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The Effects of Aerobic Exercise on Embryonic and Fetal Development in the Pregnant Mare

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THE EFFECTS OF AEROBIC EXERCISE ON EMBRYONIC
AND FETAL DEVELOPMENT IN THE
PREGNANT MARE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal Physiology

by
Jason Edward Anton
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Accepted by:
Dr. Kristine Vernon, Committee Chair
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ABSTRACT

Horses are natural athletes and are commonly competed during early pregnancy. Because horses have singleton pregnancies and are conditioned to human interaction, they may prove to be a useful model in lending greater understanding of the numerous etiologies of intra-uterine growth restriction that is found in women and other mammals. Currently, there has been little research conducted to determine the effects that stress, in the form of aerobic exercise, has on early embryonic and fetal development in the horse. More research is needed to further understand the effects that stress has on early conceptus development in the mare.

The research trial consisted of eight light breed mares that were randomly designated to a non-exercise control group and an exercise treatment group. The treatment mares were exercised from Days 16 to 80 of pregnancy. Color Doppler ultrasound was utilized in all mares to evaluate embryonic vesicle size, embryo proper growth, fetal crown-to-rump length, and uterine blood flow of both the gravid and non-gravid uterine arteries. Jugular venipuncture was performed in both groups to identify the serum cortisol and progesterone concentrations between Days 16 to 80 of gestation. Exercise treatment, ultrasonography, and blood draws ended on Day 80 of pregnancy.

Results of this study indicated that exercise treatment lead to a larger embryonic vesicle in the treatment group ($P=0.005$) than the control group, but the embryo proper and fetal crown-to-rump length were similar between groups. Uterine artery blood flow velocity was significantly greater in the gravid and non-gravid horns of the exercise mares ($P=0.001$; $P<0.001$) than the control mares. Cortisol concentrations were

significantly higher in the treatment mares following exercise ($P < 0.001$) than the control mares, but progesterone concentrations were similar between groups.

It can be concluded that the exercise treatment did not induce deleterious effects on the early embryonic and fetal development of the treatment mares. Rather, aerobic exercise led to beneficial changes in uterine blood flow and embryonic vesicle size. Furthermore, the stressed state of the exercised mares did not have an impact on pregnancy loss or led to abnormal conceptus maturation. More research is needed to determine other physiological and hormonal changes that can be attributed to aerobic exercise during pregnancy in the horse.

DEDICATION

I dedicate this thesis to my family, who have supported me through the entire process and have played an integral role in my success.

ACKNOWLEDGMENTS

I would like to thank my committee for guiding me through this process. I want to thank Dr. John Gibbons who provided continual support and expertise throughout my research project. I am very grateful for encouragement and support that my fellow graduate students provided me through the rollercoaster ride that is graduate school. Finally, I would like to thank Dr. Sandra Gray and the Endocrinology lab for their help with my laboratory work.

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CHAPTER ONE

INTRODUCTION

Mares are considered sporting animals and are commonly exercised or ridden during the early gestational period of pregnancy. How exercise, or the associated known stress response to exercise (Gordon et al., 2007), influences pregnancy maintenance and/or embryonic and fetal growth is currently unknown in the horse. However, spontaneous abortion or conceptus mortality is highest during the first third of gestation in the mare, and approximately 77% of early embryonic death (EED) incidences occur by Day 35 of pregnancy in the horse (Villahoz et al., 1985). In a more recent article, Allen et al. (2010) documented pregnancy loss in Thoroughbred mares from Days 15 to 42 of pregnancy that ranged from a low 5.9% in young mares (3-8 yrs) with as high as 18.6% in aged mares (>18 yrs).

The etiology of early embryonic or fetal death is still debated and contributes to significant losses in revenue and effort to horse owners. The relationship between the equine maternal environment and developing conceptus is critical to the outcome of pregnancy (Betteridge, 2007). Acute stressors such as rectal palpation and transportation have been found to have negligible effects on pregnancy failure in the horse (Irwin, 1975; Baucus et al., 1990). Little published research has been conducted to determine the effects that chronic stressors, such as aerobic exercise, have on embryonic and early fetal loss in the horse.

The aim of this research was to investigate the effects of exercise on the pregnant mare from Days 16 to 80 of gestation. Treatment mares, once confirmed pregnant via

ultrasound examination on Day 14, began a moderate exercise regimen that continued throughout the duration of the project. Conceptus growth parameters, such as embryonic vesicle size, embryo proper growth, and crown to rump length were measured with ultrasound as determinants of embryonic and fetal growth. Blood was sampled from each mare to determine cortisol concentrations to measure the known stress response to exercise, and to evaluate progesterone concentrations as the critical early pregnancy maintenance hormone. Blood flow velocity was monitored through both uterine arteries with the assistance of a color Doppler ultrasound to evaluate any differences. The results of this research can provide valuable information to horse owners to evaluate any detrimental or even positive effects that controlled activity has on early pregnancy maintenance and conceptus growth in mares. Furthermore, results may provide insight into stress and its influence on the above mentioned parameters during early pregnancy in the mare.

CHAPTER TWO

LITERATURE REVIEW

Early Equine Pregnancy

Fertilization and Early Conceptus Maturation

Early pregnancy in the mare is characterized by physiological and hormonal changes that are both similar to other species and unique to the horse. Fertilization occurs in the ampulla-isthmus junction of the oviduct following copulation in the horse (Flood et al., 1979). The equine conceptus travels down the oviduct and enters the uterus around Day 6 at the blastocyst stage through the uterotubal papilla, a junctional area between the oviduct and the uterus (Webel et al., 1977). During Days 6 – 17 of embryonic development, myometrial contractions move the conceptus freely within the uterine lumen, where the embryo accumulates nourishment through the exocrine secretions of the endometrial glands (Allen and Stewart, 2001). Myometrial contractions are initiated by the pulsatile secretions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the conceptus and prostaglandin E_2 from the uterine endometrium (Allen and Stewart, 2001). Allen (2001) stated that some instances of early, spontaneous abortion can be attributed to the over-secretion or hypersensitivity of the corpus luteum to $PGF_{2\alpha}$.

An important feature of equine pregnancy during the embryonic stage is the glycoprotein capsule that surrounds the blastocyst from Day 6 to Day 23 (Allen and Stewart, 2001). The capsule is formed from material secreted by the trophoblast layer of the embryo, and it envelops the embryo under the zona pellucida (Oriol et al., 1993).

This tough, elastic barrier maintains the blastocyst's spherical shape and provides structural support (Allen and Stewart, 2001). The capsule is sticky in nature allowing for the adherence of uterine proteins during the mobility phase of the embryo (Ginther, 1983). It is currently thought that embryo migration is critical to pregnancy recognition in the mare; however, the exact mechanisms of pregnancy recognition in the mare are unknown. The end of the motile phase of the embryo occurs abruptly, as the embryo becomes fixed in the uterine horn at random, either contralateral or ipsilateral to the location of ovulation. Fixation occurs around Day 17 due to the increase in size of the embryo and the embryo's inability to pass through the narrow base of the uterine horn (Allen and Stewart, 2001). The trophoblast of the embryo begins to differentiate into invasive and non-invasive tissue types and completes this differentiation by Day 35 of pregnancy (Allen and Stewart, 2001). This physiological process is the foundation for the formation of endometrial cups.

Endometrial Cup Formation

Endometrial cup formation is a physiological process that is unique to *Equus caballus*. The trophoblast of the developing embryo begins to thicken forming a chorionic girdle between Days 25 and 35 of pregnancy (Allen, 2001). This girdle forms as series of folds, increasing the surface area necessary to support the proliferating trophoblastic cells at the top of each fold (van Niekerk, 1965). Around Day 36 of pregnancy, the specialized trophoblastic cells begin to invade the maternal endometrium (Allen and Moor, 1972). Trophoblastic hyperplasia ceases around Day 40 as these cells

reach the basement membrane and stream out into the endometrial stroma (Allen, 2001). The endometrial cups become functional between Day 36 and Day 40 and begin to secrete the glycoprotein hormone, equine chorionic gonadotropin (eCG; Allen, 2001). This specific glycoprotein stimulates both follicle stimulating hormone (FSH) and luteinizing hormone (LH) biological activities leading to the ovulation or luteinization of follicles on each ovary, giving rise to accessory corpora lutea (Allen, 2001). Endometrial cups are also responsible for the up-regulation of serum-conjugated estrogens that are ovarian in origin but are secreted by luteal tissue and not follicular tissue (Allen, 2001). These specialized cups reach maximum hormone-secreting capacity between Days 60 to 70 of pregnancy and begin to degenerate shortly thereafter (Allen, 2001).

Accessory Corpora Lutea Formation and Blood Flow

The formation of the secondary corpora lutea contributes to the increase of maternal serum progesterone (Allen, 2001). Secondary corpora lutea begin to form around Day 38 of gestation in the mare. The additional progesterone produced by the newly-formed luteal tissue is important for the maintenance of pregnancy until about Day 100 of pregnancy when the equine placenta becomes capable of producing the quantity of progesterone necessary to maintain pregnancy (Holtan et al., 1979).

Color Doppler ultrasonography is a valuable diagnostic tool in determining the vascularization of the corpus luteum. The blood flow of the corpus luteum, as measured through ultrasound, appears to be positively correlated with luteal function in women (Tamura et al., 2008). Additionally, the cross sectional area of the equine corpus luteum

is related to the amount of progesterone produced from the luteal tissue (Ginther et al., 2007). Kelley et al. (2009) reported that progesterone concentrations decreased two days prior to a noticeable decrease in vascular perfusion of the corpus luteum in cows. High vascularization of the luteal tissue is necessary for the maintenance of pregnancy and deficiency in luteal development and function has been implicated as a cause for spontaneous abortion in women (Tamura et al., 2008). In addition, the reduction of progesterone concentrations in maternal circulation was found to induce abortion in mice (Arck et al., 2007). In mares, around Day 35 of pregnancy, a resurgence of the primary corpus luteum occurs due to stimulation of the original luteal tissue by eCG (Bergfelt et al., 1989). Ginther (1985) stated that the failure of the corpora lutea to produce sufficient levels of progesterone before Day 50 of gestation lead to pregnancy loss in the mare. Similarly, studies where progesterone-binding sites were blocked during early pregnancy lead to spontaneous abortion in humans, mice, and guinea pigs (Arck et al. 2007).

Placentation

The placental tissue in the mare begins to form between days 35 and 42 of pregnancy. Around Day 35, the uterine epithelium proliferates and grows over the endometrial cups to form the microcotyledonary villi (Wooding and Fowden, 2006). The microcotyledons are structures formed from two membranes, one fetal-derived and the other dam-derived (Wooding and Fowden, 2006). These specialized structures maximize the amount of surface area available for substrate delivery to the developing conceptus (Wooding and Fowden, 2006). Around Day 100, the placenta is responsible

not only for the oxygen and substrate delivery to the fetus but also for the production of the hormonal milieu and waste removal that is necessary for the preservation of the pregnancy (Allen, 2001).

The mechanism for the majority of substrate delivery to the developing fetus is the exchange of maternal blood over fetal capillary beds (Wooding and Fowden, 2006). The blood flow between the feto-placental unit of the horse is between 300 and 2200 ml/kg of placenta/min (Wooding and Fowden, 2006). Along with the rate of flow, other determinants of oxygen and substrate delivery are hemoglobin content, oxygen availability, and the concentration of nutrients in maternal circulation (Clapp, 2006). Blood flow varies from day to day, but in most species, there is a direct correlation between rate of blood flow to the feto-placental unit and the rate of development of the conceptus (Wooding and Fowden, 2006). Research has determined that foal birth weight is directly proportional to the size of the placenta (Wooding and Fowden, 2006). Clapp (2006) stated that in numerous mammalian models, the quality of the earliest form of placental tissue plays a key role in the quantity of functional placental tissue in the later stages of gestation. Furthermore, he concluded that growth rate and surface area of the placenta are useful indicators of neonate size in the human (Clapp, 2006). Little is known about the critical period when modifications to the placenta can be made without having detrimental effects in the horse.

The Stress Response and Reproduction

Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal axis (HPA) is the neuroendocrine regulator of the body's adaptive response to stress (Tsigos and Chrousos, 2002). When stimuli challenge the homeostatic condition of the animal, the HPA is activated initiating the cascade of endocrine responses to address the stressor (Tilbrook et al., 2000). The initiation of the stress response leads to an increase in respiration rate and cardiac output, an increase in catabolism, and the redirection of blood flow to areas such as the heart, muscles, and brain (Chrousos and Gold, 1992). The effects of stress are mediated through the release and action of glucocorticoids and catecholamines (Tilbrook et al., 2000). The response of the pituitary gland to corticotropin releasing hormone (CRH) stimulus is the release of adrenocorticotrophic hormone (ACTH; Tsigos and Chrousos, 2002). ACTH stimulates the adrenal cortex to secrete glucocorticoids, primarily cortisol in most mammals (Tsigos and Chrousos, 2002).

HPA Action on the Hypothalamic-Pituitary Gonadal Axis

Many studies have been conducted to determine the effect that the HPA has on the hypothalamic-pituitary-gonadal axis (HPG). In various mammalian species, different physical stressors have been evaluated based upon their ability to disrupt normal reproductive function (Tilbrook et al., 2000). Chronic stress has been shown to have inhibitory effects on reproduction through the induction of cortisol secretion and blood

flow alteration in women (Tilbrook et al., 2000). Glucocorticoids inhibit the release of pituitary gonadotropins and cause reproductive tissues, such as the gonads, to become resistant to sex steroids through receptor inactivity in women (Tsigos and Chrousos, 2002). Along with its suppressive effects on the gonads, cortisol has shown to have inhibitory effects on the mare uterus' steroid hormone receptors (Berghold et al., 2007). Finally, in their review of fetal growth restriction, McMillen et al. (2001) stated that intrauterine growth restriction results in a disproportionately high rate of perinatal morbidity and mortality. They further state that overexposure of the fetus to excess glucocorticoids may be implicated in fetal growth restriction (McMillen et al., 2001).

Cortisol

Cortisol has been determined to be an accurate indicator of stress in horses (Hoffsis et al., 1970). Bottoms et al. (1972) stated that plasma cortisol was the dominant glucocorticoid in the horse. At rest, the unstressed horse has been reported to have a plasma cortisol concentration of approximately 5 µg/100mL (Hoffsis et al., 1970). Conversely, exercised horses have been reported to have significantly higher cortisol concentrations; with peaks varying due to exercise intensity and duration (McKeever, 2002).

Nepomnaschy et al. (2006) studied pregnant women to identify a possible correlation between elevated cortisol levels and early pregnancy loss. Their findings suggested that abnormally high levels of cortisol contributed to spontaneous abortion by altering normal reproductive function at both a tissue and hormone level (Nepomnashy et

al., 2006). A study conducted in sheep determined that elevated levels of cortisol lead to the premature activation of growth regulatory mechanisms in the fetus that had both pre- and post-natal deleterious consequences (Fowden and Forhead, 2009). Previous research had determined that high levels of cortisol suppress insulin-like growth factors found in the liver, skeletal muscles, and adrenal glands in fetal sheep (Fowden, 2003). Cortisol is capable of reducing gonadotropin-releasing hormone (GnRH) receptor activity through the enhancement of negative feedback mechanisms on estradiol in sheep (Daley et al., 1999). Turner et al. (1999) conducted a study in gilts and found that chronic cortisol administration delayed ovulation through the impairment of the LH surge during the estrous cycle. Research has yet to establish a threshold for systemic circulation of cortisol before it exhibits harmful effects on equine pregnancy.

Glucocorticoid Effects on Fetal Development

Fetal development is dependent upon various hormones and their specific actions throughout gestation. Studies have shown that hormones are a contributing factor for the differentiation of the conceptus in utero (Fowden and Forhead, 2009). Hormones affect fetal growth both directly and indirectly, either through genetic programming or fetoplacental growth and maturation (Fowden and Forhead, 2009). During pregnancy, hormones are produced at both the maternal and fetal level that have direct effects on the pregnancy outcome (Fowden and Forhead, 2009). An important class of hormones that have uterine programming action are the glucocorticoids, which affect the development of the tissue and organ systems of the fetus (Fowden and Forhead, 2009). Kapoor et al.

(2008) determined that excess exposure of glucocorticoids to the developing human fetus can reprogram the fetal HPA, permanently changing the HPA's activity of the offspring throughout their lives. Specifically, alterations to the fetal HPA in utero can lead to predisposition to cardiovascular disease and diabetes in humans (Matthews et al., 2004). Fetal exposure to glucocorticoids can occur simply through the initiation of the stress response of the dam (Kapoor et al., 2008). Hypoxemia has been shown to prematurely stimulate fetal HPA (Matthews and Challis, 1996). Research has shown that elevated concentrations of glucocorticoids impair fetal growth and are a major determinant of intra-uterine growth restriction (IUGR) (Fowden and Forhead, 2009). Challis et al. (2001) found that the fetal HPA is responsible for the maturation of organ systems that are essential for post-natal survival. Endocrine changes initiated by elevated glucocorticoid levels can be transient, but some alterations persist after the glucocorticoid concentrations return to their basal values (Fowden and Forhead, 2009). These hormonal modifications are both the source and result of intrauterine programming leading to an effect on both the fetal and placental units. (Fowden and Forhead, 2009). The changes initiated by chronic exposure to glucocorticoids include underdevelopment of the fetal HPA and placental hormone deficiency (Fowden and Forhead, 2009). The critical window of fetal HPA maturation is very species-specific (Kapoor et al., 2008). Research by Fowden et al. (2008) stated that the activation of the fetal HPA is an essential process for parturition in the mare. Equine pregnancy is unique in that fetal cortisol levels increase rapidly very close to term, increasing uteroplacental prostaglandin synthesis and initiating myometrial contractions (Fowden et al., 2008). More research is needed to

determine the effects that stress has on fetal development and the fetal HPA activity necessary for parturition.

Environmental Influence on Fetal Development

A major influence on fetal growth is the intrauterine environment. Stress during pregnancy can lead to intrauterine growth restriction (Barry et al., 2008; Verkauskiene et al., 2007; Clapp, 2003; Fowden and Forhead, 2009). Complications from intrauterine growth restriction in offspring include low birth weight, decreased growth potential, transition difficulties, and metabolic disturbances (Galan et al., 2005; Ergaz et al., 2005). Ergaz et al. (2005) in a thorough review of intrauterine growth restriction stated that the uterine environment is a greater determinant of birth weight than the genetic contribution of the dam. The authors also surmised external environmental factors, such as heat stress, have been found to induce fetal growth restriction (FGR) in women. A study in pregnant ewes determined that heat stress throughout the gestational period lead to the birth of smaller neonates with underdeveloped organ systems (Thureen et al., 1992). The authors concluded this form of severe FGR was attributed to placental insufficiency. Research performed in dairy cows found that heat stress was a source for early pregnancy loss due around the time of implantation (Garcia-Ispierto et al., 2006). Recently published research determined that a hot, humid environment had a negative effect on embryo quality in exercised mares (Mortensen et al., 2009).

Exercise-Induced Stress

Moderate exercise is categorized and defined by Bouchard et al. (1994) as an exercise intensity that utilizes the aerobic energy pathway, has a duration range of 30 to 180 minutes, and has a maximal aerobic capacity of 70% in humans. This degree of exercise activated the HPA and generated a physiological and hormonal response in women (Tilbrook et al., 2000). Moderate exercise has been shown to increase cortisol up to 29% of the basal levels through the stress response in horses (Gordon et al., 2007). The plasma concentration of cortisol was more than double the normal value 60 minutes following exercise in horses (Gordon et al., 2007). In exercise-induced stress, a marked increase in cortisol concentrations in horses was attributed to exercise duration and not intensity (Hyypa, 2005). Previous studies have shown that elevated concentrations of serum cortisol after exercise bouts may alter the anabolic responses of testosterone and growth hormone in horses (Hyypa, 2005). Marc et al. (2000) examined the effects that a horse's degree of fitness had on cortisol levels when subjected to an exercise regimen. At the onset of exercise, unfit horses had a much higher concentration of serum cortisol than fit horses (Marc et al., 2000). However, as exercise progressed the cortisol levels in the initially unfit horses began to normalize and no statistical difference was found between the two groups after 24 weeks of training (Marc et al., 2000).

A study investigating the role of exercise on human pregnancy found that the redistribution of blood during exercise poses a threat to fetal well-being (Penney, 2008). This was speculated to be due to decreased utero-placental blood flow to facilitate the blood flow demand of the skeletal muscles (Penney, 2008). Clapp et al. (2000)

performed several studies analyzing the effects of exercise on fetal development and welfare in humans. His findings suggested that exercise in early gestation followed by cessation of physical activity led to the development of larger neonates with a high percentage of body fat (Clapp et al., 2000). Conversely, exercising throughout gestation resulted in taller and leaner neonates (Clapp et al., 2000).

During exercise in horses, the primary metabolic effects of cortisol are increased hepatic gluconeogenesis, mobility of free fatty acids, and lipolysis (Hyypä, 2005). Cortisol is also instrumental in suppressing the release of insulin during exercise to maximize the utilization of blood glucose (McKeever, 2002). Hackney (2006) found that exercise in humans altered the metabolic clearance rate of a hormone either through degradation or changes in the uptake of the hormone by target tissues. The hormonal response during exercise is influenced by hemodilution or hemoconcentration actions that are linked to the shifting of plasma fluids in and out of vascular beds (Hackney, 2006). Little to no research has been conducted to determine the effect of exercise stress on fetal well-being and growth in the horse during early gestation and is the focus of this research.

Placental Tissue Proliferation

Exercise is an environmental factor that has an impact on placental development, and in turn, has an effect on fetal development. Clapp et al. (2000) determined that moderate, sustained exercise in pregnant women reduced the amount of oxygen and substrate delivery to the maternal-fetal interface by more than 50%. However,

continuous bouts of moderate, aerobic exercise have been shown to stimulate placental growth during early and mid-gestation in the woman (Clapp, 2006). A prolonged exercise regimen stimulated growth factors within the placenta that lead to hypertrophic tissue growth increasing the surface area which compensated for the reduction in oxygen availability (Clapp, 2006). Routine endurance exercise during early pregnancy increases the proliferation of the intervillous space and villous tree of the placenta (Clapp, 2006). Little is known about the benefit of exercise-induced modifications to the placenta during early pregnancy in the horse.

Uterine Blood Flow

Quantitative analysis of blood flow is possible and outlined in *Ultrasonic Imaging and Animal Reproduction: Color-Doppler Ultrasonography* (Ginther, 2007). Uterine blood flow has been studied in the horse as an indicator of the amount of oxygen and substrate delivered to the conceptus (Bollwein et al., 2003). Additionally, Doppler studies in humans and sheep have shown that in pregnancies with placental insufficiency, increased vascular resistance in both umbilical and uterine arteries with decreased fetoplacental blood flow was observed (Barry et al., 2008).

Color Doppler ultrasonography is a technology being utilized to measure blood flow in individual vessels and can be reported as pulsatility and resistance indices (PI and RI, respectively). Doppler indices (PI and RI) are ratios that are useful in the assessment of blood flow velocity measurements for reproductively important tortuous vessels, such as the uterine arteries. Pulsatility index is measured as the difference between the peak

systolic frequency shift and the minimum-diastolic frequency shift divided by the time-averaged maximum frequency shift over one complete cardiac cycle (Honnens et al., 2008). The resistance index is measured as the difference between the peak systolic frequency shift and the end-diastolic frequency shift divided by the peak systolic frequency shift (Bollwein et al., 2003). The PI and RI are parameters used to determine the velocity of blood flow and intra-arterial resistance. A great deal of variation exists between the rate of blood flow in the uterine artery of pregnant mares (Bollwein et al., 2003). Blood flow in older mares tended to have a higher resistance in the uterine artery than blood flow in younger mares (Bollwein et al., 2003). Pregnancy is a transforming process that can alter uterine blood flow. Stimulation of uterine blood flow as measured by ultrasound can be credited to the presence of an embryo (Honnens et al., 2008). The embryo intensifies uterine blood flow through its production of vasoactive substances such as estrogen in sows (Honnens et al., 2008). Bollwein et al. (2003) has demonstrated that there are drastic differences in uterine blood flow between the contralateral and ipsilateral uterine horns during early pregnancy in horses. Tissue weights at birth have been shown to be directly correlated to uterine artery diameter and uterine blood flow during pregnancy in women (Jeffreys et al., 2006). Uterine blood flow is directly correlated with the number of corpora lutea located on each ovary (Honnens et al., 2008). Uterine arteries supply blood not only to the bovine pregnant uterus but also to the ovaries where secondary corpora lutea form (Honnens et al., 2008). In mares, as the nutritional demands of the developing conceptus increase, there is a marked increase in uterine artery blood flow (Bollwein et al., 2003). The physiological process of

placentation has been shown to have a direct effect on uterine artery blood flow in horses (Bollwein et al., 2003). Jeffreys et al. (2006) determined that uterine blood flow in women that exercise in late gestation is decreased during both times of rest and exercise. Little research has been conducted to determine the correlation between uterine blood flow and early embryonic and fetal loss in the horse.

Conclusions

It is not uncommon for mares to be ridden or competed during early stages of pregnancy. Research into the effects of exercise stress on the pregnant mare remains scant. More research is needed to determine the effects that a chronic stressor, such as exercise, has on early conceptus development and pregnancy loss in the horse. Previous research determined that moderate intensity exercise is sufficient to evoke the stress response via the HPA in the horse (Hyypa, 2005). A HPA-induced homeostatic mediator, such as cortisol, has been shown to have inhibitory effects on reproduction (Hyypa, 2005) and implicated as a factor in intrauterine growth restriction (Ergaz et al., 2005) and pregnancy failure (Nepomnashy et al., 2006). The measurement of uterine blood flow with color Doppler ultrasonography in pregnant mares is a novel approach to determine any correlation that may exist between blood flow and pregnancy results (Bollwein, 2002). This research is undertaken to increase our understanding of the effects that aerobic exercise-induced stress has on early pregnancy outcome in the horse.

CHAPTER THREE

MATERIALS AND METHODS

Experimental Units

Eight mares (Quarter Horse and Quarter Horse/Thoroughbred cross) ranging from 5 to 15 years (7.63 ± 4.10) were utilized in this study. Six of the eight mares were nulliparous; two other multiparous mares were divided equally between treatments. The Henneke Body Condition Score (1983) was used to determine the body condition of all mares at the beginning of the project and weight was determined with the use of a weight tape placed around the heart girth of each horse. The average BCS was 6.0 and the average weight was $531.25 \text{ kg} \pm 24.31$. There were no significant difference in BCS and body weight within groups or between groups ($P > 0.05$). Mares were kept in separate pastures according to their random assignment to either the Treatment or Control group. Concentrate diets of both groups were formulated based upon the National Research Council (NRC) recommendation for horses per exercise intensity. The concentrate fed to both groups was Southern States Reliance® textured feed (Southern States Cooperative, Inc., Richmond, VA). Mares were individually fed twice a day at 0900 and 1700 hours; the exercise group consumed 1.59 ± 0.07 kg of concentrate at each feeding, and the control group consumed 1.36 ± 0.05 kg concentrate. Coastal Bermuda grass (*cynodon dactylon*) hay and water were available ad libitum throughout the project. The mares were housed on pastures containing common Bermuda, coastal, winter rye, and

fescue grasses. This research was conducted from February to July 2009, encompassing a wide range of climate differences.

Estrus Detection, Breeding, and Pregnancy Detection

Mares naturally cycled back into estrus and were individually teased 6 days a week by a stallion to approximate the mare's stage of the estrous cycle. Mares that exhibited behavioral features denoting stallion receptivity, such as lifting their tail, winking of the vulva, and urinating were placed in breeding stocks and evaluated via ultrasound transrectally with a 10-5.0 MHz broadband 52mm linear array transducer connected to a color Doppler ultrasound (Micromaxx ®, Sonosite, Bothwell, WA USA) between 0700 and 0845 hours to determine the presence of a peri-ovulatory follicle.

Once a 35mm follicle or greater was observed on the second estrous cycle of the season, one of two proven stallions was collected with a CSU Model TM artificial vagina (Animal Reproduction Systems, Chino, CA). Semen was analyzed by an equine densimeter (591a Densimeter ®, Animal Reproduction Systems, Chino, CA) to determine concentration and recommended breeding dose. A video microscope was utilized to record sperm quality parameters such as forward motility and morphology. The perineum of each mare was cleaned in an aseptic manner and the tail wrapped in preparation for insemination. Mares received an intra-cervical insemination with a use of a sterile breeding pipette and sterile breeding sleeve. Mares were inseminated with 500 million motile sperm cells extended out to 20mL with EZ Mixin extender (Animal Reproduction Systems, Chino, CA). An injection of 1.5cc (0.2 mg/kg) of reconstituted

Deslorelin™ (deslorelin acetate, Applied Pharmacy Services, Mobile, AL) was administered intramuscularly to induce ovulation immediately following breeding.

Ultrasound was performed one day post-breeding to determine if ovulation had occurred or if a dominant follicle persisted for more than two consecutive breedings. Mares that had not ovulated were bred again or until ovulation could be detected. Confirmed ovulation was considered Day 0 of pregnancy. Mares underwent an ultrasound exam on Day 14 post-ovulation to determine the presence of an embryonic vesicle, denoting pregnancy. If a mare was not confirmed pregnant, estrus detection again commenced and the mare was bred according to the previously mentioned procedure.

Conceptus Growth Parameters

Mares confirmed pregnant on Day 14 underwent an ultrasound exam every 48 hours until Day 80 of gestation at 0700 hours. Ultrasound was utilized to measure conceptus size (mm) and uterine blood flow. The embryonic vesicle was measured as the cross section between the furthest two points of the vesicle. The measurement of the vesicle ended on Day 36 due to the prolific growth of the vesicle and the inability to obtain an accurate measurement. The embryo proper (EP) was measured when its presence could be clearly identified within the growing vesicle by approximately Day 24. The size of the EP was determined by obtaining the average of two sets of perpendicular cross-sectional measurements. At Day 40 of gestation, the EP was considered a fetus and fetal crown-to-rump measurements were recorded. These measurements were taken until

approximately Day 70 when the fetus could no longer be measured due to its position over the pelvic ridge.

Blood Flow and Perfusion Parameters

Rate of blood flow through arterial vessels was measured with the use of the color Doppler feature of the ultrasound. Blood flow through the uterine arteries is calculated and displayed as resistance index (RI) and pulsatility index (PI) by the software of the Micromaxx® Doppler ultrasound. The PI and RI are parameters used to determine the velocity of blood flow and intra-arterial resistance; these parameters also provide an objective quantitative assessment of blood flow through the targeted arterial vessels. Beginning on Day 16 of gestation, the rate of blood flow through the left and right uterine arteries was measured in replications. Approximately three cardiac cycles of the left and right uterine arteries were recorded during each ultrasound exam.

Exercise Treatment

Mares that were randomly assigned to the treatment group commenced an exercise acclimation period beginning on Day 16 of pregnancy. Mares were exercised in a free-flow exerciser (Trojan Manufacturing Inc., Iowa Park, TX USA) beginning at 1530 hours, changing directions every other day for 6 days a week and given one day of rest. The train up period lasted 14 d and consisted of the following protocol: 5 minutes at 3 m/s, 30 minutes at 9 m/s, and 5 minutes at 3 m/s. Based upon previously published

research, the treatment mares were considered to be at an acceptable level of fitness at the end of this period to begin the actual exercise regimen which consisted of the following:

Speed	3 m/s	9 m/s	13 m/s	9 m/s	13 m/s	9 m/s	3 m/s
Duration	5 mins.	10 mins.	5 mins.	10 mins.	5 mins.	10 mins.	30 mins.

Exercise ended on Day 80 of pregnancy. Heart rate, respiration rate, and rectal temperature of the treatment group were taken before and after every other exercise bout. The pre-exercise values of each vital sign of the treatment group were thought to be comparable to the control, alleviating the need to take the vital signs of the control animals. Heart rate was determined through palpation of the right front digital pulse, and respiration rate was obtained through observation. Body temperature was measured rectally with the use of a digital thermometer. Weather data, including daily average ambient temperature, was provided by the National Oceanic and Atmospheric Administration (NOAA) website. Ambient temperature was separated into five ranges as follows: Range 1 (4.44-15.56°C), Range 2 (16.1-21.1°C), Range 3 (21.7-23.9°C), Range 4 (24.4-26.7°C), and Range 5 (27.2-32.2°C).

Blood Sampling and Analysis

Blood was collected in both groups via jugular venipuncture with two 6mL BD Vacutainer® Plus plastic serum tubes (Franklin Lake, NJ) beginning on Day 16 of pregnancy. Blood collection occurred three times a day, every other day. Blood was

drawn prior to feeding at approximately 0700 hours, before exercise (approximately 1530 hours), and following exercise (approximately 1700 hours). Serum tubes were stored overnight at 4°C to initiate serum separation. Samples were centrifuged the following day for 20 minutes at 900.7g. A pipette was used to draw off the serum, and serum was transferred to 2mL plastic freezer tubes and 4mL glass freezer tubes and stored at -20°C until future analysis.

Cortisol & Progesterone

Radioimmunoassay (RIA) was performed with the use of Siemens Coat-a-Count® kits (Siemens Healthcare Products, Los Angeles, CA) for the isolation and measurement of cortisol and progesterone according to the manufacturer's instructions. The Coat-A-Count® procedure is considered a solid phase RIA that utilizes radioactive I¹²⁵ labeled cortisol or progesterone, respectively. The radioactively labeled hormone exhibits competitive binding properties with the mare serum-derived cortisol or progesterone for active antibody binding sites (Farmer and Pierce, 1974). Tubes included in the kit contain fixed antibodies on the bottom surface which allows decanting of supernatant after a fixed time to terminate the competition for binding between the two forms of the hormone (radioactive and serum derived). A gamma counter was used to determine the target hormone concentration found in each sample.

Statistical Analysis

. The R Program for Statistical Computing (version 2.10.1, University of Auckland, NZ) was used to perform an F-test and corresponding paired Student's t-test on the repeated measures for embryonic vesicle, embryo proper, crown to rump measurements, uterine artery pulsatility index (PI) and resistance index (RI), cortisol concentrations, and progesterone concentrations. The JMP program (version 8.02, SAS Institute Inc., Cary, NC) was utilized to analyze a two-way ANOVA for embryonic vesicle, embryo proper, crown to rump measurements, uterine artery pulsatility index (PI) and resistance index (RI), cortisol concentrations, progesterone concentrations, and ambient temperature effects on vital signs. The model for each parameter included the treatment, day of pregnancy, and treatment by day interaction. Significant differences were reported at $\alpha = 0.05$ and trends were reported at $0.10 \geq \alpha > 0.05$.

CHAPTER FOUR

RESULTS

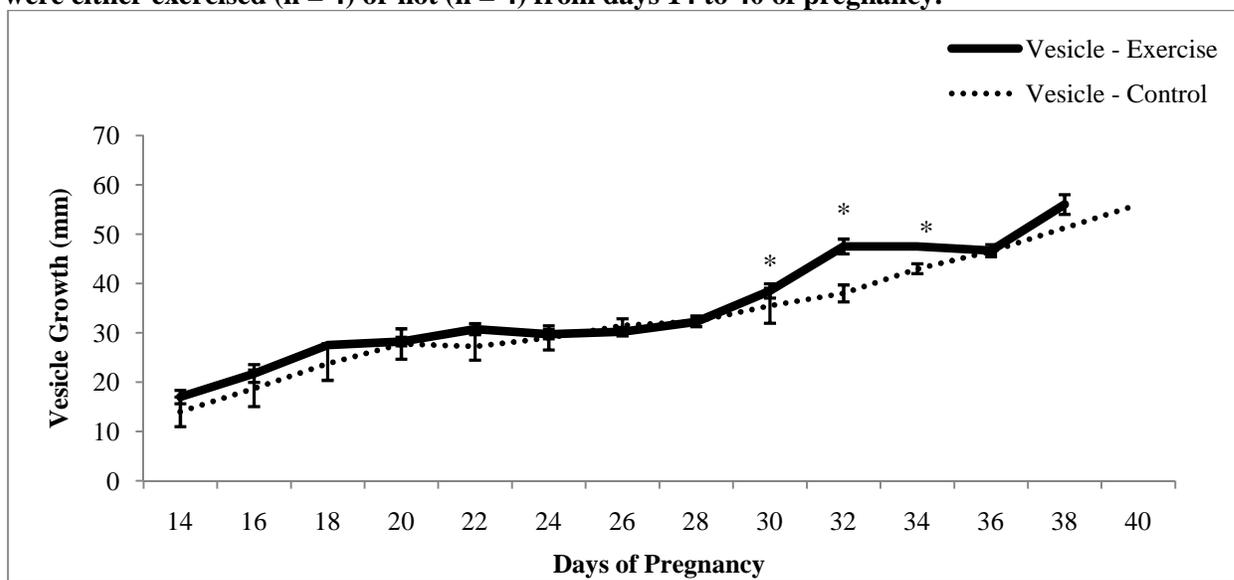
Conceptus Maturation Parameters

Embryonic Vesicle

There was main effect of vesicle size between treatment groups ($P = 0.0050$), with the exercised mares having significantly larger embryonic vesicles than the non-exercised control mares (Table 1). There was also a day effect throughout the study ($P < 0.0001$). A tendency for treatment by day interaction existed ($P = 0.0890$) and on days 30, 32, and 34, a treatment by day interaction was observed ($P < 0.0500$; Figure 1).

Figure 1.

The interaction of day and experimental treatment on embryonic vesicle size for mares that were either exercised ($n = 4$) or not ($n = 4$) from days 14 to 40 of pregnancy.



Days with * indicate significant differences between treatment groups ($P < 0.05$)

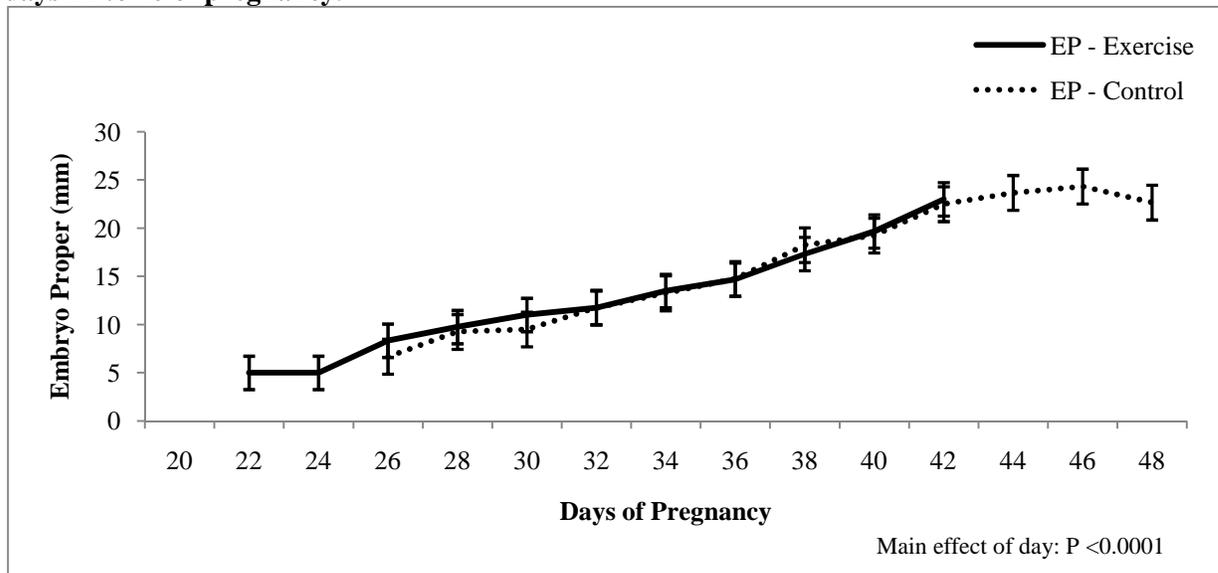
Main effect of treatment by day interaction: $P = 0.0890$

Embryo Proper

There were no differences in embryo proper size overall between groups ($P = 0.6750$; Table 1). As expected, the embryo proper for both groups combined was larger as the pregnancy progressed, with an overall main effect of day ($P < 0.0001$). No interactions of day and treatment existed ($P = 0.7390$; Figure 2).

Figure 2.

The embryonic proper size for mares that were either exercised ($n = 4$) or not ($n = 4$) from days 22 to 48 of pregnancy.



Crown to Rump Measurement

There were no differences between groups for fetal crown to rump measurements ($P = 0.1290$; Table 1). Again, as expected, when both groups were combined there was an overall day effect ($P < 0.0001$). There was no effect of a treatment and day interaction for the duration of the study ($P = 0.3910$; Figure 3).

Figure 3.

The fetal crown to rump measurement for mares that were either exercised (n = 4) or not (n = 4) from days 36 to 70 of pregnancy.

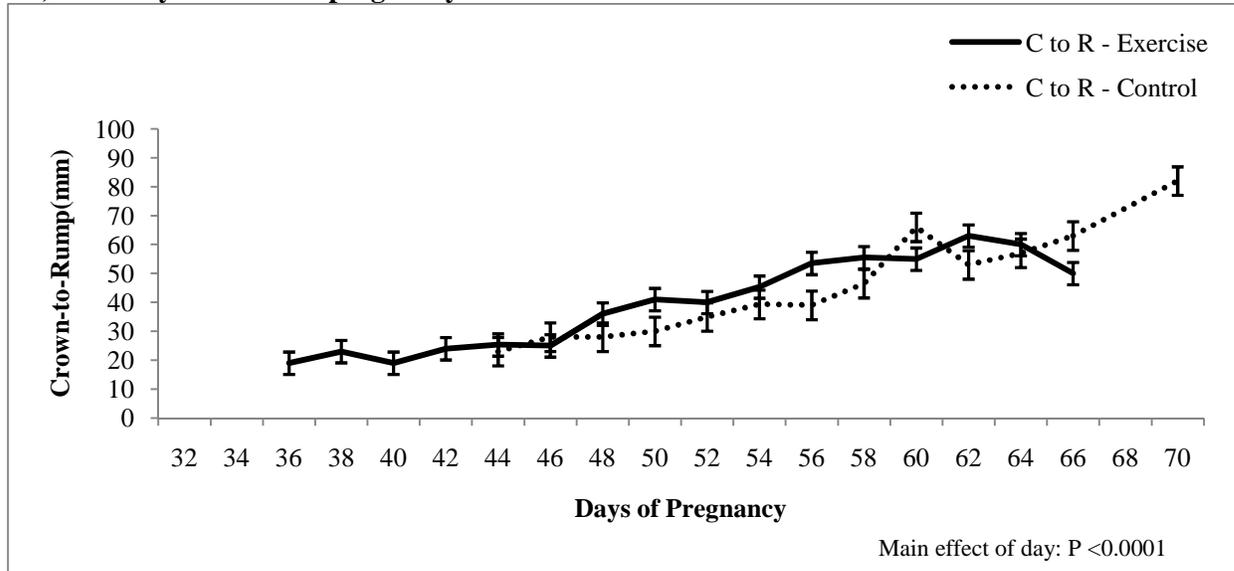


Table 1.

The treatment effect that aerobic exercise had on the measured physiological parameters of the early equine conceptus.

Parameters	Treatment LSM ± SEM	Control LSM ± SEM	Treatment Effect p- value
Vesicle	36.6 ± 0.511	33.9 ± 0.536	P = 0.005
Embryo Proper	14.870 ± 0.366	14.665 ± 0.336	P = 0.675
Crown to Rump	42.624 ± 1.496	39.345 ± 1.681	P = 0.129

Uterine Artery Blood Flow Parameters

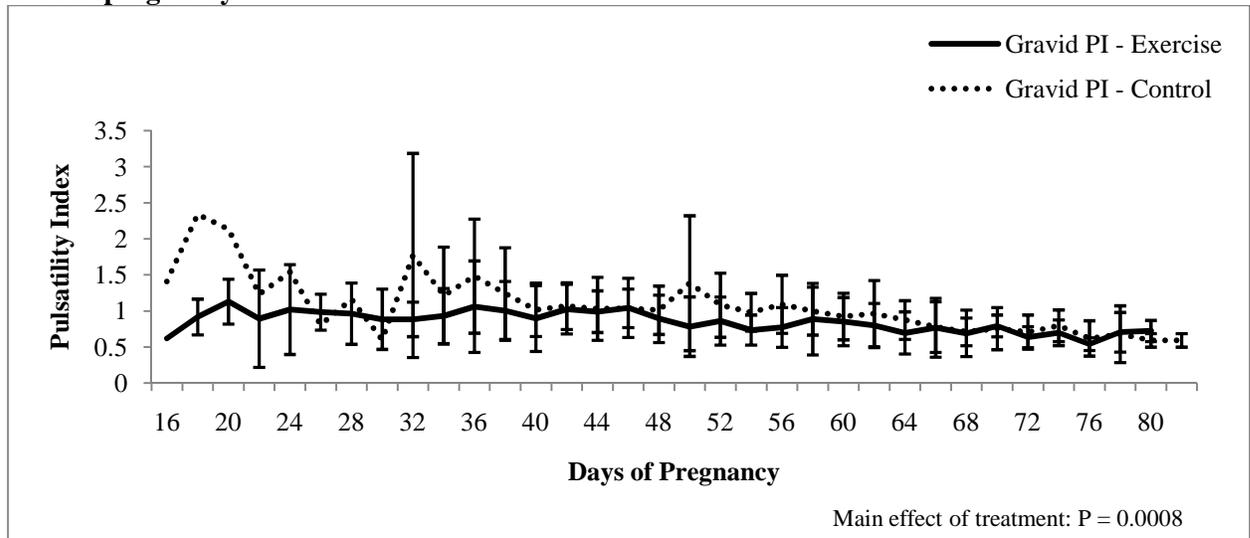
Gravid Horn Pulsatility Index (PI)

The gravid horn PI for the exercised mares was significantly lower than the non-exercised controls (P = 0.0008; Figure 4; Table 2). There was an overall day effect (P = 0.0240) for both treatments combined. However, there is no main effect of the interaction of treatment and day for the duration of the study (P = 0.7600). Also, the PI of the gravid horn was lower (exercise: 0.857 ± 0.036; non-exercise: 1.050 ± 0.041) than

the non-gravid horn (exercise: 1.084 ± 0.036 ; non-exercise: 1.372 ± 0.057) in both the exercise and non-exercise groups throughout the study ($P < 0.0001$).

Figure 4.

The gravid horn PI for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.

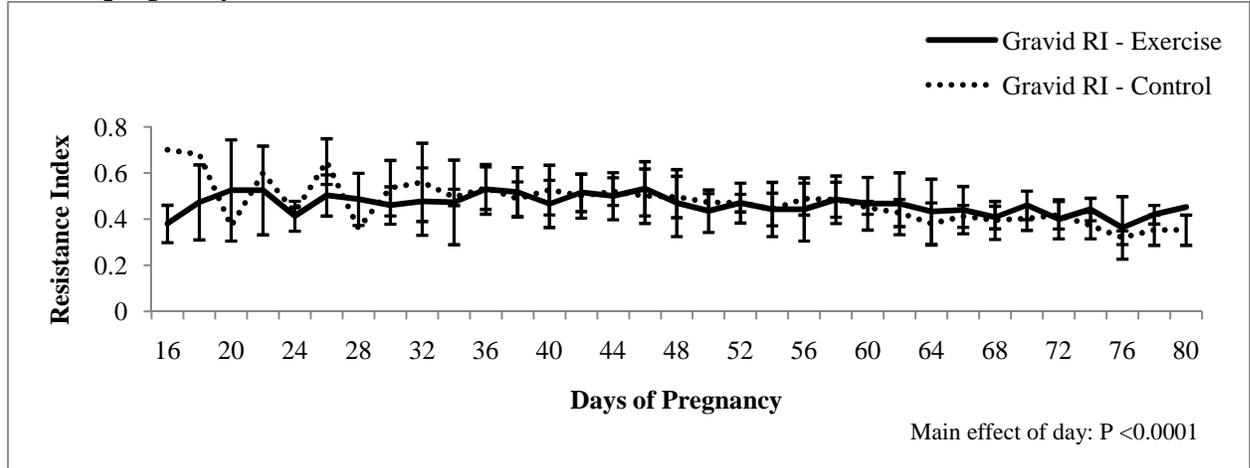


Gravid Horn Resistance Index (RI)

There was no difference in gravid horn RI measurements between groups ($P = 0.8450$; Figure 5; Table 2). There was an overall day effect ($P = 0.0150$) for both groups combined. There was no main effect of treatment by day interaction through the study ($P = 0.8280$). Also, the RI of the gravid horn was lower (exercise: 0.465 ± 0.010 ; non-exercise: 0.466 ± 0.010) than the non-gravid horn (exercise: 0.526 ± 0.010 ; non-exercise: 0.560 ± 0.013) in both the exercise and non-exercise groups ($P < 0.0001$).

Figure 5.

The gravid horn RI for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.

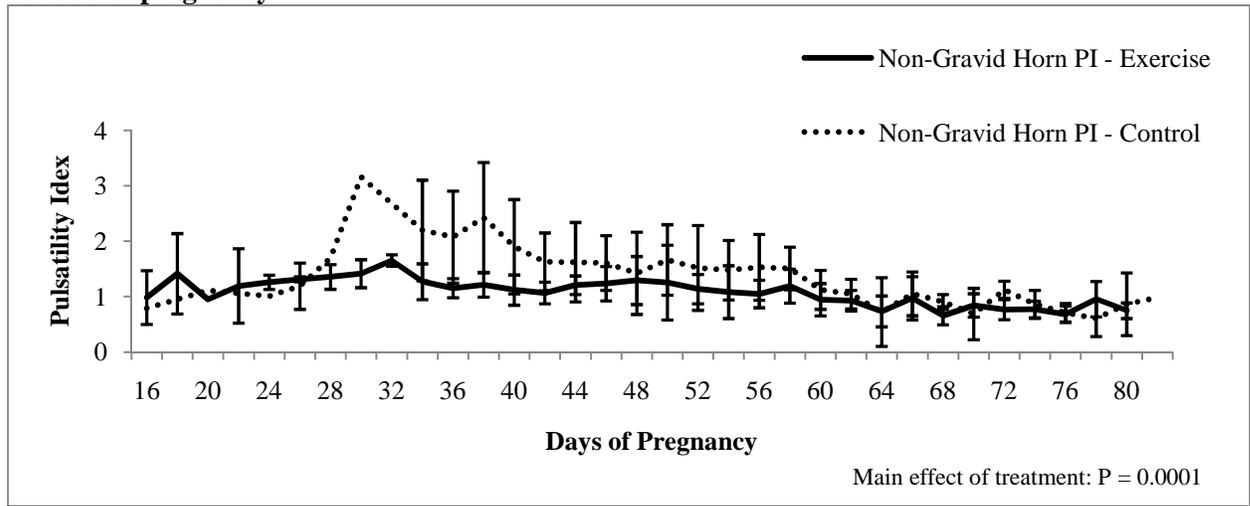


Non-Gravid Horn PI

The non gravid horn PI for exercised mares was significantly lower than the non-exercised control mares (P = 0.0001; Figure 6; Table 2) There was also a main effect of day (P <0.0001) throughout the study. There was no effect of treatment by day interaction (P = 0.6220).

Figure 6.

The non-gravid horn PI for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.



Non-Gravid Horn RI

There was a strong trend for the non-gravid horn RI measurements to be lower in the exercised mares than the non-exercised mares throughout the study ($P = 0.0560$; Table 2; Figure 7). There was an overall day effect ($P = 0.0070$), but no treatment by day interaction ($P = 0.9960$).

Figure 7.

The non-gravid horn RI for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.

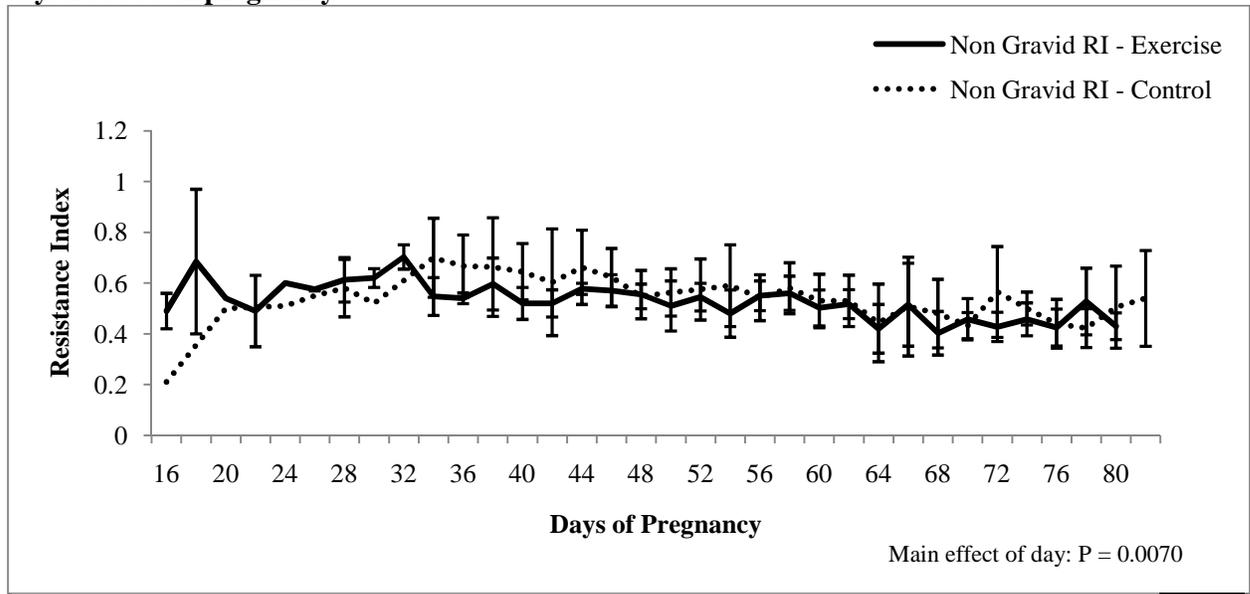


Table 2.

The treatment effect that aerobic exercise had on measured uterine arteries of both the gravid and non-gravid uterine horns during early equine pregnancy.

Parameters	Treatment LSM \pm SEM	Control LSM \pm SEM	Treatment Effect p-value
Gravid Horn PI	0.866 \pm 0.037	1.050 \pm 0.041	$P = 0.0008$
Gravid Horn RI	0.463 \pm 0.009	0.466 \pm 0.010	$P = 0.8450$
Non-Gravid Horn PI	1.089 \pm 0.049	1.372 \pm 0.057	$P = 0.0001$
Non-Gravid Horn RI	0.527 \pm 0.011	0.560 \pm 0.013	$P = 0.0560$

Hormonal Parameters

Cortisol

Serum cortisol concentrations were higher in the exercised mares than the non-exercised mares ($P < 0.0001$; Table 3). There was an overall day effect ($P < 0.0001$) for both groups combined, and a treatment by day interaction on the pre-exercise cortisol concentrations on day 26 and on the post-exercise cortisol concentrations on days 18, 32, and 40 ($P = 0.0040$; Figure 8).

At 0700 hours there was a day effect on cortisol concentrations ($P < 0.0001$), and a strong trend for the exercise group to have higher serum cortisol concentrations at 0700 hours was noted ($P = 0.0560$; Figure 9). There was no treatment by day interaction for serum cortisol at 0700 hours ($P = 0.7330$).

At 1530 hours (pre-exercise) there was a trend for a possible day effect in cortisol ($P = 0.091$) but the cortisol concentrations between groups were similar ($P = 0.4110$). There was no treatment by day interaction for 1530 hours cortisol concentrations ($P = 0.7410$).

Finally, at 1700 hours (post-exercise) there was a day effect in cortisol concentrations ($P < 0.0001$; Figure 8). Cortisol concentrations of the exercised group were significantly higher than the non-exercised group ($P < 0.0001$). There was no interaction found between treatment and day in cortisol at 1700 hours ($P = 0.9350$).

Figure 8.

The interaction of treatment by day of post-exercise cortisol concentrations for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.

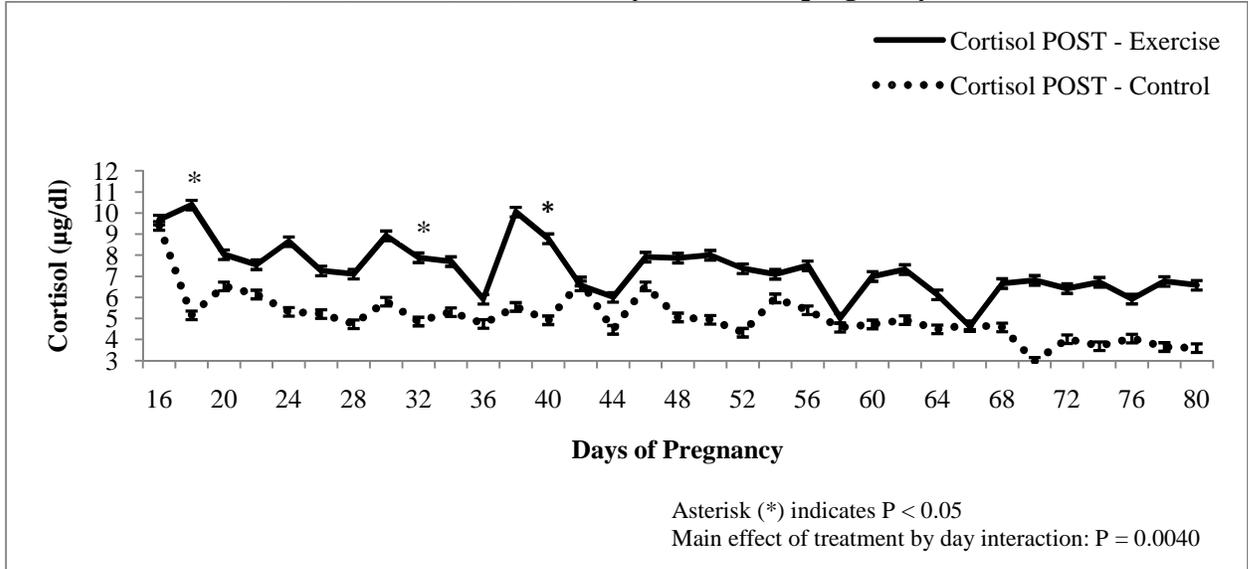
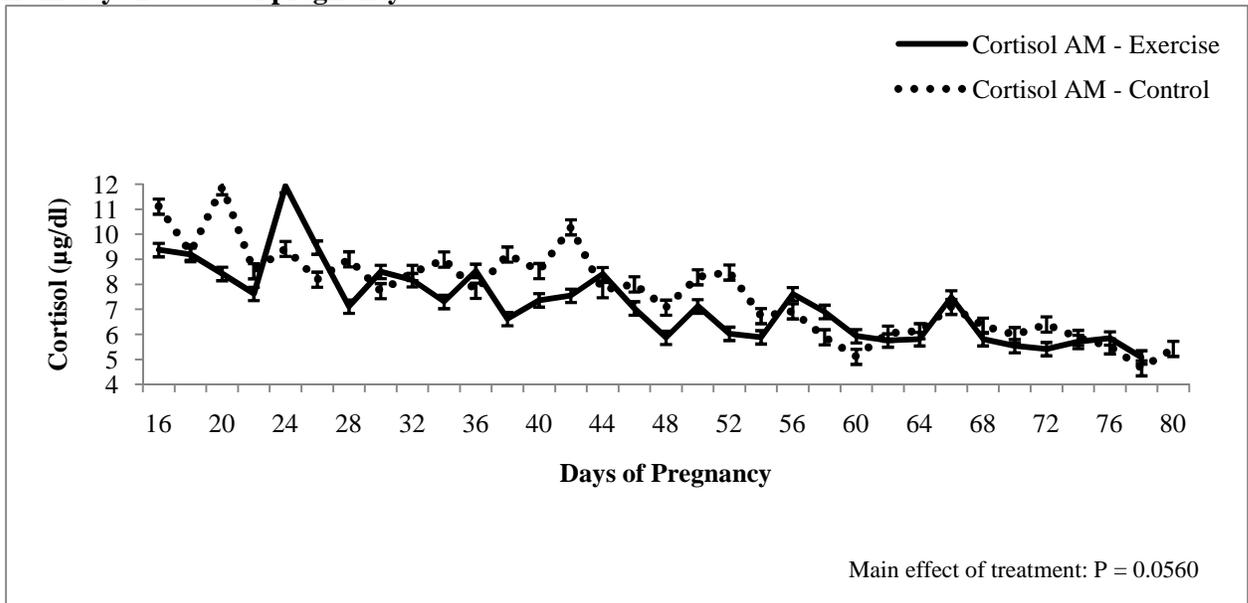


Figure 9.

The AM cortisol concentrations for mares that were either exercised (n = 4) or not (n = 4) from days 16 to 80 of pregnancy.

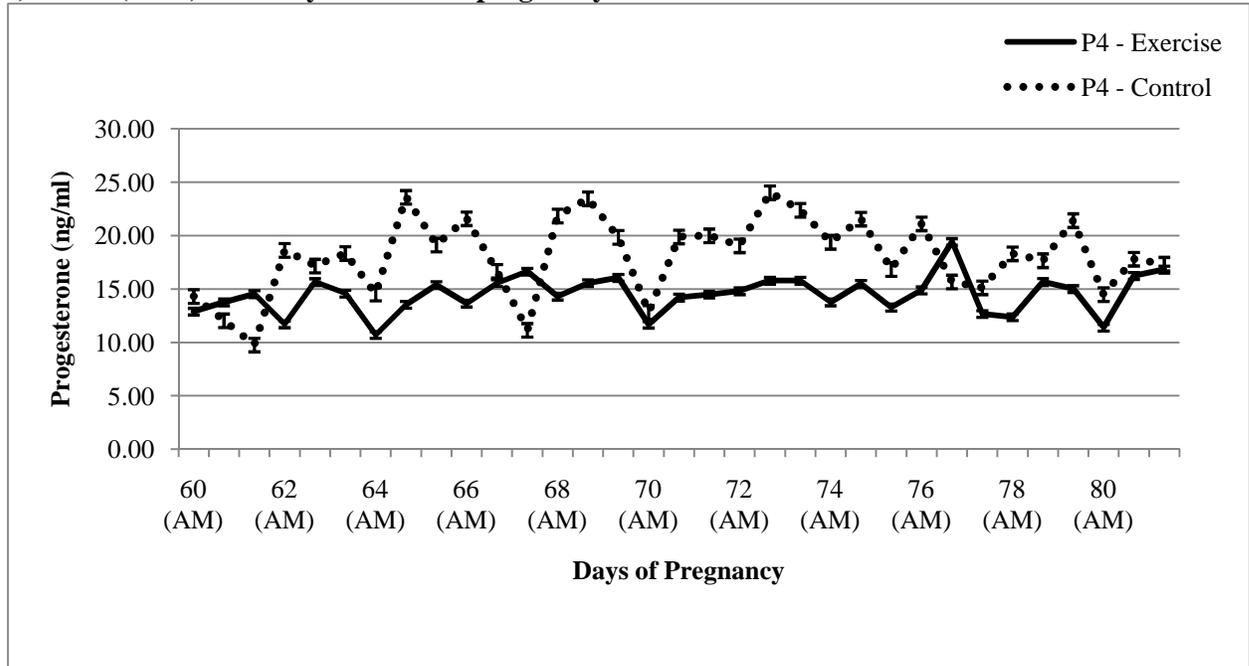


Progesterone

Progesterone concentrations between the exercise group and the non-exercise group were similar ($P = 0.1390$; Table 3). However, the generated progesterone data showed progesterone concentrations were lower in exercised mares than non-exercised mares between days 60 to 80 (Figure 10). There was a day effect for progesterone ($P < 0.0001$) over the course of this study, but there was no treatment by day interaction ($P = 0.7180$).

Figure 10.

The difference in serum concentrations of progesterone for mares that were exercised ($n = 4$) or not ($n = 4$) from days 60 to 80 of pregnancy.



At 0700 hours there was a day effect progesterone concentrations ($P < 0.0001$), and a strong trend for the exercise group to have lower progesterone concentrations at 0700 hours was noted ($P = 0.0660$, Figure 11). There was no treatment by day interaction for serum progesterone at 0700 hours ($P = 0.4780$).

At 1530 hours (pre-exercise) there was an overall day effect in progesterone ($P < 0.0001$), but progesterone concentrations between groups were similar ($P = 0.5840$).

There was no treatment by day interaction for 1530 hours progesterone concentrations ($P = 0.4720$).

Finally, at 1700 hours (post-exercise) there was a day effect in progesterone concentrations ($P < 0.0001$). Progesterone concentrations between groups were similar ($P = 0.8560$). There was no interaction found between treatment and day in progesterone at 1700 hours ($P = 0.8400$).

Figure 11.

The AM progesterone concentrations for mares that were either exercised ($n = 4$) or not ($n = 4$) from days 16 to 80 of pregnancy.

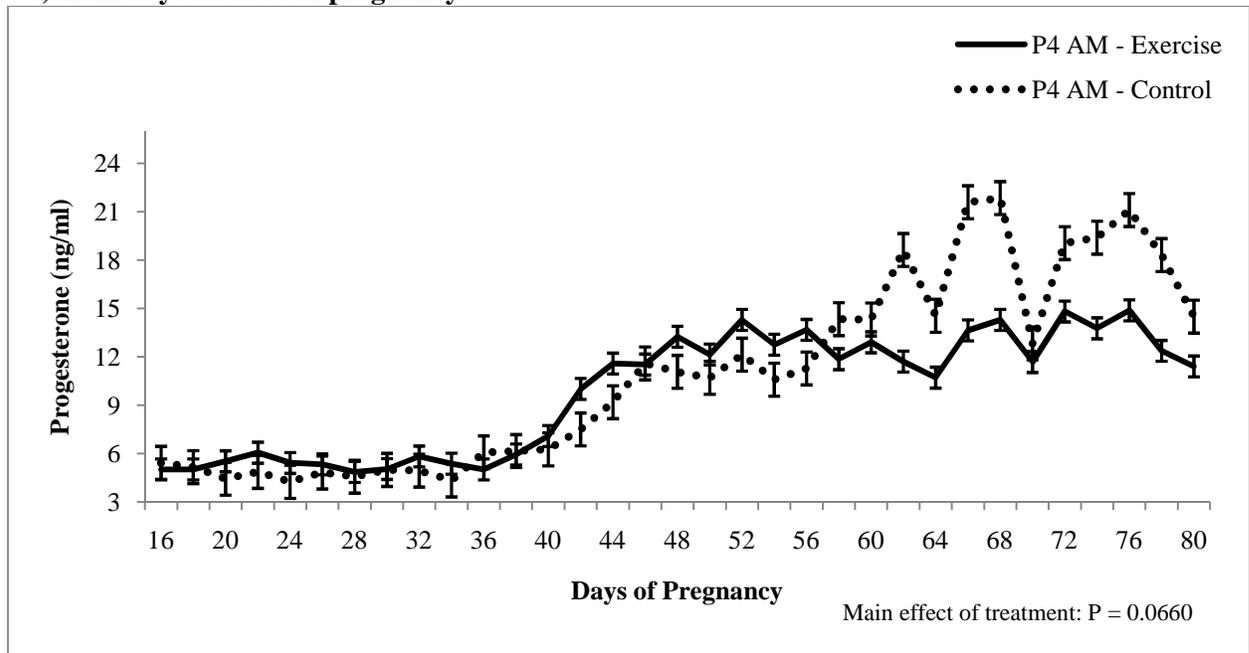


Table 3.

The treatment effect that aerobic exercise had on measured serum cortisol and progesterone concentrations during early equine pregnancy.

Parameters	Treatment LSM \pm SEM	Control LSM \pm SEM	Treatment Effect p-value
Cortisol	6.712 \pm 0.105	6.033 \pm 0.104	$P < 0.0001$
Progesterone	10.815 \pm 0.240	11.326 \pm 0.249	$P = 0.1390$

Ambient Temperature Effect

Heart Rate, Respiration Rate, and Rectal Temperature

There seemed to be minimal change in these vital signs in varying ambient temperatures over the duration of the project.

CHAPTER FIVE

DISCUSSION

Overview

The results of this research determined that aerobic exercise had a significant impact on conceptus maturation and the maternal stress response. Progesterone concentrations appeared to be different after Day 60 of pregnancy, with the exercised mares having a lower concentration than the non-exercised mares. A day effect was present in the following nine parameters: embryonic vesicle growth, embryo proper maturation, fetal crown to rump growth, uterine artery pulsatility and resistance indices (of both the gravid and non-gravid horns), serum cortisol concentrations, and serum progesterone concentrations. The day effect was most likely due to conceptus maturation, blood flow velocity increase, and hormonal differences associated equine pregnancy.

The embryonic vesicle was found to be larger in the exercise group than in the control group over the period of time that it could be successfully measured with ultrasound ($P = 0.0050$). A trend for a treatment and day interaction could be seen in the embryonic vesicle growth, with periods of significance occurring in conjunction with major physiological changes associated with equine pregnancy such as vesicle fixation at the base of the uterine horn. The embryo proper had comparable growth between groups from the point that it could be visualized via ultrasonography until the embryo proper's

transition into a fetus. The crown to rump measurements determined that the fetal growth curve was similar between the exercise group and the control group.

The two units of measure for uterine artery blood flow (pulsatility and resistance indices) were evaluated separately and by uterine horn. The pulsatility index (PI) in both the gravid and non gravid horns was found to be lower in the exercise group than the control group ($P = 0.0008$; $P = 0.0001$). The resistance index (RI), on the other hand, in the gravid uterine horn was similar between groups, and a trend could be identified in the RI of the non-gravid horn being lower in the exercise group than the control group ($P = 0.0560$).

As expected, cortisol concentrations were found to be higher in the exercise group than the control group during 1700 hours (post exercise) time point ($P < 0.0001$). Interestingly, a trend could be identified in the 0700 hours time point that indicated higher concentrations of cortisol concentrations in the exercise group compared to the control ($P = 0.0560$). The cortisol concentrations at the 1530 hours (pre exercise) time point was similar between groups. A treatment and day interaction could be identified in the cumulative cortisol data set ($P = 0.0040$). Progesterone concentrations at the 1530 and 1700 hours time points were similar between groups, but a trend could be seen in the 0700 hours time point that indicated progesterone concentrations in the exercise group were lower than in the control group ($P = 0.0660$). The heart rate, respiration rate, and rectal temperatures of the exercise group were compared to five difference temperature ranges to determine the effect of ambient temperature on these parameters. An ambient temperature effect could not be found on rectal temperature. The ambient temperature did

have an effect on respiration rate ($P = 0.0270$), and a trend was identified in the possible effect of ambient temperature on heart rate ($P = 0.0630$).

Conceptus Maturation

A major finding of this research is that exercising pregnant mares had a significant effect on embryonic and fetal growth parameters. In exercised mares, embryo proper and fetal crown to rump lengths were measurable and observed at earlier time points during pregnancy, suggesting an accelerated growth rate. Women who participate in a high intensity, aerobic exercise regime during the first half of pregnancy, and then decrease the exercise volume by 50% have given birth to larger neonates (Clapp, 2006). In women, there is evidence that in response to aerobic exercise during early pregnancy (and to meet the oxygen and nutritional demands of the developing conceptus), placental tissue proliferation was greater and fetal crown-heel length was increased (Clapp et al., 2000). The results of this research seem to corroborate these findings in women.

Embryonic Vesicle

The equine embryonic vesicle is an important structure for pregnancy detection via ultrasound, and for providing the early conceptus with a nutrient rich environment conducive for growth and survivability (Allen, 2001). Research has been performed to determine the effects that extraneous factors have on vesicle size relative to embryonic age. Cuervo-Arango et al. (2009) determined that a factor such as time of insemination,

in relation to ovulation, had a direct effect on embryonic vesicle size. Maternal age did not to have an effect on vesicle quality or vesicle size in mares.

The vesicle size difference is apparent during the mobility phase of the embryo (approximately Day 14) and the time of fixation at the base of the uterine horn (approximately Day 16). The introduction of exercise did not seem to affect the growth rate of the embryonic vesicle, and the continued growth rate difference may be due to the increased level of activity of the mare.

Embryo Proper

The embryo proper is located within the embryonic vesicle and is one of the first structural and measurable forms of the conceptus that can be evaluated via ultrasound. Ginther and Silva (2005) conducted a study to determine factors affecting the orientation of the embryo proper within the vesicle. Their findings suggested that the tensile strength of the vesicle wall, the turgidity of the uterine wall on the vesicle, and the massaging action of uterine contractions are some factors that directly affect embryo proper orientation and visualization through ultrasound. Denker (2000) discovered that in most mammalian species, the embryonic vesicle and endometrium contain adhesive properties that contribute to the final vesicle orientation in the uterine horn.

The exercise group had a measureable embryo proper four days earlier than the control group, which may be a result of an increased growth rate in the exercised mares. A potential increase in mobility of the vesicle in the exercise group could have lead to an earlier finalized orientation of the embryonic vesicle, increasing the possibility of early

embryo proper detection and measurement via ultrasound. Also, the results show that uterine blood flow was greater in the exercised mares; greater volume of blood flow would increase the amount of available nutrient and substrate delivery to the conceptus. This, in turn, could lead to an increase in embryo proper size. Measurements of the embryo proper continued to be recorded after day 40 in the non-exercised mares due to the inability to identify the fetal crown to rump orientation that was observed in the exercised mares.

Crown to Rump Measurements

Equine embryologists consider the developmental transition of the embryo proper into a fetus to occur on Day 40 of pregnancy. At this time point, a crown to rump fetal orientation begins to become apparent through ultrasound. The fetal crown to rump measurement in the exercised mares was observed sooner than the control mares, similar to the embryo proper and embryonic vesicle. The ability to measure the crown to rump orientation ended earlier in the exercise group due to fetal migration over the pelvic ridge before Day 70 and thus an inability to further monitor via trans-rectal ultrasound.

Clapp (2006) found that aerobic exercise during early pregnancy in women increased the levels of insulin-like growth factors found in circulation, and this growth difference could be attributed to a change in circulating IGFs. The exercised mares could have a greater concentration of IGFs in systemic circulation, which would contribute to larger neonate size. This hypothesis is supported by Clapp (2006) who found that moderate intensity, aerobic exercise during early human pregnancy lead to a 2

cm increase in crown to heel measurements and a 330 gram neonate weight difference compared to women who did not exercise.

Uterine Artery Blood Flow

Uterine Artery PI and RI

The uterine arteries provide the blood supply to the uterus in the pregnant and non-pregnant animal. During pregnancy, the uterine arteries are also responsible for the blood supply to the developing placenta (Silver et al., 1982). Previous published research determined blood flow velocity through the uterine arteries changes substantially during early equine pregnancy (Bollwein et al., 2003). Bollwein et al. (2003) observed, as pregnancy progressed, the RI and PI decreased (indicative of an increase in blood flow velocity). In contrast, Rafla and Etokowo (1998) found that women who exercised while pregnant had a transient rise in the PI following individual exercise bouts, but no harmful effects on the fetus could be determined. Erkkola et al. (1992) performed a study to determine the wave flow velocities of the uterine artery in normal human pregnancy during exercise. They determined that, due to the observed ratio of systolic to diastolic pressures of a cardiac cycle, blood flow through the uterine artery may decrease during exercise (Erkkola et al., 1992). In human pregnancy, sustained aerobic exercise bouts resulted in an acute reduction of oxygen and nutrient delivery to the fetoplacental unit, but exercise increased the maternal systemic plasma volume at rest (Clapp, 2003). An increase in the PI and RI during early pregnancy in women has been linked with reduced placental volumes and fetal growth restriction (Hafner et al., 2001). Conflicting research

published by Clapp et al. (2000) determined that unfit women that began an aerobic exercise regimen during early pregnancy had increased placental volumes, which, in turn, lead to increased weights of the neonates compared to the unexercised control. They further determined that an exercise-induced stimulus of the placenta during early pregnancy had lasting positive effects on the placenta villi proliferation throughout gestation but did not affect gestational length (Clapp et al., 2000).

In the current research trial, the uterine artery was located via ultrasound by the artery's position relative to the aorta, distal circumflexure artery, and the external iliac artery (Ginther, 2007). As pregnancy progressed, the uterine arteries become more prominent due to increased vessel diameter. This increase in vessel diameter aided in locating and measuring the blood flow velocity through the uterine arteries. The PI of the gravid horn was lower than the non-gravid horn in both groups. This is in agreement with the results of Bollwein et al. (2003) who reported similar differences in the gravid and non-gravid horns of pregnant mares. Additionally, these results are interesting in that this may indicate local factors produced by the developing conceptus may act to increase blood flow to the horn of pregnancy. Hoos and Hoffman (1980) found that the presence of a blastocyst in the rabbit uterus induced production of vasoactive substances such as prostaglandins which increased vascularization at time of implantation. A more recent study determined that trophoblastic cell production of vascular endothelial growth factor (VEGF) not only contributed to placental growth but had vasodilation properties and increased blood flow to the site of pregnancy (Valdes et al., 2009).

The difference in uterine artery blood flow velocity between the exercised mares and the non-exercised mares may lead to an increase in the amount of functional placental surface area to support a larger fetus. As stated previously, women who exercise during pregnancy have greater placental growth that results in a longer fetus (Clapp et al., 2000). In a recent report, Weissgerber et al. (2010) reported that exercising pregnant women had higher levels of placental growth factors, such as vascular endothelial growth factor, and lower levels of anti-angiogenic factors, such as IGF binding proteins. These findings would support the hypothesis that placental size is directly correlated with neonate size (Clapp et al., 2000).

Aerobic exercise has been found to cause the upregulation of vascular growth factors such as vascular endothelial growth factor (VEGF; Breen et al., 1996). This research would support the finding of the trend that was observed of the resistance index of the non-gravid horn in the exercised mares being lower than the control. This difference could be attributed to greater vascularization due to exercise and increased fitness. Prior et al. (2004) found that exercise increases the shear stress on vessels causing the diameter of the artery to increase. Shear stress has been considered the primary vasodilatory stimulus that initiates vessel enlargement (Prior et al., 2004). Therefore, exercise increases the blood flow velocity beyond the expected increases that could be attributed to pregnancy.

Hormonal Parameters

Cortisol

Aerobic exercise affects the homeostatic condition of the horse, initiating the release of cortisol into systemic circulation (Gordon et al., 2007). A diurnal variation in plasma cortisol levels exist in the horse, with the highest levels observed at 1000 hours and the lowest levels at 2000 hours (Zolovick et al., 1966). The results of this work corroborate these findings. For both groups of this study, cortisol concentrations were at their highest at the 0700 hours blood draw when compared to the 1530 hours blood draw.

Exercise in horses has been shown to increase cortisol concentrations (McKeever, 2002) and the increase in cortisol concentrations vary with the duration and intensity of exercise (Gordon et al., 2007; Hyypa, 2004; McKeever, 2002). Research has determined that aerobic exercise induced-stress lead to a peak in blood cortisol concentration 30 to 60 minutes following the exercise bout (Gordon et al., 2007). Mares exercised under this study's conditions experienced significant rises in cortisol concentrations similar to those changes previously reported.

The elevated levels of cortisol in the exercise group at 0700 hours and 1700 hours began to normalize over the duration of the exercise period (64 days). Additionally, the cortisol levels in the control group began to lessen over the project. The normalization trend to systemic cortisol in the exercise group could be due to the acclimation to the exercise regimen. Irvine and Alexander (1994) found that Thoroughbred race horses exhibited an adaptation effect to cortisol levels due to the horses' acclimation to environment and training regimen. Marc et al. (2000) determined that a normalization

effect occurred in cortisol concentrations of initially unfit horses that underwent an aerobic exercise regimen over a 24-week period. More recent research found that mares experienced an adaptation reaction to plasma cortisol levels within the peripartal period of pregnancy (Teixeira et al., 2008). However, a study into hormonal adaptations to exercise in men found that cortisol variations induced by seasonality muted any possible adaptation effect to exercise (Gouarne et al., 2005). The normalization to systemic cortisol levels in this study could be attributed to the increase in day length, leading to less pronounced changes in the diurnal variation of cortisol in both the exercised and non-exercised mares. Another possible explanation for the less pronounced trend of lower cortisol concentrations in the control group is the acclimation to jugular venipuncture and handling. Social stress could have been reduced in both groups, as mares were grouped together immediately prior to this experiment. For future research, it may be beneficial to group mares earlier, before the beginning of the experiment to lessen any chance of social stress.

Progesterone

Progesterone is considered the essential steroid hormone for the maintenance of early equine pregnancy (Squires and Ginther, 1975). Plasma progesterone levels are at their lowest by Day 35 in equine pregnancy, reaching nadir concentrations of 3 – 5 ng/ml (Pashen, 1984). Approximately Day 36 of equine pregnancy, endometrial cups began to form and produce equine chorionic gonadotropin (eCG), a hormone responsible for biological activity similar to luteinizing hormone and follicle-stimulating hormone, that

induces maturation and ovulation of follicles (Allen et al., 1973). The formation of secondary corpora lutea sharply increase plasma progesterone concentrations between Days 38 to 40, and progesterone concentrations continue to increase due to the continued eCG activity on follicles of successive follicular waves (Stewart et al., 1976). The endometrial cups reach their maximum eCG-secreting capacity between days 60 to 70 of pregnancy, and these cups become non-functional by approximately day 100 of pregnancy (Allen, 1969).

The serum concentrations of progesterone in both the exercised and non-exercised mares followed patterns during certain time points that were previously identified in other research. Yet, it seems that the progesterone concentrations in the exercised mares were lower than the non exercised mares between Days 60 to 80. The data from this research showed a leveling off of progesterone concentrations in the exercised mares, while progesterone concentrations in the non-exercised mares continued to increase. Concentrations of eCG in maternal serum rise sharply from Day 40 to reach a variable peak around Days 60 to 70 (Allen and Stewart, 2001). Additionally, serum progesterone peaks around weeks 11 to 12 (Day 80) of pregnancy (Allen and Stewart, 2001). The findings of this research may suggest that exercise has a direct effect on endometrial cup formation and function. A possible effect could be the redirection of blood flow away from the reproductive organs to the skeletal muscles during exercise bouts. As mentioned above, previous research in women has determined that the placental villi underwent compensatory hyperplasia to combat the reduction in blood flow to the uterus during aerobic exercise. The endometrial cups begin to form and regress over a period of

approximately 35 days, a time frame that may not allow for compensatory growth. Furthermore, aerobic exercise may affect the efficacy of secreted eCG because of the redirection of blood flow and the limited (0.2 – 6.0 hours) half life of plasma eCG (Aggarwal and Papkoff, 1981). The liver and kidneys are responsible for the degradation of eCG (Aggarwal and Papkoff, 1981). During exercise bouts in the treatment mares, the liver may have degraded the eCG at a faster rate than considered normal due to increased gluconeogenesis that takes place primarily in the liver. This metabolic process supercedes the need to regulate reproductive function during exercise in horses (Gordon et al., 2007).

The Ambient Temperature Effect

Heart Rate, Respiration Rate, and Rectal Temperature

Research has determined that ambient temperature significantly impacts heart rate, respiration rate, and rectal temperature during and following aerobic exercise in horses (Hargreaves et al., 1999). Competition horses that were transitioned into a hot, humid environment from a cool, dry environment did not exhibit a significant change in heart rate immediately following exercise bouts, but respiration rate and rectal temperatures were significantly higher following exercise (Marlin et al., 2001). Hydration status of horses exercised in a hot environment has been shown to have a direct effect on heart rate and rectal temperatures following exercise, with elevated heart rate and temperatures observed in under hydrated horses compared to hydrated horses (Geor and McCutcheon, 1998). Exercise intensity and heat acclimation have been shown

to affect the heart rate of exercised horses; unacclimated horses had a significantly higher heart rate than acclimated horses (Marlin et al., 1999). Warm up periods prior to endurance exercise was found to lower the heart rate in horses after the actual exercise regimen was completed (Lund et al., 1996).

The treatment mares in the current study were subjected to aerobic exercise over a wide range of ambient temperatures (4°C to 32°C). The variability of temperatures during which the research project was conducted may have eliminated an acclimation effect. A difference in heart rate, respiration rate, and rectal temperature before and after exercise was found to be directly correlated to ambient temperature. Heart rate, respiration rate, and rectal temperature were recorded after a 30-minute cool down period, and this interval may have masked any differences in these rates and temperatures that could have been observed prior to the cool down period. The results show that a change in respiration rate is the least transient change when compared to heart rate and rectal temperature. Dissipated heat lost in the form of sweat production may account for the negligible difference observed in rectal temperature taken before and after exercise. To more accurately determine differences pre and post exercise in these vital signs, they should have been taken before a cool down period was allowed.

Conclusions

In this current research, it was hypothesized that aerobic exercise would act as a chronic stressor and have detrimental effects on embryonic and fetal development. Embryonic vesicle size, embryo proper growth, and fetal crown to rump

measurements were evaluated via ultrasound, because of the correlation these areas have with embryonic and fetal viability and maturation. The results of aerobic exercise on these parameters indicate possible alteration to gestational age due to accelerated growth of the conceptus. Trans-abdominal ultrasound would be necessary to determine if the growth curve of the exercised mares remained accelerated or became similar to the control. An important physiological parameter that should be considered for measurement in future studies is the placenta, due to its vital role in fetal survivability. Any alterations to the placenta due to aerobic exercise could have a direct effect on fetal development.

The measurement of uterine artery blood flow through color Doppler technology proved to be a useful tool when assessing the effects of aerobic exercise on blood flow velocity. The blood flow results indicate that aerobic exercise increases blood flow velocity in both uterine horns and ultimately to the conceptus and placenta. This noted increase could provide insight into the difference observed in the growth rates of the conceptus between the exercised mares and non-exercised mares. It appears from this research that increased blood flow to the uterus, due to exercise, contributed to accelerated growth pattern observed here. Further research examining increased uterine blood flow, minus stress factors, would be beneficial in describing this correlation further.

The endocrine components measured in this research, cortisol and progesterone, were important to determine the stress state and the pregnancy status of the mares. Cortisol proved to be an accurate predictor of stress, and cortisol's levels ultimately

decreased over the duration of the project, indicating a possible acclimation effect of the mares. Progesterone increased in intervals due to the formation of accessory corpora lutea that occurred in waves. Aerobic exercise seemed to have an inhibitory effect on progesterone levels when the highest concentrations of systemic progesterone were expected but not seen in the treatment mares. Therefore, it is possible that exercise disrupted either the formation of the endometrial cups, the potency of eCG, or altered luteal tissue formation in pregnant mares.

Ambient temperature did not seem to affect conceptus development, uterine artery blood flow, and hormonal levels. The treatment mares were exercised in wide range of temperatures, and a possible acclimation reaction could be identified. In the future, the vital signs of the control mares should be taken to provide data for comparison and determination of a treatment effect.

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