

Effects of resveratrol supplementation on metabolic health and reproductive performance in
obese mares on pasture

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ABSTRACT

Resveratrol, a naturally-occurring phytoestrogenic stilbene derivative, has been shown to elicit shifts in physiology of obese animals consuming a high calorie or ad libitum diet toward that of lean counterparts. This study was designed to evaluate effects of oral resveratrol supplementation on parameters of metabolic health and reproductive cyclicity in obese mares on pasture. Seventeen healthy, mares were matched by age and assigned to obese control (OBC; n=5, mean BCS=7.4±0.3), obese supplemented with 5g/d resveratrol (OBR; n=6, mean BCS=7.4±0.2) or non-obese control (NOC; n=6, mean BCS=5.4±0.1) treatments. Control horses received the resveratrol carrier paste. Across three consecutive estrous cycles, morphometric measurements were collected biweekly and follicular dynamics were evaluated via transrectal ultrasonography every other day. Frequently-sampled intravenous glucose tolerance tests were conducted pre- and post- treatment. Insulin and glucose kinetics were analyzed via minimal model. Resveratrol supplementation had no discernible effect on reproductive parameters ($P>0.05$), however obese mares had more (6 vs. 0) hemorrhagic anovulatory follicles. Neither resveratrol treatment nor time on study influenced morphometric measurements or minimal model parameters (raw data or data adjusted for animal size). As a whole, horses became more insulin resistant over time (Si value < 0.78 (1/[mU/L·min])). NOC horses had lower ($P=0.01$) acute insulin response to glucose relative to OBC or OBR. Although resveratrol supplementation did not elicit detectable responses in this study, promising results in other species warrant further investigation of the compound in horses, including exploration of bioavailability and possible effects at the tissue or cellular levels.

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CHAPTER 1: INTRODUCTION

With increasing prevalence in the industry, obesity is a serious problem in horses often resulting in several maladaptive metabolic co-morbidities associated with increased adiposity including insulin resistance (IR), oxidative damage, chronic systemic inflammation, and laminitis. Obesity is even more of an issue in the broodmare, proven to be deleterious for reproductive performance often resulting in aberrant cyclicity, inhibiting ovulation, and decreasing overall fertility (Vick et al., 2006). Conventional management strategies that work to reduce obesity such as caloric restriction, limited access to pasture and/ or forced exercise, may not be appropriate for broodmares due to certain nutritional and management requirements during gestation. Therefore, there is a need to investigate other potential management options to improve metabolic health and reproductive performance in broodmares on pasture.

A principal component in the pathogenesis of obesity, IR, is caused by insulin insensitivity at the cell surface (before, at or distal to the cell receptor) which affects glucose metabolism inside the cell, or from insulin ineffectiveness as a result of disrupted glucose cellular metabolism (Kronfeld et al., 2005). A strong association of obesity and IR has been established (Frank et al., 2011), implicating a potential role of adipocytes as mediators of insulin sensitivity at the tissue level (Despres and Lemiux, 2006; Tinworth et al., 2010).

Several metabolic maladies associated with obesity have been shown to further exacerbate IR in overweight or obese horses. Increased free fatty acid (FFA) release associated with increased adiposity, has a direct impact on IR evidenced by increased hepatic glucose production and decreased skeletal muscle uptake of glucose (Tinworth et al., 2010). Insulin-stimulated glucose transport is also inhibited by increased FFA release which leads to a decrease in insulin sensitivity (Rajala and Scherer, 2003). Excessive FFA circulation results in oxidative

stress, which can result in abnormal changes in intracellular signaling leading to chronic inflammation and IR in horses (Kronfeld et al., 2005). Chronic low-grade inflammation is associated with increased concentrations of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF) and decreased concentrations of anti-inflammatory cytokine, such as interleukin 10 (IL-10) in adipose tissue (Suagee et al., 2011;2013).

Promising areas of investigation focus on the potential use of therapeutic agents such as pharmaceuticals and nutraceuticals for the treatment of metabolic co-morbidities associated with EMS. These include several insulin sensitizing drugs used for the treatment of diabetes and metabolic dysfunction in humans, including metformin (Tinworth et al., 2012) and pioglitazone (Wearn et al., 2012); however, no discernible effects of the drugs on insulin sensitivity were detected. Levothyroxine sodium (synthetic thyroxine) administration to healthy euthyroid mares resulted in a significant increase in insulin sensitivity (Si) and glucose effectiveness (Sg) ($P < 0.01$) over a 48 wk treatment period (Frank et al., 2008a,b). Nutraceutical supplementations, such as omega 3 (Ω -3, n-3), fatty acids have been hypothesized to improve glucose metabolism in the horse by increasing peripheral tissue response, both the receptor and signaling aspects, to insulin secretion (Hess et al., 2012). An evident effect of Ω -3 supplementation on insulin sensitivity (Si) has not been identified, however trending data suggests that an improvement of Si is seen with supplementation of fatty acids in insulin resistant horses (Hess et al., 2012).

Emerging trends in research focus on the use of polyphenolic anti-oxidant compounds for treatment of insulin resistance and associated systemic inflammation. Resveratrol (RSV), a natural polyphenolic compound derived from the skins of red grapes was discovered to reduce the occurrence of mortality due to coronary heart disease in individuals consuming high fat diets, a phenomenon known as the “French Paradox” (Renaud and Delorgeril, 1992; Catalgol et al.,

2012). This compound has undergone considerable evaluation over the past 10 years, and a diverse range of biological benefits have been described; including several aspects of maladaptive metabolic conditions, including obesity, insulin resistance, and inflammation (Catalgol et al., 2012). Additionally, RSV has been proposed as a promising treatment for insulin resistance due to its ability to upregulate SIRT1 activity in adipocytes (Costa et al., 2010), a key factor in signaling pathways that mimic protective mechanisms induced by caloric restriction.

A small number of studies have evaluated the effects of RSV in horses (Equithrive, 2012; Zambito, 2011; Person. Comm., A. Adams). In an exercise model, a low (2.5 g/d trans-RSV) and high (5 g/d trans-RSV) dose had no effect on glucose tolerance, insulin sensitivity or overall lipid peroxidation in six, quarter horse geldings (Zambito, 2011). However, RSV treatment (1g/d) in 20 geriatric horses for 4 wk resulted in decreased equine inflammatory cytokine production both *in vitro* and *in vivo* (Equithrive, 2009). The effects of RSV have not been previously evaluated in the broodmare; however, promising results of oral RSV supplementation in other models warrant further investigation in the equine model. The purpose of the study reported here was to determine the effects of oral RSV supplementation on improving parameters of metabolic health and simultaneously enhancing reproductive performance in obese broodmares. We hypothesized that oral RSV supplementation (5g/d) administered to obese broodmares would significantly improve glucose effectiveness and insulin sensitivity, decrease BW, improve morphometric measurements, and enhance reproductive cyclicity and hormone profiles during three consecutive estrous cycle.

CHAPTER 2: REVIEW OF LITERATURE

Obesity

The worldwide prevalence of obesity in humans is reaching staggering proportions. This epidemic has been intensely studied in the human. According to the World Health Organization (WHO), over two-thirds of adults are overweight or obese. Obesity is defined as the accumulation of excess body fat often resulting in associated co-morbid health conditions, including insulin resistance, oxidative stress damage, chronic low-grade systematic inflammation, and reproductive dysfunction. Collectively these conditions, known as metabolic syndrome (MetS), are risk factors assessed to predict the occurrence of two major metabolic maladies, type II diabetes mellitus and cardiovascular disease (Treiber et al., 2006). Today, obesity is the fifth leading risk for deaths globally, and the leading cause of death from non-communicable diseases (WHO, 2013). Despite large scale financial investments to increase awareness and education about this disease, the numbers of obese individuals in today's society continue to escalate. Current efforts to ameliorate this growing trend through exercise programs, dietary programs, gastric surgeries, and various drug therapies have yet to provide long term results.

Obesity in the horse

The recent rise in equine obesity has mirrored that observed among humans. Studies in the United States and United Kingdom reveal the prevalence of equine over weight or obesity now exceeds 50% (Sillence et al., 2002; Wyse et al., 2008; Argo, 2009; Thatcher et al., 2012). Common causes of obesity among the equine population are similar to those observed for humans; namely, increased caloric intake and inadequate physical exercise.

Increased adiposity is a predisposing factor for equine metabolic syndrome (EMS). Similar to the metabolic condition of humans, EMS is considered a predictive factor in identifying major co-morbid medical conditions in the horse. These include insulin resistance, laminitis, hypertriglyceridemia, hyperleptinemia, arterial hypertension, altered reproductive cycling in mares, and chronic low grade systematic inflammation (Frank et al., 2011).

White adipose tissue (WAT) is an active secretory tissue that has wide ranging paracrine and endocrine effects throughout the body (Bastard et al., 2006; Vick et al., 2007), and plays a vital role in equine health. Given the importance of WAT in regards to equine health, it is critical to understand the basic biology, including its role in overall body composition and its regional distribution (Dugdale et al., 2011a). In humans, excessive visceral (abdominal) fat is linked to risks for several metabolic conditions including diabetes (Type II) and cardiovascular disease, and has been associated with an increased production and secretion of inflammatory proteins by adipose tissue (Alberti et al., 2006; de Luca and Olefsky, 2008; Suagee et al., 2011). These pro-inflammatory cytokines such as tumor necrosis factor α (TNF α), IL-1, and IL-6 are used to assess inflammatory status, and have been correlated with regional fat adiposity and obesity in the horse (Vick et al., 2007). A similar relationship between increased WAT and risk for metabolic disease such as insulin resistance (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007), laminitis in ponies (Carter et al., 2009b), and altered reproductive activity in mares (Vick et al., 2006) has been reported in the horse.

Methods of Assessing Adiposity

Several attempts have been made to quantify body fat composition in horses using objective measures, including morphometric data, ultrasound measurements of superficial fat

thickness, and body condition scoring (Westervelt et al., 1976; Kane et al., 1987; Kearns et al., 2002a,b ; Donaldson et al., 2004; Frank et al., 2006; Dugdale et al., 2010); however, validation of methods has been limited. Several factors hinder the advancement of these methods and prove challenging for the development of a universal body condition assessment system capable of quantifying fat content (Thatcher et al., 2007; Wyse et al., 2008).

Body Condition Score

Current methods of assessing equine ‘body fatness’ have focused on subjective evaluation of body condition score (BCS; Dugdale et al., 2011a). However, unlike the similar scoring protocols used in other agricultural species, the equine method has been modified continually with indirect indices (rump fat thickness) of total body fat mass, to compensate for variances seen between breeds of horses (Dugdale et al., 2011a). The first system designed to assess regional fat deposition was developed in 1983 by Henneke and colleagues as a subjective assessment of subcutaneous fat cover. This scoring system was developed in pre-parturient Quarter Horse mares and has been shown to correlate with subcutaneous fat deposition in a variety of breeds (Henneke et al., 1983; Gentry et al., 2004; Carter et al., 2009). Fat deposition is categorized on a scale of 1 (extremely thin) to 9 (extremely fat) and assessed by physical palpation and visual evaluation of six areas of the body: neck, withers, shoulders, ribs, loin, and tail head (Kohnke, 1992; Suagee et al., 2008, modification of Henneke et al., 1983, Table 2.1).

Table 2.1: Characteristics of Individual Body Condition Scores (Adapted from Suagee et al., 2008). Used under fairuse, 2013

BCS	<u>CHARACTERISTICS</u> Neck Area	Wither Area	Shoulder Area	Rib Area	Loin Area	Tailhead Area
Anatomic Description	From the poll to the 3 rd cervical vertebrae	From the top of the shoulder blade to the top of the spinous processes of the 3 rd , 4 th , and 5 th vertebrae	Between scapula/triceps muscle and barrel	The side of the horse; including the 6 th through 12 th ribs	Lumbar vertebrae	Caudal sacral vertebrae to tuber ischii
4	Small amount of fat deposited on the crest of neck with slight depression in front of withers, otherwise flat between the poll and withers	Wither prominence depends on conformation. Shape of spinous processes visible. Fat begins to accumulate around transverse processes	Point of shoulder easily identified. Top of scapular spine may be visible. Some fat felt but area is concave in appearance.	Central ribs visible (ribs 6-12) with others easily felt.	Some filling around sides but tops of spinous process - easily visible.	Tailhead prominence depends on conformation. Spinous processes of sacral vertebrae visible with little fat fill on sides. When viewed from side, some fat accumulation but concave in appearance.
5	Even deposition of fat along crest of neck, creating a smooth nearly flat line between the poll and the withers.	Fat accumulating from top of shoulder blade to point of withers lending a nearly flat appearance.	Fat fill in shoulder area creates smooth transition from shoulder blade to barrel though slightly concave. Can no longer identify scapular ridge.	Ribs cannot be visually distinguished, but can be easily felt.	Fat fill along spinous processes make loin area level.	Fat filled along either side of tailhead, spinous processes of sacral vertebrae no longer visible. Fat along tailhead results in flat appearance when viewed from the side.
6	Fat cover on crest of the neck slightly increases height of neck (eg. A "cresty" neck beginning to develop).	Fat fill results in flat appearance of withers.	Fat fill appears convex and increasing in size ventrally.	Fat laid down between ribs, making them difficult to distinguish from each other. Can be felt with direct pressure.	Fat beginning to accumulate above spinous processes, creating a slight depression.	Fat fill slightly convex in appearance.
7	Obvious crest with fat fill increasing the width of the neck. Fat fill along crest filled in cranially and caudally. Fat laid down in front of shoulder, at point where neck and body meet.	Fat fill convex in appearance.	Fat fill causes obvious convexity, and has increases in size ventrally to encompass the area just behind the point of the elbow.	Noticeable filling between and on top of ribs. Individual ribs can be felt but difficult, even with direct pressure.	Fat accumulated above spinous processes creating an obvious depression on the loin area.	Fat fill above level of body processes of tailhead.

There remains the capacity to improve upon the BCS system originally developed for Quarterhorse broodmares. The application of this system to horses or ponies of other breeds is cautioned (Suagee et al., 2008; Dugdale et al., 2011a, b), warranting the need for the development universal body scoring system. Current applications of this system lack the ability to access changes in visceral fat mass; rather scoring is based upon external appearance of subcutaneous, inter- and intra-muscular fat (Dugdale et al., 2012) resulting in a non-linear relationship between BCS and actual body fat mass as determined by the ‘gold standard’ (post mortem measurements of body lipid measured by chemical composition analysis). Observer error and undistinguished ‘grade descriptors’, also prove challenging for subjective quantification of total body fat (Dugdale et al., 2012) with the BCS system.

BCS provides an index of total somatic (skeletal-associated soft) tissue; however, when compared with cadaver dissection or chemical composition analysis, its usefulness in predicting total body fat mass (TBFM) and discerning between fat and muscle is less effective (Dugdale et al., 2011a). The contribution of WAT to overall somatic tissue was evaluated in obese pony mares, revealing an increase in total WAT content with BCS, however a loss in sensitivity of BCS for body WAT quantification is seen when BCS exceeds 5-6, suggesting a need for a more precise objective measurement of body fat in moderate to obese animals (Dugdale et al., 2010, 2011a, and 2012).

Previous studies show that BCS correlates with glucose tolerance (Frank et al., 2006) and insulin sensitivity (Vick et al., 2007). Glucose and insulin concentrations are influenced by myriad factors including feed consumption, diurnal variations in cortisol, feed type, excitement and stress, reproductive status, illness, genetics and obesity (Ralston, 2002). Most studies evaluating insulin and glucose regulation show positive association with consumption of diets

that contain high concentrations of enzymatically digestible carbohydrates such as starches and sugars (Treiber et al., 2005). The association between insulin and glucose dynamics in forage carbohydrates is less understood.

Deuterium oxide (D₂O) dilution

A minimally invasive, yet expensive, deuterium oxide (D₂O) dilution method has been developed to estimate TBFM *in vivo*, allowing for the quantification of body fat mass in living animals (Dugdale et al., 2011b). When this method was compared with the ‘gold standard’ (post mortem measurements of body lipid measured by chemical composition analysis), D₂O derived estimates of TBFM were strongly associated with proximate analysis and dissection derived values ($r^2 > 0.97$, $P < 0.0001$; Dugdale et al., 2011b). When compared with the BCS system, a non-linear relationship was reported for D₂O dilution method, in agreement with previous work finding a non-linear association between equine BCS and TBFM measured by gross carcass dissection (Martin-Rosset et al., 2008; Dugdale et al., 2011a). This non-linear relationship suggests that large changes in body fat content are associated with relatively small changes in BCS (Dugdale et al., 2010), confirming previous findings in which body fat evaluation was less sensitive and therefore less accurate for ‘upper’ and ‘lower’ regions of the BCS system. The D₂O dilution method is believed to provide a more accurate overall estimate of total body fat mass in combination with the BCS system, however it is to be noted that greater variation is seen between individual scorers so it is recommended to use a single observer to reduce the variation (Dugdale et al., 2012).

Morphometric measurements

Linear and circumferential measurements have been utilized and correlated with BCS to provide additional indices for overall body fat composition in horses. Measurements include: rump width, heart girth, belly girth, and neck circumference (Dugdale et al., 2010, Table 2.2).

Morphometric measurements were correlated with condition scores resulting in weight ($r^2=0.50$, $P<0.01$), girth circumference ($r^2=0.40$, $P<0.05$), weight: height ($r^2=0.58$, $P<0.01$), and girth: height ($r^2=0.51$, $P<0.01$) (Henneke et al., 1983), with the most significant correlations seen between girth: height ratio to BCS ($r^2=0.64$, $P<0.001$; horses; $r^2=0.83$, $P<0.001$ in ponies) (Carter et al., 2009). However, current application of morphometric measurements in assessing adiposity is minimal.

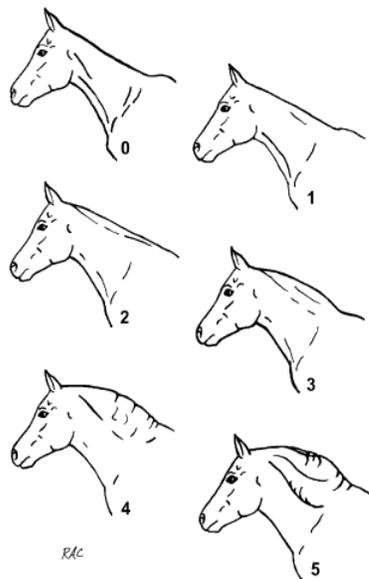
Table 2.2: Specific anatomical sites used to obtain morphometric measurements which were retrospectively associated with changes in body mass (adapted from Dugdale et al., 2010). Used under fair use, 2013.

Linear or circumferential measure	Detailed description where necessary	Coefficient of variation
Rump Width	Point of left hip to point of right hip, measured over the contour of the dorsum.	1%
Heart Girth	Measured during the end expiratory pause, caudal to the points of the elbows, and immediately behind the caudal extremity of the withers.	0.1%
Belly Girth	Measured during the end expiratory pause, at the widest part of the belly: approximately 2/3 of the linear distance between the point of the left shoulder and the point of the left hip	0.2%
Neck circumference at mid-crest	Measured perpendicularly to the top line of the crest, approximately half of the crest length caudal to the poll; approximately at the position of cervical vertebrae 3/4.	0.8%

Cresty Neck Score

A cresty neck score was developed to assess the regional deposition of adiposity along the nuchal ligament (Carter et al., 2009). Fat stores within the neck offer the most appreciated superficial index of adiposity in the pony (Carter et al., 2009; Dugdale et al., 2010). With a range from zero (no visual appearance of crest) to five (crest is large and droops to the side) this system quantifies the amount of regional fat deposition and is used to evaluate the overall assessment of adiposity in the horse (Carter et al., 2009, Figure 2.1). Crest height or neck circumference: height ratio are suitable morphometric measurements for assessment of apparent neck adiposity, with a strong association shown between these measurements and cresty neck score ($r_s = >0.50$, $P < 0.01$; Carter et al., 2009).

Cresty neck scoring may provide indirect assessment of whole body adiposity and be associated with metabolic conditions such as insulin resistance (Znamirowska, 2005; Carter et al., 2009). In 2006, Frank and colleagues correlated measurements of neck circumference with glucose ($r=0.71$; $P=0.015$) and insulin ($r=0.88$; $P<0.001$) areas under the curve during fasting combined glucose-insulin tests, suggesting that crest scoring systems may serve as indices of insulin resistance in native pony mares (Dugdale et al., 2010).



- 0- No visual appearance of a crest (tissue apparent above the *ligamentum nuchae*). No palpable crest
- 1- No visual appearance of a crest, but slight filling felt with palpation
- 2- Noticeable appearance of a crest, but fat deposited fairly evenly from poll to withers. Crest easily cupped in one hand and bent from side to side
- 3- Crest enlarged and thickened, so fat is deposited more heavily in middle of the neck toward poll and withers, giving a mounded appearance. Crest fills cupped hand and begins losing side to side flexibility
- 4- Crest grossly enlarged and thickened, and can no longer be cupped in one hand or easily bent from side to side. Crest may have wrinkles/creases perpendicular to topline
- 5- Crest is so large it permanently droops to one side

Figure 2.1: Illustrations of cresty neck scores (From Carter et al., 2009). Used under fairuse, 2013.

Subcutaneous fat deposition measurement by ultrasonography

Subcutaneous fat deposition has been shown to have a high correlation to BCS ($r^2=0.65$, $P<0.001$) and is used to calculate body fat percentage in the horse (Westervelt et al., 1976; Gentry et al., 2004; Carter et al., 2009). Two superficially accessible fats depots (rump and rib-eye), are measured to the nearest 0.01mm by transcutaneous ultrasonography to provide predictive indices of overall body fat percentages verified by concurrent ‘gold standard’ carcass dissection and chemical composition analysis (Westervelt et al., 1976; Gentry et al., 2004, Figure 2.2; Dugdale et al., 2010). During these processes, total body WAT content is determined from 3 regional adipose deposits: intra-abdominal belly wall-associated (retroperitoneal), subcutaneous and intramuscular (Dugdale et al., 2011a), and contributes to total somatic soft tissue. Total somatic tissue (WAT + non-WAT) contributions to overall recovered body mass via gross anatomical evaluation, has been almost perfectly linearly described ($r^2=1.00$, $P<0.001$) by subjective measurements of BCS (Dugdale et al., 2011a). Additionally, linearly-ordinal increments in BCS were associated with an exponential increase ($r^2=0.96$) in proportions of

WAT in regards to recovered body mass (Dugdale et al., 2011a). Retroperitoneal (internal) and subcutaneous and intramuscular (external) contribute equally to BCS- associated changes in total WAT (Dugdale et al., 2011a).

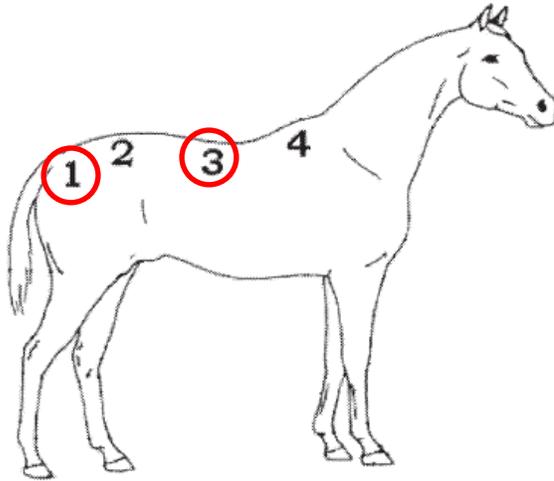


Figure 2.2: Two main locations used to measure subcutaneous fat thickness in the horse. (From Gentry et al., 2004). Used under fairuse, 2013.

Ultrasound measurements are taken 5 cm lateral to the midline on the right side of the horse; with site one occurring over the middle gluteal muscle between point of hip and center of tail-head root approximately 7.62 cm anterior from the tail-head (Gentry et al., 2004; Dugdale et al., 2010). The second site being parallel with the 12th intercostal space centered 15 cm lateral to the dorsal midline denoted as the ‘rib-eye’ (Dugdale et al., 2010). Subcutaneous fat measurements at the tailhead are the highest correlated ($r^2=0.87$, $P < 0.02$) ultrasound measurement to overall BCS scores, however due to a large amount of variation in BCS accounted for in this region it is recommended to combine measurements of the 13th rib area with the tailhead area to help reduce variability (Gentry et al., 2004).

Insulin Resistance

Insulin resistance (IR), a hallmark phenotype of EMS, is marked by a reduction in insulin sensitivity or responsiveness of peripheral tissues to the metabolic action of insulin, reducing glucose uptake notably in muscle, adipose tissue and liver, resulting in increased circulating blood glucose levels (Kahn, 1978; Kronfeld et al., 2005; Hoffman, 2009). Target tissues in muscle, adipose and liver require larger quantities of circulating insulin to elicit glucose removal from the blood. This desensitization can refer to inefficient insulin signaling at the cell surface or disruption of signaling pathways within the cell (Kronfeld et al., 2005). In humans IR is fundamental in the pathology of Type II diabetes mellitus and is shown to be etiologically associated with cardiovascular disease, obesity, hypertension and dyslipidemia (Sinaiko and Caprio, 2012). Diminution of cellular response could be attributed to rapid insulin degradation, neutralization by antibodies, and interference at the cell surface of the insulin receptor or during translocation of transporters to the cell surface (Kronfeld et al., 2005). Deficiencies in these steps result in increased glucose output via gluconeogenesis, resulting in higher circulating glucose concentrations which can ultimately result in insulin resistance.

In a healthy state, following a grain meal or oral glucose load, hyperglycemia occurs; provoking insulin secretion from pancreatic beta cells, resulting in suppression of hepatic glucose production and stimulation of blood glucose uptake in skeletal muscle and adipose tissue. A state of insulin resistance implies that either the peripheral (skeletal muscle or adipose tissue) or central (liver) tissues are insensitive to insulin action or the pancreatic release of insulin in response to hyperglycemia is weakening (Johnson, 2002). With Type I diabetes, pancreatic beta cell failure is responsible for chronically elevated levels of plasma glucose, often requiring exogenous insulin supplementation to sustain glucose homeostasis. Type II diabetes is

often characterized by the state of insulin resistance, in which decreased tissue sensitivity to insulin results in persistent elevations in plasma glucose rendering the pancreas exhausted often leading to islet beta cell failure (Johnson, 2002; Kronfeld et al., 2005).

Carbohydrate digestion and absorption

Horses evolved consuming primarily fermentable forage carbohydrates; however, dietary needs have changed due to evolving energy demands of the ever-changing performance lifestyles. Forage is a main source of nutrition for most horses and is composed of approximately 75% of structural carbohydrates (cellulose and hemicellulose); the remaining composition being non-structural carbohydrates (mono/disaccharides, starches, and fructans; Hoffman, 2009; Shepard et al., 2012). Seasonal and diurnal changes have a significant impact on nutritional composition of forages, where higher non-structural carbohydrates (fructans, glucose, fructose and sucrose) are typically seen during the early spring months (Kagan et al., 2011a,b). Due to this variation in plant composition, management of forage consumption in the equine is critical due to potential detrimental metabolic issues, such as laminitis, associated with consumption of feedstuff high in non-structural carbohydrates.

Structural carbohydrates are generally digested in the large intestine by microbes (Shepard et al., 2012); however, NSC digestion occurs mainly in the small intestine via enzymatic hydrolyzation. Limited hydrolyzation occurs before carbohydrates reach the small intestine, due in part to limited enzymatic activity of saliva and minimal effect of gastric acids in the stomach (Hoffman, 2009). Hydrolyzation occurs in the small intestine with the initial secretion of pancreatic α -amylase resulting in cleavage of α -1,4 linkages, and is followed by the cleavage of α -1,6 linkages via amylopectinase resulting in disaccharides and oligosaccharides

(Hoffman, 2009). No free sugars are yielded in the lumen; however, when these products are exposed to disaccharidases (sucrose, lactose, and maltase) along the brush border cells free sugars (glucose, galactose, and fructose) are liberated resulting in a high energy yield (Dyer et al., 2002; Hoffman, 2009).

Glucose transportation and absorption

Once glucose is made available in the small intestine it is absorbed into the blood and utilized or stored in the liver and muscle as glycogen. Glucose absorption occurs along the brush border via the high affinity, low capacity, Na⁺/glucose co-transporter type I(SGLT1) across the concentration gradient by active transport of Na⁺ and the Na⁺/K⁺-ATPase into the enterocyte (Dyer et al., 2002; Hoffman, 2009; Fig. 2.3). Facilitative glucose transporters (GLUT) carry the accumulated sugars in the enterocytes through the basement membrane into systemic circulation (Joost and Thorens, 2001; Hoffman, 2009). Upon entering circulation glucose is either oxidized directly to produce ATP or it is stored in the muscle or liver as glycogen where it will be available for the production and release of glucose during exercise (Pagan, 1999).

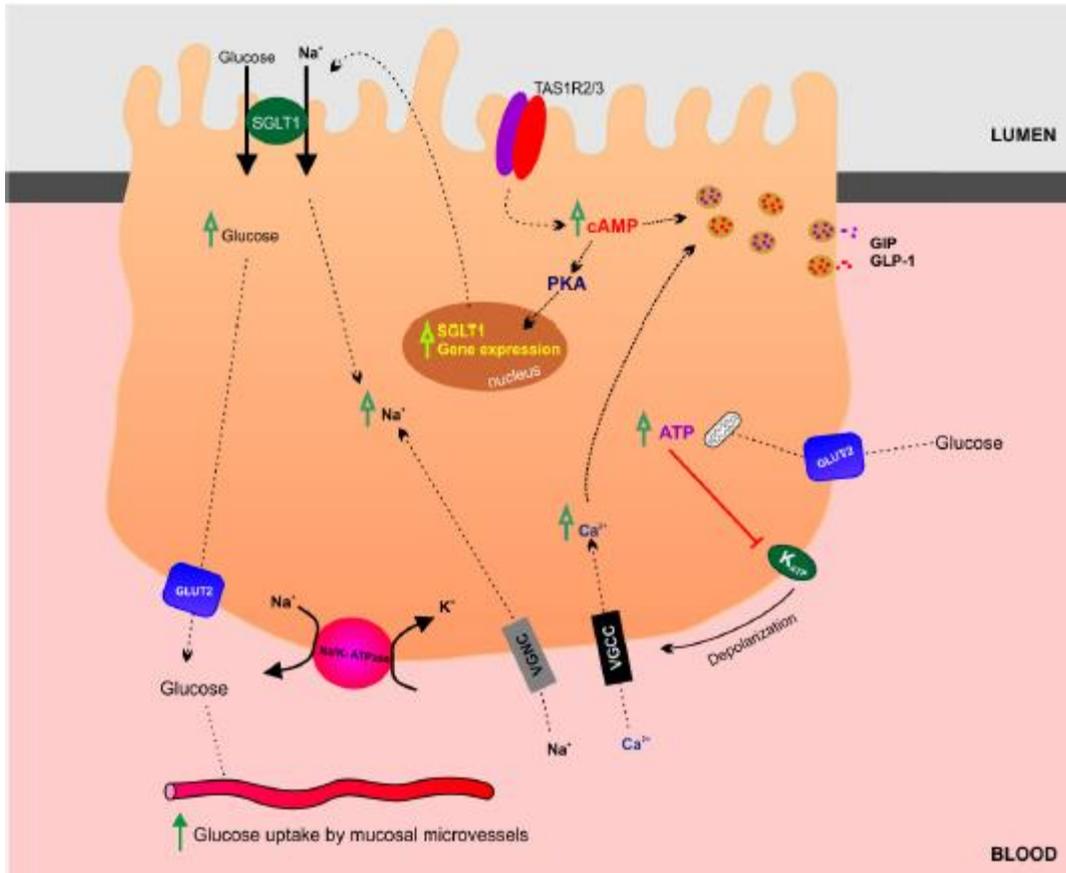


Figure 2.3: Glucose transport across enterocyte (From Mobasher, 2012). Used under fairuse, 2013.

Insulin and Glucose Homeostasis

Insulin is a peptide hormone produced by the beta cells of the islets of Langerhans within the pancreas and regulates carbohydrate, lipid and protein metabolism within the body. A major metabolic action of insulin is the maintenance of whole body glucose homeostasis and facilitation of efficient glucose utilization (Geor, 2008). Fasting blood glucose concentrations in horses are usually between 60 and 90 mg/dL (Ralston, 2002). This concentration is balanced via glucose absorption from the intestine, production by the liver and metabolism by peripheral tissues (Saltiel and Kahn, 2001). Insulin serves as the primary regulator of glucose homeostasis by stimulating uptake and storage of glucose in skeletal muscle and adipocytes and inhibiting the

production and release of glucose by the liver through halting gluconeogenesis and glycogenolysis (Saltiel and Kahn, 2001). Insulin is essential for intracellular transport of glucose into insulin-dependent skeletal muscle and adipose tissues (Wilcox, 2005).

An increase in blood glucose concentration stimulates secretion of insulin into circulation (Fig. 2.4). Glucose uptake at the cellular level is either insulin dependent or independent. Insulin independent glucose uptake relies on certain transporter proteins, such as GLUT 1 which are found in most brain cells. These proteins are able to move glucose intra-cellularly without facilitation via insulin action. For insulin-dependent glucose uptake, which occurs mostly in skeletal muscle or adipose tissue, insulin stimulates the translocation of the glucose transporter GLUT4 from intra-cellular vesicles to the plasma membrane, allowing for glucose diffusion down a concentration gradient into the cell. Glucose uptake into the muscle accounts for 60-70% of whole-body glucose uptake and allows for glycogen synthesis and storage to be utilized as an energy source for muscle contraction (Wilcox, 2005). Intra-cellular glucose transport into adipocytes accounts for 10% of whole body glucose uptake, and promotes lipogenesis while suppressing lipolysis and allowing for free fatty acid flux into circulation to be utilized by other organs for energy (Wilcox, 2005).

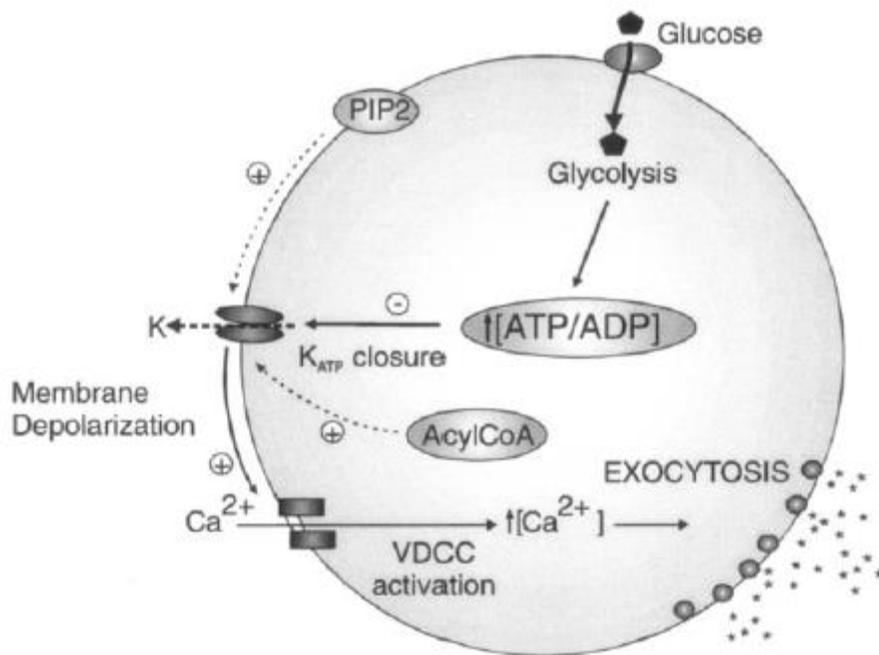


Figure 2.4: Glucosensing in the pancreatic β cell (From Koster et al., 2005). Used under fairuse, 2013.

Methods of Measuring Insulin Sensitivity

Glucose homeostasis abnormalities can be attributed to pancreatic dysfunction or reduced tissue response to circulating glucose concentrations. There are two methods for evaluating insulin sensitivity: non-specific indications or quantitative measurements of insulin sensitivity (Kronfeld et al., 2005). Non-specific indications include: basal glucose and insulin concentrations, tolerance testing (oral glucose tolerance test (OGTT), intravenous glucose tolerance test (IVGTT), frequently sampled intravenous glucose tolerance test (FSIGT), and insulin tolerance test (ITT)(Carter, 2008). Quantitative methods such as the hyperinsulinaemic euglycaemic clamp (HEC) and the minimal model (MinMod) analysis using FSIGT data, have the ability to separate and identify whether abnormalities are due to pancreatic beta cell function or from peripheral tissue response.

Basal insulin and glucose concentrations

Fasting glucose and insulin concentrations are clinical tools used to identify horses with suspected insulin resistance (Kronfeld et al., 2005; Treiber et al., 2006). The accuracy of these values are questioned due to the individual daily variability of basal concentrations due to several factors including feeding and stress (Treiber et al., 2006; Firshman and Valberg, 2007). Ratios of basal glucose-to-insulin are effective in identifying the source of glucose homeostasis abnormalities. Glucose-to-insulin ratios describe insulin sensitivity of peripheral tissues and insulin-to-glucose ratios describe pancreatic action in response to elevated plasma glucose levels (Firshman and Valberg, 2007).

Oral glucose tolerance test (OGTT)

The OGTT assesses intestinal ability to absorb the glucose challenge, endocrine function of the pancreas, and sensitivity of peripheral tissues to insulin (Ralston, 2002; Firshman and Valberg, 2007). This test has been recently modified to eliminate the use of a nasogastric tube, and replaced with an oral glucose bolus given in the form of corn syrup (Karo Light syrup, Ach Food Companies Inc, Cordova, TN, USA) allowing for assessment of horses in a field environment (Frank, 2011). This test is conducted after a 12- to 16- h fast, followed by a dose of 150mg/kg body weight of corn syrup. One blood sample is taken 60-90 min post administration of bolus, and evaluated for plasma insulin concentration. An insulin concentration greater than 60 $\mu\text{U}/\text{mL}$ at any time point between 60 and 90 min suggests insulin resistance (Frank, 2011).

Intravenous glucose tolerance test (IVGTT)

First designed by Mehring and Tysnik in 1970, IVGTT has since been utilized to evaluate the impact of diet and metabolic differences between different breeds of equids (Firshman and Valberg, 2007). This test evaluates the pancreatic action and sensitivity of peripheral tissues to insulin secretion (Garcia and Beech, 1986). After a 12-24 h fasting period, horses are infused with 0.5 g glucose/kg BW bolus via an intravenous catheter. Blood glucose and insulin concentrations are determined at 0, 5, 15, 30, 60, and 90 min and then hourly for 6 h after initial glucose bolus (Firshman and Valberg, 2007). An immediate spike in blood glucose concentrations will occur and peak (242 ± 17 mg/dL) around 15 min post injections, returning to baseline within 1 hr (Ralston, 2002). Insulin concentrations will parallel glucose concentrations, with a peak (157 ± 11 μ U/mL) observed 30 min post injection (Ralston, 2002). As seen in horses with IR, a high peak in blood glucose is accompanied by a delayed return to baseline concentrations (Firshman and Valberg, 2007).

Insulin tolerance test (ITT)

ITT is a direct measurement of responsiveness of peripheral tissues to exogenous insulin induced hypoglycaemia. Depending on the dosage of insulin (ranges between 0.2 to 0.6 IU/kg BW) response will vary; however, a 50% drop in blood glucose concentration should be observed within 20 to 30 min and return to baseline within 90 to 120 min (Kaneko, 1989; De la Corte et al., 1999). Responses to this test are highly influenced by an array of factors; more specifically age, diet and stress. In insulin resistant animals, blood glucose levels do not fall as quickly or as low as found in the normal response curve, and a return to baseline concentrations

is seen sooner (Firshman and Valberg, 2007). A major disadvantage of all aforementioned tests is the endogenous insulin fluctuation that occurs and the impact it has on glucose homeostasis.

Euglycemic-Hyperinsulinemic Clamp

In 1966 a clamp technique was developed by Andres and colleagues in which blood glucose levels are held at a predetermined level and the endogenous glucose-insulin negative feedback loop is interrupted (Pratt et al., 2005; Firshman and Valberg, 2007). This procedure remains one of the most accurate means to determining the effects of hyperglycaemia and hyperinsulinaemia on tissue sensitivity; however, pitfalls to this technique include placing the animal in a non-physiologic environment and overall challenges with performing the procedure (Kronfeld, 2005; Kronfeld et al., 2005; Vick et al., 2006; Firshman and Valberg, 2007). With the hyperglycaemic clamp, a fixed level of plasma glucose is maintained for 2 h, suppressing hepatic production. Therefore the glucose infusion rate becomes a measure of pancreatic insulin secretion, allowing for quantification of pancreatic beta cell sensitivity to glucose (Firshman and Valberg, 2007). The hyperinsulinaemic euglycaemic clamp provides a steady state of insulin concentrations during which insulin sensitivity of skeletal muscle and adipose tissues is measured as the rate of glucose infusion occurring to maintain euglycaemia during the clamp (Firshman and Valberg, 2007). Both techniques provide and accurate methods for understanding the dynamics of insulin resistance in the equine; however they prove to be technically difficult requiring special equipment and rather expensive.

Frequently sampled intravenous glucose tolerance test (FSIGT)

The FSIGT provides advantages over several other methods, as it is able to estimate both insulin-dependent and insulin-independent glucose utilization as well as evaluate pancreatic beta

cells sensitivity to circulating glucose. During this procedure horses are fasted for a 12 h period prior to jugular catheter placement, and subsequent glucose bolus infusion at a dosage of 0.33g/kg BW, followed by an insulin dose 20 min post glucose bolus (Giraudet et al., 1994; Hoffman et al., 2003). During the 3-h duration of the test, blood samples are collected via catheter at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 21, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min after the first glucose bolus (Hoffman, 2003). Exaggerated glucose responses during this test can be attributed to failure of beta cell secretion of insulin or impaired glucose disposal in peripheral muscle and tissues (Kronfeld et al., 2005). Results of the FSIGT are analyzed with Minimal Model Software, providing several methods over other methods of analysis, most notably, the ability to differentiate between insulin-mediated glucose disposal and glucose-mediated glucose disposal (Bergman et al., 1979).

Minimal model analysis of glucose-insulin dynamics

Minimal model analysis is a nonlinear mathematical model that is fitted to data obtained via the aforementioned FSIGT. This method generates calculated parameters that call for physiologic interpretations including glucose effectiveness (S_g : capacity of peripheral cells to take up glucose without insulin mediation), insulin sensitivity (S_i : efficiency of insulin to accelerate glucose uptake by peripheral cells), acute response of insulin to glucose (AIRg: the increase in plasma insulin above basal concentrations from 0 to 10 min post glucose bolus), disposition index (DI; product of AIRg and S_i , and is the appropriateness of beta cell response relative to the degree of insulin resistance within the tissue) (Bergman et al., 1979; Hoffman et al., 2003, Fig. 2.5; Kronfeld, 2005; Treiber, 2005). These parameters are determined by fitting glucose and insulin curves to the data obtained from FSIGTs. This method has been applied to

horses to identify insulin resistance in obese mares (Treiber et al., 2006), and has been validated to identify insulin resistance with and without pancreatic action (Kronfeld et al., 2005). There are five mathematical equations used to calculate these parameters (Bergman et al., 1979; Hoffman et al., 2003). Sg (min^{-1}) is calculated using the following equation:

$$\dot{G}(t) = -(X + Sg) \times G(t) + (Sg \times Gb)$$

Where $\dot{G}(t)$ represents the net rate of change in plasma glucose clearance; X represents insulin action on the acceleration of glucose disposal associated with insulin concentrations above basal; $G(t)$ represents plasma glucose concentrations (mg/dL) at time t , with $G(0)$ being the theoretical glucose concentration at time 0; and Gb represents basal glucose concentration (mg/dL) as a result of hepatic production (Hoffman et al., 2003).

S_i ($\text{L} \cdot \text{mU}^{-1} \cdot \text{min}^{-1}$) is determined by use of the following equations:

$$\dot{X}(t) = -p_2 \times X(t) + (p_3 \times [I(t) - I_b])$$

$$S_i = p_3/p_2$$

where $\dot{X}(t)$ (min^{-2}) represents the change in insulin action over time; p_2 represents the rate of insulin action decline (min^{-1}); $X(t)$ quantifies insulin action and its acceleration of glucose disposal at time t associated with insulin concentrations above basal (min^{-1}); parameter p_3 represents the rate of insulin introduction to interstitial space (min^{-1}); $I(t)$ represents the insulin concentration (mU/L) at time t ; and I_b is basal insulin concentration (mU/L) (Hoffman et al., 2003; Treiber, 2005). All assumptions were $X(0) = 0$ and $[I(t) - I_b] = 0$ if $I(t) < I_b$ (Hoffman et al., 2003).

AIRg ($\text{mU} \cdot \text{min} \cdot \text{L}^{-1}$) is calculated using the following equation:

$$\text{AIRg} = \int [I(t) - I_b] \times dt$$

where $I(t)$ represents insulin concentration at time t and I_b represents basal insulin concentrations (Hoffman et al., 2003). In accordance with the definition of the term AIRg (Bergman, 1997), this equation was integrated from $0 \leq t \leq 10$ minutes (Hoffman et al., 2003). DI is determined as follows:

$$DI = AIRg \times Si$$

Analysis of these parameters allows for differentiation of compensated or uncompensated insulin resistance (IR) (Kronfeld et al., 2005). With compensated IR, pancreatic insulin release is due to a decrease in insulin sensitivity whereas with uncompensated, the decrease in sensitivity cannot be matched by pancreatic secretion (Carter, 2008).

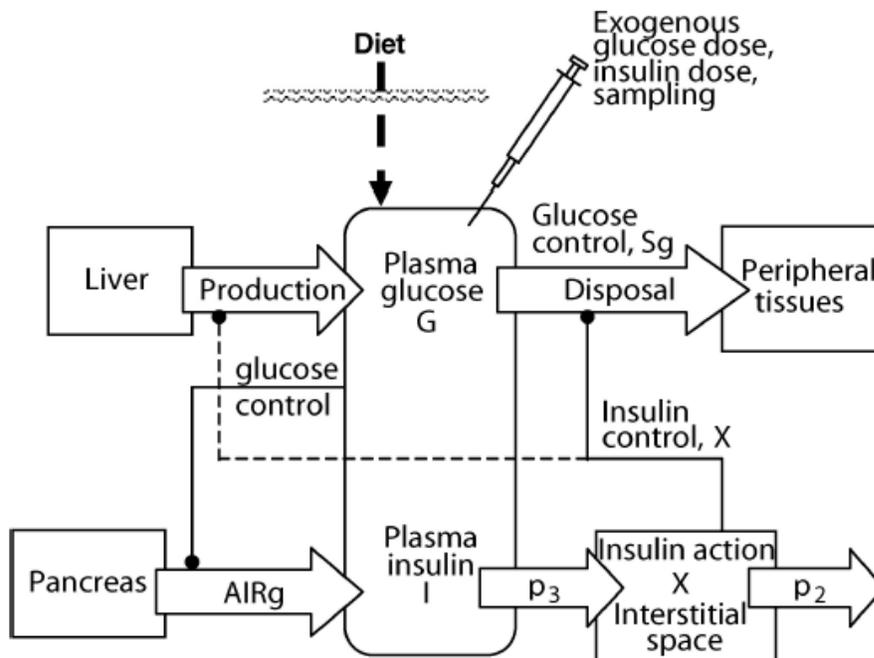


Figure 2.5: Diagram of minimal model components (From Hoffman et al., 2003). Used under fairuse, 2013.

Basal proxies

Proxies have been developed using basal glucose and insulin concentrations to determine insulin sensitivity and pancreatic responsiveness. These include the reciprocal of the insulin square-root index (RISQI), which estimates insulin sensitivity as relative to the amount of insulin secretion required to consistently maintain glucose homeostasis (Treiber et al., 2005). The second proxy, modified insulin response to glucose (MIRG) is calculated as $(800 - 0.30 [\text{insulin} - 50]^2) / (\text{glucose} - 30)$, estimating the ability of pancreatic beta cells to compensate for exogenous glucose secretion (Treiber et al., 2005). These proxies are surrogate tests serving as comparisons of insulin sensitivity and pancreatic responsiveness to be comparable to results found with minimal model analysis.

Adiposity and concurrent inflammation

An association between BCS and insulin sensitivity has been reported showing lower insulin sensitivity (80%) in obese horses (BCS: 7-9) compared to lean counterparts, with similar MinMod analysis results; obese horses tend to have lower Si and higher Sg values (Hoffman et al., 2003). Similarly, a decrease in insulin sensitivity is seen with an increase in percent body fat (% FAT) in obese mares (Vick et al., 2007). Several physiological changes attributed to increased adiposity are to be considered when evaluating the impact of BCS on insulin sensitivity. Obesity is now also described as a physiological state of mild but chronic inflammation (Das, 2001; Ramos et al., 2003). Human studies provide evidence that evidence that elevated inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), IL-1 (He et al., 2006) and IL-6 (Vojarova et al., 2001) play vital roles in obesity-associated insulin resistance (Dandona et al., 2004; Krogh-Madsen et al., 2006). As depicted in the figure below, an inter-

relationship is seen between obesity, inflammatory cytokines, and insulin sensitivity or insulin resistance.

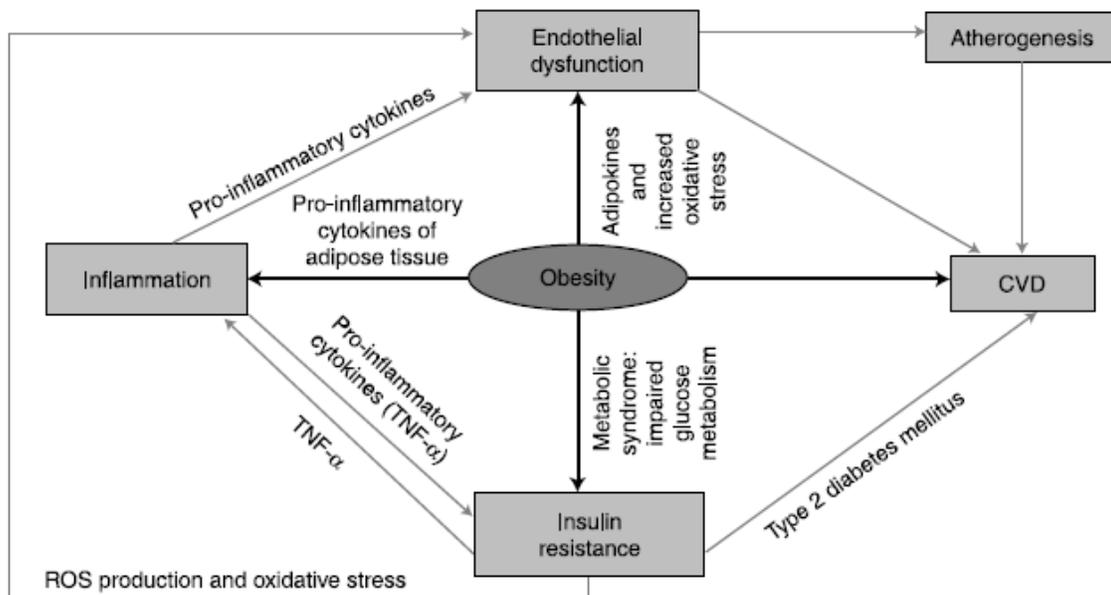


Figure 2.6: Schematic diagram of the inter-relationship of the most prominent co-morbidities associated with obesity. (From van der Spuy and E. Pretorius, 2009). Used under fairuse, 2013.

Similar results have been seen in obese mares with high BCS and % FAT and elevated circulating concentrations of insulin with decreased insulin sensitivity. Increased inflammatory response as demonstrated by elevated circulating TNF- α and increased blood mRNA expression of TNF- α was shown in 60 obese horses, identifying an interrelationship between obesity and inflammation (Vick et al., 2007). Increased insulin concentrations associated with obesity related insulin resistance, may influence circulating TNF- α and IL-6 concentrations in horses (Suagee et al., 2011). TNF- α has been shown to correlate with sex ($r=-0.26$, $P=0.011$) with higher levels seen in mares, and IL-6 is correlated with sex ($r=-0.24$, $P=0.021$) and age ($r=0.32$, $P=0.001$) (Suagee et al., 2013); however, to date only TNF- α has been shown to correlate with BCS in

horses (Vick et al., 2007). These results are consistent with findings in other species; however the mechanism behind the interrelationship of cytokine/insulin resistance interaction in horses is not fully elucidated, warranting further investigation.

Obesity and Reproduction

The relationship between metabolic status and reproductive function is well established in the horse (Gentry et al., 2002; Vick et al., 2006; Waller et al., 2006; Gastal et al., 2011a,b). A bilateral relationship exists between adiposity and gonadal function, often resulting in a cascade of metabolic and reproductive hormonal interactions that result in decreased reproductive performance. Growing evidence suggests associations between metabolic status, measured as body fat percentage and/or energy expenditure, and reproductive activity during winter months (Fitzgerald et al., 2002; Vick et al., 2006; Salazar-Ortiz et al., 2011).

Reproductive Cycle in the Mare

The mare is a seasonal polyestrous animal, in which estrous cycles typically occur in non-pregnant animals throughout spring and summer months, followed by a reproductive quiescence during winter months. This phenomenon is influenced primarily by photoperiod, but is also affected by an array of factors including age, exogenous hormone administration and nutritional status of the mare. The estrous cycle is defined as the period from onset of estrus (sexual receptivity) to the next onset of estrus, with an average length of 21 d, but with a range from 18 to 23 d with estrus comprising 4-7 of these days (Ginther et al., 2002). The estrous cycle is divided into two phases, the ovulatory (follicular) and interovulatory (luteal) phases. During the ovulatory phase, commonly referred to as the follicular phase in which behavioral estrus is observed, the mare is sexually receptive to the stallion and the reproductive tract

prepares for insemination and fertilization. The interovulatory phase, or luteal phase, is the phase during which the uterus prepares for pregnancy. The time in which mares enter into reproductive quiescence is known as the anovulatory period; characterized by minimal follicular activity elicited by follicles <20 mm and decreased production of gonadal hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and inhibin (Donadeu and Ginther, 2002).

Endocrinology of the Estrous Cycle

The regular pattern of estrous cycles depends on a delicate balance of hormones produced by the pineal gland, hypothalamus pituitary, ovaries and endometrium of the uterus (Brinsko et al., 2003). Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus after stimulation via melatonin production from the pineal gland and is responsible for the stimulation of gonadotropin synthesis, (FSH and LH), from the anterior pituitary gland. Estrogen produced by maturing follicles of the ovary has a positive feedback on GnRH production from the cyclic center of the hypothalamus hence LH release from the anterior pituitary, while simultaneously promoting negative feedback in conjunction with inhibin on the production of FSH release (Brinsko et al., 2003). Progesterone, a naturally occurring steroid hormone, is produced by luteal tissue and is responsible for sustaining gestation. Progesterone concentrations are greatest during diestrus and fall significantly during estrus (Fig. 2.7). Estrogen, LH, and FSH concentrations rise during estrus, with an additional rise in FSH during diestrus associated with follicular recruitment and selection for the subsequent ovulation (Fig. 2.7). Prostaglandin F₂alpha (PGF₂α) is released from the uterine endometrium and results in luteolysis, causing a direct decline in circulating progesterone concentrations and promoting follicular maturation and the onset of estrus. Ovulation occurs approximately 24-48 h after the onset of estrus, and is marked by a surge in endogenous LH secretion from the anterior pituitary (Fig. 2.7).

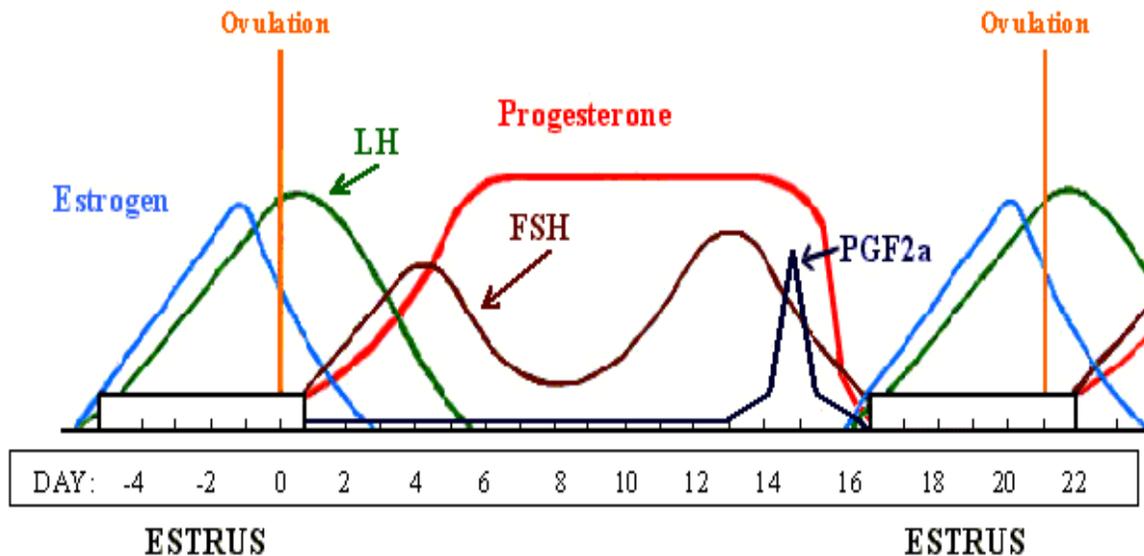


Figure 2.7: Endocrinology of the estrous cycle (From equine-reproduction.com). Used under fairuse, 2013.

Follicular Development

Follicular development in the mare is characterized by waves of small follicles, providing a cohort of follicles that grow in synchrony until a dominant follicle emerges. Similar to other species, the mare is born with a predetermined number of primordial follicles (Ginther, 1992), and will typically ovulate one dominant follicle per estrous cycle. Follicular development occurs in three phases: recruitment, selection (deviation), and dominance. Follicles develop in response to tonic levels of FSH and LH and are categorized according to size: small (2-10 mm), medium (11-24 mm), and large (≥ 25 mm) (Ginther, 1992). Recruitment is characterized by small antral follicle growth and estradiol production, and is followed by selection of follicles that do not undergo atresia. The selected follicles will proceed to develop until one or more large

preovulatory follicles dominate other antral follicles from the cohort exerting an inhibitory effect. These phases are collectively termed follicular waves.

There are various types of waves that occur during different phases of the reproductive cycle in the mare. Major waves, with a dominant follicle size ≥ 25 mm, occur during the ovulatory season or natural breeding season in the mare. During major waves, follicles grow in synchrony, with eventual divergence or selection of a dominant follicle for continued development. Minor waves, characterized by the absence of a dominant follicle, occur during diestrus and during the anovulatory season. All mares will have a follicular wave during the estrous cycle, and occasionally will have two. The first wave generally occurs early diestrus, rarely resulting in an ovulation, followed by the second or 'primary' wave that occurs mid-diestrus and results in a dominant ovulatory follicle (Ginther, 1992, Fig. 2.8). Emergence of follicular waves during the ovulatory season is associated with FSH surges (Ginther and Bergfelt, 1993); however, this association has not been established during the anovulatory period in which minor follicular waves occur regularly with the absence of dominant follicle development. An increase in inhibin production by follicles during the selection phase results in reduction of FSH secretion from the anterior pituitary and in increase in LH production. Concentrations of LH reach a transient plateau around the time of ovulation with a peak noted 1 d post ovulation (Brinkso et al., 2003), and recede to baseline levels shortly after suggesting that the gradual rise in LH concentration is responsible for ovulation rather than a peak concentration as seen in other species. Several factors are believed to differentially enhance FSH and LH responsiveness of the future dominant follicle, including intrafollicular concentrations of estradiol, insulin-like growth factor 1 (IGF1), inhibin-A, and activin-A (Gastal et al., 2010). Increased concentrations of these factors occur in the dominant follicle prior to ovulation and are

thought to prepare the follicle for decreased FSH availability and increased LH concentrations (Gastal et al., 2010).

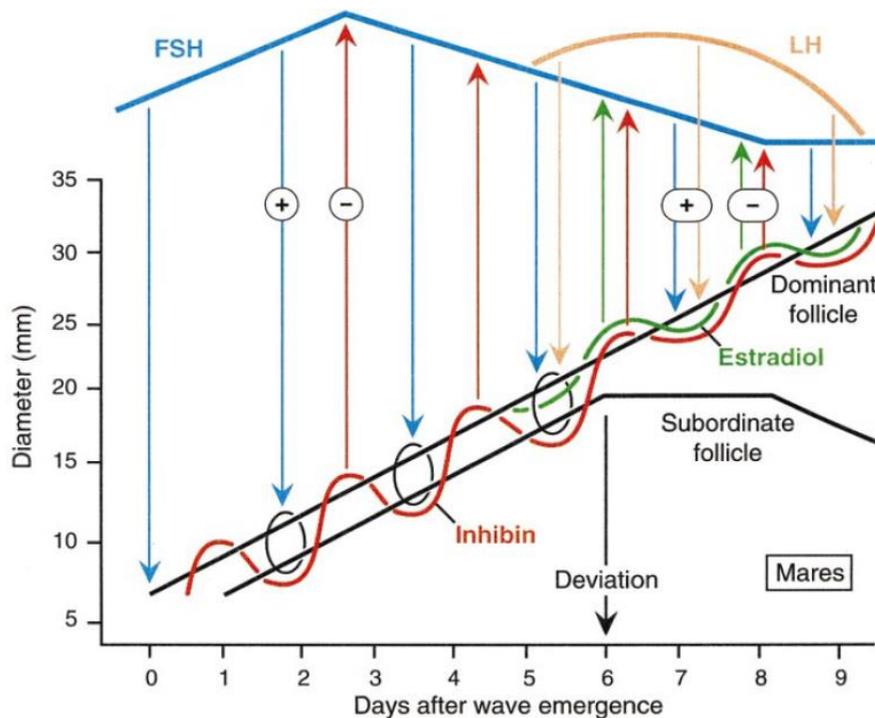


Figure 2.8: Follicle development and hormone regulation (From Ginther et al., 2001). Used under fairuse, 2013.

Similarities between horses and humans

The characteristics regarding follicle development and systemic hormones between mares and women have been reviewed with striking similarities reported. Several aspects of follicular development and hormonal regulation have been reviewed, some of the similarities include: (1) intervals between follicle development; (2) percentage growth of follicles; (3) presence of major and minor waves; (4) surges in FSH on the day of follicular wave emergence; (4) estradiol and progesterone profiles during periovulatory period; (5) longer duration of follicular phase; (6)

increase in length of the follicular phase and inter-ovulatory interval with age; and (7) anovulatory disturbances such as hemorrhagic anovulatory follicles (HAFs) (Ginther et al., 2004; Ginther et al., 2005; Gastal et al., 2006; Carnevale, 2008; Ginther et al., 2008; Baerwald, 2009; Gastal, 2009; Cuervo-Arango and Domingo-Ortiz, 2011; Gastal, 2011a,b). These similarities in follicle dynamics could prove useful in studying folliculogenesis in women, and evaluation of reproductive disorders such as polycystic ovarian syndrome (PCOS) (Gastal et al., 2011c). There is strong evidence suggesting that the mare could serve as an appropriate animal model to investigate the interrelationships between ovarian follicular development, reproductive endocrinology, and metabolic status at whole-animal down to cellular level providing insight on some causes of ovarian disturbances observed in several species, notably the woman (Gastal et al., 2011c).

Impact of Obesity on Cyclicity and Follicular Development

As seen in humans and rodents, metabolic changes associated with obesity such as increases in free fatty acid (FFAs) production, irregular adipokine production, elevated inflammatory cytokine production, and insulin resistance have a notable impact on reproductive function in the mare (Sessions et al., 2004; Vick et al., 2007; Gastal et al., 2011c). Altered metabolic status resulting from excess energy balance and adiposity has been shown to lead to a continuation of reproductive activity during normal anovulatory seasons (Fitzgerald et al., 2002; Gentry et al., 2002; Salazar-Ortiz et al., 2011) and longer interovulatory intervals and luteal phases (Vick et al., 2006). The mechanisms leading to this disruption of the estrous cycle isn't clear, however an association between elevated insulin concentrations and disrupted gonadotropin secretion has been observed in mice (Bruning et al., 2000), pigs (Barb et al., 2001; Mao et al., 2001), sheep (Bucholtz et al., 2000), and humans (Nestler, 2000). Induction of

transient insulin resistance via infusion of heparinized lipid emulsion in the mare increased luteal phase length, reduced peak plasma progesterone concentrations during the luteal phase, and significantly increased interovulatory length (Sessions et al., 2004). The underlying mechanisms are not clear; however, elevations in insulin and reduced insulin sensitivity are highly correlated with both obesity and PCOS in humans and suggest an arrest of follicular development to be directly influenced by insulin (Balen, 2004; Sessions et al., 2004; Panidis et al., 2006). The cessation of follicular growth is believed to be the result of enhanced estradiol production by small follicles, in the presences of insulin, inhibiting further growth and arrest of the follicles in immature stages (Diamanti-Kandarakis and Bergiele, 2001). This ultimately increases follicular phase length as a result of slowed follicular development (Sessions et al., 2004).

The ovary has been suggested as an alternate site of insulin action in the human, due to the presence of insulin receptors (Poretsky et al., 1984) and the ability of insulin to stimulate steroidogenesis in ovarian cells in vitro (Barbieri et al., 1983). Though this relationship has not been established in the horse, recent studies suggest a similar relationship may exist. Changes in insulin sensitivity during the estrous cycle have been observed with lower sensitivity ($Si \times 10^{-4} \text{ L} \cdot \text{min}^{-1} \cdot \text{mIU}^{-1}$) seen during the luteal phase compared to the follicular phase ($P < 0.001$) (Cubitt, 2007). Infusion of insulin during the mid to late luteal phase (7-17d post ovulation) did not alter estrous cycle length, corpus luteum diameter, or plasma LH concentrations; however plasma progesterone concentrations tended to be lower (Rambags et al., 2008). These results conflict with early findings that development of insulin resistance resulted in increased plasma progesterone concentration during the estrous cycle (Sessions et al., 2004), with similar results found in women during the menstrual cycle (Pulido and Salazar, 1999). Evidence of stimulatory effect of insulin on steroidogenesis in other species (Cox et al., 1994; Mao et al., 2001), suggests

that insulin may in fact exert a stimulatory effect on progesterone production by the corpus luteum and have an inhibitory effect on steroidogenesis in its absence in the horse (Sessions et al., 2004). A positive correlation between follicular fluid insulin and plasma insulin ($r= 0.25$, $P < 0.001$) has been shown in mares, with significantly higher insulin concentrations ($P < 0.01$) observed in large ($> 25\text{mm}$) follicles ($1.4 \pm 0.1 \text{ mUI/L}$) when compared to medium (16-25mm) or small ($\leq 15\text{mm}$) follicles (Cubitt, 2007). Collectively, these results from other species and recent findings in the horse suggest a potential ovarian site of action for insulin, however further research is warranted to elucidate the mechanisms behind these actions.

Resveratrol

Resveratrol (3, 4', 5-trihydroxystilbene, RSV, Fig. 2.9) a polyphenolic stilbene derivative, is produced in response to stress, infection, and injury and is commonly found in the skin of red grapes, wine, apples, peanuts, blueberries and cranberries (Wenzel and Somoza, 2005; Lee et al., 2012). The importance of this non-flavonoid compound was revealed when an inverse relationship between wine consumption and the incidence of cardiovascular diseases was discovered, termed as the “French Paradox” (Renaud and de Lorgeril, 1992; Di Castelnuovo et al., 2002; Lee et al., 2012). Several epidemiologic studies revealed that moderate wine drinkers were healthier than their heavy or nondrinking counterparts with respect to coronary heart disease (CHD) (Constant, 1997; Soleas et al., 1997; Bhat et al., 2001b). It is believed that polyphenols present in plants are one of the components attributed to the cardioprotective effects conveyed by this phenomenon, and high amounts of RSV in wine have been speculated to nominate it a vital component (Lee et al., 2012). *Polygonum cuspidatum*, one of the most abundant natural sources of resveratrol, was first discovered in the early 1980's among Japanese

scientists and was used in a traditional Asian folk medicine (Kojo-kon) to treat a wide range of afflictions including fungal diseases, skin inflammations, and heart, liver and blood disease (Wenzel and Somoza, 2005). Several other major biological impacts of this compound include antioxidative, anti-inflammatory, anti-aging, and estrogenic effects in addition to anticancer and chemopreventative *in vivo* and *in vitro* (Frémont, 1999; Bhat et al., 2001a; Wenzel and Somoza, 2005; Das and Maulik, 2006; Cucciolla et al., 2007).

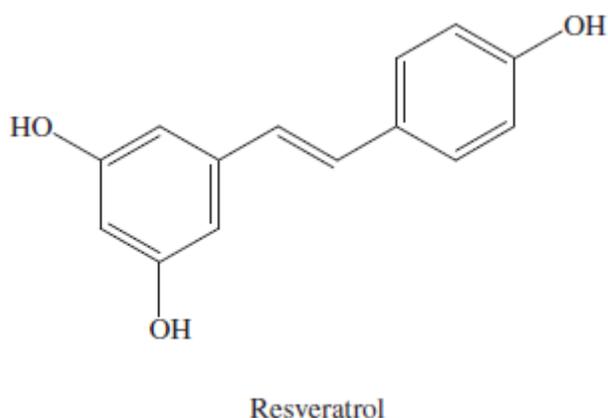


Figure 2.9: 3,4',5-Trihydroxytrans-stilbene (Resveratrol) (From Lin et al., 2012). Used under fairuse, 2013.

Metabolism and bioavailability

Metabolism of bioactive food components is sometimes hindered by the protective mechanisms of the intestine, in an attempt to prevent the entry of potentially harmful components of the intestinal lumen into circulation (Planas et al., 2012). This can at times provide a barrier in the absorption of bioactive compounds ultimately reducing their bioavailability. When RSV crosses the intestinal epithelium (Fig. 2.10) it enters the enterocytes by simple diffusion, with only approximately 75% of the administered dose entering the

enterocyte and only 1.5% of unmodified polyphenol reaching the blood (Planas et al., 2012). Once RSV enters the enterocyte it is modified by UGT and SULT yielding glucuronide and sulfate conjugates, with only approximately 17% of glucuronide and 1.5% of sulfate reaching the bloodstream (Juan et al., 2010). The entry of RSV metabolites into circulation from the enterocytes is regulated by intestinal ATP-binding cassette (ABC) transporters, which are acknowledged as major determinants governing drug metabolism and bioavailability (Planas et al., 2012). The fraction of RSV that is able to bypass this intestinal line of defense and reach the blood is minimal, and believed to be the main causative factor in its low oral bioavailability. ATP-binding cassette transporters also influence regional distribution of RSV after it has successfully evaded the intestinal barriers. Resveratrol that arrives at the liver undergoes additional metabolism (Maier-Salamon et al., 2008; Planas et al., 2012) and subsequent excretion of conjugates through bile (Hebbar et al., 2005; Lancon, et al., 2007; Planas et al., 2012).

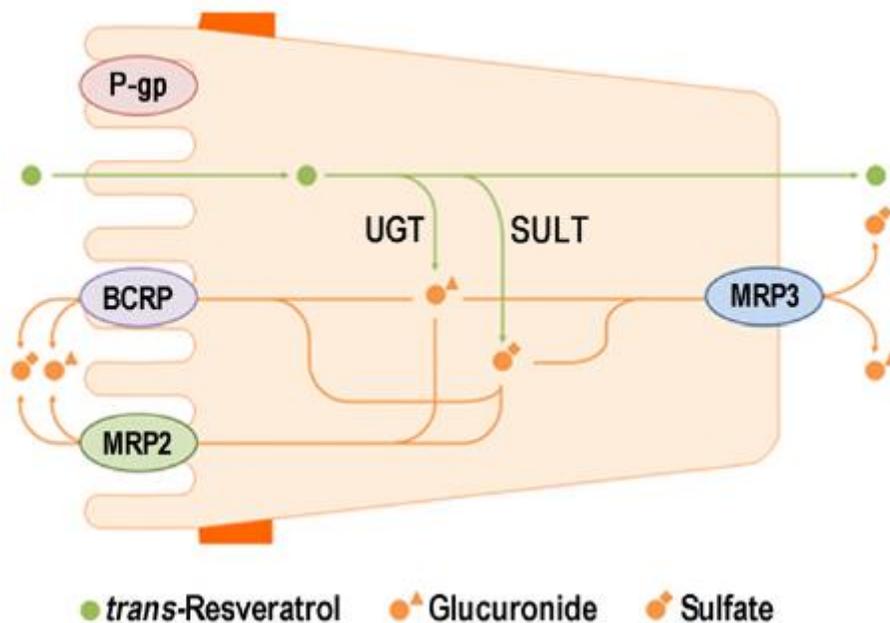


Figure 2.10: Resveratrol uptake and metabolism in the enterocyte. (From Planas et al., 2012). Used under fairuse, 2013.

The bioavailability of a nutrient is defined by the degree to which it becomes available to the target tissue after administration (Maid-Kohnert, 2001; Wenzel and Somoza, 2005). Several *in vitro* and *ex vivo* studies have contributed to the current knowledge on metabolism and bioavailability of RSV in humans and animals. The oral bioavailability of RSV is very low due to rapid and extensive metabolism of this compound and elimination through ABC transporters and incomplete intestinal absorption (Andlauer et al., 2000; van de Wetering et al., 2009; Juan et al., 2010; Planas et al., 2012). This has proved very challenging to the scientific community and their attempts to study the several beneficial biological activities of this compound.

Biological Effects of Resveratrol

Obesity

A metabolic syndrome of major interest in the scientific community is obesity and the several co-morbid conditions that are attributed to this disease. RSV has been extensively investigated as a potential treatment in metabolic syndrome and several promising findings warrant further investigation of the role and mechanisms of actions for this compound and its treatment of obesity.

A significant reduction in total body fat content and decreased weights of epididymal, inguinal, and retroperitoneal white adipose tissue pads has been observed after RSV treatment in mice (Lagouge et al., 2006). A substantial reduction in visceral fat and liver mass indices and fasting serum insulin concentrations were observed in RSV treated mice (Shang et al., 2008; Xu and Si, 2012). However, conflicting results were observed by Rivera and colleagues, who reported a slight decrease in body weight after RSV treatment (10mg/kg body weight for 8 wks) but a reduction in body weight gain was not seen (Rivera et al., 2009). In a recent study, RSV treatment resulted in lower weight gain, visceral fat-pad weights, plasma triglyceride levels, free fatty acids, and glucose (Kim et al., 2011) when compared to control subjects who did not receive treatment. Several mechanisms of action by which RSV exerts favorable effects have been proposed, however the exact molecular mechanism remains unclear (Xu and Si, 2012). Activation of SIRT1 has been indicated as the primary mechanism of action for RSV's effects on obesity (Lagouge et al., 2006; Xu and Si, 2012). Through the activation of peroxisome proliferator-activated receptor- γ co-activator (PGC)-1 α , SIRT1 induces gluconeogenic genes and hepatic glucose output (Rodgers et al., 2005). Resveratrol action is believed to be mediated

through AMPK signaling, due to its in-ability to increase metabolic rate and reduce fat mass in AMPK-knockout mice (Um et al., 2010; Xu and Si et al., 2012).

Diabetes

RSV treatment has demonstrated potential beneficial effects in people with diabetes by protecting β -cells through the inhibition of inflammatory cytokines (TNF- α , IL6, nuclear factor- κ , and light-chain-enhancer of activated B cells) which are responsible for damage in pancreatic islet cells (Lee et al., 2009). Furthermore, RSV treatment has been attributed to an increase in insulin sensitivity in vitro in a SIRT1-dependent manner, and in vivo by attenuating high-fat diet-induced insulin resistance (Sun et al., 2007). RSV effect is not only seen in a high calorie diet model but genetic diabetes animal models evidenced by the insulin sensitizing effect observed in obese Zucker rats (Rivera et al., 2009), ob/ob mice (Sharma et al., 2010), and db/db mice (Kitada et al., 2011; Minakawa et al., 2011) treated with RSV (Xu and Si, 2012).

Reproduction

While extensive research has been done to better understand the pharmacologic and therapeutic properties of RSV on metabolic disorders, little is known regarding the role of this compound in reproduction function. Current research has revealed a potential therapeutic use of RSV in conditions associated with highly vascularized theca-interstitial hyperplasia and abnormal angiogenesis, such as polycystic ovary syndrome commonly found in women (Ortega et al., 2012). RSV treatment of ovarian cancer cells *in vitro* revealed the potential inhibitory effect of this compound on ovarian cancer cell growth by downregulating cross-talk between estrogen receptor α and insulin-like growth factor-1 receptor (Kang and Choi, 2012).

Additionally, RSV treatment in non-obese, immature rats resulted in increased thickness of

endometrial tissue indicating the potential influence of this compound on fertility and pregnancy (Singh, 2011).

Negative effects of RSV treatment have been attributed to its elicited phytoestrogen actions (Bowers, 200; Bhat et al., 2011a; Henry and Witt, 2002). Phytoestrogens are compounds produced by plants that interact with estrogen receptors (ER), potentially eliciting estrogenic effects. Structurally, RSV is very similar to estrogen and binds to both ER- α and ER- β (Henry and Witt, 2002; Fig. 2.11). A comparable binding affinity has been noted, but a 7000 fold lower affinity than estradiol (Bowers, 2000) suggests that RSV may act as an estrogen agonist by binding to ER, resulting in weaker elicitation of estrogenic effects. It is hypothesized that RSV may be capable of altering gene transcription in estrogen sensitive tissue such as uterine endometrium (Henry and Witt, 2002). RSV's effects can be characterized as estrogenic or anti-estrogenic, dependent upon the ratio of phytoestrogens to estrogens present. This competition between phytoestrogens such as RSV, and endogenous steroid hormones leads to a predominantly anti-estrogenic effect in humans (Adlercreutz et al., 1991), but interestingly results in an estrogenic effect in sheep (Adams, 1995).

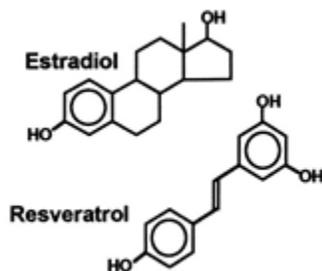


Figure 2.11: Resveratrol and endogenous estradiol are very similar in molecular structure (From Gehm et al., 1997). Used under fairuse, 2013.

Application in the Equine Industry

Research efforts in the equine industry have focused on gaining a better understanding of the effects of resveratrol supplementation on *in vitro* and *in vivo* anti-inflammatory activity in the “inflamm-ageing” geriatric horse model, and glucose-tolerance and insulin sensitivity while reducing oxidant damage in an exercise model (Zambito, 2011; Equithrive, 2012). RSV treatment has been shown to decrease equine inflammatory cytokine production; however its effects on glucose-tolerance and insulin sensitivity still remain unclear. Current studies are focusing on the role of resveratrol in the treatment of EMS through the activation of SIRT 1 and its ability to increase inflammation and decrease inflammation and deposition of fat (Equithrive, 2012). Despite increasing popularity of RSV use in the equine industry and incorporation of this compound into supplements used to promote health and improve athletic performance, the current understanding of the metabolism and bioavailability of this compound is limited, hindering the marketability of the compound. Evidence in human and rat studies provide support for further investigation of this compound and its effects in the equine in regards to metabolic and reproductive impact. Similarities between human females and the mare encourage the use of the equine model in studying reproductive dysfunction as a result of poor metabolic health and potential therapeutic treatments.

In order to fully understand the capabilities of RSV current research should focus on the metabolism and bioavailability of this compound in the horse, and further elucidate the mechanism of action at a whole organism and cellular level. Better understanding of the biological function and effects of RSV will further support the validation of this compound as a potential candidate for treatment of metabolic conditions. Furthermore, therapeutic benefits as a

result of improved metabolic status evidenced in other species, such as enhanced reproductive performance could be further investigated in the horse.

CHAPTER 3: EFFECTS OF RESVERATROL SUPPLEMENTATION ON METABOLIC HEALTH AND REPRODUCTIVE CYCLICITY IN OBESE MARES

Objectives

The long term goals of this study were to evaluate the pathophysiology of obesity-related reproductive dysfunction in the mare, and discover potential therapeutic agents to improve overall health and productivity. Specifically, the aim of this study is to determine the role of resveratrol in improving metabolic status while simultaneously improving reproductive function in obese mares. The specific objectives and hypothesis for the work contained herein were:

1. Evaluate the effect of resveratrol supplementation on improving parameters of metabolic health in obese mares.
2. Assess the effect of resveratrol supplementation on enhancing reproductive performance in obese horses

Hypothesis: Oral supplementation of resveratrol to obese mares will elicit an enhanced metabolic profile while simultaneously improving reproductive function.

Materials and Methods

Horses

Twenty-one healthy, light horse (Warmblood and Thoroughbred) mares were used in this study. Fourteen mares were group-housed on a 12 hectare mixed-grass (fescue, bluegrass, white clover) pasture with *ad libitum* access to forage, water, and shelter. Seven mares were group-housed in 2 hectare dry lot with *ad libitum* access to water and shelter, and were offered mixed-grass hay to maintain appropriate body condition (BCS= 5.5 ±0.5) throughout the duration of the

study. Four horses were dropped from the protocol prior to the study due to health reasons. Summary of forage analysis can be found in Table 3.1 and Table 3.2. All animals were recruited from the existing research herd maintained at the Middleburg Agricultural Research and Extension Center in Middleburg, Virginia, and the experimental protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee. All horses received routine veterinary and farrier care in accordance with the university and USDA standards. Age and reproductive history of the mares is presented in table 3.3.

Table 3.1: Nutrient analysis (DM basis Equi-Analytical, Ithaca, NY) for hay, fed on an *ad libitum* basis throughout study.

Nutrient Content (DM Basis)	Pre	Mid-Trt	Post
Digestible Energy (DE, Mcal/kg)	1.9	1.9	1.9
Crude Protein (CP, g/kg)	63.5	74.1	57.6
Acid Detergent Fiber (ADF, g/kg, %)	45.4	47.2	47.7
Neutral Detergent Fiber (NDF, g/kg, %)	70.9	72.3	71.7
Water Soluble Carbohydrates (WSC, g/kg)	103.0	73.1	77.2
ESC (Simple Sugars, g/kg)	28.6	12.0	17.8
Crude Fat (g/kg)	13.9	11.7	10.4
Calcium (g/kg)	3.1	2.5	2.1
Phosphorous (g/kg)	2.4	1.8	2.3

Table 3.2: Nutrient analysis (DM basis Equi-Analytical, Ithaca, NY) for pasture, fed on an *ad libitum* basis throughout study.

Nutrient Content (DM Basis)	Pre	Post
Digestible Energy (DE, Mcal/kg)	2.0	1.9
Crude Protein (CP, g/kg)	125.1	126.2
Acid Detergent Fiber (ADF, g/kg)	36.7	39.5
Neutral Detergent Fiber (NDF, g/kg)	62.6	64.4
Water Soluble Carbohydrates (WSC, g/kg)	107.9	91.0
ESC (Simple Sugars, g/kg)	99.9	73.7
Crude Fat (g/kg)	24.9	23.6
Calcium (g/kg)	3.3	3.1
Phosphorous (g/kg)	3.0	3.6

Table 3.3: Mean age \pm range and parity for each treatment group.

Treatment Group	Age (Yr)	Parity
OBR (n=6)	=10 \pm 7	3 -Nulliparous 3-Multiparous
OBC (n=5)	=10 \pm 6	2-Nulliparous 1-Primiparous 2-Multiparous
NOC (n=6)	=11 \pm 2	4-Nulliparous 2-Multiparous

Resveratrol Supplementation

Previous studies performed on several mammalian species, including humans, have generally regarded resveratrol as safe (GRAS). Obese horses were matched by age and assigned

to three treatment groups: obese control (OBC; n=5, mean BCS=7.4±0.3) or obese supplemented with 5g/d resveratrol (OBR; n=7, mean BCS=7.4±0.2). Lean, or non-obese control horses (NOC; n=6, mean BCS 5.4±0.1) served as negative control. Resveratrol dosage was determined based on previous studies (Zambito, 2011; Pers. Comm., A. Adams) using product supplied by the same manufacturer. The supplement was compounded (Equithrive, KY) with a concentration of 1 g of 99% pure micronized resveratrol per 10 g polyethylene glycol-based paste which served as the administration vehicle. The resveratrol micronization process ensures that 90% of particles measure less than 10 µm, and when combined with polyethylene glycol, makes the compound more water-soluble to increase bioavailability. Control mares received the vehicle paste (polyethylene glycol-based). Supplementation began the day of ovulation after the mares exited winter anestrus and continued throughout the duration of the experiment (3 estrous cycles: A, B, and C). Mares received 30 mL of either RSV or carrier paste daily, dependent upon which treatment group they were assigned to.

Sample collection and analysis

Objective 1. Evaluating the effect of resveratrol on improving parameters of metabolic health in obese mares. In order to assess resveratrol's effect on mobilization and storage of energy substrates, we evaluated three indicators of metabolic function: morphometric measurements, glucose tolerance, and circulating biomarkers.

Morphometric measurements. Morphometric measurements were taken prior to the initiation of the study and on d 6-8 and d 19 of each estrous cycle. Body weight and body condition were evaluated weekly to ensure body condition scores were maintained throughout the study. Body weight was assessed via electronic scale. Body condition score (Henneke et al.,

1983) was assessed by two independent, experienced scorers. Mid-neck circumference, height, body length, and girth were determined with a flexible tape. Subcutaneous rump-fat thickness was measured 8cm dorsal to the tailhead and 8 cm lateral to the dorsal midline, using an Aloka SSD-900 ultrasound scanner with a 5-MHz probe (Aloka Co. Ltd., Tokyo, Japan).

Glucose tolerance. All mares were subjected to a frequently sampled intravenous glucose tolerance test (FSIGT, as described by Hoffman et al., 2003) prior to supplementation for treatment assignment and on the 6-8 d of cycle C.

Briefly, horses were weighed using an electronic scale and catheterized (Abbocath, 14g, 5.5 in, Abbott Laboratories, Abbott Park, IL) using a aseptic technique and lidocaine anesthesia between 1800 and 2000 the night before the test. Baseline blood samples (30mL) were collected 30 min prior to the start of each test. A glucose bolus of 0.3g/kg BW (dextrose solution, 50%, Phoenix Pharmaceutical, Inc, St. Joseph, MO) was infused via catheter over a 2 min period. Twenty min after the glucose dose, an insulin bolus (Humulin R, Williw Birch Phamaceutical, Inc, Taylor, MS) of 30mlU/kg BW was administered via catheter. The test was conducted for 3.5 h, during which blood samples (30mL) were collected at: -30, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min post glucose administration. Blood samples were placed in serum tubes (1- 10 mL tube per time point; Vacutainer, Franklin Lakes, NJ) and sodium fluoride tubes containing potassium oxalate (2- 6 mL tubes per time point; Vacutainer, Franklin Lakes, NJ) and stored on ice until centrifugation at 3000 x g for 10 minutes. Between each blood sample, the catheter and extension set were flushed with heparinized saline (15- 20 cc) to prevent clot formation and reduce contamination of successive samples. Aliquots were collected from both serum and plasma tubes, and stored in microcentrifuge tubes at -80° C until analysis.

Plasma insulin concentration were analyzed, in duplicate, using Siemens Coat-A-Count Insulin Radioimmunoassay kit (Siemens, Los Angeles, CA), previously validated for horses (Reimers et al., 1982; Freestone et al., 1991; 1992). Detection range was from 2 to 350 μ IU/mL. After the samples were decanted, they were placed on a gamma counter (Packard Instruments, Cobra II Auto Gamma- Counter, Downers Grove IL). Plasma glucose was analyzed by enzymatic assay using the YSI 2700 Select Biochemistry Analyzer (Yellow Springs, OH) with YSI 2365 glucose membranes, YSI 2747 glucose/L-Lactate standard, and YSI 2357 buffer concentration kit.

Glucose and insulin curves were applied to the minimal model (Boston et al., 2003) using MinMod Millennium software (Kennett Square, PA). Four minimal model parameters were evaluated: acute insulin response (AIRg), the animals pancreatic response to a glucose bolus; insulin sensitivity (Si), the sensitivity of peripheral tissues to insulin; glucose effectiveness (Sg), glucose mediated glucose disposal; and disposition index (DI), the product of Si and AIRg. Basal proxies (Treiber et al., 2005) MIRG (β -cell responsiveness) and RISQI (indication of Si) were calculated from basal blood samples. MIRG was measured as $(800-0.30[\text{insulin} - 50]^2) / (\text{glucose} - 30)$. RISQI, the reciprocal inverse square of basal insulin was measured as $\text{insulin} - 0.5$.

Circulating biomarkers. Blood samples were taken prior to treatment and on d 6-8 and d 19 post-ovulation for all three cycles via jugular venipuncture and analyzed for concentrations of plasma TNF- α and IL-10 concentrations. Blood samples were collected via venipuncture into serum and EDTA tubes, immediately centrifuged (3000 x g for 10 min), and aliquoted into microcentrifuge tubes that were stored at -80 ° C for analysis. TNF- α and IL-10 were analyzed using a commercially available equine specific ELISA kit (Genorise Scientific Inc. Paoli, PA).

Objective 2. Assess the effect of resveratrol supplementation on enhancing reproductive performance in obese mares.

Resveratrol's effects on the reproductive system were evaluated by comparing differences between treatment and control mares in ovarian follicular dynamics and circulating steroid hormone levels.

Ovarian follicular dynamics. In order to retrieve mares from winter anestrus, compounded controlled-release progesterone preparation was administered twice, two intramuscular injections a week apart, prior to the initiation of the study. This method has proven effective in inducing ovulation within 7 to 10 d in seasonally anestrus mares, without the use of any other treatments (e.g. artificial photoperiod), having follicle sizes greater than 20mm (Staempfli et al., 2011). The first day of ovulation (ovulation A) and ovarian dynamics were determined via transrectal ultrasonography using an Aloka SSD-900 ultrasound scanner with a 5-MHZ probe (Aloka, Japan). The first estrous cycle was treated as a loading period, in which all mares were ultrasounded as necessary to detect the next estrus period and ovulation (ovulation B). Sample and data collection occurred during three consecutive estrous cycles. Parameters measured included the length of the estrous cycle, length of estrus, maximum diameter of corpus luteum, and maximum diameter of the pre-ovulatory ovarian follicle.

Circulating levels of reproductive hormones. Blood samples were collected every-other-day for three full estrous cycles for measurement of serum progesterone concentrations. Blood samples were collected via jugular venipuncture into EDTA and serum blood tubes and immediately centrifuged (3000 x g for 10 min), and the serum and plasma were aliquoted into microcentrifuge tubes and stored at -80°C until subsequent hormone analyses.

Radioimmunoassays (Siemens Coat-A-Count; Diagnostics Products Corporation)- were used for measuring equine serum progesterone.

Data analysis. Results of the Shapiro-Wilk test revealed that not all variables were normally distributed; therefore data was transformed as necessary. Presented means \pm SE are raw data, and statistically analyzed data is transformed. Spearman rank correlation coefficients were used to quantify association between variables. All data were analyzed using the PROC MIXED procedure of SAS (9.2, Cary, NC). Fixed effects (independent variable) considered in the full model included treatment (TRT), time (TIME) and TRT*TIME. Glucose effectiveness (Sg), insulin sensitivity (Si), and acute insulin response to glucose (AIRg) and disposition index (DI) were calculated using minimal model software (MinMod Millennium, Version 6.02) as previously described (Hoffman et al., 2003). Any animal that did not fit the model was removed prior to statistical analysis. The null hypothesis was rejected at $\alpha= 0.05$ or main effects and interactions with trends defined when $P \leq 0.15$. Where OBR and OBC means were not significantly different, data were collapsed to examine differences between obese (O) and lean (L).

Results

Body weight (BW) and body condition score (BCS)

BW's were evaluated on a biweekly basis and BCS were evaluated weekly and are presented in Table 3.4. Mean difference in body weight (kg) with SEM from pre- to post-treatment was 10.4 ± 3.5 , 5.4 ± 1.8 , and 7.6 ± 1.8 for OBR, OBC, and LNC, respectively. Mean difference in BCS (1-9) with SEM from pre- post- treatment was 0.25 ± 0.11 , 0.30 ± 0.12 , and 0.42 ± 0.15 for OBR, OBC, and NOC respectively. No significant effect for treatment ($P=0.15$), time ($P=0.20$) or treatment by time interaction ($P=0.80$) was observed for BW. Horses remained healthy throughout the study, with no adverse effects observed with the different treatments.

Table 3.4: Mean body weight (kg) and mean BCS (score 1-9) ± SEM pre- and post- treatment for Obese supplemented with 5g RSV/d (OBR), Obese control (OBC) and Non-obese control (NOC) treatment groups.

Variable	BW (Kg, Pre)	BW (Kg, Post)	BCS (Score, Pre)	BCS (Score, Post)
OBR	596.9 ± 7.8	591.8 ± 11.7	7.7 ± 0.1	7.8 ± 0.2
OBC	645.7 ± 13.3	644.7 ± 15.4	7.9 ± 0.1	7.8 ± 0.1
NOC	611.9 ± 21.5	608.0 ± 24.1	5.3 ± 0.8	4.9 ± 0.8

Morphometric measurements

Mean rump fat thickness (RFT, cm) and mean neck circumference (NC, cm) were evaluated biweekly. Mean RFT decreased but not significantly from pre- to post- treatment for all treatment groups. Mean NC decreased for OBC and NOC treatment groups, and increased for OBR horses. There was no significant treatment effect ($P>0.05$) for RFT.

Circulating biomarkers

RSV had no significant treatment effect on circulating levels of TNF- α ($P=0.37$) and IL-10 ($P=0.84$) (Table 3.5), however a significant time effect was observed for TNF- α ($P=0.05$).

Table 3.5: Mean values for TNF- α (OD) and IL-10 (OD) for each treatment group.

Item	TRT			P-Value			P-Value (Obese vs. Lean)		
	OBR	OBC	NOC	TRT	TIME	TRT*TIME	TRT	TIME	TRT*TIME
TNF-α (OD)				0.37	0.05*	0.58	0.38	0.11	0.29
Pre	1.26	1.60	1.07	-	-	-	-	-	-
Post	1.42	1.96	1.10	-	-	-	-	-	-
IL-10 (OD)				0.84	0.60	0.61	0.94	0.75	0.72
Pre	0.90	0.73	0.42	-	-	-	-	-	-
Post	0.83	0.79	0.43	-	-	-	-	-	-

* $P<0.05$

FSIGT

Minimal model analysis of FSIGT results, along with basal insulin, glucose concentrations evaluations revealed no overall treatment effect of RSV ($P>0.05$). The parameters that resulted from the minimal model analysis are presented in Table 3.6. For acute insulin response to glucose (AIRg) a significant difference ($P=0.005$) was observed between NOC and OBC horse for AIRg values. Although there was no significant difference ($P=0.06$) between NOC and OBR horses, a trend is apparent for AIRg value between the two treatment groups. Insulin sensitivity (S_i) was not significantly affected by treatment ($P=0.15$); however a significant time effect ($P=0.02$) was evident between pre- and post- treatment values. A significant time effect ($P=0.02$) was seen for the disposition index (D_i) which is a function of S_i * AIRg. There was no significant treatment ($P=0.85$), time ($P=0.97$), or treatment by time ($P=0.47$) for glucose effectiveness (S_g).

Minimal model analysis of FSIGT results were also compared between obese (BCS > 7 , $n=12$) and non-obese (BCS < 7 , $n=5$) as well as insulin resistant (IR, S_i values below 0.78 ($1/[mU/L \cdot min]$), $n=9$) and non-insulin resistant mares (Non-IR, S_i value above 0.78 ($1/[mU/L \cdot min]$), $n=8$). A significant time effect ($P=0.02$) for D_i between obese horses pre- and post- treatment was seen. A significant difference ($P=0.01$) for AIRg values existed when compared between obese and lean. No significant treatment ($P>0.05$), time ($P>0.05$) or treatment by time ($P>0.05$) was seen for RISQ or MIRG proxies for all three treatment groups. A significant relationship ($P<0.01$) between AIRg values and BCS was observed, with an increase in AIRg values seen with increasing BCS scores. A statistical difference ($P < 0.01$) was observed in S_i values for IR and Non-IR horses, with IR horses have a S_i value less than 0.78 ($1/[mU/L \cdot min]$) and non-IR horses having S_i values well above 0.78 ($1/[mU/L \cdot min]$), validating

current literature suggesting that IR horse have a Si value lower than 0.78 (1/[mU/L·min]) (Hess et al., 2012).

Basal glucose (mg/dL) concentrations were significantly different (P=0.02) between NOC and OBR groups, and numerically different between OBC and OBR horse with a trending p- value (P=0.10). No significant time (P=0.13) or treatment by time (P=0.52) effect was observed for basal glucose concentrations across all treatment groups. Glucose concentrations (ng/dL) for pre-treatment are as follows: OBR (91.9 ± 14.31), OBC (83.2 ± 2.11), and NOC (80.02 ± 3.51). Numerical differences were observed in basal glucose concentrations for obese and non-obese horses, where higher concentrations were seen in OBR and OBC groups when compared to NOC. Basal insulin values, measured as μ IU/mL did not reveal any significant treatment (P=0.19), time (P=0.40) or treatment by time (P=0.79) effect. Basal insulin values were 2-fold higher in obese horses than lean.

Table 3.6: Minimal model parameters Si (insulin sensitivity), Sg (glucose-mediated transport), AIRg (β -cell pancreatic response), DI (disposition index) and basal proxies – RISQI (insulin sensitivity) and MIRG (β pancreatic response).

Item	TRT			P-Value			P-Value (Obese vs. Lean)		
	OBR	OBC	NOC	TRT	TIME	TRT*TIME	TRT	TIME	TRT*TIME
INS (μU/mL)				0.19	0.40	0.79	0.07	0.54	0.50
Pre	5.95	7.58	2.92	-	-	-	-	-	-
Post	6.68	10.2	4.75	-	-	-	-	-	-
GLU (mg/dL)				0.02*	0.13	0.52	0.02*	0.18	0.93
Pre	92.0	83.0	80.1	-	-	-	-	-	-
Post	92.5	87.8	81.8	-	-	-	-	-	-
Si, 1/(mU/L·min)				0.16	0.02*	0.84	0.09	0.03*	0.62
Pre	0.94	1.27	1.98	-	-	-	-	-	-
Post	0.17	0.48	1.61	-	-	-	-	-	-
Sg, 1/min				0.85	0.97	0.47	0.99	0.71	0.22
Pre	0.02	0.01	0.01	-	-	-	-	-	-
Post	0.01	0.01	0.01	-	-	-	-	-	-
AIRg, 1/(mU/L)·min				0.02*	0.73	0.58	0.01**	0.98	0.31
Pre	178.6	302.5	93.6	-	-	-	-	-	-
Post	183.3	352.7	90.4	-	-	-	-	-	-
DI, AIRg·Si				0.32	0.02*	0.65	0.96	0.02*	0.71
Pre	163.1	267.3	219.9	-	-	-	-	-	-
Post	28.6	179.9	116.9	-	-	-	-	-	-
RISQI				0.26	0.64	0.32	0.11	0.95	0.27
Pre	0.4	0.6	0.6	-	-	-	-	-	-
Post	0.4	0.4	0.8	-	-	-	-	-	-
MIRG				0.53	0.70	0.49	0.25	0.99	0.37
Pre	3.9	5.9	4.2	-	-	-	-	-	-
Post	3.9	5.5	3.3	-	-	-	-	-	-

*P <0.05, **P<0.01

Assume no statistical difference, collapsed values looking at obese vs. lean horses: obese horses (n=12) and lean horse (n=5).

GLU, basal glucose; INS, basal insulin

Table 3.7: Spearman correlation coefficients for individual pairs of items measured.

Item ¹	BW	Si	AIRg	MIRG	RISQI
BCS ²	NS ³	-0.29†	0.52**	NS	NS
BW, kg		NS	NS	NS	NS
Si			NS	NS	0.28†
AIRg				0.40*	-0.30†
MIRG					-.98
RISQI					

¹BCS= Body condition score (score); BW=body weight (kg); Si=insulin sensitivity (1/(mU/L·min)); AIRg=Acute insulin response to glucose (1/(mU/L)·min); MIRG and RISQI= basal proxies.

²The average of two scores from separate observers; values ranged from 1 to 9.

³NS=not significant.

† P< 0.10, *P<0.05, **P<0.001

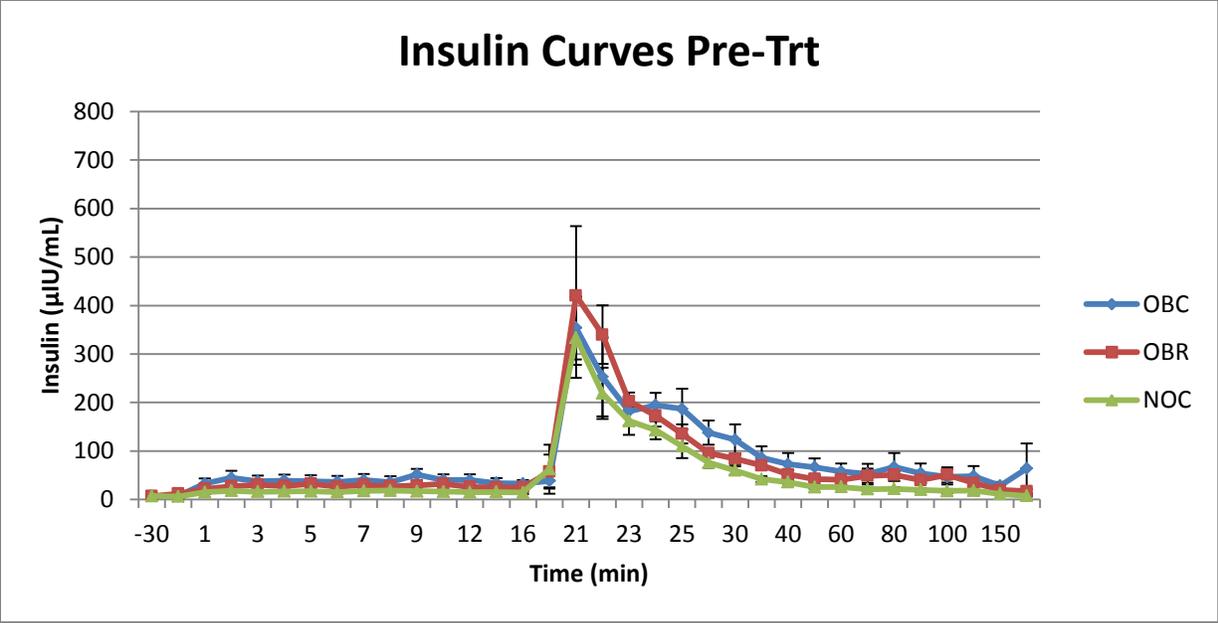


Figure 3.1: Mean insulin concentrations \pm SEM from a frequently sampled intravenous glucose tolerance test (FSIGT) for the obese supplemented (OBR, n=6), obese control (OBC, n=5), and non-obese control (NOC, n=6) pre-treatment.

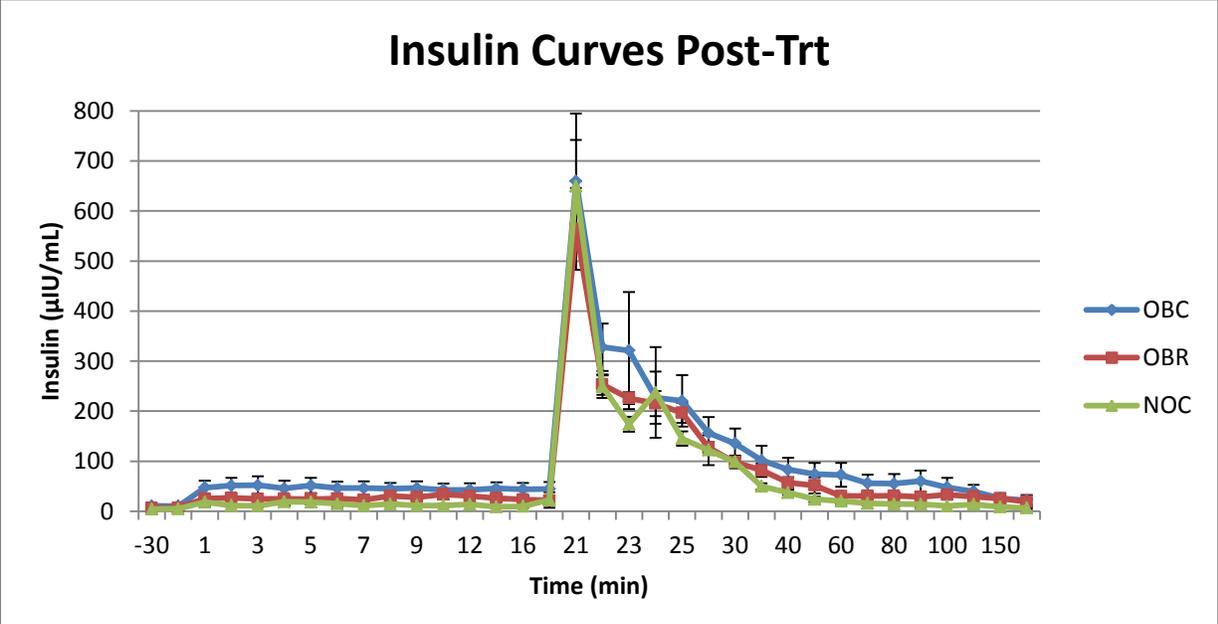


Figure 3.2: Mean insulin concentrations \pm SEM from a frequently sampled intravenous glucose tolerance test (FSIGT) for the obese supplemented (OBR, n=6), obese control (OBC, n=5), and non-obese control (NOC, n=6) post-treatment.

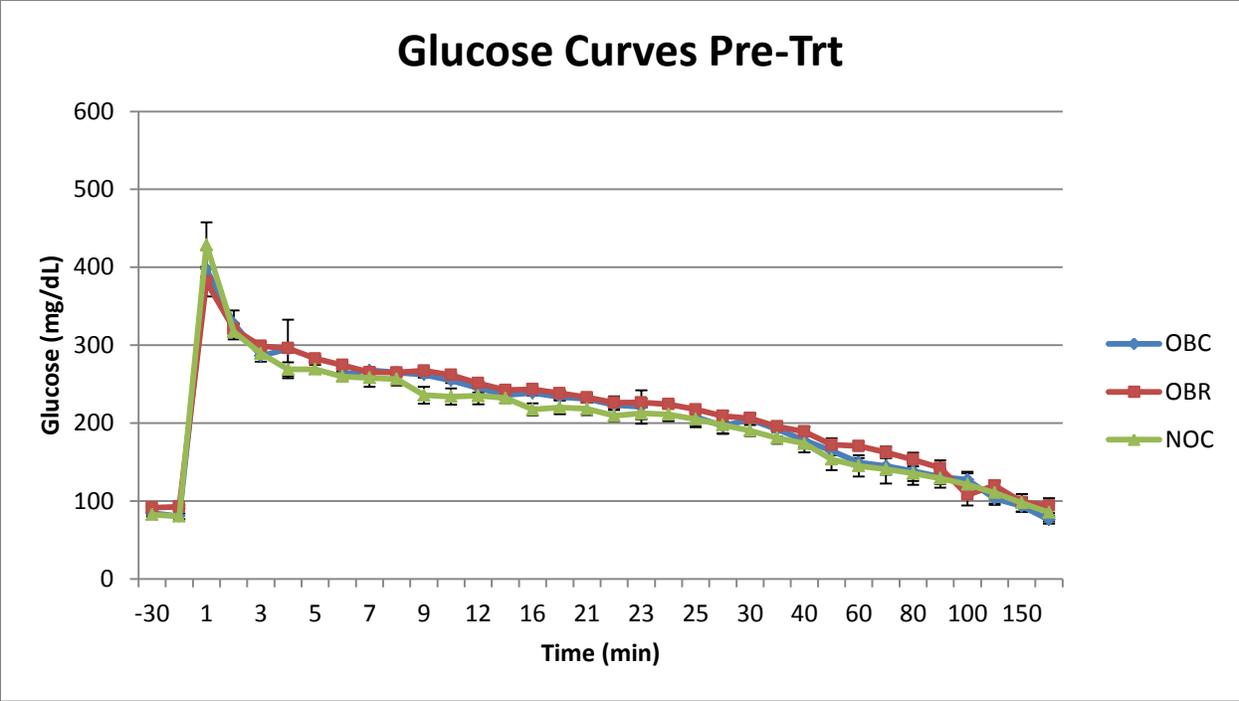


Figure 3.3: Mean glucose concentrations \pm SEM from a frequently sampled intravenous glucose tolerance test (FSIGT) for the obese supplemented (OBR, n=6), obese control (OBC, n=5), and non-obese control (NOC, n=6) pre-treatment.

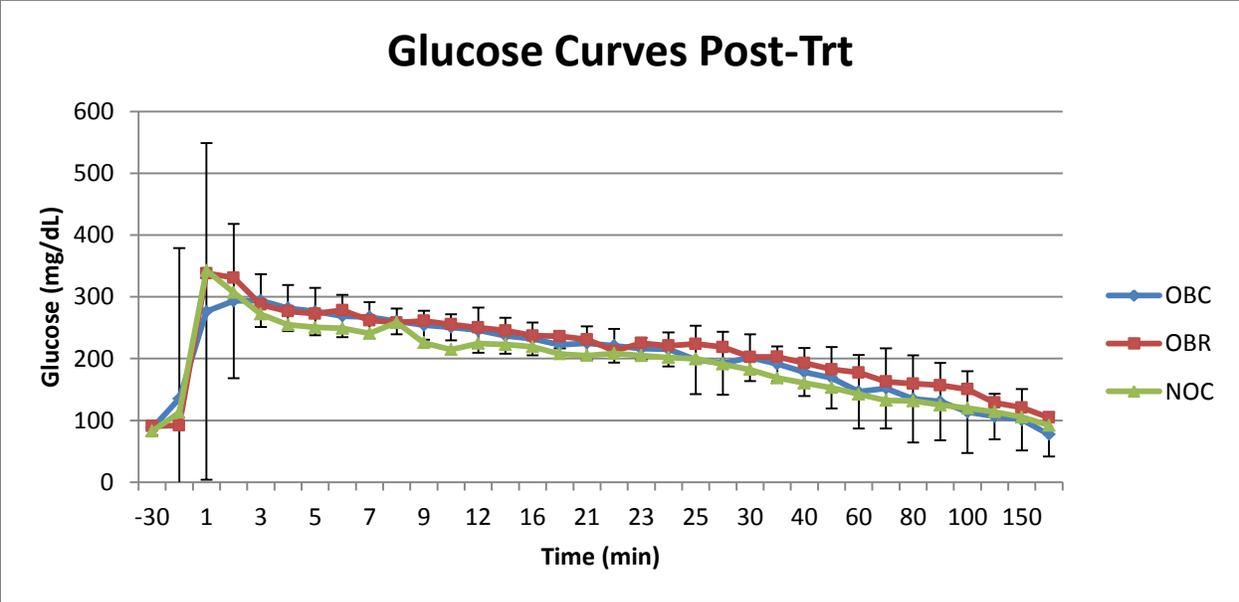


Figure 3.4: Mean glucose concentrations \pm SEM from a frequently sampled intravenous glucose tolerance test (FSIGT) for the obese supplemented (OBR, n=6), obese control (OBC, n=5), and non-obese control (NOC, n=6) post-treatment.

Reproductive Parameters

Preovulatory follicle size, measured in mm over three consecutive estrous cycles, did not show any significant treatment ($P=0.72$), treatment by time ($P=0.22$), or body condition (0.300) effect. Interovulatory period (d), did not reveal any significant treatment ($P=0.80$), treatment by time ($P=0.35$), or body condition ($P=0.86$) effect. Mean \pm SEM interovulatory cycle length for each estrous cycle (1-3) were 19.6 ± 1.1 , 21.3 ± 1.2 , and 21.8 ± 1.3 d respectively (Figure 3.6). Average \pm SEM interovulatory intervals for each treatment group (NOC, OBC, and OBR) were 20.8 ± 1.1 , 19.8 ± 1.2 , and 20.7 ± 1.3 respectively (Figure 25). Regardless of treatment (OBC or OBR), obese mares had more (6 vs. 0) hemorrhagic anovulatory follicles. Reproductive endocrine data (progesterone and estradiol profiles) were not reported in this study to time and financial limitations.

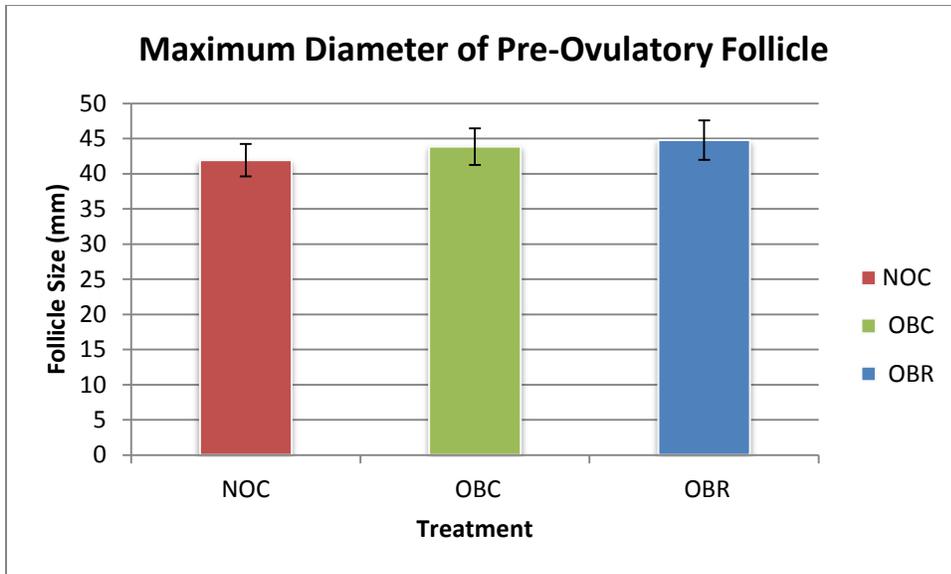


Figure 3.5: Mean \pm SEM for maximum diameter of pre-ovulatory follicle for three treatment groups (NOC: n=6, OBC: n=5, and OBR: n=6) over 3 consecutive estrous cycles. No significant treatment effect ($P=0.72$) was observed for maximum diameter of pre-ovulatory follicle.

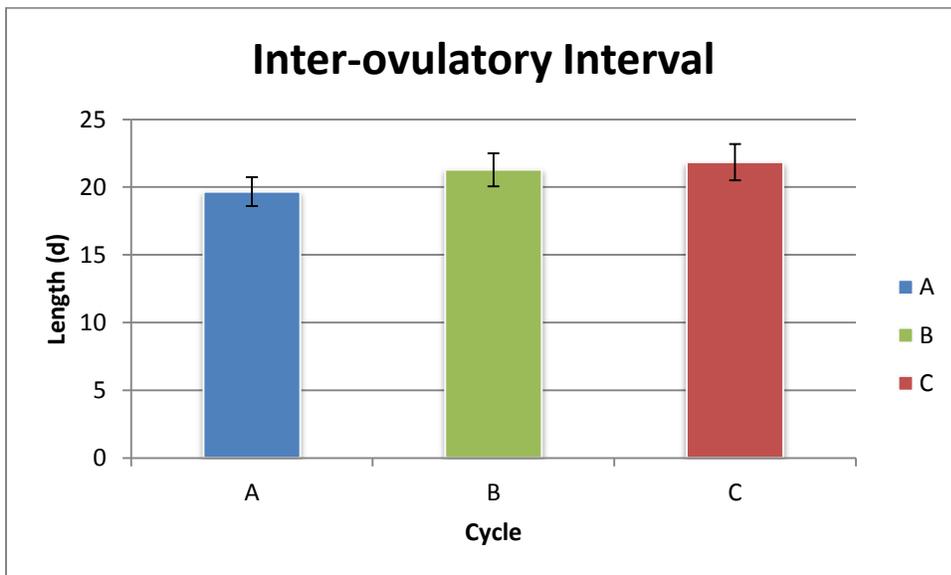


Figure 3.6: Mean \pm SEM for length of interovulatory period for each consecutive estrous cycle. There was no significant difference observed for interovulatory period for obese (n=11) and lean (n=6) mares ($P=0.86$).

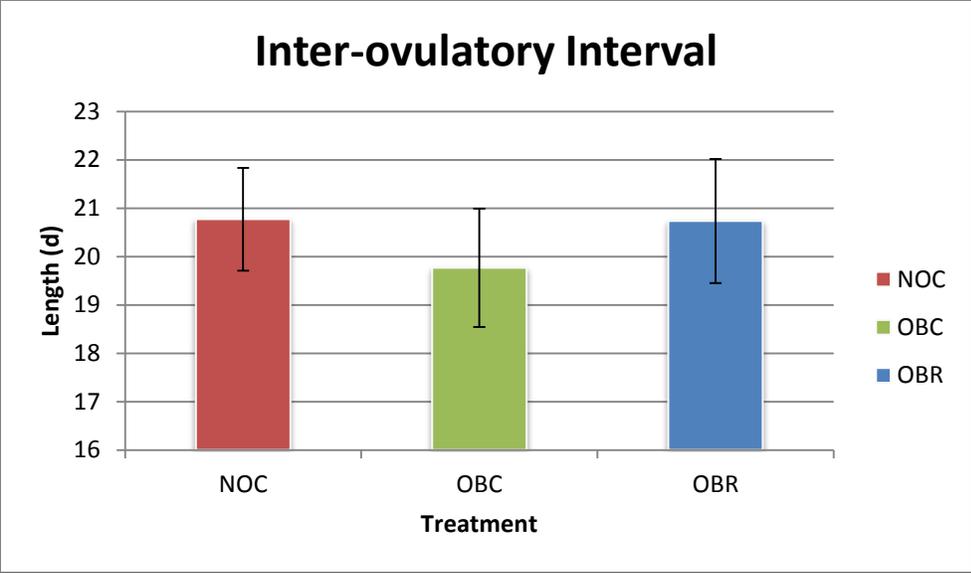


Figure 3.7: Mean \pm SEM for interovulatory interval length for each treatment group (NOC: n=6, OBC: n=5, and OBR: n=6) over 3 consecutive estrous cycles. No significant treatment effect ($P=0.80$) was observed for interovulatory interval length.

CHAPTER 4: DISCUSSION AND IMPLICATIONS

Evidence *in-vitro* and *in-vivo* from other species, supports the therapeutic role of RSV in the treatment of metabolic maladies such as insulin resistance, oxidative stress damage, and low-grade systemic inflammation. While an effect of resveratrol was not determined in this current study, the question still remains valid. There are several promising results to be taken and further investigated to determine the potential therapeutic role of this compound in the obese broodmare.

In order to properly identify the effects of a compound on metabolic and reproductive aspects in any species, it is essential to understand the pharmacokinetics of the substance. To this writer's knowledge, no study to date has been conducted to look at the pharmacokinetics of RSV in the horse model. Attempts have been made to improve the proposed effects of this compound, including micronization through an anti-solvent precipitation process (Kim et al., 2012) that enhances saturation solubility and dissolution rate by decreasing particle size and degree of crystallinity. The effectiveness of this process to increase bioavailability in the equine model specifically still remains to be validated, however it is an essential step that will lead to a better understanding of the mechanisms of absorption and distribution of this compound, chemical changes of the substance, and effects and routes of excretion of the metabolites in the body.

A small number of reports (Zambito, 2011; Pers. Comm., A. Adams) have investigated the effects of RSV; however, minimal desired effects were reported. The dosage used in this study (5g trans-RSV/d) was based on the dosages used in prior studies (Zambito, 2011; Pers. Comm., A. Adams) however, reported dose regimens have not been validated by pharmacokinetic evaluation to accurately determine the dosage required to elicit desired therapeutic effects.

Additionally, the plasma steady-state kinetics of RSV should be determined in order to establish the clinically relevant half-life ($t_{1/2}$) which has a direct impact on dosing regimens. By gaining a better understanding of the metabolism, absorption, and distribution of this compound in the equine metabolic system, the understanding of metabolic effects of RSV would be enhanced, and the proposed therapeutic effects could be achieved with scientifically supported, specific dosage regimens.

One of the most marketable therapeutic effects of RSV in other species is its ability to mimic the effects of caloric restriction without requiring the individual to undergo the physical means of reducing adiposity without simultaneous weight loss. This characteristic would specifically be ideal for the treatment of obesity and associated insulin resistance in broodmares with pending increases in energy demands for placental and fetal growth and mobilization of substrates for fetal use (George et al., 2011). In a recent study evaluating the effects of acute nutrient restriction of mares during mid-gestation, a decrease in maternal glucose and insulin concentrations was seen with enhanced insulin secretion in their neonatal foals (Ousey et al., 2008; George et al., 2011). These findings further exacerbate the need for a therapeutic treatment for obese broodmares that will improve metabolic health and simultaneously not have a negative impact on the gestation.

In this investigation, whole-body parameters were evaluated and though a significant treatment effect was not evident, a time effect was noted for AIRg and Si. Positive correlations were observed between AIRg and BCS in this study, conforming previously published relationships identified between insulin responses and BCS in other studies of obese equids (Carter et al., 2009; Dugdale et al., 2010; Suagee et al., 2013). The hyperinsulinemia observed in obese horse is most likely a negative feedback component associated with insulin resistance,

resulting in overcompensation of pancreatic output to recompense for decreased peripheral insulin effectiveness on glucose uptake (Suagee et al., 2013). BCS and Si showed a significant negative correlation, supporting previous findings (Vick et al., 2007) showing an increase of insulin sensitivity associated with decreasing BCS.

Basal proxies have been validated as significant indicators of their respective minimal model parameters (Hess et al., 2012), and were also shown to be correlated in this study. MIRG and AIRg were significantly correlated, however the correlation coefficient ($r=0.39$) was much smaller than reported in previous studies (Treiber et al., 2005, $r=0.75$; Hess et al., 2012, $r=0.60$). A positive correlation was also found between RISQI and Si in this study, however not significant and with a much smaller correlation coefficient ($r=0.29$), when compared to other findings (Treiber et al., 2005, $r=0.77$). This could be a result of the larger population sizes utilized in previous studies, allowing for higher correlation coefficients.

The relationship between obesity, pro-inflammatory cytokine production and insulin sensitivity has been investigated (Vick et al., 2007; Suagee et al., 2013), revealing TNF- α as one of the most influential cytokines on insulin sensitivity in the obese horse. Higher TNF- α production as a result of increased adiposity associated with obesity, have been shown to not only regulate immune/inflammatory response but also insulin sensitivity (Arner, 2005; Tilg and Moschen, 2006; Vick et al., 2007). These findings could help justify results of the current study in which the overall TNF- α production for all three treatment groups increased pre- to post-treatment, in addition to an overall increase in the number of IR horses ($Si \text{ value} < 0.78 \text{ 1/[mU/L}\cdot\text{min]}$) in each group. Even though BCS remained consistent throughout the duration of the study for all three treatment groups, it is possible that the whole body measurements taken

and parameters evaluated were unable to detect metabolic and degree of adiposity changes that would otherwise be more likely apparent at a cellular or tissue level.

Slight decreases in body weight were observed over the duration of the study and could have been confounded by the differences in feed intake between all three groups and/or individual intake. Feed intake was not controlled for the two obese groups (OBR and OBC), because the horses were provided with *ad libitum* access to pasture. Diurnal and seasonal variations in pasture composition over the course of the study could have confounded the FSIGT results (Kagan et al., 2011a, b). The non-obese control group was maintained on a dry lot with *ad libitum* access to round bales that were a first cutting from the field the other horses were housed on. Due to the differences in forage consumed between groups and individuals, it was nearly impossible to assess effects of diet on treatment groups. Additionally, levels of resveratrol were not determined in forage analysis process.

The reproductive parameters evaluated for this study were not affected by treatment or time. There are several factors that could have influenced these outcomes, specifically data collection methods and variability in age, parity, and last foaling date between mares in each treatment group. Ultrasound data was collected by four individuals with varying levels of experience. Additionally, in accordance with original protocol's uterine biopsy samples were collected for the first estrous cycle, however due to drastic changes noted in estrous cycle length the samples were halted. Later investigation of the literature revealed a potential confounding effect of date of biopsy and inter-estrus interval, which has been previously described in horses (Snider et al., 2011). Due to the large variability between individual horse characteristic including age (range: 3- 16 yr), parity (range: maiden- greater than 3), and last foaling date (range: 2009- 2011) it is impossible to separate treatment effects from basic biological effects.

Ideally, these variables would need to be distributed evenly across treatment groups and blocked for accordingly. The reproductive results from this study are better utilized a preliminary data, and can provide insight for future studies looking to investigate the influence of improved metabolic status on reproductive parameters in the horse.

Resveratrol supplementation in the obese mare showed no overall treatment effect on parameters of metabolic health and reproductive function. However, the question still remains as to whether RSV treatment will benefit metabolic and reproductive parameters in the obese broodmare. Several aspects of current literature describing the role of RSV in other species elicit roles of this compound that were not able to be investigated in this study due to time and financial limitations. One such role, is the activation of the metabolic regulators silent information regulator 2 homolog (SIRT), a major player in many vital processes, such as DNA repair, cell survival, gluconeogenesis, muscle cell differentiation, cell cycle regulation, lipid metabolism, fat mobilization, and insulin sensitivity (Brooks and Gu, 2009). However, controversial experimental data challenge this proposed mechanism of action. There is limited supporting evidence for direct interaction with SIRT1 as a mechanism of resveratrol's biological effects (Stuart and Robb, 2013). However, due to the broad range of SIRT1 molecular targets it is proposed that the signalling pathways regulated by SIRT1 and RSV overlap. These targets include various aspects of cellular stress response (FOXO transcription factors), growth and development ($ER\alpha$), and cell metabolism (PGC 1- α) (Stuart and Robb, 2013). By identifying the direct mechanisms of SIRT1 activation and simultaneous down regulation of molecular targets, such as PGC 1- α , the biological effects of this compound could be further elucidated.

The molecular mechanisms responsible for the proposed effects of resveratrol continue to be the subject of much debate within the scientific community. Due to the striking parallels that

exist between the effects of resveratrol and those of estrogens, this compound is believed to elicit its effects through stimulation of estrogen receptor (ER) signalling pathways (Stuart and Robb, 2013). Resveratrol has been shown to bind to and activate gene transcription via the estrogen receptor (ER) subtypes ER α and ER β in vitro (Henry and Witt, 2002, Stuart and Robb, 2013); however, in vivo experiments have demonstrated that its agonistic effects may only be present in gonadally intact females (Henry and Witt, 2002). Estrous cycle disruption was noted, with abnormally long or continuous cyclicity occurring, which could possibly explain the variations in estrous cycle length observed in this study (Henry and Witt, 2002). Additionally, binding affinity of phytoestrogens is believed to be preferentially higher for ER β than ER α (Goldberg et al., 1996). This is important to note due to the distinct biochemical properties each subtype has on different biological functions. ER β is expressed primarily in the uterus, lung, paraventricular nucleus of the hypothalamus (PVN), and granulosa cells of developing follicles (Henry and Witt, 2002). ER β agonists prevent increases in body mass and white adipose tissue mass associated with high fat diet in normal mice (Yepuru et al., 2010), which is strikingly similar to that reported for dietary resveratrol supplementation in mice (Lagouge et al., 2006; Stuart and Robb, 2013). The binding affinity of RSV to ER β has not been investigated in the horse; however, based on literature in other species, it could be speculated that this agonistic effect could potentially disrupt reproductive function. Nonetheless, this overwhelming concordance between the cellular and systemic effects of resveratrol and those elicited by endogenous estrogens in the body warrant further investigation of this proposed mechanism of action (Stuart and Robb, 2013).

Current research suggests RSV effects are exerted most abundantly at the cellular level. The overall design of the current study focused on evaluation of whole-body level parameters,

which severely limited the ability to detect treatment effects that could potentially exist. Due to the short length of treatment for each group, it would be difficult to identify effects at the whole body level. Although resveratrol supplementation did not elicit detectable responses in this study, promising results in other species warrant further investigation of the compound in horses, including exploration of bioavailability and possible effects at the tissue or cellular levels.

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