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Follicular Dynamics in Insulin Resistant Mares

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To the Graduate Council:

I am submitting herewith a thesis written by Julio Cesar Prado entitled "Follicular Dynamics in Insulin Resistant Mares." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Follicular Dynamics in Insulin Resistant Mares

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ABSTRACT

Obesity and insulin resistance have been linked to prolonged interovulatory period, aberrations in the estrous cycle, and continuous reproductive activity during the non-breeding season. EMS has been determined to influence the intrafollicular environment of mare ovaries. In humans, insulin resistance has been linked to polycystic ovaries as part of Polycystic Ovarian Syndrome (PCOS). A study was conducted to determine 1) the impact of insulin resistance on follicle growth and size at ovulation, and 2) whether predicted ovulatory follicles respond to hCG administration in Insulin-resistant (IR) mares. Mares were selected for the study based on insulin sensitivity and separated into an IR group (n=6) and a IS group (n=6); their ovaries and uterus were examined via ultrasound at regular intervals during a spontaneous cycle and a PGF2a shortened synchronized cycle. The dominant follicles (F1) had similar size and F1 size at ovulation between groups. Insulin-resistant mares had more subordinate follicles than Insulin-sensitive (IS) mares ($P < 0.05$). The second largest follicle (F2) of IR mares was larger in diameter ($P < 0.05$) than the F2 of IS mares which may signify a lack of dominance by the largest follicle. Administration of Human Chorionic Gonadotropin (hCG) injections induced ovulation before 48 h in 2 of 4 IR mares while inducing ovulation on all (n=4) IS mares although difference in time to ovulation after hCG administration did not differ statistically. Results observed in this study may provide caution to practitioners working with insulin resistant mares with regards to numbers and sizes of secondary follicles and the effectiveness of hCG for induction of ovulation. The results of this study may support information that the mare could be used as a model to study human ovarian pathologies.

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CHAPTER 1: INTRODUCTION

Insulin resistance has become a concern among horse owners in the United States and is part of Equine Metabolic Syndrome which is associated with laminitis (Frank, Geor et al. 2010). Obesity and insulin resistance have been linked to prolonged interovulatory period (Vick, Sessions et al. 2006), aberrations in the estrous cycle (Vick, Sessions et al. 2006), and continuous reproductive activity during the non-breeding season (Morley and Murray 2014). The ovarian intrafollicular environment in the mare is influenced by insulin resistance (Sessions-Bresnahan and Carnevale 2014). In humans, insulin resistance has been linked to polycystic ovaries as part of Polycystic Ovarian Syndrome (PCOS) (Ehrmann 2002). Follicle manipulation therapy using drugs like human chorionic gonadotropin (hCG) and gonadotropin releasing hormone (GnRH) is common among practitioners and producers trying to induce ovulation in synchrony with insemination (Bergfelt 2000). Although hCG is effective in a high percentage of mares, it is still not understood why ovulation fails or is delayed in some mares (Gastal, Silva et al. 2006).

The present study was designed in an attempt to further understand how insulin resistance affects follicle dynamics, ovulation times and the impact of follicle manipulation therapy. The null hypothesis was that Insulin-resistant (IR) mares would have similar follicular dynamics to Insulin-sensitive (IS) mares. The objectives of the current study were to determine 1) the impact of insulin resistance on follicle growth and size at ovulation, and 2) response of predicted ovulatory follicles to hCG administration in insulin resistant mares.

CHAPTER 2: LITERATURE REVIEW

Mare Reproduction

Normal Estrous Cycle

The estrous cycle in the mare has an average length of 21 days of which 4 to 7 days the mare is receptive to the stallion and considered in estrus (Ginther 1992). Luteinizing hormone (LH) and estradiol as well as progesterone (P4) play different roles during different stages of the estrous cycle of the mare in a similar way as other species (Ginther 1992). In the ovaries, LH induces androgen synthesis by theca interna cells and follicle-stimulating hormone (FSH), and LH stimulate aromatase activity by granulosa cells and this joint action by those two hormones facilitates the production of estradiol (Liu and Hsueh 1986) which in turn stimulates the production of more granulosa cells and increases their sensitivity to other gonadotropins (Ginther 1992). Higher levels of estradiol also cause edematous hyperplasia of the endometrial folds and this edema increases progressively at the beginning of estrus but decreases near ovulation (Dascanio and McCue 2014).

Luteinizing hormone (LH) plays a role on the follicle wall during ovulation although with a lower surge as seen in cows (Dieleman, Kruip et al. 1983). A few days before estrus, LH levels start to rise and then decrease 1 or 2 days after ovulation (Ginther 1992). Luteinizing Hormone also has a function immediately after ovulation acting on the granulosa cells which luteinize and start to produce progesterone. Luteinizing Hormone maintains the CL (Corpus Luteum) which forms from the remnants of an ovulatory follicle and produces in turn progesterone (P4) which plays a large role in the maintenance of pregnancy (Ginther 1992).

Follicular dynamics

Mares can have one or more follicular waves during an interovulatory interval. Major waves have a follicle with diameter 28 mm and greater; whereas, in minor follicular wave, follicles rarely reach sizes greater than 23 mm (Ginther 1992). Follicles that grow larger than 28 mm are considered dominant-sized follicles. In the presence of FSH, follicles are recruited at the beginning of a follicular wave. A follicular wave can have 4 to 8 follicles initially; and as follicles grow, one is selected, the dominant follicle, which may or may not become the ovulatory follicle (Pycock, Samper et al. 2006). Once a dominant follicle grows to a certain size, other follicles in the wave regress and diminish (Ginther 1992, Ginther, Beg et al. 2004).

Follicle selection is the process in which a particular follicle in a follicular wave is selected to become a dominant and potentially ovulatory follicle (Ginther, Beg et al. 2004). In the mares, deviation is characterized by growth of a specific follicle that will be called the dominant follicle preferentially; while at the same time other follicles (subordinate follicles) in the follicular wave regress. The dominant follicle has higher increasing levels of of inhibin-A, activin-A, insulin-like growth factor-1 (IGF1) and estradiol which are believed to play a role in follicle deviation (Ginther, Beg et al. 2004) but IGF1 is thought to be crucial to the initiation of deviation as demonstrated by causation of dominance of the second largest follicle by injecting recombinant IGF1 into it (Ginther, Gastal et al. 2008). Ovulation occurs in follicles at 35 to 55 mm in diameter (Pierson and Ginther 1985, Ginther 1992).

Follicle manipulation therapy

Human chorionic gonadotropin (hCG) is commonly used in equine practice for the induction of ovulation in mares after observation of a dominant follicle 35 mm and larger in the

presence of edema, hCG acts as a LH analog resulting in ovulation within 24 to 48 h (Gastal, Silva et al. 2006). The size of the follicle of at least 35 mm ensures the follicle is the expected ovulatory follicle and presence of edema ensures the mare is at estrus and the uterus is prepared to receive an embryo (Ginther 1992, Gastal, Silva et al. 2006).

Seasonal Anestrous

As described by Nagy et al (Nagy, Guillaume et al. 2000) and Williams et al (Williams, Thorson et al. 2012), during winter months, due to decreasing amounts of daylight hours, mares enter into a period of anestrus and few mares ovulate during this period. The pineal gland translates the environmental signal to an endocrine signal by secreting melatonin during the periods of darkness which is associated with a decrease in gonadotropin secretion and consequently a decrease in ovarian activity. In the northern hemisphere, the mare's natural cycling season runs from April to September with a maximal percentage of ovulations occurring in the summer months. By the end of September, a transitional period begins, ovulation ceases, and levels of LH start to decrease. Follicular waves and recruitment continue during anestrus and mares may exhibit exterior signs of estrus but ovulation does not commonly occur. During seasonal anestrus, dominant-sized follicles grow but fail to ovulate. In the period of resurgence from anestrus, follicles grow and regress until one ovulates following increases in LH pulse frequency (Nagy, Guillaume et al. 2000, Williams, Thorson et al. 2012).

Ultrasound and Reproductive Examination in the Mare

Transrectal ultrasound imaging (UI) has become the standard for evaluating the mare reproductive tract (Ginther 2014). A 5 mhz linear array probe is normally used to scan the uterus and ovaries, even though it has less penetration, the greater resolution provides better details of

the ovarian structures (Ginther 2014). Ultrasound imaging if done only once, provides a snapshot of the reproductive organs at that time. Unless combined with prior history or behavioral characteristics of the mare, one could find it very difficult to assess reproductive status of the mare with just a single ultrasound examination. For breeding and research purposes repeated ultrasound examinations are performed to determine changes in the characteristics of the reproductive organs (Ginther and Pierson 1983).

A routine reproductive examination begins with a scan of the body of the uterus and then each uterine horn to determine uterine health and normalcy and characterize the presence of edema. The next step involves evaluation of the ovaries and ovarian structures. Follicles are identified, counted and measured with the presence or absence of a corpus luteum noted. Comparison of results from a series of reproductive examinations will provide an idea of the reproductive status in the mare. If a dominant follicle is identified, and in subsequent ultrasound imaging examinations growth of that follicle can be established in the presence of uterine edema, the assumption can be made that the mare is in estrus. A large follicle (35 to 55mm) that is absent at the subsequent ultrasound imaging examination is assumed to have ovulated; especially if edema in the uterus is subsiding (Ginther and Pierson 1983).

Insulin Resistance

Definition

In adipose tissue, insulin decreases lipolysis and free fatty acid (FFA) efflux from adipocytes; in liver, it inhibits gluconeogenesis and induces glucose uptake in skeletal muscle (Saltiel and Kahn 2001). The rate of glucose transport is dependent on the concentration of Glut4 at the surface of the cell, insulin stimulates the translocation of these proteins to that site (Chang,

Chiang et al. 2004). Insulin resistance is an impaired sensitivity to insulin by its main target organs that include adipose tissue, liver and muscle (Zeyda and Stulnig 2009). Insulin resistance leads to increased concentrations of FFAs and ectopic fat accumulation that impede insulin mediated glucose uptake in skeletal muscle and elevated glucose production in liver (Zeyda and Stulnig 2009).

Equine Metabolic Syndrome and Insulin Resistance

Equine metabolic syndrome is a clinical syndrome that occurs in horses and is characterized by increased regional or general adiposity, a predisposition towards laminitis (clinical or subclinical) that develops in the absence of recognized causes and insulin resistance (Frank, Elliott et al. 2006, Frank, Geor et al. 2010). Insulin resistance in horses is determined by a variety of tests including Oral Sugar Testing (OST), Combined Glucose and Insulin Test (CGIT) and others (Eiler, Frank et al. 2005, Andrews 2007, Schuver, Frank et al. 2014).

Etiology

The etiology of insulin resistance and equine metabolic syndrome is not well understood. In humans, insulin resistance is a product of decreased insulin stimulated glycogen synthesis attributed mostly to decreased glut4 transport activity (He, Barak et al. 2003). In horses, other responsible mechanisms mentioned are a reduction of the density of insulin receptors in the cell surface, malfunctions of said receptors or defects in internal signaling pathways (Frank 2006). There seems to be a genetic component in equine insulin resistance as there is a higher prevalence among some breeds and ponies (Frank 2006). Environmental factors that are believed to play a role in the development of insulin resistance in horses are high sugar and starch diets and lack of exercise (Nadeau 2006).

Symptoms and effects of insulin resistance in horses

Insulin resistance in horses is associated with an increase in regional adiposity; especially in the neck and base of tail. Horses may or may not be obese but are generally called "easy keepers" by their owners which implies higher body condition scores on less feed than other horses (Frank 2006, Nadeau 2006). Insulin resistance is also thought to pre-dispose horses to laminitis by impairing glucose delivery to the hoof tissue (Pass, Pollitt et al. 1998). Insulin-resistant horses generally have higher body condition scores, neck circumference, glucose concentration, insulin concentration, glucose to insulin ratio than normal horses (Frank 2006, Frank, Elliott et al. 2006). Insulin resistance has also been identified as a risk factor or component of hyperlipidemia, pituitary adenoma, and osteochondrosis (Treiber, Boston et al. 2005).

Glucose and Insulin in the ovary

As described in a review by Dupont and Scaramuzzi, insulin and glucose play important roles in the normal ovarian function. Glucose, as an energy substrate, nurtures the cyclic development of follicles, maturation and ovulation of the dominant follicle and the formation and maintenance of the corpus luteum. Insulin affects follicular development and possibly through interactions between their signaling pathways can have gonadotropin-like actions (Dupont and Scaramuzzi 2016).

Insulin Resistance and Reproduction in Mares

In mares, insulin resistance has been associated with a prolonged interovulatory period, possibly by a stimulatory effect of insulin on progesterone production by the corpus luteum (Sessions, Reedy et al. 2004); in association with high body condition scores elevated insulin increased reproductive activity during the non-breeding season (Gentry, Thompson et al. 2002)

and produced other alterations of the estrous cycle (Vick, Sessions et al. 2006). A recent study by Sessions and Carnevale, demonstrated that the ovarian intrafollicular environment in the mare is influenced by insulin resistance (Sessions-Bresnahan and Carnevale 2014). Insulin-resistant mares are also thought to be at higher risk of complications during pregnancy (George, Staniar et al. 2011).

Insulin Resistance and Reproduction in Other Species

Studies addressing changes in insulin receptor signaling cascade and metabolic mediators in Suffolk sheep found a reduction in granulosa cell expression of adiponectin in the ovary (Ortega, Rey et al. 2010), which is thought to induce periovulatory changes in follicular cells (Ledoux, Campos et al. 2006). Exogenous insulin increased IGF1 in fluid of medium follicles in gilts, increased the number of small and medium follicles and reduced the number of atretic follicles (Matamoros, Cox et al. 1991). Hyperinsulinemia as a result of feed-induced high body condition scores in cattle has been associated with impaired oocyte quality and embryo development, increased IGF1 levels in plasma at the time of estrus and higher numbers of small and medium follicles (Adamiak, Mackie et al. 2005). Insulin insufficiency has been observed in cystic ovarian disease in cattle (Opsomer, Wensing et al. 1999) indicating insulin could be a factor in the pathogenesis by reduced gonadotropin release (Vanholder, Opsomer et al. 2006).

Polycystic ovary syndrome (PCOS) is a common disorder of women characterized by hyperandrogenism and chronic anovulation (Dunaif 1997) in women. It has been associated with insulin-resistance (Dunaif and Hoffman 1988, Ehrmann 2002) and decreased circulating adiponectin (Ardawi and Rouzi 2005). Polycystic ovary morphology observed in ultrasonographic examinations is defined by the presence of 8 or more subcapsular follicular cysts ≤10 mm and

increased ovarian stroma (Dunaif 1997). In PCOS there is insufficient FSH causing an arrest in follicular growth and anovulation (Ledoux, Campos et al. 2006). There are also reports of impaired oocyte quality associated with insulin resistance in women (Maheshwari, Stofberg et al. 2007).

In summary and of interest to our research, insulin resistance could cause an increased number of follicles, elevated IGF1 levels in the ovary and in plasma (Matamoros, Cox et al. 1991, Adamiak, Mackie et al. 2005), decreased circulating adiponectin (Ardawi and Rouzi 2005, Ortega, Rey et al. 2010, Sessions-Bresnahan and Carnevale 2014), a prolonged interovulatory period (Sessions, Reedy et al. 2004), and other alterations of the estrous cycle in mares (Vick, Sessions et al. 2006).

CHAPTER 3: MATERIALS AND METHODS

Mares

Animal Care and Use

This study was approved by East Tennessee Clinical Research's Institutional Animal Care and Use Committee, protocol number ETCR-13-0142. General health observations of all study animals were conducted once daily for the duration of the study beginning at acclimation. Observations included an assessment of general health, behavior/attitude, fecal consistency, and appetite. A physical examination of each candidate was conducted during the acclimation period.

General Description

Subjects were commercially acquired mares, cooperative with study procedures. Breeds were evenly distributed among groups. Ages ranged from 9 to 11 in the Insulin-resistant (IR) group and 6 to 12 in the Insulin-sensitive (IS) group, there were no significant differences in mean ages between groups ($P = 0.51$). Candidate mares were determined in good general health by physical exam prior to the beginning of acclimation and daily general health observations. Body condition scores (Henneke, Potter et al. 1983), ranged from 7 to 8 in the IS group and 7 to 8 in the IR group and did not differ between groups ($P = 0.59$). Individual body weights (kg) were measured prior to the start of acclimation and was also similar ($P = 0.18$) between groups, IS mares ranged from 490 to 526 kg IR mares ranged from 446 to 526 kg. The livestock scale used for measuring body weights was certified by a licensed scale service within three months before initiation of the study. Before and after each weighing session, accuracy of the scale was verified with standard test weights ranging from ~45 to ~363 kg.

Husbandry

Mares were maintained in individual 12 ft. by 12 ft. stalls throughout the study. Stalls were constructed of metal panels and/or solid wooden walls. Stall flooring was packed limestone bedded with pine/hardwood shavings and sawdust. Each stall was equipped with a metal feeder designed to offer grain and hay simultaneously, and water was provided in two 16-liter buckets that were filled twice daily. Mares were fed daily standard portions of a commercial 11% protein concentrate; quantities of concentrate were measured by volume and provided equally in a.m. and p.m. Mares also received ~1.5% of body weight of a mixed-grass hay daily, divided into similar portions that were provided in a.m. and p.m. Stalls were cleaned daily and bedding was replaced as needed.

Insulin Resistance and PPID

Insulin-resistance for the Insulin-resistant group was determined by a combined glucose insulin test (CGIT) (Eiler, Frank et al. 2005) or an oral glucose test (OGT) (Schuver, Frank et al. 2014) in previous studies conducted by our group; Insulin-sensitive mares had negative screening CGIT or OST testing results. In baseline blood samples measured IR mares had significantly higher ($P < 0.01$) fasted insulin levels than IS mares (Mean 25.72 ± 5.42 and 7.03 ± 3.83 respectively). Prior to initiating acclimation, each mare was tested for the absence of pituitary pars intermedia dysfunction (PPID) by means of a dexamethasone suppression test (Andrews, Frank et al. 2004).

Ultrasound Examinations

A Universal Ultrasound (Bedford Hills, New York) model DP6600Vet was used with a 4.0/5.0/6.0/7.5 MHz ultrasonic transducer (model 50L60EAV) attached and set to 5.0 MHz. Each

mare was evaluated by transrectal ultrasound approximately every 48 h until a 28 mm follicle was recorded; then every 12 to 24 h until ovulation. During each ultrasound session, identifiable ovarian structures were measured and recorded, and uterine edema was characterized.

Hormonal Therapy

Five days after the first recorded ovulation for each mare, prostaglandin $F_{2\alpha}$ (Lutalyse, Pfizer, Kalamazoo, MI; 10mg IM) was administered after ultrasound examination to produce lysis of the CL and thus shorten the diestrus period for completion of the second objective. After treatment with prostaglandin $F_{2\alpha}$ and a follicle reached ≥ 35mm in size, hCG was administered to the mare (Chorulon, Intervet, Madison, NJ; 3,000 Units IV) to induce ovulation.

Experimental design

Treatment Groups

Six Insulin-resistant (IR group) and six Insulin-sensitive (control group) mares were selected from a pool of 18 candidates; mares that were not cooperative to study procedure or that were showing signs of estrus were excluded from the study (n=6).

Periods of Observation

The study was divided into two stages: spontaneous cycle and synchronized cycle. The spontaneous cycle included evaluations up to 7 days prior to the first ovulation. The synchronized cycle began five days after the first ovulation when mares were treated once with prostaglandin $F_{2\alpha}$; at observation of the presence of a 35 mm diameter follicle, mares were treated with a dose of hCG to induce ovulation (Figure 1).

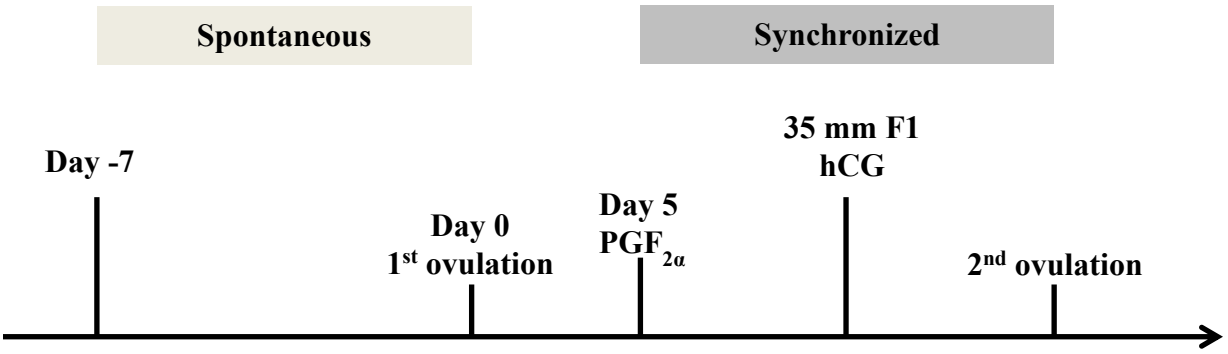

Figure 1. A schematic of the periods of observation. The spontaneous cycle was normalized so all enrolled mares were evaluated within the same number of days before ovulation.

Variables of interest

Follicles were counted as: total follicles observed, ≥ 10mm, ≥15mm, ≥20mm, ≥25mm, ≥30mm. The largest (F1) and second largest (F2) follicles recorded in a single ultrasound session were recorded separately for data analysis. Follicles with two or more measurements were averaged to obtain an approximate diameter. Growth rate of the follicles were calculated using the times and dates of each ultrasound imaging examination and the measured sizes of F1 and F2 for the respective examination within each cycle. Uterine edema was scored as: 0 = absent (no edema), 1 = slight, 2 = moderate or 3 = heavy (Ferris and McCue 2010). For analysis, estrus was defined as edema score of 2 or 3 and a follicle greater than or equal to 28 mm in diameter (Ginther, Gastal et al. 2008). The time of ovulation (disappearance of the predicted ovulatory or largest follicle) was determined on a case by case basis based on observations during ultrasound sessions.

Statistical Analyses

Analysis of variance and comparison of least squares means were performed via mixed models, with autoregressive repeated measures on mares for the above variables during the Spontaneous Cycle, when the mare was in estrus in the spontaneous cycle, during the

Synchronized Cycle and when the mare was in estrus in the synchronized cycle. Fixed effects for a combined analysis were Group, Cycle, Estrus status and their interaction. Regression analysis were also conducted to determine influence of F1 size on the variables during the same periods of observation.

For variables in the examinations at each ovulation, at the time of prostaglandin administration, and at the time of hCG administration, analysis of variance and comparison of least squares means were performed via mixed models.

The software program “R” version 3.2.3 (2015-12-10; "Wooden Christmas-Tree"; The R Foundation for Statistical Computing) was used to analyze the data. Significance level was set at P = 0.05 for all analysis and trends were set at P = 0.10.

CHAPTER 4: RESULTS

Number of follicles

Insulin-resistant mares had significantly more total follicles ($P < 0.01$; Figure 2, Table 1), follicles 10 mm or larger ($P < 0.01$; Figure 2, Table 1), follicles 15 mm and larger ($P< 0.05$; Figure 2, Table 1) as well as follicles 20 mm or larger than insulin-sensitive mares ($P < 0.01$; Figure 2, Table 1). The number of ≥25 mm follicles of insulin resistant mares were or tended to be higher than the number of ≥25 mm follicles of insulin sensitive mares (Figure 2, Figure 3, Table 1). There were no significant differences between the number of 30 mm and greater follicles between groups (Table 1). Total number of follicles ($P = 0.0226$, Table 1), numbers of follicles ≥15 mm ($P = 0.0289$, Table 1), ≥25 mm and ≥30 mm were different between the spontaneous and the synchronized cycles and there was a significant interaction of insulin sensitivity, cycle and estrus status (Table 1). Only the number of follicles ≥25 mm and ≥30 mm were different ($P < 0.01$, Table 1) if mares were or were not in estrus; there was a significant ($P < 0.05$) interaction between insulin sensitivity, cycle and estrus status in the case of the number of follicles ≥30 mm (Table 1).

Dominant follicle and F2 Sizes

Size of the dominant follicle (F1) did not differ between insulin resistant and the insulin sensitive mares during the spontaneous cycle ($P = 0.8031$; Table 2). During the synchronized cycle, insulin resistant mares tended to have larger F1 sizes than insulin sensitive mares ($P = 0.0519$, Table 2), there was a significant interaction of insulin sensitivity and cycle ($P = 0.0004$) in relation to F1 size.

Table 1. Effects of Insulin sensitivity, Cycle and Estrus status on numbers of follicles. a, b: denotes a significant effect $P < 0.05$.

	Total	≥10mm	≥15mm	≥20mm	≥25mm	≥30mm
Insulin sensitivity						
Insulin resistant	11.37[a]	8.11[a]	4.44[a]	2.34[a]	1.29[a]	0.90
Insulin sensitive	7.03[b]	4.18[b]	2.43[b]	1.00[b]	0.70[b]	0.51
SEM	1.21	0.93	0.51	0.27	0.21	0.21
Cycle						
Spontaneous	9.91[a]	6.21	3.89[a]	1.81	1.06[a]	0.77
Synchronized	8.49[b]	6.08	2.97[b]	1.54	0.93[b]	0.64
SEM	0.80	0.60	0.43	0.24	0.16	0.15
Estrus status						
In estrus	9.52	6.15	3.38	1.81	1.22[a]	0.91
Not in estrus	8.88	6.14	3.48	1.54	0.77[b]	0.50
SEM	0.48	0.35	0.30	0.18	0.11	0.09
ANOVA P-values						
Insulin sensitivity	0.0012	0.0001	0.0002	< 0.0001	0.0062	0.0622
Cycle	0.0226	0.8374	0.0289	0.1234	0.0288	0.0200
Estrus status	0.1504	0.9866	0.7155	0.1556	0.0001	0.0001
Insulin sensitivity x Cycle	0.0706	0.2436	0.7461	0.8558	0.2289	0.6067
Insulin sensitivity x Estrus status	0.9997	0.8929	0.8904	0.7609	0.5505	0.1498
Cycle x Estrus status	0.5731	0.5094	0.3397	0.4769	0.1098	0.0584
Insulin sensitivity x Cycle x Estrus status	0.0229	0.4054	0.7973	0.7127	0.1218	0.0162

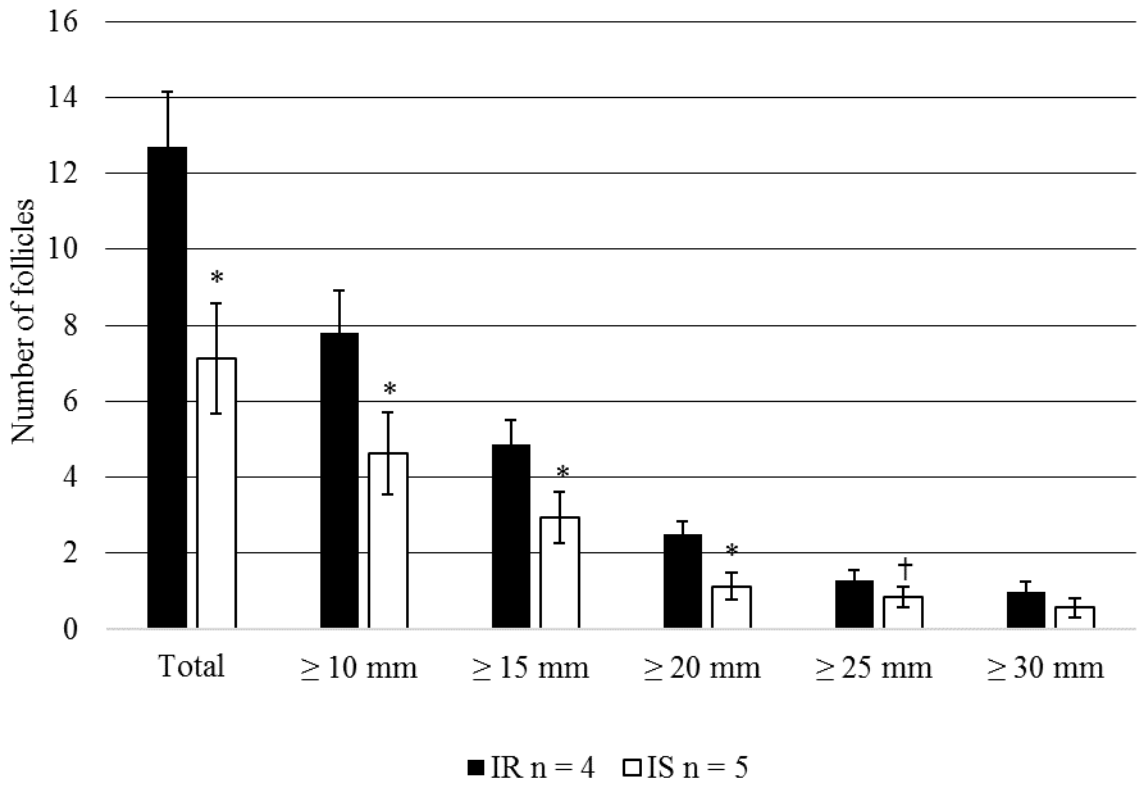

Figure 2. Number of follicles (mean ± SE) of the different size classes during the spontaneous cycle. IR: Insulin-resistant, IS: Insulin-sensitive. * denotes $P < 0.05$, † denotes $P < 0.10$ within a follicle size class.

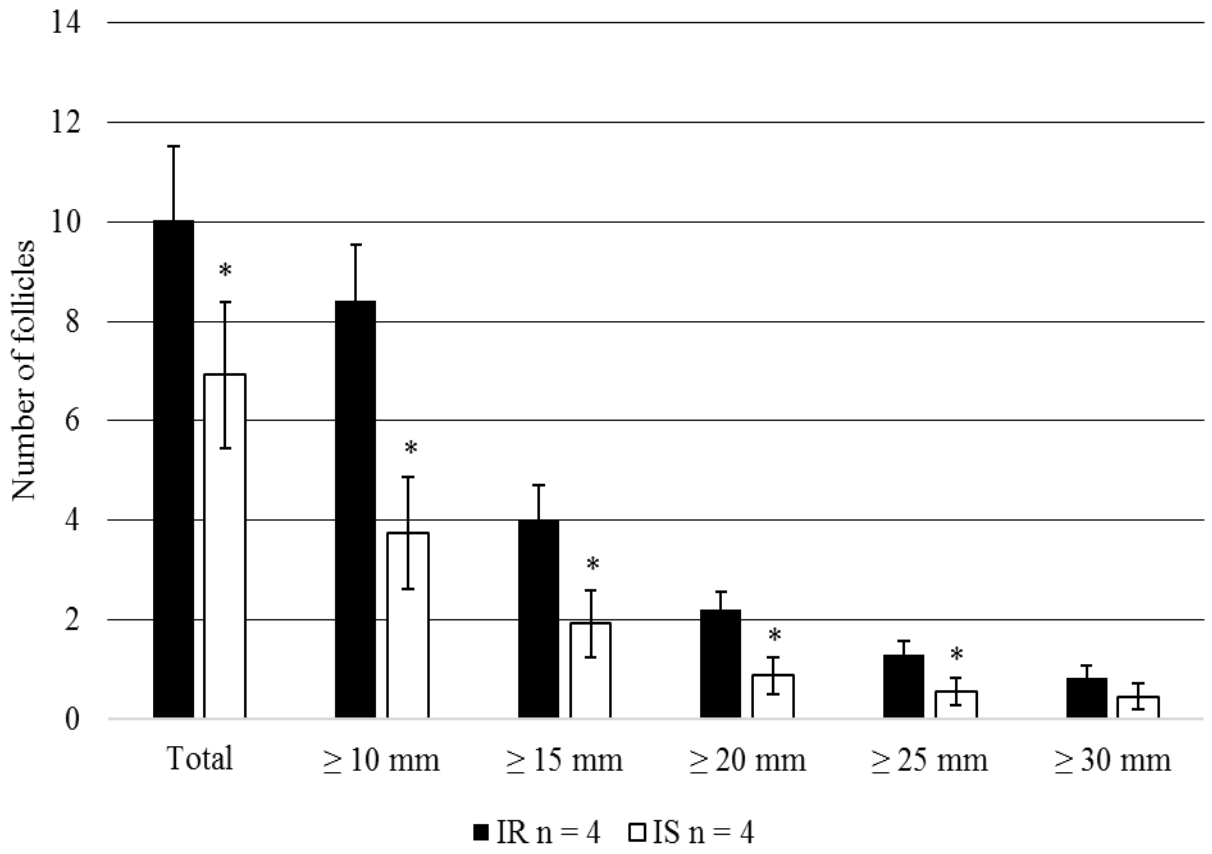

Figure 3. Number of follicles (mean ± SE) of the different size ranges during the synchronized cycle. IR: Insulin-resistant, IS: Insulin-sensitive. *: denotes $P < 0.05$ within a follicle size class.

Table 2. Effects of insulin sensitivity, cycle and estrus status on follicle size and growth rate during the spontaneous and synchronized cycles. a, b: denotes a significant effect $P < 0.05$.

	Follicle size (mm)		Growth rate (mm/day)	
	F1	F2	F1	F2
Insulin sensitivity				
Insulin resistant	34.68	25.72[a]	2.06	0.45
Insulin sensitive	27.71	16.69[b]	2.06	-0.31
SEM	5.87	2.92	0.62	0.64
Cycle				
Spontaneous	36.96[a]	22.69[a]	1.78	0.11
Synchronized	25.43[b]	19.72[b]	2.34	0.02
SEM	2.08	1.39	0.63	0.65
Estrus status				
In estrus	34.19[a]	21.49	2.47	-0.69[a]
Not in estrus	28.20[b]	20.92	1.65	0.83[b]
SEM	1.09	0.76	0.68	0.70
ANOVA P-values				
Insulin sensitivity	0.3502	0.0048	0.6052	0.2319
Cycle	<.0001	0.0101	0.6608	0.1591
Estrus status	<.0001	0.4195	0.3122	0.0140
Insulin sensitivity x Cycle	0.0004	0.4254	0.4577	0.6605
Insulin sensitivity x Estrus status	0.0826	0.1623	0.9178	0.0695
Cycle x Estrus status	0.0987	0.0218	0.1177	0.0054
Insulin sensitivity x Cycle x Estrus status	0.2286	0.0933	0.5137	0.8268

Size of the second largest (F2) follicle was larger ($P < 0.01$) in insulin resistant mares compared to insulin sensitive mares (Table 2). There was a significant effect of cycle on F2 sizes ($P < 0.05$; Table 2) and a significant cycle by estrus status interaction ($P < 0.05$; Table 2) on the size of F2.

Growth rate of the F1 follicle did not differ between groups during the study (Table 2); however, growth rate of the F2 follicle significantly differed between groups when mares were in heat, ($P < 0.05$; Table 2), with a positive growth rate in insulin resistant mares (0.39 mm/day ± 0.68) and a negative growth rate (-1.77 ± 0.64) on the insulin sensitive mares.

Association between F1 size and number of follicles, F2 size and edema.

The size of F1 had a significant association to the number of ≥ 25 mm follicles, the number of ≥ 30 mm follicles during both cycles (Table 3). There was also a significant association of the size of F1 to the edema scores of both groups during the spontaneous cycle and to the edema scores of the insulin sensitive mares during the synchronized cycle (Table 3). With the exception of the slopes for the number of follicles ≥ 10 mm during the synchronized cycle, regression slopes did not differ between groups (Table 3).

Time to Spontaneous Ovulation

Insulin resistant mares ovulated 75.31 h ± 16.87 after appearance of a 35 mm follicle and insulin sensitive mares 47.82 h ± 15.09 after, but these times did not differ ($P = 0.26$). Insulin resistant mares tended to take significantly less time ($P = 0.08$) to ovulate once an edema score of 2 or more was recorded (76.78 h ± 12.36, range: 42.17 to 96.27 h) than insulin sensitive mares (109.94 h ± 11.05, range: 77.6 to 148.75 h).

Table 3. Regression slopes on size of the F1 follicle for different classifications of follicle sizes (in number of follicles/F1 mm), edema scores (in score/F1 mm) and F2 size (in mm/F1 mm) of Insulin-resistant (IR) and Insulin-sensitive (IS) mares. Slopes different from 0 are marked with * ($P < 0.05$). a, b indicates slopes differ between groups ($P < 0.05$).

	Spontaneous cycle		**Synchronized cycle**	
	IR n = 4	IS n = 5	IR n = 4	IS n = 4
Number of follicles				
Total	-0.04	0.09	-0.07	0.02
≥ 10 mm	-0.03	0.01	-0.14*	-0.02
≥ 15 mm	-0.07	-0.01	-0.04[a]	0.04[b]
≥ 20 mm	0.01	0.01	0.03	0.05*
≥ 25 mm	0.07*	0.03*	0.04*	0.04*
≥ 25 mm	0.05*	0.05*	0.05*	0.04*
Edema Score	0.08*	0.08*	0.00	0.05*
F2 Size	0.22	0.02	0.43*	0.18

Time from $PGF_{2\alpha}$ administration to a 35 mm follicle

Time from administration of $PGF_{2\alpha}$ to the presence of a 35mm follicle was similar (P = 0.79) between mares, 136.07 h ± 33.21 for IR mares and 148.86 h ± 46.98 for IS mares.

Time from hCG administration to ovulation

Insulin resistant mares (n = 4) ovulated 59.53 h ± 13.51 after hCG administration (range 28.75 h to 112.58 h). Insulin sensitive mares (n = 4) ovulated 32.80 h ± 19.11 after hCG treatment (range 12.00 to 35.50 h), but the difference between the average times to ovulation of the groups was not significant (P = 0.15).

CHAPTER 5: DISCUSSION

The mares included in the study were of very similar size and weight, were generally healthy and had no differences in body condition scores. Mares were selected based on their Insulin-sensitive status and the fact that they were similar in other characteristics establishes that results reported in this study can be attributed to their Insulin-resistant or Insulin-sensitive status.

Number of subordinate follicles

Insulin resistant mares had significantly more subordinate (<25 mm) follicles than insulin sensitive mares, which could be attributed to changes in the ovary that have been observed in other species (Matamoros, Cox et al. 1991, Dunaif 1997). In cattle with feed-induced hyperinsulinemia, higher numbers of small and medium follicles were observed (Adamiak, Mackie et al. 2005). In women, Polycystic Ovary Syndrome (PCOS) is characterized by multiple medium sized follicles that fail to ovulate (Dunaif 1997) (see Figure 4). When administered exogenous insulin, prepubertal gilts exhibited increased numbers of small and medium follicles possibly due to an increase in IGF1 levels (Matamoros, Cox et al. 1991). The intrafollicular fluid of IR mares has been shown to present similar characteristics as that of women with PCOS (Dunaif 1997, Sessions-Bresnahan and Carnevale 2014). In women with PCOS, high levels of insulin in the ovary is thought to contribute to steroidogenic abnormalities that can affect LH and FSH release (Dunaif 1997). Abnormal levels of LH and FSH in the insulin resistant mare could affect recruitment of follicles and dominance causing an increase in the number of antral follicles and a decrease in atresia (Matamoros, Cox et al. 1991, Ginther, Gastal et al. 2008), but a more likely explanation lies in the possibility that insulin resistance causes an increase of IGF1 in the follicular environment (Salazar-Ortiz, Monget et al. 2014) and these higher IGF1 levels stimulate the growth

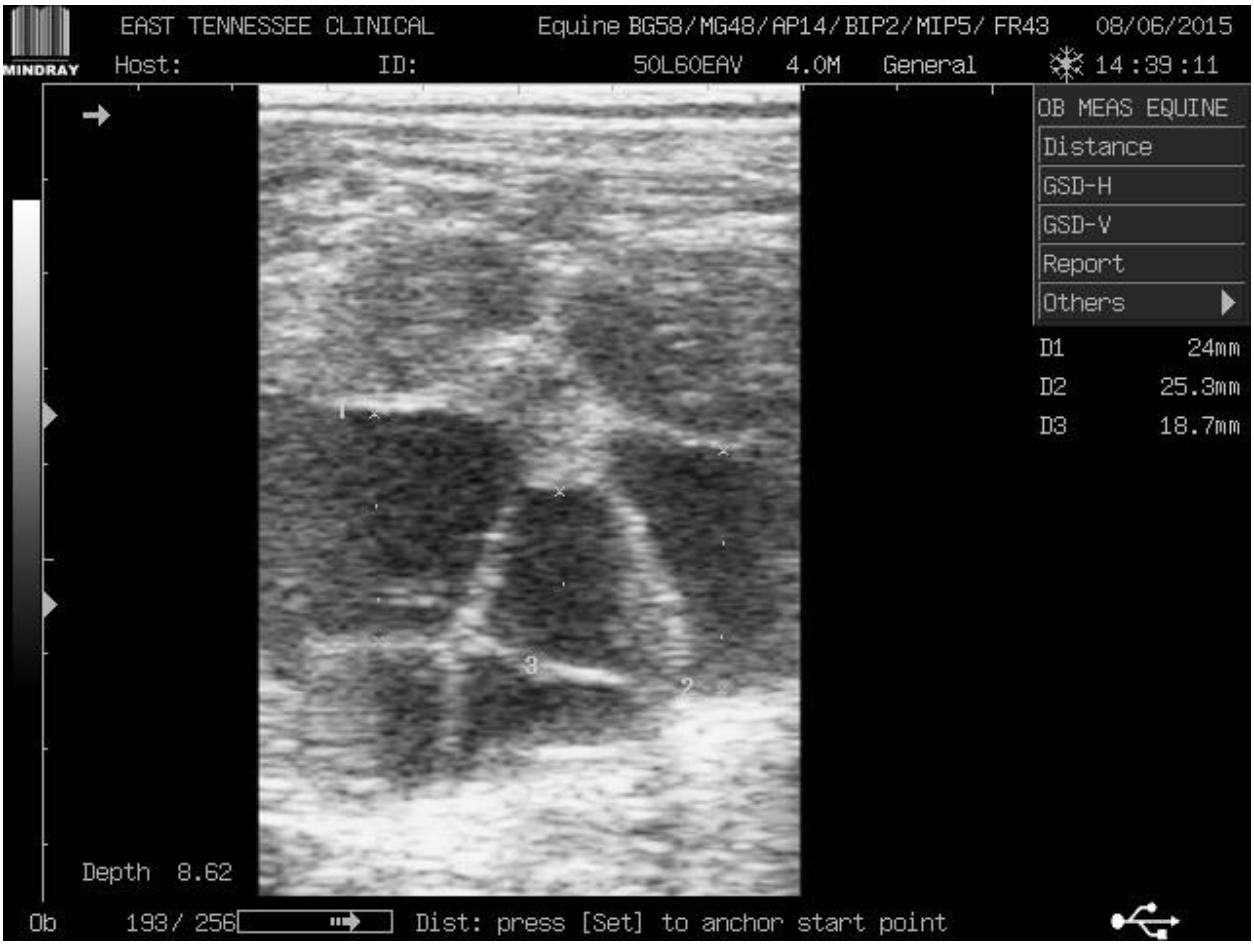

Figure 4. Representative ultrasound image of a "Pomegranate Ovary" of an Insulin Resistant mare. The largest follicle observed (D2 in image) in this image has an approximate diameter of 25 mm while the second largest (D1 in image) measures 24 mm. another medium sized follicle (D3 in image) measured 18 mm. 4 additional follicles are present in this image.

of secondary follicles (Silva, Figueiredo et al. 2009). Further research is needed to correlate ovarian follicular dynamics with hormone levels in IR mares.

The size of F1 had a negative influence on the number of ≥10 mm, ≥15mm of IR mares but no influence on the number of follicles of those sizes of IS mares. Increasing size of the dominant follicle, and in this case the second largest follicle also being larger in size, could have made it difficult to identify and count smaller follicles (Pierson and Ginther 1987).

Size of dominant and second largest follicles

During all phases of the study, the second largest follicle (F2) observed during ultrasound examinations of insulin resistant mares was or tended to be larger than the F2 of the IS mares (Figure 4; Figure 5). During estrus, normally one would observe regression of subordinate follicles and the size of F2 would continue to decrease (Ginther, Beg et al. 2004). This did not happen in the case of insulin resistant mares. This apparent lack of dominance of the F1 follicle towards the F2 has also been reported in swine (Matamoros, Cox et al. 1991) where it was attributed to an increase in IGF1 in medium follicles that may cause a co-dominance of F1 and F2 (Figure 5). When recombinant IGF1 was injected into the second largest follicle of mares, it outgrew the dominant follicle and became the dominant follicle (Ginther, Gastal et al. 2008), insulin resistant mares may have higher insulin and IGF1 levels in the follicular environment (Salazar-Ortiz, Monget et al. 2014) and thus IGF1 may be causing more than normal growth of the F2 and other subordinate follicles.

The size of the F1 follicle was similar among groups throughout the study except during the interovulatory period between the spontaneous and the induced ovulations, when there was a

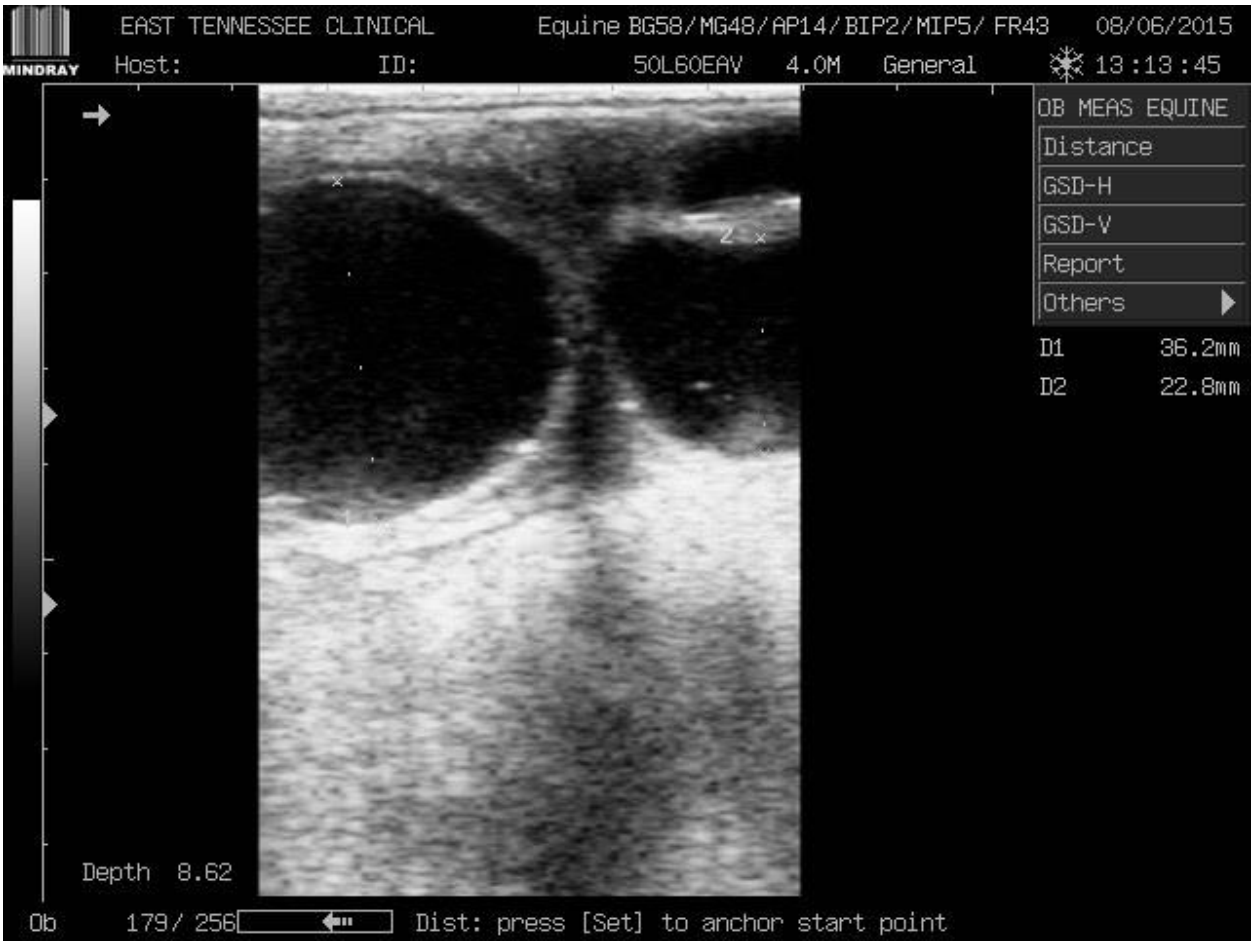

Figure 5. Representative ultrasound image of co-dominance between F1 and F2 of an insulin resistant mare. The right ovary of this mare in estrus presents 2 large follicles, the F1 (dominant) follicle measured 40 mm (36 x 44 mm) and the second largest follicle measured 28 mm (34 x 23 mm). Yet another large follicle was present in the left ovary of this mare at the time measuring 25 mm and had a total of 8 follicles of at least 10 mm.

tendency of the F1 of the insulin resistant mares to be larger than that of the insulin sensitive mares. In our estimation, this could be attributed to the F2 from the previous ovulation still being present on the ovaries of the insulin resistant mares after the first ovulation and was counted as the F1 on subsequent ultrasound examinations. Follicle ablation studies in insulin resistant mares could provide better understanding of this observation.

Influence of F1 size on edema score

During the synchronized cycle, F1 size did not have a significant positive influence on the edema scores of insulin resistant mares. As expected (Pierson and Ginther 1987, Ginther 1992), the edema score of insulin sensitive mares increased as the F1 size increased. Since uterine edema in the mare is related to estradiol production by the dominant follicle (Samper 1997), it is possible estradiol production in the F1 of insulin resistant mares is affected in some way by the presence of larger subordinate follicles.

Time from $PGF_{2\alpha}$ administration to a 35 mm follicle

There were no differences between the groups in the time elapsed from prostaglandin injection to when the F1 reached 35 mm in size which is interesting due to the fact that previous studies had suggested insulin resistant mares had increased interovulatory period (Sessions, Reedy et al. 2004). Although that may still be the case, the present results would reinforce the belief that if insulin resistance affects the interovulatory period it would be in diestrus, or in relation to the luteal phase as described by Vick et al with obese mares (Vick, Sessions et al. 2006), and not when the mare is approaching estrus. The difference between our research and previous reports is that the current project used naturally occurring insulin resistant mares and others had used induced

insulin resistance by the hyperinsulinemic-euglycemic clamp procedure (Sessions, Reedy et al. 2004), and obese mares compared with feed restricted mares (Vick, Sessions et al. 2006).

Time from hCG administration to ovulation

Although times to ovulation after hCG administration between insulin resistant and insulin sensitive mares did not differ, we believe with larger numbers of mares that a difference may have been revealed. The expected time to ovulation after administration of hCG to a mare with a follicle greater than 35 mm and in the presence of edema is 12 to 48 h. The insulin sensitive mares in the present study all ovulated within that timeframe as would be expected (Gastal, Silva et al. 2006). However, two of the insulin sensitive mares ovulated within the timeframe, one ovulated a few hours past the expected time and the other had not ovulated at the time of finalization of the study. It is possible that as seen in women with PCOS (Ehrmann 2002) some follicles of insulin resistant mares do not ovulate. Cellular changes in the ovarian follicular cells may also prevent them from responding to hCG in the way normal ovaries respond but these mares all seem to respond to ovulation signals in the same way as insulin sensitive mares since they all ovulated during the spontaneous cycle.

Implications and Conclusion

Practitioners working with mares that may be insulin resistant or present signs of equine metabolic syndrome, must be mindful of the possibility that these mares may have greater numbers of small or medium follicles, increased sizes of secondary follicles and that the effectiveness of hCG for induction of ovulation may be decreased.

More research is necessary to elucidate the differences in the follicular characteristics of insulin resistant mare ovaries and their relationship to reproductive hormones. In women, by

reducing (with metformin) hyperinsulinism, a reduction in intraovarian androgens was produced leading to a reduction in E2 levels and favoring orderly follicular growth in response to exogenous FSH (De Leo, La Marca et al. 1999). Although metformin has been tested as a potential treatment in horses to combat the symptoms of insulin resistance, it was not effective at the doses tested (Vick, Sessions et al. 2006). Other potential treatments for insulin resistance need to be evaluated in the mare.

The observation of these differences in the follicular dynamics of insulin resistant mares in relation to insulin sensitive mares further stresses the value that the mare model could have in studying the effects of insulin on ovarian function (Gastal, Gastal et al. 2011, Ginther 2012, Alves, Alves et al. 2016).

LIST OF REFERENCES

Adamiak, S., K. Mackie, R. Watt, R. Webb and K. Sinclair (2005). "Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle." Biology of reproduction **73**(5): 918-926.

Alves, K. A., B. G. Alves, G. D. Gastal, S. G. de Tarso, M. O. Gastal, J. R. Figueiredo, M. L. Gambarini and E. L. Gastal (2016). "The mare model to study the effects of ovarian dynamics on preantral follicle features." PloS one **11**(2): e0149693.

Andrews, F. M. (2007). "Combined dexamethasone suppression/thyrotropin-releasing hormone stimulation test for detection of equine cushings disease." Proceedings of the 29th Bain Fallon Memorial Lectures: Behaviour, Lameness and Medicine, Fremantle, Western Australia, Australia, 1-7 July 2007: 46-48.

Andrews, F. M., N. Frank, C. S. Sommardahl, H. Eiler, B. W. Rohrbach and M. McCracken (2004). Diagnostic value of a combined dexamethasone suppression/thyrotropin-releasing hormone stimulation test in Equine Cushing's disease. Proceedings of the 50th Annual Convention of the American Association of Equine Practitioners, Denver, Colorado, USA, 4-8 December, 2004. Lexington, American Association of Equine Practitioners (AAEP)**:** 338-340.

Ardawi, M. S. M. and A. A. Rouzi (2005). "Plasma adiponectin and insulin resistance in women with polycystic ovary syndrome." Fertility and sterility **83**(6): 1708-1716.

Bergfelt, D. R. (2000). "Estrous synchronization." Equine breeding management and artificial insemination. Saunders Company, Philadelphia: 165-177.

Chang, L., S.-H. Chiang and A. Saltiel (2004). "Insulin signaling and the regulation of glucose transport." MOLECULAR MEDICINE-CAMBRIDGE MA THEN NEW YORK- **10**(7/12): 65.

Dascanio, J. and P. McCue (2014). Equine Reproductive Procedures, John Wiley & Sons.

De Leo, V., A. La Marca, A. Ditto, G. Morgante and A. Cianci (1999). "Effects of metformin on gonadotropin-induced ovulation in women with polycystic ovary syndrome." Fertility and sterility **72**(2): 282-285.

Dieleman, S., T. A. Kruip, P. Fontijne, W. De Jong and G. Van der Weyden (1983). "Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone." Journal of Endocrinology **97**(1): 31-NP.

Dunaif, A. (1997). "Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis 1." Endocrine reviews **18**(6): 774-800.

Dunaif, A. and A. Hoffman (1988). "Insulin resistance and hyperandrogenism: clinical syndromes and possible mechanisms." Biorhythms and stress in the physiopathology of reproduction. Hemisphere Publishing Co, Washington, DC: 293-317.

Dupont, J. and R. J. Scaramuzzi (2016). "Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle." Biochemical Journal **473**(11): 1483-1501.

Ehrmann, D. A. (2002). "Insulin resistance and polycystic ovary syndrome." Curr Diab Rep **2**(1): 71-76.

Eiler, H., N. Frank, F. M. Andrews, J. W. Oliver and K. A. Fecteau (2005). "Physiologic assessment of blood glucose homeostasis via combined intravenous glucose and insulin testing in horses." American Journal of Veterinary Research **66**(9): 1598-1604.

Ferris, R. and P. McCue (2010). "The effects of dexamethasone and prednisolone on pituitary and ovarian function in the mare." Equine veterinary journal **42**(5): 438-443.

Frank, N. (2006). Insulin resistance in horses. AAEP Proceedings.

Frank, N., S. B. Elliott, L. E. Brandt and D. H. Keisler (2006). "Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance." Journal of the American Veterinary Medical Association **228**(9): 1383-1390.

Frank, N., R. Geor, S. Bailey, A. Durham and P. Johnson (2010). "Equine metabolic syndrome." Journal of Veterinary Internal Medicine **24**(3): 467-475.

Gastal, E., L. Silva, M. Gastal and M. Evans (2006). Effect of different doses of hCG on diameter of the preovulatory follicle and interval to ovulation in mares. Animal Reproduction Science, ELSEVIER SCIENCE BV PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS.

Gastal, E. L., M. Gastal, A. Wischral and J. Davis (2011). "The equine model to study the influence of obesity and insulin resistance in human ovarian function." Acta Sci Vet **39**: 57-70.

Gentry, L., D. Thompson, G. Gentry, K. Davis, R. Godke and J. Cartmill (2002). "The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period." Journal of animal science **80**(10): 2695-2703.

George, L. A., W. B. Staniar, T. A. Cubitt, K. H. Treiber, P. A. Harris and R. J. Geor (2011). "Evaluation of the effects of pregnancy on insulin sensitivity, insulin secretion, and glucose dynamics in Thoroughbred mares." American journal of veterinary research **72**(5): 666-674.

Ginther, O. (1992). "Reproductive Biology of the Mare. Equiservices." Cross Plains, WI: 174-175.

Ginther, O., E. Gastal, M. Gastal and M. Beg (2008). "Intrafollicular effect of IGF1 on development of follicle dominance in mares." Animal reproduction science **105**(3): 417-423.

Ginther, O. J. (2012). "The mare: a 1000-pound guinea pig for study of the ovulatory follicular wave in women." Theriogenology **77**(5): 818-828.

Ginther, O. J. (2014). "How ultrasound technologies have expanded and revolutionized research in reproduction in large animals." Theriogenology **81**(1): 112-125.

Ginther, O. J., M. A. Beg, M. O. Gastal and E. L. Gastal (2004). "Follicle dynamics and selection in mares." Animal Reproduction (Belo Horizonte) **1**(1): 45-63.

Ginther, O. J., E. L. Gastal, M. O. Gastal and M. A. Beg (2008). "Dynamics of the Equine Preovulatory Follicle and Periovulatory Hormones: What's New?" Journal of Equine Veterinary Science **28**(8): 454-460.

Ginther, O. J. and R. A. Pierson (1983). "Ultrasonic evaluation of the reproductive tract of the mare; principles, equipment, and techniques." Journal of Equine Veterinary Science **3**(6): 195-201.

He, W., Y. Barak, A. Hevener, P. Olson, D. Liao, J. Le, M. Nelson, E. Ong, J. M. Olefsky and R. M. Evans (2003). "Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle." Proceedings of the National Academy of Sciences **100**(26): 15712-15717.

Henneke, D. R., G. D. Potter, J. L. Kreider and B. F. Yeates (1983). "Relationship between condition score, physical measurements and body fat percentage in mares." Equine Veterinary Journal **15**(4): 371-372.

Ledoux, S., D. B. Campos, F. L. Lopes, M. Dobias-Goff, M.-F. Palin and B. D. Murphy (2006). "Adiponectin induces periovulatory changes in ovarian follicular cells." Endocrinology **147**(11): 5178-5186.

Liu, Y. and A. Hsueh (1986). "Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera." Biology of reproduction **35**(1): 27-36.

Maheshwari, A., L. Stofberg and S. Bhattacharya (2007). "Effect of overweight and obesity on assisted reproductive technology—a systematic review." Human reproduction update **13**(5): 433-444.

Matamoros, I. A., N. M. Cox and A. B. Moore (1991). "Effects of exogenous insulin and body condition on metabolic hormones and gonadotropin-induced follicular development in prepuberal gilts." Journal of animal science **69**(5): 2081-2091.

Morley, S. A. and J.-A. Murray (2014). "Effects of Body Condition Score on the Reproductive Physiology of the Broodmare: A Review." Journal of Equine Veterinary Science **34**(7): 842-853.

Nadeau, J. (2006). "Insulin Resistance in Horses." Extension Articles: 4.

Nagy, P., D. Guillaume and P. Daels (2000). "Seasonality in mares." Animal Reproduction Science **60**: 245-262.

Opsomer, G., T. Wensing, H. Laevens, M. Coryn and A. de Kruif (1999). "Insulin resistance: the link between metabolic disorders and cystic ovarian disease in high yielding dairy cows?" Animal reproduction science **56**(3): 211-222.

Ortega, H. H., F. Rey, M. M. Velazquez and V. Padmanabhan (2010). "Developmental programming: effect of prenatal steroid excess on intraovarian components of insulin signaling pathway and related proteins in sheep." Biology of reproduction **82**(6): 1065-1075.

Pass, M., S. Pollitt and C. Pollitt (1998). "Decreased glucose metabolism causes separation of hoof lamellae in vitro: a trigger for laminitis?" Equine Veterinary Journal **30**(S26): 133-138.

Pierson, R. and O. Ginther (1985). "Ultrasonic evaluation of the preovulatory follicle in the mare." Theriogenology **24**(3): 359-368.

Pierson, R. and O. Ginther (1987). "Follicular population dynamics during the estrous cycle of the mare." Animal Reproduction Science **14**(3): 219-231.

Pycock, J., J. C. Samper and A. O. McKinnon (2006). Current therapy in equine reproduction, Elsevier Health Sciences.

Salazar-Ortiz, J., P. Monget and D. Guillaume (2014). "The influence of nutrition on the insulin-like growth factor system and the concentrations of growth hormone, glucose, insulin, gonadotropins and progesterone in ovarian follicular fluid and plasma from adult female horses (Equus caballus)." Reproductive Biology and Endocrinology **12**(1): 1.

Saltiel, A. R. and C. R. Kahn (2001). "Insulin signalling and the regulation of glucose and lipid metabolism." Nature **414**(6865): 799-806.

Samper, J. C. (1997). Ultrasonographic appearance and the pattern of uterine edema to time ovulation in mares. Proceedings of the 43rd Annual Convention of the American Association of Equine Practitioners.

Schuver, A., N. Frank, K. A. Chameroy and S. B. Elliott (2014). "Assessment of insulin and glucose dynamics by using an oral sugar test in horses." Journal of Equine Veterinary Science **34**(4): 465-470.

Sessions-Bresnahan, D. R. and E. M. Carnevale (2014). "The effect of equine metabolic syndrome on the ovarian follicular environment." J Anim Sci **92**(4): 1485-1494.

Sessions, D., S. Reedy, M. Vick, B. Murphy and B. Fitzgerald (2004). "Development of a model for inducing transient insulin resistance in the mare: preliminary implications regarding the estrous cycle." Journal of animal science **82**(8): 2321-2328.

Silva, J., J. Figueiredo and R. Van den Hurk (2009). "Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis." Theriogenology **71**(8): 1193-1208.

Treiber, K., R. Boston, D. Kronfeld, W. Staniar and P. Harris (2005). "Insulin resistance and compensation in Thoroughbred weanlings adapted to high-glycemic meals." Journal of animal science **83**(10): 2357-2364.

Vanholder, T., G. Opsomer and A. De Kruif (2006). "Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review." Reproduction Nutrition Development **46**(2): 105-119.

Vick, M. M., D. R. Sessions, B. A. Murphy, E. L. Kennedy, S. E. Reedy and B. P. Fitzgerald (2006). "Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity." Reproduction, Fertility and Development **18**(6): 609-617.

Williams, G. L., J. F. Thorson, L. D. Prezotto, I. C. Velez, R. C. Cardoso and M. Amstalden (2012). "Reproductive seasonality in the mare: neuroendocrine basis and pharmacologic control." Domestic Animal Endocrinology **43**(2): 103-115.

Zeyda, M. and T. M. Stulnig (2009). "Obesity, inflammation, and insulin resistance--a mini-review." Gerontology **55**(4): 379-386.

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