potential for omega-3 fatty acid supplementation to alter the post-ovulatory uterine environment and improve reproductive performance in the overweight or obese mare.

Conceptus Gene Expression

The adverse effects of obesity on reproduction are likely in response to negative effects on the uterine environment and the resulting problems from compromised embryonic development. To the authors knowledge this is the first report that indicates an alteration of embryonic gene expression as a result of omega-3 supplementation to mares.

It has been hypothesized that compromised embryonic development, observed in obese individuals, is a result of aberrations in oocyte development. Recent reports have demonstrated an alteration in the ovarian follicular environment in obese horses with equine metabolic syndrome. Changes in the follicular fluid of EMS mares are consistent with increased inflammatory cytokines that are common in obese horses. Additionally, follicular fluid from EMS mares contained increases in insulin and leptin, molecules that have been demonstrated to alter reproductive activity. Further, granulosa cells obtained from mares with EMS displayed alterations in gene expression related to tissue remodeling, an important and necessary mechanism during ovulation (Sessions-Bresnahan and Carnevale, 2014). The alterations in expression patterns of inflammatory genes from endometrial samples presented in this report demonstrate that the effects of omega-3 supplementation are directly on reproductive tissues.

Due to the delayed implantation observed in equine embryonic development, the milieu of hormones and factors secreted from the endometrium plays an important role in modulating embryonic health. Embryos obtained from mares supplemented with DHA displayed differential expression of genes related to lineage differentiation. Embryos obtained from supplemented mares had increased mRNA abundance of *GATA3, ELF3,* and *TFAP2A*, genes that are associated with trophectoderm lineage differentiation. Trophectoderm cells are responsible for deriving extra-embryonic tissues, namely the placenta as well as endometrial cups, which are unique to equine pregnancy (Iqbal et al., 2014). The increased expression of trophectoderm-related genes, indicates either increased activity of those specific cells or increased numbers or trophectoderm-specific cells. Since *CDX2* and HAND1 were not differentially expressed in conceptuses, it is

more likely that the activity of TE cells was enhanced in those samples obtained from DHA supplemented mares. Both *CDX2* and HAND1 are constitutively expressed in the conceptus and the effects of DHA supplementation are likely on more inducible factors downstream. As the interaction between the outer trophectoderm cells and the endometrium of the mare is critical to pregnancy maintenance and establishment, it can be hypothesized that this increase potentially improved the viability of the embryos produced in DHA supplemented mares.

Embryos obtained from supplemented mares displayed further differential expression of genes related to the differentiation of primitive endoderm. Embryos obtained from supplemented mares displayed increased transcript abundance of both *GATA4* and *GATA6.* The primitive endoderm, which will eventually differentiate into the visceral and parietal endoderm is responsible for the formation of internal fetal structures as well as the yolk sac that supports early pregnancy (Prendiville et al., 1994). As obesity is known to reduce the quality of embryos and potentially delay embryonic development (Norman, 2010), increases in expression of these genes could indicate a mitigation of the harmful effects of obesity on embryonic development through omega-3 fatty acid supplementation.

Recently the role of fatty acids in embryonic development has become an area of intense research. Growing evidence suggests that fatty acids may play a role in the proper development of mammalian oocytes and embryos. Evidence suggests that omega-3 fatty acids improve oocyte development and embryo quality in *in-vitro* studies (Marei et al., 2010). Mammalian embryos naturally contain low levels of DHA, however supplementation of culture media with DHA increased the amount of DHA in embryos in

addition to increasing embryo quality indicating a potential benefit of DHA on overall embryonic development (McEvoy et al., 2000). Recent work demonstrates that alterations in the ratio of fatty acids in culture media will alter embryonic fatty acid composition, highlighting the importance of an optimum fatty acid ratio in the maternal diet (Van Hoeck et al., 2011). While the overall effects of fatty acids on embryo development are unknown, it is apparent that fatty acid composition is crucial for proper embryonic development. Much of the understanding of the role of fatty acids in embryonic development comes from *in-vitro* derived embryos, a technology that as of yet is unsuccessful in horses. Utilization of *in-vivo* derived embryos is crucial to better understand embryonic development in horses.

Conclusions

The current study demonstrated that supplementation of an algae-derived omega-3 fatty acid supplement to overweight mares during the pre and peri-conceptual period altered uterine composition, endometrial gene expression and embryonic gene expression. This study is the first to demonstrate in a live animal model that supplementation of omega-3 fatty acids alters the fatty acid composition and resulting health of endometrial tissue in the mare.

The main objectives of this study were to elucidate potential mechanisms by which alterations of the maternal uterine environment, due to over conditioning and nutritional supplementation during the periconceptual period may alter embryonic development. It was hypothesized that nutritional supplementation of omega-3 long chain polyunsaturated fatty acids (LCPUFA) from an algal source during the periconceptual period would alter endometrial composition and gene expression. Further, it was predicted that alterations in endometrial gene expression and competency would result in marked differences in gene expression of conceptus tissues relating to embryonic development.

There are currently few recommendations for feeding strategies for mares during the pre- and peri-conceptual period. The results from this study indicate that alterations in maternal reproductive health through nutritional supplementation are feasible. While specific recommendations for feeding strategies cannot be made solely from this research, it is important to consider further exploration of this concept.

Acknowledgments

This study was financially supported in part by Cooperative Research Farms Inc. (Richmond, VA). In addition, the DHA supplement was provided by DHA Gold (DSM Nutritional Products). The authors would like to thank the staff and undergraduate and graduate interns of the Virginia Tech Middleburg Agricultural Research and Extension Center for handling and care of the mares used in this study. Additionally, we express our gratitude to Dr. Kevin Dippert of Equine Reproduction Concepts (Amissville, VA) for his guidance in developing the embryo flush techniques for this project. Finally, we would like to thank the University of Missouri Agricultural Experiment Station Chemical Laboratories for their fatty acid analysis of the endometrial tissue.

Tables and Figures

Table 3-1- Nutrient composition of feedstuffs

			Pasture		
Nutrient	**Concentrate**	**DHA Supplement**	**Mid Summer[1]**	**Late Summer[2]**	**Fall[3]**
DM %	90.20	97.20	32.20	36.50	34.80
CP %	14.40	10.20	16.15	12.10	14.70
ADF %	19.40	1.50	35.00	36.20	32.40
NDF %	35.70	2.50	52.00	54.10	53.80
Ca %	1.12	0.03	0.87	0.82	1.82
P %	0.71	0.15	0.28	0.22	0.24
Zn mg/kg	211.00	4.00	ND	ND	ND
Cu mg/kg	55.00	5.00	ND	ND	ND
Total Fatty Acids, DM %	4.72	52.28	1.48	0.95	1.15
C18:2 n-6 (LA)	2.47	ND	0.21	0.15	0.15
C18:3 n-3 (ALA)	0.21	ND	0.77	0.50	0.65
C20:5 n-3 (EPA)	ND	0.81	ND	ND	ND
C22:6 n-3 (DHA)	ND	20.76	ND	ND	ND

Except for DM, all values presented on a 100% DM basis
DM= dry matter; CP= crude protein; ADF= acid detergent fiber; NDF= neutral detergent fiber; ND= not detected
[1]Mid Summer= mean of samples obtained in June and July
[2]Late Summer= mean of samples obtained in August and September
[3]Fall= mean of sample obtained in October and November

Table 3-2- Body weights and body condition scores

	Start of Supplementation[1]		End of Supplementation[2]	
	DHA	Control	DHA	Control
BCS (1-9)	6.93±0.2	7.00±0.28	7.07±0.3	7.08±0.31
Body Weight (kg)	645±54	652±45	642±52	661±30

[1] June 20, 2013
[2] October 24, 2013

Body weights and body condition scores of mares from DHA supplemented group and non-supplemented control group displayed as mean ± SEM. Supplementation began June 20, 2013 and continued through October 24, 2013 for a duration of supplementation of 126 days.

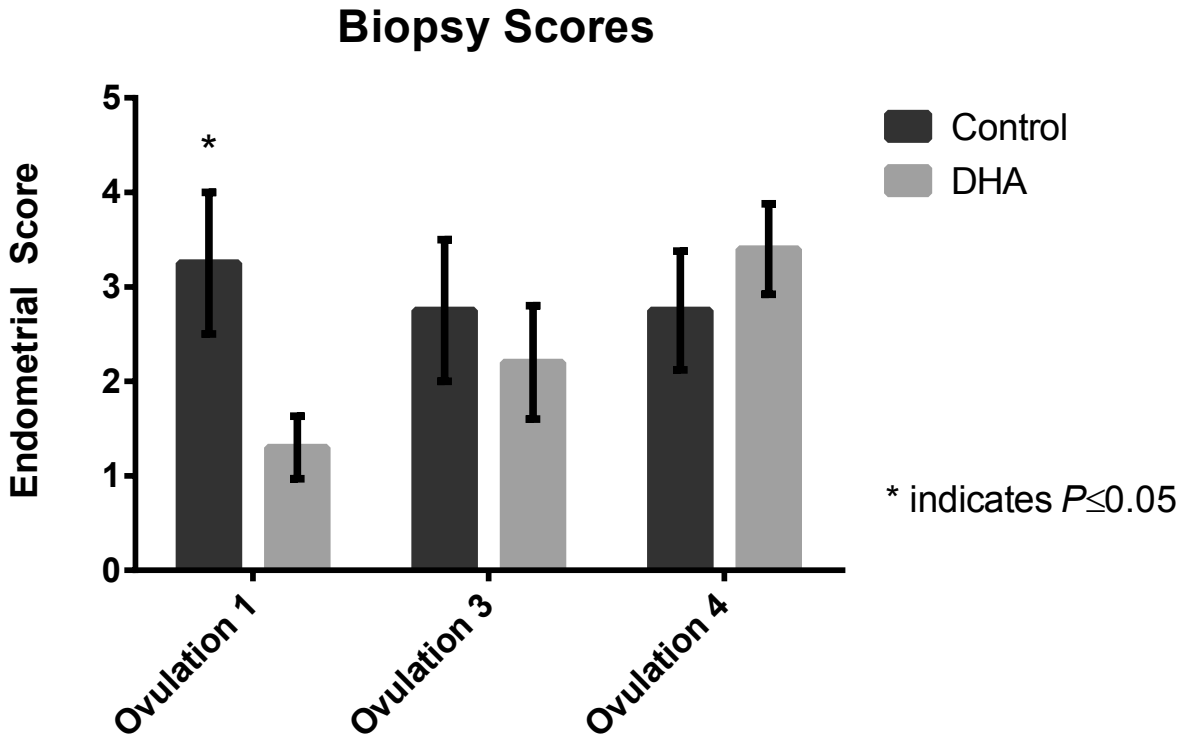
Table 3-3- Endometrial fatty acid composition

	Control	DHA	*P-Value*
DHA (ug)	6.41±2.37	24.99±2.45	0.0002
DHA (% Total Fat)	1.80±0.35	3.29±0.38	0.0155
DHA (% Wet Weight)	8.15±2.02	19.62±2.18	0.0027
[1]Omega 3 (ug)	14.79±3.16	42.20±3.42	0.0001
[1]Omega 3 (% Total Fat)	4.02±0.35	5.48±0.37	0.0154
[1]Omega 3 (% Wet Weight)	18.77±2.35	32.73±2.54	0.0020
[2]Omega 6 (ug)	100.51±17.21	202.46±18.59	0.0020
[2]Omega 6 (% Total Fat)	26.63±0.67	26.01±0.73	0.5506
[2]Omega 6 (% Wet Weight)	15.55±2.01	12.69±1.89	0.1011
[1]Omega 3 : [2]Omega 6	0.15±0.02	0.21±0.02	0.0174

[1]Omega 3= Linolenic (18:3n3) + Clupanodonic (22:5n3) + DHA (22:6n3)
[2]Omega 6= Linoleic (18:2n6) + Arachidonic (20:4n6)

Endometrial tissue fatty acid composition (mean ± SEM). As analyzed by the University of Missouri ESCL.

Figure 3-1- Endometrial biopsy scores



Endometrial biopsy scores as analyzed by an independent investigator at Auburn University College of Veterinary Medicine utilizing a modified Kenney Scale. Scores represent a scale of 1-5 with a score closer to 1 being indicative of healthier tissue. Tissues scoring 2a and 2b were reassigned a score of 3 or 4 respectively to allow for statistical analysis.

Table 3-4- Forwards and reverse primer sequences for genes utilized for qRT-PCR analysis of endometrial tissues.

Gene	Forward (F) and Reverse (R) Primer (5'-3')	Function
PTGS1	F: GCCTGACTCCTTCAGAGTGG R: TCTCGGGATTCCTTGATGAC	Prostaglandin Signaling
PTGS2	F: TATCCGCCCACAGTCAAAGACA R: TGTTGTGTTCCCGCAGCCAAAT	Prostaglandin Signaling
PTGES	F: GGAACGACATGGAGACCATCTAC R: GAAGGGATGCCCAATCCCCTAG	Prostaglandin Signaling
PTGFS	F: AAGCCAGGGCTCAAGTACAA R: AGCACCGTAGGCAACTAGGA	Prostaglandin Signaling
HPGD	F: GTTGCACAGCAGCCTGTTTA R: CATCGATGGGTCCAAAATTC	Prostaglandin Signaling
SLCO2A1	F: CGTTTTCTCTCTGCAAACCA R: GAGCGGTACTCCACTCCATT	Prostaglandin Signaling
PPARA	F: AGTGGTCCAGGATCAGATGG R: AGGCATGAACTCCGTAATGG	Prostaglandin Signaling
PPARD	F: ACGACATCGAGACATTGTGG R: TGATCTCCTTGTAGGGTGGC	Prostaglandin Signaling
PTGER2	F: CCTCCAAGCCCTTAGGTTTC R: TATCCACAAGGGCCAGCTAC	Prostaglandin Signaling
PTGFR	F: CGTGTGCTTGTTTGCTGTTT R: ATGGCATTGCACAAGAATGA	Prostaglandin Signaling
PLA2G4A	F: AGGGACAGCAACATTTACCCT R: GAGGTCTGGGCACGAACAAA	Prostaglandin Signaling
OXTR	F: TCTTCTTCGTGCAGATGTGG R: ACAGCATGTAGATCCAGGGG	Prostaglandin Signaling
PLA2G3	F: CACAGACTGTCTCGCCCTTT R: CTGGAACCTGGCATCACAGT	Prostaglandin Signaling
ESR1	F: GATAATCGACGCCAGGGAGG R: CTTCGTAGCATTTGCGGAGC	Steroid Signaling
ESR2	F: TCCTTTCTCACGTCAGGCAC R: GCCGTCTTTGCTCTCACTCT	Steroid Signaling
PGR	F: CCCAGCATGTCGCCTTAGAA R: TGATCAGTGGGGGCATCAAC	Steroid Signaling
FABP3	F: GGTCAAGTCCCTTGTGACACT R: GAGGCAATCTGGTGCTGAGT	Fatty Acid Metabolism
APOA1	F: GGGAAAACAGCTGAACCTGA R: GGAAATCGTCCAGGTAGGGC	Fatty Acid Metabolism
SAA	F: GTCATCAGCGATGCCAGAGA R: GTACTTGTCAGGCAGGCCAT	Fatty Acid Metabolism

CRP	F: GCAGCCGGTGCAAGATAGAA R: TTCCAAATCCCCAGGCCATC	Inflammatory Signaling
IL1B	F: CGGCCGGGACATAACTGACT R: GCCTGCAGCATGTTCAAACC	Inflammatory Signaling
IL6	F: GGCACCCAGTCTGAGAACAG R:TCTCAGGCTGAACTGCAGGAA	Inflammatory Signaling
IL10	F: GGCACCCAGTCTGAGAACAG F: TGGCAACCCAGGTAACCCTTA	Inflammatory Signaling
TNFA	F: GGCCCAGACACTCAGATCAT R: TTGGGGGTTTGCTACAACAT	Inflammatory Signaling
NFKB	F: GCCAACCCAAGTCTCTCTCC R: ATTACTGACAGCCCTTGCCC	Inflammatory Signaling
UTEROCALIN	F: CCCGGATGTCATGTGGATGT R: GTGGAGGCACCGATCAGTTT	Nutrient Transport
BACTIN	F: GGGACCTGACGGACTACCT R: CCGTGGTGGTGAAGCTCTA	Reference Gene
GAPDH	F: CATCATCCCTGCTTCTACTGG R: TCCACGACTGACACGTTAGG	Reference Gene

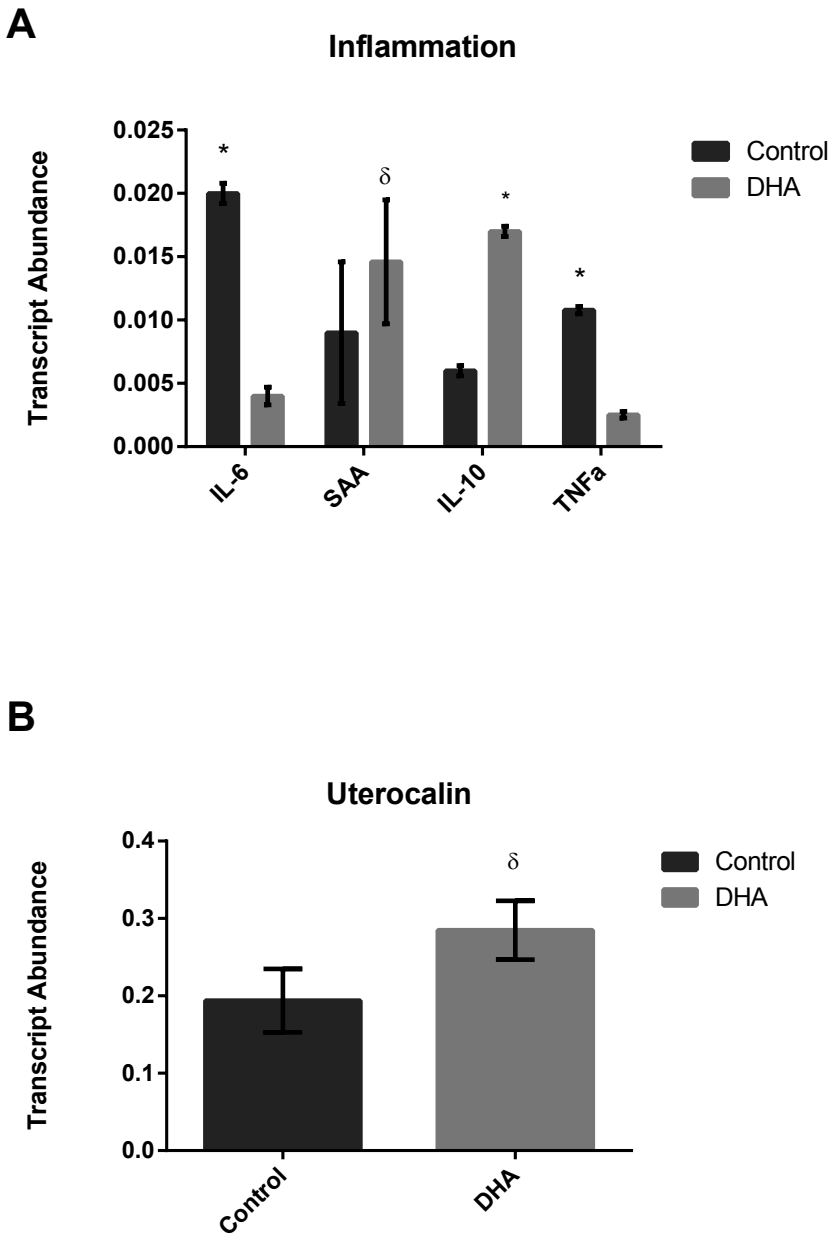
Table 3-5- Forwards and reverse primer sequences for genes utilized for qRT-PCR analysis of embryonic tissues.

Gene	Forward (F) and Reverse (R) Primer (5'-3')	Function
SLC17A5	F: GCAGATTTTGGAGTTGGAGC	Capsule Formation
	R: AAGCTTGCTTCTCTCAAGCG	
TGM3	F: GGTGTCTGTGAACATGACGG	Capsule Formation
	R: ATGAGATCTTCACGGGATGC	
REX1	F: GACGGGAAAGGCCTGGATAGAAG	ICM Differentiation
	R: GGCGGTAAGAAGCTGTTGAGAAAGG	
SALL4	F: AGAACTTCTCGTCTGCCAGC	ICM Differentiation
	R: TTTCCTTGGGAAACATCTCG	
CDX2	F: GGAGCTGGAGAAGGAGTTTC	TE Differentiation
	R: GGAGGGAAGACACAGGACTC	
HAND1	F: TGCCCACGAGGTTCATGTTG	TE Differentiation
	R: GCAGAACTCAAGAAGGCGGA	
GATA3	F: GCATCCAGACCAGAAACCGA	TE Differentiation
	R: ATGGTGAGGTCCGAAGGAGA	
ELF3	F: ACAGCAAGCTCTTCTCCAGC	TE Differentiation
	R: GACACTTCTCCAGGCAGACC	
EOMES	F: CTAAAAGAAGGTGCCAAAGC	TE Differentiation
	R: CTTAAGACCCAGCCCTTCTC	
TFAP2A	F: AATGCTTTGGAAACTGACGG	TE Differentiation
	R: ATTGACCTACAGTGCCCAGC	
GATA6	F: CGAGCGCTGTTTGTTTAGGG	Endoderm Differentiation
	R: ACTTCTAGCTCCTCGGGTGG	
GATA4	F: CTGACAAAGCCCAGAAGACC	Endoderm Differentiation
	R: CGTGGATTTCCTGACAGACC	
PTGS1	F: GCCTGACTCCTTCAGAGTGG	Prostaglandin Signaling
	R: TCTCGGGATTCCTTGATGAC	
PTGS2	F: TATCCGCCCACAGTCAAAGACA	Prostaglandin Signaling
	R: TGTTGTGTTCCCGCAGCCAAAT	
PTGES	F: GGAACGACATGGAGACCATCTAC	Prostaglandin Signaling
	R: GAAGGGATGCCCAATCCCCTAG	
PTGFS	F: AAGCCAGGGCTCAAGTACAA	Prostaglandin Signaling
	R: AGCACCGTAGGCAACTAGGA	
HPGD	F: GTTGCACAGCAGCCTGTTTA	Prostaglandin Signaling
	R: CATCGATGGGTCCAAAATTC	
SLCO2A1	F: CGTTTTCTCTCTGCAAACCA	Prostaglandin Signaling
	R: GAGCGGTACTCCACTCCATT	
PPARA	F: AGTGGTCCAGGATCAGATGG	Prostaglandin Signaling
	R: AGGCATGAACTCCGTAATGG	
PPARD	F: ACGACATCGAGACATTGTGG	Prostaglandin Signaling

	R: TGATCTCCTTGTAGGGTGGC	
PLA2G3	F: CACAGACTGTCTCGCCCTTT	Prostaglandin Signaling
	R: CTGGAACCTGGCATCACAGT	
FABP3	F: GGTCAAGTCCCTTGTGACACT	Fatty Acid Metabolism
	R: GAGGCAATCTGGTGCTGAGT	
SAA	F: GTCATCAGCGATGCCAGAGA	Fatty Acid Metabolism
	R: GTACTTGTCAGGCAGGCCAT	
APOA1	F: GGGAAAACAGCTGAACCTGA	Fatty Acid Metabolism
	R: GGAAATCGTCCAGGTAGGGC	
IL10	F: GGCACCCAGTCTGAGAACAG	Inflammatory Signaling
	R: TGGCAACCCAGGTAACCCTTA	
CRP	F: GCAGCCGGTGCAAGATAGAA	Inflammatory Signaling
	R: TTCCAAATCCCCAGGCCATC	
IL1B	F: CGGCCGGGACATAACTGACT	Inflammatory Signaling
	R: GCCTGCAGCATGTTCAAACC	
GAPDH	F: CATCATCCCTGCTTCTACTGG	Reference Gene
	R: TCCACGACTGACACGTTAGG	
18S	F: AACGACACTCTGGCATGCTAACTA	Reference Gene
	R: CGCCACTTGTCCCTCTAAGAA	

TE indicates trophectoderm. ICM indicates inner cell mass.

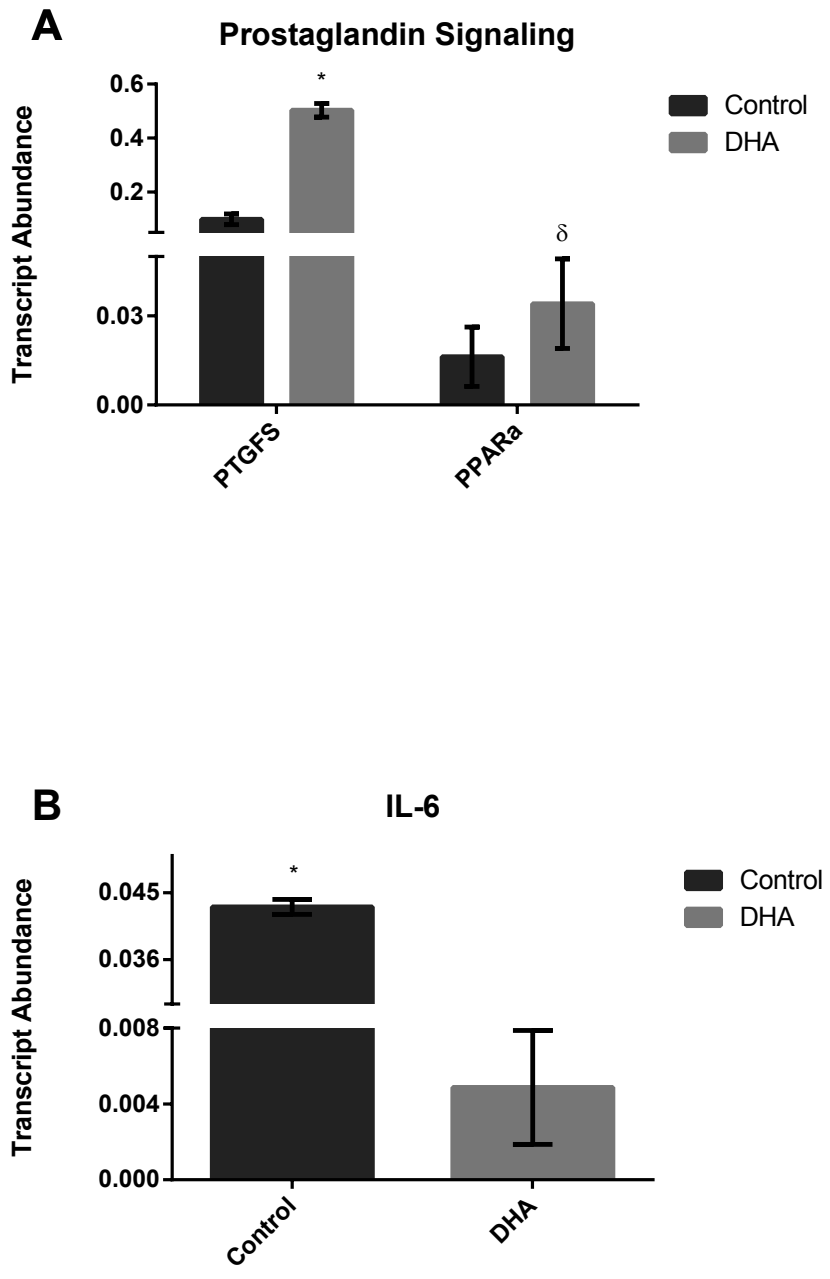
Figure 3-2- Overall gene expression



* indicates $P \leq 0.05$
δ indicates $P \leq 0.10$

Genotypic evaluation of equine endometrial tissues as analyzed between treatment groups regardless of time point. Values are expressed as relative transcript abundance with *BACTIN* used as the reference gene. **A)** Differentially expressed transcripts related to inflammatory pathways in endometrial tissue. **B)** Differential expression of *UTEROCALIN* in endometrial tissue.

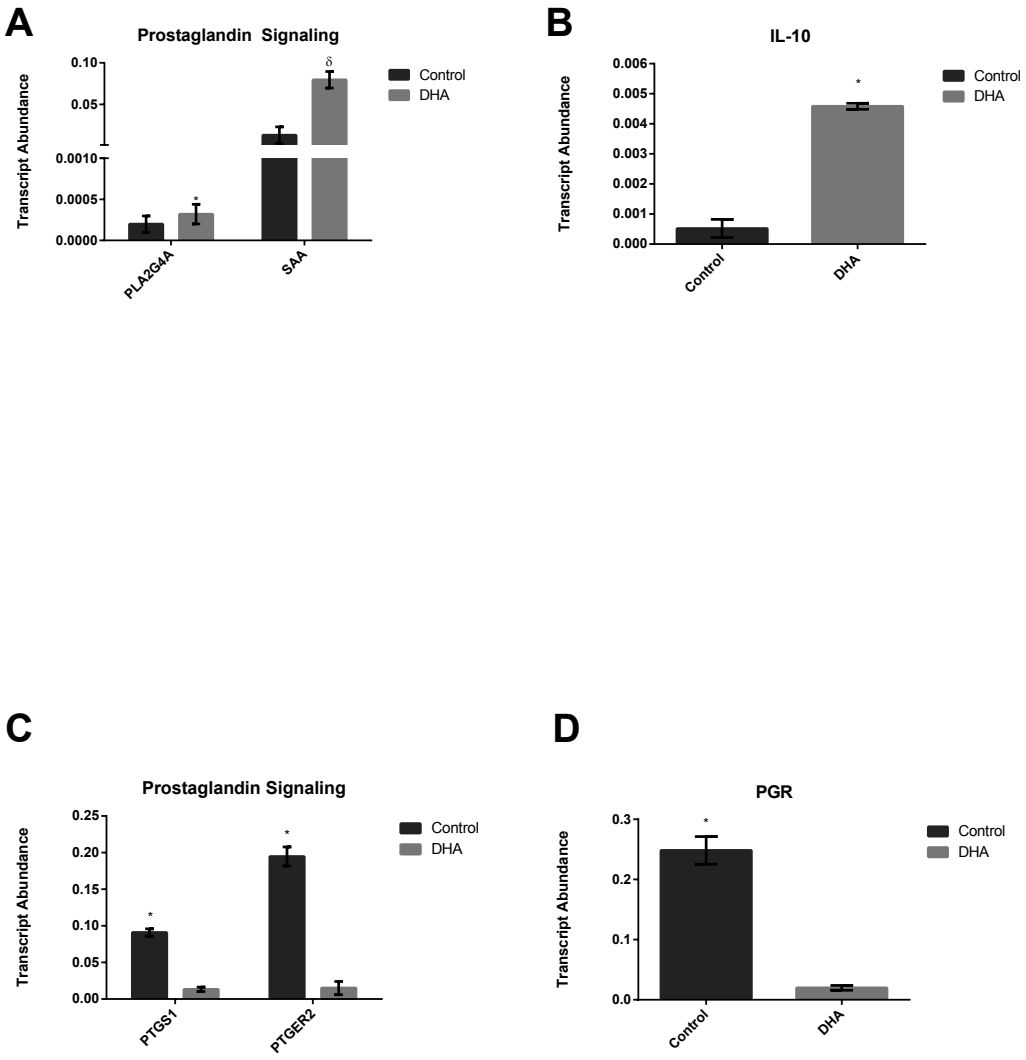
Figure 3-3- Endometrial gene expression in cyclic mares



* indicates $P \leq 0.05$
δ indicates $P \leq 0.10$

Genotypic evaluation of equine endometrial tissues obtained from supplemented and control animals post-ovulatory cycle 1. Values are expressed as relative transcript abundance with *BACTIN* used as the reference gene. **A)** Differentially expressed transcripts related to prostaglandin signaling. **B)** Differential expression of *IL6* in endometrial tissue.

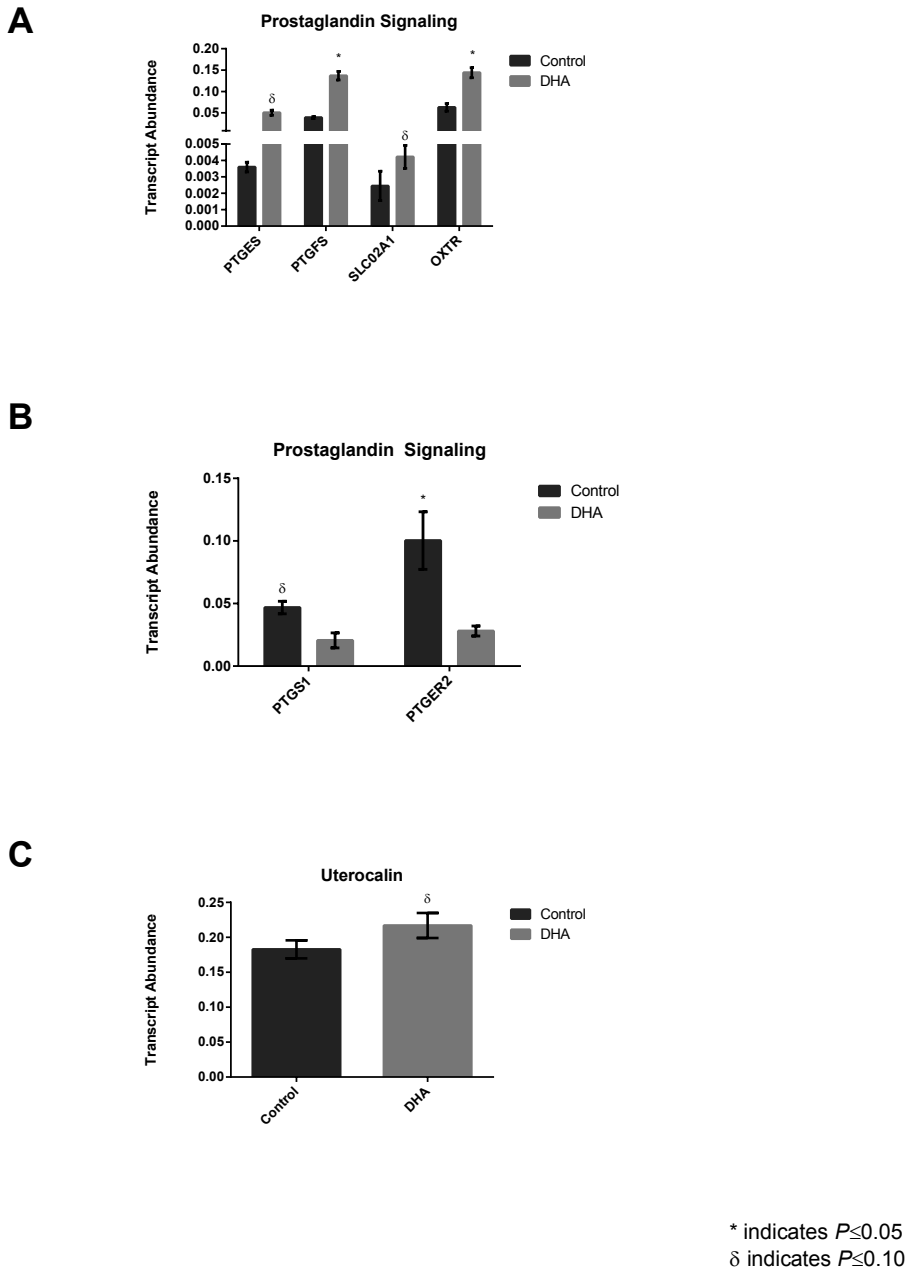
Figure 3-4- Post-ovulatory cycle 3 endometrial gene expression



* indicates $P \leq 0.05$
δ indicates $P \leq 0.10$

Genotypic evaluation of equine endometrial tissue obtained from pregnant supplemented and control animals post-ovulatory cycle 3. Values are expressed as relative transcript abundance with *BACTIN* used as the reference gene. **A)** Differentially expressed transcripts related to prostaglandin signaling in endometrium obtained from pregnant mares approximately 12.5 days post ovulation. **B)** Differential expression of *IL10* in endometrium obtained from pregnant mares approximately 12.5 days post ovulation. **C)** Differential expression of genes related to prostaglandin signaling in endometrium obtained from mares bred but not pregnant approximately 12.5 days post ovulation. **D)** Differential expression of *PGR* in endometrium obtained from mares bred but not pregnant approximately 12.5 days post ovulation.

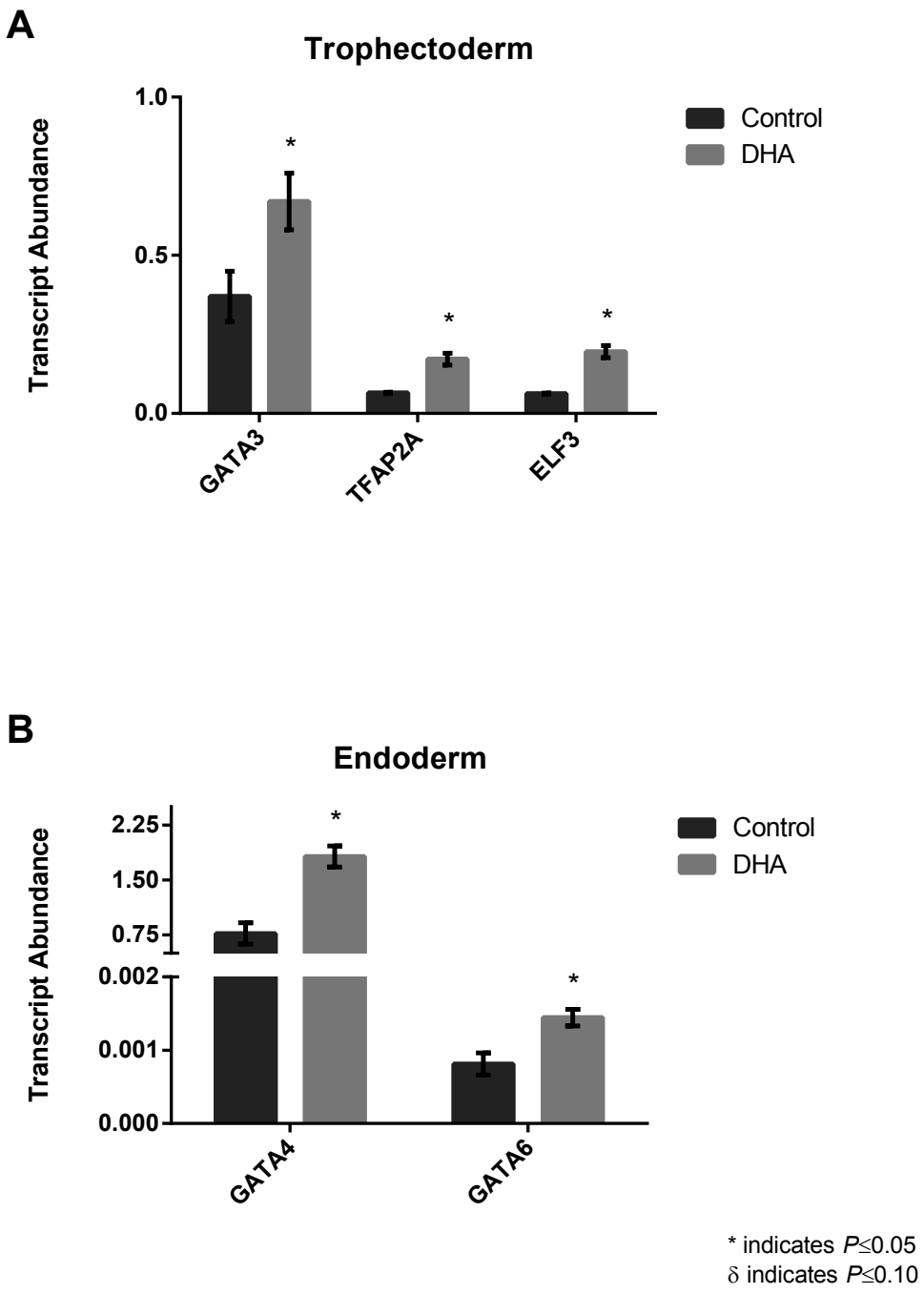
Figure 3-5- Post-ovulatory cycles 3 and 4 endometrial gene expression



* indicates $P \leq 0.05$
δ indicates $P \leq 0.10$

Genotypic evaluation of equine endometrial tissue obtained from pregnant supplemented and control animals post-ovulatory cycles 3 and 4. Values are expressed as relative transcript abundance with *BACTIN* used as the reference gene. **A)** Differentially expressed transcripts related to prostaglandin signaling in endometrium obtained from pregnant mares approximately 12.5 days post ovulation. **B)** Differentially expressed transcripts related to prostaglandin signaling in endometrium obtained from mares bred but not pregnant approximately 12.5 days post ovulation. **C)** Differential expression of *UTEROCALIN* in endometrium obtained from mares bred but not pregnant approximately 12.5 days post ovulation.

Figure 3-6- Conceptus gene expression



Genotypic evaluation of day 12.5 equine conceptuses obtained from supplemented and control animals. Values are expressed as relative transcript abundance with *GAPDH* used as the reference gene. **A)** Differentially expressed transcripts related to trophectoderm differentiation in embryos obtained from mares approximately 12.5 days post ovulation. **B)** Differentially expressed transcripts related to endoderm differentiation in embryos obtained from mares approximately 12.5 days post ovulation.

CHAPTER 4
IMPLICATIONS AND FUTURE RESEARCH

As our knowledge regarding metabolic imprinting and programming increases it would be irresponsible to disregard the role of early-life nutrition on long-term physiological outcomes. The pre and peri-conceptual period may represent a unique window in which nutritional intervention could be used to improve lifelong health. The prevalence of obesity across species is rising at an astounding pace. While research into weight loss strategies and early intervention continue, it is necessary that we utilize the resources available to us to better understand the role of nutrition in modulating embryonic development and developmental programming. In order to do so, it is necessary to use both *in vitro* and *in vivo* models to elucidate the role of maternal nutrition in lifetime disease risk in the horse.

The previous chapters described the beneficial effects of feeding an algae-derived omega-3 long-chain polyunsaturated fatty acid supplement to mares during the pre and peri-conceptual periods. To the authors knowledge this is the first study to report the alteration of fatty acid composition of endometrial tissue as a result of supplementation. Additionally, uterine health may have been improved following supplementation indicating the potential for nutritional recommendations regarding feeding the overweight broodmare. Analysis of gene expression in endometrial tissue revealed striking differences regarding inflammation signaling genes that could further highlight the beneficial reproductive benefits of omega-3 fatty acid supplementation. Finally, the alterations in embryonic gene expression emphasize the effects of nutritional intervention on overall development of offspring. While these data provide keen insight into the role of maternal nutrition on reproductive health and embryonic

development, there is still much to learn. Currently there are very few recommendations for feeding the horse during the pre and peri-conceptual period. This absence presents a wide-open area for research into potential dietary interventions that could lead to long-term improvements in offspring health. Results of the previously presented research may provide groundwork for developing supplements that could be used to improve uterine health in overweight and metabolically compromised mares.

This chapter will describe broad research goals that will hopefully direct my future career objectives. My career research goals aim to improve the reproductive health of horses while developing a better understanding of the mechanisms responsible for developmental programming and maternal-fetal interaction. Through my research, I hope to develop feeding strategies that would utilize programming, and eventually reprogramming, to improve health through nutritional intervention.

The increased accessibility to next-generation technologies has opened up a world of research that was once thought to be too expensive or even unattainable. The existing samples from the work presented in this dissertation represent a unique opportunity to utilize the advancements in next-generation RNA sequencing to provide a broad overview of the effect of nutritional supplementation on both endometrial health and embryonic development. The vast amount of data garnered from this analysis could provide further evidence as to the beneficial effects of omega-3 fatty acid supplementation on uterine health and embryonic development. Investigating pathways with a similar expression profile in both endometrial and embryonic tissues may provide targets for intervention strategies. Various growth factors related to angiogenesis, differentiation, implantation and placentation have already been implicated for their

potential role in developmental programming and deep sequencing analysis would provide a far-reaching approach to investigate these complex mechanisms. While these analyses would allow for evaluation of an increased number of transcripts and more in-depth pathway analysis there is still much to be investigated.

The use of an *in vitro* model for the study of maternal fetal interaction has been used in large ruminants. Unfortunately, to date, an immortalized equine endometrial cell line has yet to be established. The development of an immortalized cell line would allow further investigation of the mechanisms underlying nutritional regulation of endometrial health as well as provide a controlled environment to investigate maternal-embryo communication in the horse. Simply developing the cell line could prove challenging however ovine, bovine and porcine models exist that would provide a template for the cell line development. Once developed, an immortalized cell line could be used to investigate the effects of various treatments mimicking common *in utero* stressors. In addition, the treatment of these cells with various nutrients and nutraceuticals may prove efficacious in generating candidate compounds that could be used in whole-animal studies. Finally, interaction between an endometrial cell line and *in vivo* derived embryos may provide a method to investigate, in a cleaner and more controlled environment, the complexities of maternal-embryo communication.

Endometrial explants are another tool that has been used to evaluate the effects of various treatments on uterine health. Recently researchers utilized endometrial explants to observe the effects of various challenges and treatments associated with inflammation and prostaglandin synthesis. Explants would allow for another resource to evaluate effects of nutrition on uterine health without the compounding factors

associated with *in vivo* research. I propose utilizing an explant system in which samples are derived from obese and non-obese mares. These samples would then be cultured with and without embryos derived from both sets of mares to evaluate the effects of nutritional status on embryonic development and gene expression. While the development and collection of embryos *in vivo* may prove difficult there are currently no other options, as *in-vitro* fertilization has been met with several difficulties in the horse. Better understanding of the maternal-fetal communication pathways would allow us to better predict the predisposition of offspring to various diseases.

Finally, the use of an *in-vivo* model for equine obesity that results in live foals participating in lifelong and longitudinal studies would provide the best model to evaluate the effects of obesity on reproduction. This approach, which has been utilized in small-scale equine studies, has displayed profound differences in metabolic status, weight and overall health of offspring produced following various *in utero* stressors including nutritional mismanagement. A large scale, controlled study utilizing obese and non-obese mares bred to a single stallion would potentially provide strong evidence as to the role of nutrition on overall health. The offspring produced from these pairings could be evaluated at various timepoints postpartum. Additionally, the offspring could be subjected to various treatments to help further elucidate the overall effects of maternal nutrition and body condition on disease status in adult life. The difficulties and pitfalls of a trial of this scale are great. It would require extensive resources and manpower to accomplish, but the benefits could prove increasingly valuable.

Metabolic programming is a concept that will only increase in relevance due to a greater understanding and advanced technologies. Increasingly it appears that no

species is immune to the effects of metabolic programming. As the prevalence of obesity increases, and future generations of equine athletes, companions and research animals are born it is imperative that research continue to progress in this area. The equine industry represents a large economic footprint nationally and internationally and horse owners and breeders are increasingly interested in metabolic dysfunction across generations. It is my hope that the information presented in the previous dissertation as well as the research proposed in this chapter can be utilized to provide a healthier future for the equine species.

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Appendix 1- Modified Frequently Sampled Intravenous Glucose Tolerance Test (FSIGTT) Protocol

Catheter Insertion:

Supplies Needed: (per catheter)

- 1-prep razor
- ¼ sleeve 4 x 4 sponges
- 1-25 gauge needle
- 3 cc lidocaine
- ¼ bag clorhexidine scrub
- ¼ bag alcohol scrub
- 1- extension set
- 1- catheter
- 1- 60 cc syringe
- 1 bag heparinized saline (1 cc heparin/liter)
- 1- suture
- 1 roll elastikon
- 1 roll vetwrap
- Sterile gloves

1- Shave area with prep razor approximately 3 x 3 in square
2- Aseptically clean area with clorhexidine scrub and alcohol scrub
3- Inject 3 cc lidocaine subcutaneously on top of jugular
4- Insert catheter
5- Attach extension set (prefilled with heparinized saline)
6- Suture catheter and extension set in pace with three sutures
7- Flush catheter with heparinized saline
8- Cover with clean 4 x 4 sponge
9- Wrap in vetwrap and elastikion

FSIGTT Supplies (per horse)

- 2- 60 cc syringes
- 2- 20 cc syringes
- 2- 10 cc syringes
- 1-1 cc syringe
- 2 bags heparinzed saline (1 cc heparin/liter)
- 33 red top serum collection tubes
- 33 grey top plasma collection tubes
- 132 microcentrifuge tubes (2 aliquots serum and 2 aliquots plasma)
- Dextrose (approximately 3 500 mL bottles/horse)
- Insulin (1 bottle total/approximately 1cc/horse)

For all blood collection timepoints use the following protocol:

1- Give 10 second warning to waste reminder
2- Collect approximately 5 cc waste blood and discard 10 seconds prior to timepoint
3- Collect approximately 20 cc blood at timepoint
4- Flush catheter with approximately 12 cc heparinized saline
5- Aliquot blood into 1 grey top tube for plasma collection and 1 red top serum tube

FSIGTT Protocol:

1- Weigh all horses
2- Prepare glucose and insulin
    a. Glucose- 0.3 g glucose/kg BW
    b. Insulin- 30 mU/kg BW
3- Put glucose in 60 cc syringes
4- Put insulin in 1 cc syringe
5- Fast horses for at least 12 hours
6- Flush catheter with heparinized saline
7- Collect sample 30 minutes prior to starting
8- Take 0 minute blood sample
9- Immediately begin glucose infusion
    a. All glucose must be infused within 45 seconds (Usually 5-6 60 cc syringes)
10- Take blood samples at the following timepoints using above protocol
    a. 1,2,3,4,5,6,7,8,9,10,12,14,16,19 minutes (ALL TIMEPOINTS ARE POST GLUCOSE INFUSION)
11- Infuse insulin at 20 minutes
12- Take blood samples at the following timepoints using above protocol
    a. 22,23,24,25,27,30,35,40,50,60,70,80,90,100,120,150,180 minutes (ALL TIMEPOINTS ARE POST GLUCOSE INFUSION)
13- Remove catheter
14- Cover catheter site with biozide and monitor for inflammation for 48 hours
15- Allow serum to clot at room temperature
16- Spin plasma and serum at 5000 rpm for ten minutes
17- Alliquot into microcentrifuge tubes

Pertinent Calculations:

Glucose dose= kgBW x 0.3
Volume of dextrose= glucose dose x (100/50)
Insulin dose= (kgBW x 30)/1000
Volume of insulin= insulin dose/ 100

Appendix 2- Endometrial Biopsy Protocol

Supplies Needed:

- 2-Uterine biopsy forceps
- 2-2000 mL graduated cylinders
- Sterile lubricant (1 tube per biopsy session)
- Sterile palpation sleeves (2 per horse per biopsy)
- Surgical gloves (1 pair per horse per biopsy)
- 1-inch needle (1 per horse per biopsy)
- Palpation sleeve (1 per horse per biopsy)
- Nonsterile lubricant
- Tail bag (1 per horse per biopsy)
- Latex gloves (2 pairs per horse per biopsy)
- Ivory liquid soap
- Paper towels
- Cidex (1 gallon per biopsy session)
- Endozime AW Plus
- Sterile water (1 liter per 4 horses per biopsy session)
- Microcentrifuge tubes (2 per horse per biopsy)
- Liquid nitrogen canister

Procedure:

1- Gather all supplies and place on clean cart
2- Place both uterine biopsy forceps in Cidex solution 45 minutes prior to biopsy
3- Prepare graduated cylinder with Endozime Plus AW (3.7 cc per 1872 mL)
4- Fill liquid nitrogen canister
5- Sedate mare
6- Place mare in stocks and place tail in tail bag
7- Remove all feces per rectum using clean palpation sleeve
8- Using liquid ivory soap and warm water cleanse the perineum. Work from the internal area to the external to minimize contamination of perineum and vulva. Once the area has been scrubbed at least three times dry the area.
9- Utilizing sterile gloves the assistant will remove the biopsy forceps from Cidex and individual performing biopsy sill rinse with 35cc of sterile water ensuring basket is thoroughly rinsed
10- Individual performing biopsy will put on sterile palpation sleeve and apply sterile lubricant to the back of their hand
11- Assistant will hand the biopsy forceps to the individual doing the biopsy
12- Ensuring the basket is kept closed during positioning, the biopsy forceps will be introduced into the vagina with the individual's hand covering the tip of the instrument
13- The forceps will be manually guided through the cervix into the uterine lumen

14- Forceps will be kept in place with the external hand while the gloved hand is withdrawn and inserted into the rectum to manually position the basket of the biopsy instrument
15- The forceps jaws are opened and the uterine tissue will be pressed into the basket and the jaws are closed
16- Once the sample is taken, the forceps are carefully removed
17- The sample is then gently teased from the basket by an assistant wearing a sterile glove utilizing a needle
18- Sample is immediately placed into a labeled microcentrifuge tube and flash frozen in liquid nitrogen and stored at -80°C
19- Once the sample has been taken the mare's perineum is rinsed with warm water and the tail bag is removed
20- The mare is then put back into a stall with no hay until the effects of sedation wear off
21- Biopsy instruments are rinsed with 35cc of sterile water and placed in the Endozime AW Plus solution for 20 minutes. The instrument is then taken from the solution and rinsed with 35cc sterile water and placed in Cidex solution for 20 minutes.

Appendix 3- Modified Embryo Collection for Day 12 Equine Embryos

Supplies Needed:

- 1- modified nasogastric tube approximately 3 feet long
- Collection vessel
- 2 liters embryo flush media
- Sterile palpation sleeves
- Non-sterile palpation sleeves
- Sterile lubricant
- Non-sterile lubricant
- Tail wrap
- Cryovial
- Liquid nitrogen

Procedure:

1- Assemble apparatus while maintaining sterility
2- Assemble apparatus while maintaining sterility
3- Prepare embryo flush fluid (2L)
    a. Complete fluid (has surfactant)
    b. 32 degrees Celsius
4- Prepare all necessary equipment
    a. Collection plate
    b. Freeze medium
    c. Liquid nitrogen
5- Bring mare to stocks and sedate
6- Assemble apparatus at stocks with fluid elevated above the floor
7- Evacuate fecal material
8- Ultrasound to determine presence of embryo (d 12 or later)
9- Bag tail
10- Clean anus and vulva
    a. Utilizing a gloved hand and ivory soap clean from anus down and out two to three inches and rinse
    b. Continue cleaning until adequate
    c. Dry with paper towel from vulva towards anus
    d. Spray with dilute betadine solution and wipe from vulva towards anus
11- Utilizing a sterile gloved hand enter through vulva and place NG tube into cervical os
12- Infuse 1/3 of solution into the uterus
13- Utilizing a gloved hand and barrier between anus and vulva manipulate fluid through entire uterus
14- Flush fluid from uterus through collection cup
15- Repeat this process two more times
16- Once embryo has been flushed it will be visible in collection cup
17- Flush all fluid out of tubing through collection cup

18- Inject 20 IU oxytocin IV
19- Wait three minutes
20- Flush remaining fluid
21- Bring collection cup to laboratory and flush all fluid into a sterile search plate
    a. Utilize flush media to flush all areas of collection cup and lid
22- Remove catheter from the mare by removing air from Foley catheter bladder
23- Clean mare with hose or paper towel
24- Wait until mare is awake and return her to herd
25- Process embryo for freezing
26- Sterilize parts of apparatus that can be sterilized and dispose of all others