ABSTRACT

GOERTZEN, ELLEN WOODRING. Effects of Chromium Supplementation on C-peptide and Hair of Quarter Horse Geldings, and of Low and High Glucose Doses During an Intravenous Glucose Tolerance Test. (Under the direction of Dr. Shannon Pratt-Phillips).

An experiment was conducted to determine the effect of dietary chromium supplementation on plasma C-peptide kinetics following a meal or intravenous glucose tolerance test (IVGTT) (EXPT1A), and changes in chromium hair concentration in mature idle quarter horse geldings (EXPT1B). A second experiment (EXPT2) evaluated the effect of glucose dose on plasma C-peptide concentrations during an IVGTT.

In EXPT1A, twenty-four horses were divided equally among two 42 d periods. Within each period, horses (n=12) were assigned to one of four treatments: 0 mg (CON; n=3), 2 mg (CR2; n=3), 4 mg (CR4; n=3) and 8 mg (CR8; n=3) of dietary chromium per d supplied by a chromium propionate supplement. On d-0 and 28 of each period, horses were fed a grain-mix concentrate at 0.2 kg/100 kg BW as fed, and blood samples were collected via venipuncture immediately before feeding (time 0 h), and at 2 h and 4 h post feeding. On d-42, IVGTT were administered using 100 mg dextrose/kg BW, and jugular venous blood samples were collected via catheter at frequent intervals following glucose infusion. Plasma C-peptide concentrations were analyzed using a human double antibody radioimmunoassay kit validated for use in horses. Concentrations for d-28 and 42 and AUC for d-42 were analyzed using SAS PROC MIXED with repeated measures. Day 28 plasma C-peptide concentrations peaked at 2 h and decreased after. No main effects of treatment or period were observed on d-28 or 42. However, visual contrasts suggested that AUC on d-24 was lower for CR4 than the other treatments.

EXPT1B utilized the same design as EXPT1A, with an additional period of horses (n=12) included. On d-0, 28, and 42, hair samples were collected from 3 regions of the mane and stored in airtight bags at room temperature. Samples were washed, dried and cut to represent the length of growth during the supplementation period, and analyzed by graphite furnace on an atomic absorption spectrophotometer for chromium content. Chromium content for d-0, 28, and 42 were analyzed by ANOVA in SAS PROC MIXED; the model included TRT, Day, and Period. Main effects of TRT ($p=0.0461$) and Day ($p=0.0393$) were observed, and no TRT*Day interaction was observed. Overall chromium content decreased from d-0 to 28 and increased from d-28 to 42; concentrations on d-0 and 42 were not different. Overall, chromium content was lower for CR4 than CR2 ($p=0.0062$). No clear pattern of chromium bioaccumulation in hair during the supplementation period was observed.

EXPT2 utilized a crossover design completed on two days, seven days apart. On d-1, four horses were randomly assigned to one of two IV glucose dose treatments: low dose (100 mg/kg BW), or high dose (300 mg/kg BW); on d-2, treatments were reversed. Intravenous GTT were administered and venous jugular blood samples were collected via catheter at frequent intervals following glucose infusion. Plasma C-peptide was analyzed as described in EXPT1A, serum analyzed for insulin using a radioimmunoassay kit, and plasma glucose measured using a colorimetric assay. Data were analyzed using ANOVA for repeated measures design in SAS PROC MIXED. Plasma C-peptide and serum insulin concentrations**,** insulin:glucose ratio, insulin:C-peptide ratio, and peak glucose were lower for the low dose than the high dose, using all sample times ($p=0.0182$, $p<0.0001$, $p=0.0257$, $p<0.0001$, $p=0.0356$). Plasma C-peptide increased visually following IV glucose

administration, but this increase was not significant. Serum insulin, insulin:C-peptide ratio, and insulin:glucose ratio were higher for d-1 than d-2 ($p<0.0001$, $p=0.0036$, $p=0.0002$), and plasma glucose was lower for d-1 than d-2 ($p=0.0006$). Because this topic is incompletely understood, the findings of this study contribute to overall understanding of characteristic equine insulin and C-peptide dynamics following IV glucose administration.

Effects of Chromium Supplementation on C-peptide and Hair of Quarter Horse Geldings, and of Low and High Glucose Doses During an Intravenous Glucose Tolerance Test

by
Ellen Woodring Goertzen

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Animal Science

Raleigh, North Carolina

2015

APPROVED BY:

_____	_____
Dr. Paul Siciliano	Dr. Jerry Spears

_____
Dr. Shannon Pratt-Phillips
Committee Chair

DEDICATION

This thesis is dedicated to my family, my closest friends, my mentors, and my riding students. I would like to thank my parents for their undying support and for always having complete faith in me. To my dearest friends, David Hull and Sarah Lyons, who continue to love and support me unconditionally, even as we travel in different directions in life. To my most influential mentors in my career with horses, including: Vernell Falgout, Denise Blanks, Anne Brzezicki, and Jessica Roberson. To my riding students, thank you for reminding me every day why we do the work that we do. Your courage to rise to every challenge, dedication to your sport, and intense love for your horses are clear and strong in everything you do. Thank you for serving as a reminder to always do what you love, and to love it wholeheartedly. Finally, this thesis is dedicated to Whit, who taught me to be patient and never give up.

BIOGRAPHY

Ellen Woodring Goertzen has always had a love for all animals, but her strong passion for horses took hold when she began taking riding lessons in Petal, Mississippi at the age of 9. She continued to take lessons and rode every horse she could, eventually convincing her parents that this was not a phase, but a lifelong passion. With their support, she owned a Thoroughbred gelding in college and trained him from the racetrack to novice level eventing before his retirement. Ellen earned a Bachelor of Science in Animal Science (Horse concentration) in 2013 from Middle Tennessee State University, where she competed actively on the Equestrian Team in Intercollege Horse Show Association events. She then moved to Raleigh, North Carolina to pursue a Master of Science degree in Animal Science, specializing in equine nutrition, under the guidance and direction of Dr. Shannon Pratt-Phillips. Ellen currently teaches riding lessons in Chapel Hill, NC, where she lives with her dog Nero and cat Scully.

ACKNOWLEDGMENTS

I could not be more aware that I owe my current success entirely to the support of the following people:

Dr. Pratt-Phillips, thank you for always being a source of encouragement and direction;

Missy Lloyd, without whom my entire project would have fallen apart, thank you for your undying patience as I attempted to gain a small part of your incredible knowledge;

Dr. Siciliano and Dr. Spears, thank you for supporting me in this process;

Lawson Walston, thank you for your helpful and willing attitude, and for taking such good care of all of our horses;

Tabatha Wilson, thank you for your stories, your hard work, and your incredible way with the horses;

Marian Correll, thank you for always being willing to help, and for always having the answer;

The research team: Caitlin Pritchett, Caroline Chang, Morghan Hastings, Daniel Adams, Alex Albright, Shannon Steiner, Kathryn Nilsson, and Lizzie Treece. I am forever inspired by your work ethic, attitude, and commitment to making this project the best it could be. Thank you so much for all your help.

The research horses, thank you for providing me with an exciting job every day, for participating in the study, and for allowing us to complete the study successfully.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: LITERATURE REVIEW

1.1 Digestion, Insulin and C-peptide

1.1 a. Carbohydrate Digestion in Horses

Horses are natural grazers that utilize a variety of forage carbohydrates for energy through hindgut fermentation. The digestive system of the horse evolved to utilize a diet of hydrolysable and fermentable forage carbohydrates; additionally, a large percentage of modern horses are supplemented with high amounts of cereal grains or other concentrated feeds high in energy. Several disorders have been associated with feeding concentrated feeds, including colic, gastric ulcers, developmental orthopedic disease, and polysaccharide storage myopathy, among others. Metabolic disorders of great significance resulting from feeding high amounts of concentrated feeds include obesity, laminitis, and insulin resistance, the combination of which is known as equine metabolic syndrome (Hoffman 2013, Frank et al. 2010).

For purposes of discussing equine digestion, carbohydrates can be categorized into two main groups: those hydrolyzed to simple sugars in the small intestine (with α-1,4 glycosidic linkages), and those which cannot be hydrolyzed and are therefore fermented to volatile fatty acids by microflora in the hindgut (with β-1,4 glycosidic linkages) (Hoffman 2013). Simple carbohydrates are hydrolyzed in the stomach by gastric acid, and further in the small intestine by enzymes including pancreatic amylase, glucosidase, and galactosidase. Brush border disaccharidases continue this hydrolysis to produce free simple sugars such as glucose, fructose, and galactose. Glucose is absorbed by the SGLT1 (Na+/K+-ATPase, glucose cotransporter) at low capacity, and by facilitative glucose transporters (GLUT) at high capacity (Hoffman 2013).

Complex carbohydrates include fiber sources hemicellulose, cellulose, and lignin, which are digested by microbial populations in the hindgut. These carbohydrates can be fed in large amounts without resulting in adverse effects. However, when simple carbohydrates are fed in excess of the enzymatic and absorptive capacities of the small intestine, hydrolysable carbohydrate overload results. Rapid fermentation in the hindgut produces changes in microbial populations, resulting in a lower cecal pH due to accumulation of lactic acid. The normal range of cecal pH is 6.4 to 6.7 in grazing horses. A pH of less than 6 indicates acidosis, associated with a number of conditions such as diarrhea, increased risk of endotoxemia, colic, and laminitis. Overconsumption of fructans, found in cool season grasses, also results in carbohydrate overload and induces laminitis. It is currently recommended to limit starch intake to less than 2 g of starch per kg body weight for each meal to avoid overload. (Hoffman 2013).

1.1 b. Obesity, Laminitis, and Insulin Resistance

Obesity is defined as a disease involving an expanded mass of adipose tissue in the body to an extent in which health is adversely affected. Generally, obesity is a result of caloric intake in excess of requirements. Obesity is associated with many genetic and environmental factors, including the overfeeding of grain-based concentrates, genetic predisposition, high caloric intake due to carbohydrate-rich available forage in certain times of year, and lack of physical activity (Geor and Harris 2013). Obesity in horses is identified through use of the Henneke body condition scoring system (Henneke et al. 1983). This system uses a scale of 1-9 to assess overall body fat deposition, with 1 being extremely emaciated and 9 being extremely obese. Horses scoring a 7 are considered overweight, while horses scoring an 8 or 9 are considered obese. This system requires both visual and palpable

evaluation and does involve some subjective judgements, and can be done without any specialized equipment. Ultrasonic measurement of fat thickness taken over the withers, ribs, rump, and tailhead is another non-invasive tool to quantify overall body fat composition, but requires specialized equipment. A strong correlation exists between body condition scores and actual subcutaneous fat; therefore, body condition scoring can be considered an accurate and easily performed measurement of overall body fat composition (Gentry et al. 2004).

Obesity has been associated with insulin resistance in horses. Several studies have determined a relationship between high body condition (horses which are overweight or obese) and insulin resistance (Vick et al. 2007, Frank et al. 2006, Powell et al. 2002). In a study on Arabian geldings, insulin sensitivity was reduced to one quarter of the baseline concentration after the horses gained 20% of their body weight, increasing on average in BCS from a 6 to an 8 (Carter et al. 2009). Weight loss in obese horses results in improved insulin sensitivity (Van Weyenberg et al. 2008). In a study by Hoffman et al. (2003), insulin sensitivity was 80% lower in obese horses than horses of healthy weight. It is important to note that while high BCS is often correlated with insulin resistance, this condition can occur in horses of normal BCS, and not all obese horses exhibit insulin resistance (Geor and Harris 2013b).

Laminitis refers to a systemic condition in the hoof associated with severe pain and lameness, which involves deterioration of the bond between the inner hoof wall and the coffin bone. Laminitis can be acute or chronic, and chronic severe laminitis can result in the rotation of the coffin bone, known as founder. Laminitis has a variety of causes, including weight overload due to injury to other limbs, metabolic disorders such as insulin resistance, and inflammatory conditions, which may be due to carbohydrate overload. Laminitis can

also be induced by prolonged hyperinsuminemia (De Laat et al. 2010). Horses at high risk for laminitis associated with carbohydrate overload include those on rich pasture and those that have had the opportunity to gorge themselves on concentrated feeds, particularly if these horses are already overweight (Geor and Harris 2013a).

Insulin resistance is defined as an abnormal metabolic state in which normal circulating concentrations of insulin do not elicit a sufficient physiologic response in target tissues (Hoffman et al. 2003). Though the incidence of diabetes is very rare in horses, the pathology of insulin resistance is similar to that of type 2 diabetes in humans. Cells in insulin resistant tissues require a greater concentration of insulin to stimulate uptake of glucose. Insulin resistance is associated with diets high in simple sugars, and the common practice of feeding carbohydrate-rich concentrates to horses may increase its incidence (Hoffman et al. 2003). Insulin resistance is a risk factor in obese and sedentary horses, especially those with Cushing's disease, grass founder, equine metabolic syndrome, and laminitis. Recent research suggests that starch should be limited to less than 1 g starch per kg body weight per meal to limit the incidence of these conditions, and to less than 0.3 g starch per kg body weight per meal in horses known to have one or more of these conditions (Vervuert et al. 2009, Hoffman et al. 2009).

The development of insulin resistance results from the combination of several factors. In horses, increased simple carbohydrate intake results in downregulation of GLUT4. It also results in an imbalance of many factors such as free fatty acids, adipokines, and glucocorticoids, and adiponectin. Insulin resistance is highly associated with obesity. Because adipose tissue plays an endocrine role in regulation of glucose, FFA, and other

hormones, overabundance and subsequent dysfunction of adipose tissue can play a major contributing role in the development of insulin resistance (Mlinar et al. 2007).

1.1 c. Insulin and C-peptide Metabolism

Insulin is a polypeptide hormone that serves to regulate circulating blood glucose concentrations through the combination of its effects on carbohydrate, protein, and lipid metabolism (Moller and Flier 1991). Insulin is a small protein weighing about 6,000 daltons, comprised of two peptide chains bound by disulfide bonds. It is synthesized in the islets of Langerhans in the pancreas. Its first precursor molecule is a single chain, preproinsulin, which becomes proinsulin after the removal of the signal peptide. This process occurs rapidly, as preproinsulin is not found in pancreatic β-cells (Bonser and Garcia-Webb 1984). Proinsulin consists of A and B chains, connected by a peptide known as C-peptide. C-peptide is cleaved from proinsulin by endopeptidases in the endoplasmic reticulum of the β-cell, producing active insulin. Insulin and C-peptide accumulate into secretory granules in the cytoplasm, and are released from the cell by exocytosis when stimulated (Akers and Denbow 2013).

Insulin and C-peptide are secreted from the β-cells of the pancreas in equimolar amounts upon stimulation by increased blood glucose concentrations (Steiner 2004). Normal blood glucose concentrations range from 3.3 to 5 mmol/L in healthy horses, and must be maintained within this fairly narrow range (Ralston 2002). When blood glucose is elevated, glucose is transported into cells in target tissues such as liver and muscle by facilitated diffusion by transporter proteins. As the glucose concentration increases intracellularly, a change in membrane polarization occurs, inducing an influx of extracellular calcium into the cell. This influx is a primary stimulator for exocytosis of insulin and C-peptide from the

pancreatic β-cells (Akers and Denbow 2013). In general, increases in glucose result in increases in insulin secretion and decreases in hepatic extraction (Duckworth et al. 1998).

Glucose is taken into the target cells by facilitated diffusion via carrier proteins (GLUT) in the cell membrane. Insulin stimulates glucose uptake in adipose tissue and skeletal muscle, where it stimulates GLUT4 to translocate from vesicles within the cell to the cell membrane in order to transfer circulating glucose into the cell. Of these two tissues, glucose disposal takes place mainly in skeletal muscle (Deems et al. 1994). As well as promoting glucose uptake into cells, insulin also suppresses glucose secretion by the liver, and the combination of these actions results in lower circulating blood glucose levels (Moller and Flier 1991).

Insulin elicits its effect on by binding to a single-transmembrane domain receptor, consisting of both extracellular ligand binding and intracellular tyrosine kinase domains. The insulin receptor is a heterotetramer found in cells in liver tissue, adipose tissue, and skeletal muscle. When insulin binds to the extracellular portion, the kinase activity of the receptor results in a complex signaling cascade through a second-messenger system (Schinner et al. 2005). The binding of insulin results in phosphorylation of the receptor on tyrosine residues, which subsequently results in activation of downstream signal proteins in one of three possible pathways: phosphoinositide-3-kinase (PI3K), CAP/Cbl/TC10, and mitogen-activated protein kinase (MAPK). In the PI3K pathway, phosphorylated tyrosine residues on insulin receptor substrates (IRS) interact with PI3K, resulting in phosphorylation of PIP2 to PIP3. PIP3 acts as a second messenger and activates PDK-1, which phosphorylates PKB. PKB then inactivates GSK-3 by phosphorylation, which results in glycogen synthesis. PKB also regulates fatty acid synthase production, downregulates PEPCK and other related genes,

and induces the translocation of GLUT4 to the plasma membrane. The CAP/Cbl/TC10 pathway reinforces this translocation. The MAPK pathway regulates gene transcription, resulting in cell proliferation and differentiation (Mlinar et al. 2007).

In human individuals with insulin resistance, the action of insulin is impaired by its inactivation in circulation, as well as faults at the receptor level and in insulin signaling. Type A insulin resistance is an inherited syndrome and is generally more severe than Type B, though only Type B has been observed in horses to the author's knowledge. Type A insulin resistance is associated with defects in the insulin receptor and its capacity to produce adequate insulin signaling (Moller and Flier 1991). It is attributed to a mutation involving decreased phosphorylation of the tyrosine residue on the β subunit of the insulin receptor after binding of insulin to the receptor. Type B insulin resistance is attributed to overproduction of autoantibodies to the insulin receptor (Mlinar et al. 2007). In rare cases, the insulin receptor substrates (IRS) may possess mutations that contribute to insulin resistance. Reduced translocation of the insulin-regulated glucose transporter GLUT4 to the plasma membrane, can also be a major contributing factor. In the insulin signaling cascade, defects may be caused by increased levels of TNF-α and FFA, which increase serine phosphorylation of IRS and thus impair signal transduction. Other important factors in normal insulin signaling are phosphatases such as protein-tyrosine-phosphatase 1B (PTP1B) which terminate insulin signaling at the appropriate time in healthy individuals, and unusually high levels of these phosphatases are conducive to insulin resistance. It is not unusual for many of these factors fall within the overall phenotype of obesity, which continues the degeneration of healthy insulin metabolism (Mlinar et al. 2007, Schinner et al. 2005). While it is not completely known whether the mechanism of acquiring insulin

resistance in humans is identical to that in horses, it appears to be similar, and is also highly correlated with obesity in horses.

Several studies have shown the secretion of insulin and C-peptide in a biphasic pattern, though the literature regarding horses specifically is very limited. Across many species, the magnitude and duration of the peaks depend on mode of administration (oral or IV) and amount of glucose administered. If glucose is administered IV, the first phase is an acute and dramatic peak 1-2 minutes after glucose administration, and the second is more gradual with a lower, flatter curve (Henquin et al. 2002, Kjems et al. 2001). The effect of oral glucose administration on insulin kinetics is not completely known, but it is thought that if the biphasic pattern exists, the peaks are much less dramatic and exhibit a lower, flatter curve. Caumo et al. (2004) report that plasma insulin response to oral administration does not follow a biphasic pattern in humans, though it may be that the response curves are too moderate to exhibit a clear visual pattern.

In addition to differences in response pattern between oral and IV glucose, magnitude of insulin and C-peptide responses are variable depending on the mode of glucose administration. Ahrén et al. (2008) report that peaks and AUC for insulin and C-peptide were higher in mice given oral glucose vs. IV glucose. This effect may be attributed to incretin, a hormone that upregulates insulin secretion in response to oral glucose administration, and neural responses to ingestion of nutrients (Ahrén et al. 2008, Caumo et al. 2004). In contrast, Shapiro et al. (1987) found no differences in insulin secretory response between oral and IV mode of administration in humans. However, it is difficult to quantitatively compare responses based on AUC due to difficulties in equalizing the amount of glucose given orally vs. IV. Duckworth et al. (1998) reported that ingested glucose also

increases insulin uptake by the liver, while glucose administered intravenously does not. This effect is likely due to signaling from the gastrointestinal system and confounding factors such as glucose absorption rates.

The origins of insulin secretion for the first and second phases are not completely clear. It has been proposed that the first phase represents the release of stored insulin and C-peptide from the pancreatic β cell, while the second phase represents secretion of stored as well as newly synthesized insulin and C-peptide (Wilcox 2005). Other research suggests that secretions contributing to the second phase are released from a reserve pool within the pancreatic β cell (Henquin et al. 2002). In horses, the exact timing of the second phase has not been determined, but may be approximately 40-60 minutes after glucose administration (Pratt et al. 2005). Giraudet et al. (1994) did not find evidence of the second phase, but endogenous insulin secretion was examined for only 30 minutes. Further research is needed to confirm the exact shape of the insulin response. The lack of a second phase in some cases may also be due to glucose concentrations being too low to elicit a visible second peak (Tóth et al. 2010). These variations may be due largely to factors involved in oral glucose such as gastric emptying, glucose absorption, gastrointestinal motility, hormones, and neural input that serve to modify the insulin response (Caumo et al. 2004). It is also important to note the range of glucose concentrations administered in the aforementioned studies on horses. When an IVGTT was performed in horses, doses of glucose administered ranged from 300-500 mg glucose per kg horse body weight. Tóth et al. (2010) and Hoffman et al. (2003) used 300 mg/kg; Giraudet et al. (1994) used 330 mg/kg; and Pratt et al. (2005) used 500 mg/kg.

After secretion by the pancreas, insulin passes through the liver. A significant percentage of insulin is extracted by the liver for local use in carbohydrate metabolism, and

what is not used is stored for release upon stimulation into the portal blood to aid in metabolism in peripheral tissues. Therefore, the amount of insulin in circulation is determined by both pancreatic secretion and hepatic extraction, and measurement of plasma insulin is not necessarily representative of pancreatic secretion alone. The amount of insulin extracted by the liver during this first pass varies due to blood glucose concentration, amount of insulin presented to the liver, and overall blood flow. When pancreatic insulin secretion is high, hepatic extraction typically occurs at a higher rate than when insulin secretion is low (Field 1973). First-pass hepatic extraction in humans is consistently measured between 50-90%, though some of this insulin is released from the liver and re-enters circulation (Meier 2005, Duckworth et al. 1998). C-peptide is extracted by the liver at relatively low efficiency. Instead, it is cleared efficiently by the kidneys by both glomerular filtration and peritubular extraction, and is not stored in muscle or adipose tissue. Insulin has a relatively short half-life of 4-6 minutes; while insulin is removed from circulation rapidly by the liver, C-peptide remains stable in circulation for greater than 30 minutes (Duckworth et al. 1998, Polonsky et al. 1986, Verchere et al. 1996). Therefore, plasma C-peptide is representative of pancreatic insulin secretion and β-cell function.

When insulin reaches target tissues, it is taken up into the cell mainly by receptor-mediated endocytosis. Insulin binds to its receptor and is then internalized by endocytosis either into endosomes or delivered within the cell to other sites including the cytosol, nucleus, Golgi, and others. These endosomes acidify upon formation, triggering the release of insulin from its receptor. Internalized insulin is either released back into circulation either intact or partially degraded, or is degraded by insulin-degrading enzyme (IDE), which is located mainly in the cytosol and in smaller concentrations in lysosomes. IDE is also found

in small amounts on the plasma membrane, indicating that small amounts of insulin may be degraded on the membrane as well as within the cell. Insulin that is released from the cell is cleared from circulation by the kidney. Insulin which escapes degradation by the liver and kidney is degraded by other insulin-sensitive tissues such as muscle and adipocytes. The rate of insulin clearance is affected by obesity and diabetes, as well as other factors including location of fat deposition, growth hormone concentrations, sex hormone concentrations, free fatty acids, and overall liver function (Duckworth et al. 1998).

1.1 d. C-peptide

C-peptide is a biologically active endogenous peptide hormone consisting of 31 amino acids, and serves to connect the A and B chains of proinsulin. Endopeptidases cleave C-peptide from proinsulin to produce active insulin. Both insulin and C-peptide are then secreted from the β-cells of the pancreas and appear in plasma in equimolar amounts (Bonser and Garcia-Webb 1984).

C-peptide functions in insulin formation by promoting efficient polypeptide folding and correct pairing of the A and B chains of proinsulin. It assists in the sorting of proinsulin into storage granules within the pancreatic β-cells, and also facilitates enzymatic cleavage of insulin from proinsulin. Its most significant function is its clinical use in assessing pancreatic β-cell function; because it is secreted from the pancreas in equimolar amounts with insulin, it serves as an independent indicator of insulin secretory rate. C-peptide is relatively stable in circulation and exhibits a relatively slow rate of turnover, taking greater than 30 minutes to be cleared from circulation. In humans, insulin has a half-life of 3 minutes or less (Steiner 2004). C-peptide has been found to be elevated in obese individuals, a finding consistent with that of elevated insulin in obese individuals (Bonser and Garcia-Webb 1983). While it

was previously assumed that C-peptide had no known biological function, there now exists a large body of evidence supporting a variety of biological functions including regulating insulin secretion, upregulating Na^+,K^+-ATPase activity, and stimulating blood flow, and administration of exogenous C-peptide has numerous beneficial effects for individuals with diabetes (Rigler et al. 1999, Ohtomo et al. 1998).

To the author's knowledge, only three studies have measured C-peptide in horses. Johnson et al. (2005) report that serum C-peptide concentrations were abnormally low in a mare with suspected diabetes mellitus. Researchers used a commercial double antibody RIA kit to measure C-peptide concentrations; however, no validation for the kit was reported in this study, so results should be interpreted with caution. In a 2010 study, Tóth et al. (2010) measured both insulin and C-peptide to assess pancreatic function and determine C-peptide kinetics such as clearance rate and plasma half-life. They performed a validation for a human double antibody radioimmunoassay (RIA) kit for the assessment of horse C-peptide in serum. However, it is important to note that the validation was not entirely successful (spike and recovery ratios were on average 123 ± 8% rather than the target range of 100 ± 20%). The kit was adequate for the researchers' purpose of measuring change in C-peptide concentrations, and relative to insulin, but did not provide an accurate measure of serum C-peptide concentrations. Tóth et al. (2010) determined that endogenous insulin and C-peptide secretion were stimulated by dextrose infusion, and partially suppressed by somatostatin. Geor et al. (2010) also found that C-peptide concentrations were suppressed by somatostatin infusion and used the same RIA kit as Tóth et al (2010). By suppressing endogenous C-peptide secretion, Tóth et al. (2010) were able to determine that the clearance rate of serum C-peptide human equivalents was 0.83 ml/min/kg body weight, with a range of 0.15-1.61

ml/min/kg body weight. They found that the ratio of insulin to C-peptide in horses with insulin resistance was significantly higher than that ratio in healthy horses; insulin resistant horses had insulin concentrations 5% higher than C-peptide concentrations, while healthy horses had insulin concentrations 74% lower than C-peptide concentrations. While insulin and C-peptide are secreted in equimolar amounts, circulating concentrations of insulin are lower than C-peptide in healthy horses due to efficient clearance by the liver. Insulin concentrations are higher in insulin resistant horses; this may be due to reduced efficiency of hepatic clearance, but that conclusion relies on the assumption that C-peptide clearance is unaffected by insulin resistance. Median C-peptide concentrations in insulin resistant horses were 1.5-fold higher, and median insulin concentrations were 9.5-fold higher than corresponding values in healthy horses. These findings indicate that elevated plasma insulin concentrations (hyperinsulinemia) can occur due to both increased pancreatic insulin secretion and decreased insulin clearance by the liver, and that the balance of insulin to C-peptide is compromised in insulin resistant horses by reduction of the body's ability to clear insulin (Tóth et al. 2010).

1.2 Chromium

1.2 a. The Role of Chromium in Insulin Dynamics

Chromium is an essential nutrient required in trace amounts for normal metabolism in mammals. Chromium serves as a cofactor for reactions involved in the breakdown of carbohydrates, proteins, and fats. It is thought to have a beneficial effect on metabolic syndrome, cardiovascular disease, and insulin sensitivity by upregulating insulin signaling. Subclinical chromium deficiency results in elevated blood glucose and insulin and decreased

high-density lipoproteins. Clinical chromium deficiency results in nerve and brain disorders, which are typically reversed by chromium supplementation. Individuals with type 2 diabetes are reported to have relatively low levels of circulating chromium, which indicates that chromium may play a role in maintaining a healthy level of insulin sensitivity. Chromium exists in the highest concentration as trivalent chromium, and in a highly toxic, less abundant form of hexavalent chromium. Dietary supplements of chromium are commonly available in its most stable and bioavailable form of chromium picolinate, as well as chromium propionate, chromium nicotinate, and chromium chloride (Hummel et al. 2007).

Approximately 0.4% to 1.5% of ingested inorganic chromium is absorbed, while chromium in yeast may be absorbed at a rate of 10-15% (Anderson et al. 1989, Jeejeebhoy 1999). Chromium that is not absorbed is excreted in the feces (Anderson et al. 1989). Chromium is absorbed into mucosal cells of the small intestine. The mechanism of absorption is not completely known. However, chromium absorption appears to be saturable but not requiring energy, indicating that it is most likely absorbed by facilitated transport (Ducros 1992).

A number of factors influence absorption. Oxalates, iron and zinc deficiency, and diabetes increase absorption, while phytates and increased age decrease absorption. Chromium binds to transferrin and to albumin to a lesser extent (Jeejeebhoy 1999). Chromium is distributed and stored throughout the body, particularly in the liver, spleen, and kidney, as well as soft tissue and bone (Hummel et al. 2007). It is stored in pools of varying turnover rates: rapid, with a half life of 0.5-12 h; medium, with a half life of 1-14 d; and slow, with a half life of 3-12 months. Chromium is thought to be well controlled metabolically and typically does not accumulate in toxic amounts. In many clinical trials

administering dietary chromium ranging from 200-1000 μg per d, very few cases of adverse effects were reported (Jeejeebhoy 1999). In other studies, high doses of chromium picolinate have resulted in production of hydroxyl radicals, mild oxidative stress, and chromosomal damage. Chronic exposure to high levels of chromium in the environment can induce respiratory and gastrointestinal cancer (Saner et al. 1984). Chromium is transported in the blood by the iron transport protein transferrin, and competes with iron for binding sites, thereby inhibiting iron uptake (Stearns 2000). Chromium is mobilized from stores and utilized in response to elevated blood glucose concentrations. Chromium that is utilized by the body is not recycled, but rather is rapidly excreted primarily in the urine. Small amounts are excreted in the hair, sweat, and bile, which may be good indicators of overexposure to chromium (Kobla 2000). The amount excreted is elevated by factors such as high sugar intake, exercise, trauma, pregnancy, and lactation (Anderson 1989).

Chromium has been used in human medicine to relieve symptoms of type 2 diabetes since the 1960s, when it was found that rats fed a diet deficient in chromium lacked the ability to mediate blood glucose concentrations, an effect that was reversed upon reintroduction of chromium to the diet (Vincent 2000). It is marketed as a nutritional supplement to assist in weight loss by lowering blood glucose, reducing fat accumulation in the body, suppressing appetite, and stimulating heat production to increase energy expenditure.

Chromium may affect glucose uptake and insulin dynamics in a number of ways; however, no literature exists on the effect of chromium on C-peptide dynamics. It is generally thought that chromium is necessary for normal action of tyrosine kinase, which is required for downstream phosphorylation in the insulin-signaling cascade. Chromium also

allows the insulin receptor to remain active by the inhibitory effect of on phosphotyrosine phosphatase, which inactivates the insulin receptor (Anderson 1998). Chromium is thought to elicit its activities as its biologically active form, low-molecular-weight chromium-binding-substance (LMWCr). This substance is a complex of four equivalents of chromic ions connected by anionic ligand bridges, though the exact amino acid sequence and structure are unknown. It is thought that upon transport into the cell, chromium results in the production of LMWCr, which binds to the insulin-activated insulin receptor. This binding stimulates tyrosine kinase activity and increases the stability of the active insulin receptor. After blood insulin concentrations normalize, LMWCr activity is terminated by its clearance from the cell and it is excreted from the body in the urine. Increased tyrosine kinase activity increases downstream phosphorylation and ultimately results in increased insulin sensitivity (Vincent 2000). Davis et al. suggest that insulin resistance and type 2 diabetes resulting from chromium deficiency may be due to insufficient receptor kinase activity, as a result of insufficient quantities of chromium to generate enough LMWCr (1997).

Wang et al. (2006) proposed another mechanism by which chromium may elicit its effect. They reported that chromium supplementation resulted in increased GLUT-4 translocation from an intracellular compartment to the plasma membrane in adipocytes, thereby increasing insulin-stimulated glucose transport across that membrane and ultimately resulting in increased insulin sensitivity.

Another possible mechanism in adipocytes is chromium affecting cholesterol homeostasis to upregulate the glucose transporter system. Chromium picolinate decreases plasma membrane cholesterol content and increases membrane fluidity, the combination of which allow a greater number of GLUT4 transporters to be present in the membrane, and

thereby increase glucose uptake by the cell. This effect was seen in obese animals with an overabundance of cholesterol in the plasma membrane, and may not be present in healthy individuals (Pattar et al. 2006). Chromium may also act directly on the plasma membrane. Individuals who consumed chromium picolinate and nicotinate supplements had increased membrane fluidity. This may be due to chromium inserting into the plasma membrane, decreasing hydrogen bonding and increasing membrane fluidity (Evans and Bowman 1992). These studies propose several possible mechanisms for the positive effect of chromium on insulin sensitivity: insulin causing chromium to move into the cell and form LMWCr, which then stimulates insulin signaling by its effect on downstream proteins; increased GLUT-4 translocation to the plasma membrane; by decreasing plasma cholesterol to upregulate the glucose transporter system; and by inserting into the cell membrane to increase membrane fluidity. It is likely that the effect of chromium can be attributed to the combination of these effects.

The effects of chromium supplementation have been studied in a variety of species and chromium forms, including chromium picolinate, chloride, and propionate. In humans, chromium has proved to be a very promising therapy for individuals with type 2 diabetes. Anderson et al. (1997) found that supplementing chromium picolinate to individuals with type 2 diabetes resulted in lower fasting plasma glucose and insulin concentrations. Cefalu et al. (1999) found that a similar supplement resulted in a significant increase in insulin sensitivity in obese individuals who were at high risk for type 2-diabetes due to family history, when compared to a control group. Numerous other studies have determined beneficial effects of supplemental chromium picolinate in individuals with type 2 diabetes including increased glucose clearance rates (Ghosh et al. 2002), decreased fasting plasma

glucose (Anderson et al. 1997), decreased fasting plasma insulin (Anderson et al. 1997), improved glucose control (Martin et al. 2006, Ghosh et al. 2002), and overall improved insulin sensitivity (Cefalu et al. 1999, Martin et al. 2006, Wilson and Gondy 1995). Chromium picolinate was found to increase glucose uptake by adipocytes cultured in diabetic conditions (Pattar et al. 2006). Chromium in brewer's yeast was also found to lower fasting plasma glucose (Racek et al. 2013). However, these results are not always consistent across studies; chromium had no effect on glucose tolerance in people with impaired glucose tolerance (Uusitupa et al. 1992). Despite the literature supporting the benefits of chromium in people with type 2-diabetes, its effects have not been substantiated in non-diabetic individuals. Many studies report no beneficial effects of supplementation in non-obese, non-diabetic individuals on fasting glucose concentration (Wilson and Gondy 1995) and insulin sensitivity (Amato 2000). In one study, chromium picolinate actually resulted in a decline in insulin sensitivity in healthy, non-obese subjects (Masharani et al. 2012).

The effects of dietary chromium in various forms, both organic and inorganic, have been extensively studied in a variety of animal species, including cats, dogs, pigs, cattle, rats, chickens, and horses. One of the most commonly observed effects of chromium supplementation is increased glucose tolerance as indicated by increased glucose clearance rates, decreased glucose half life, and decreased plasma glucose concentrations. Increased glucose clearance rates following IV glucose administration have been observed when feeding chromium as chromium picolinate to growing calves (Bunting et al. 1994), growing pigs (Amoikon et al. 1995), insulin resistant rats (Striffler et al. 1998, Wang et al. 2006), and chromium methionine to cows (Kegley et al. 2000). This effect as well as decreased glucose half life was also seen following feeding a concentrate diet to pigs fed chromium yeast (Guan

et al. 2000). Glucose half life after IV glucose administration decreased in additional studies feeding chromium picolinate to cats (Appleton et al. 2002) and chromium methionine to cows (Kegley et al. 2000). Decreased circulating plasma glucose has been observed following both IV and feeding concentrate when feeding chromium picolinate to cats, pigs, and chickens (Appleton et al. 2002, Woodworth et al. 2007, Lien et al. 1999). Chromium chloride has reduced circulating plasma concentrations of glucose, insulin, and cortisol (Kumar et al. 2013). Chromium picolinate and nicotinate have also had effects on insulin dynamics, such as decreased fasting plasma insulin in pigs (Amoikon et al. 1995), rats (Striffler et al. 1998, Kim et al. 2002), and chickens (Lien et al. 1999). Chromium as yeast has also decreased half life and increased rate of clearance of insulin and C-peptide in pigs (Guan et al. 2000). In diabetic dogs, chromium picolinate had no effect on any glucose or insulin parameters, though no glucose challenge was administered (Schachter et al. 2001). Chromium as yeast may actually increase plasma insulin (Guan et al. 2000), but this effect does not equate to decreased insulin sensitivity when combined with increased glucose clearance rate. Table 1 is a summary of studies on various types of dietary chromium in non-horse species.

Research on the effects of chromium propionate suggest its potential to improve glucose tolerance and insulin sensitivity. A summary of these studies is shown in Table 2. Rajalekshmi et al. (2014) report that chromium propionate reduced serum glucose levels in broiler birds, though no glucose challenge was administered in this study. When fed to growing dairy heifers, chromium propionate increased fasting glucose and decreased fasting insulin in a dose-dependent manner, as well as increasing glucose clearance rate (Sumner et al. 2007). However, in a study by Król et al. (2014), chromium propionate did not attenuate

development of insulin resistance in rats fed a high-fat diet. Chromium propionate was recently approved for use in cattle diets at levels of to 0.5 mg/kg dry matter (Spears et al. 2012). They determined that chromium requirements of growing heifers can be met by supplementing chromium propionate at 3 mg CR/d, equivalent to 0.47 mg CR/kg DM.

Chromium propionate appears to be safe, but levels of toxicity have not been determined. It does not appear to be deposited into animal products meant for human consumption. This was determined in cattle by Lloyd et al. (2010), in a study supplementing lactating dairy cows at 4 times the level permitted to be fed to dairy cattle by the FDA, with no changes in chromium concentration in milk, muscle, or fat. Ma et al. (2014) found no abnormal levels of deposition of chromium in eggs of laying chickens fed 4 concentrations of a CRprop supplement. In an attempt to determine LD_{50}, Staniek et al. (2010) administered 2000 mg/kg BW to rats with no adverse side effects.

Literature regarding chromium supplementation in horses specifically has varying results, and merits further investigation. Table 3 is summary of current research of chromium in horses. Chromium picolinate supplementation in healthy, non-obese horses had no apparent effects on resting plasma glucose or glucose response following IV glucose administration (Uyanik et al. 2008, Gentry et al. 1999). Chromium picolinate resulted in an increase in serum insulin following IV glucose administration (Gentry et al. 1999). In yearlings fed 3 levels of the supplement for 112 d, chromium picolinate increased glucose clearance rate and decreased glucose half-life following IV glucose administration (Ott and Kivipelto 1999). Frape (1998) reported that chromium as yeast decreased plasma glucose and insulin concentrations in healthy, non-obese horses fed 12 mg/d, though no challenge was reported. A similar supplement decreased insulin response following feeding in non-

obese horses fed 5 g/d, but only when combined with exercise (Pagan et al. 1995). However, in another study where the horses were exercised, chromium as yeast had no effect on insulin or glucose parameters, though no glucose challenge was administered in this study (Vervuert et al. 2006). A supplement containing chromium as yeast plus magnesium had no effect after IV glucose administration on obese, laminitic horses that were not exercising (Chameroy et al. 2011). Chromium propionate fed to hyperinsulenemic horses had no effect on glucose and insulin parameters following IV glucose administration (Cartmill et al. 2004).

Conclusions from the comparison of these studies are difficult to determine due to wide variations in physiological state of the horses, type of chromium, and concentration of chromium. It may be that chromium supplementation only elicits benefits when combined with exercise, or that the above listed studies did not feed the supplements at a high enough level to elicit benefits. Due to the lack of established research on the effect of chromium in horses, there is no well recognized dietary requirement for chromium in horse diets. However, Kentucky Equine Research has set the requirement somewhat arbitrarily at 5 mg/d (Jackson 1997).

While it is generally accepted that chromium elicits beneficial effects for individuals with glucose intolerance, hyperglycemia and type 2 diabetic individuals, current research is conflicting regarding its effect on glucose metabolism and insulin resistance in individuals without these conditions. Additionally, while there is significant literature regarding chromium supplementation in cases of deficiency, any benefit to those not deficient is highly variable and questionable and has not been consistently observed. Pagan et al. (1995) suggest that chromium plays an important role as a nutrient, but should not be considered a drug, as it elicits metabolic responses only in individuals and animals that are deficient.

Additionally, many of the studies reporting the efficacy of chromium have been funded by commercial companies, so results must be thoroughly examined and interpreted with caution.

1.2 b. Chromium in Hair

Hair analysis has been used as a bioindicator of heavy metal status in animals since the early 1960s, and has continued to evolve in the past 10 years as new analytical methods have been developed. Modern methodologies allow for detection of a wide variety of elements in hair samples; thus, the study of metal contaminants in animal hair is a relatively new and promising field. Heavy metals of endogenous origin are incorporated into the proteins in hair during formation. These elements are metabolically inert and are not removed by washing procedures, as opposed to exogenous metals from environmental contact. While it may be possible for exogenous metals to incorporate into the hair structure, thorough washing with effective solutions helps ensure the accuracy of the assay used (Chyla and Żyrnicki 2000). Heavy metal levels in wild animals can serve as an indicator of human population exposure, as well as an indicator of individual exposure to specific metals including copper, cadmium, mercury, manganese, nickel, lead, zinc, chromium, and others. Heavy metal status can also be assessed by measurement of concentrations in hair, skin, and chromium storage tissues such as the kidney and liver. Hair is advantageous because of its ease of collection from animals and because it serves as a measure of long-term exposure. Heavy metal levels in hair are often highly correlated with those in tissues, and more highly concentrated. Hair serves as a useful, non-invasive bioindicator of heavy metal status and of early warning signs of potential heavy metal toxicity and other physiological issues, as well as assessment of nutritional status (Burger et al. 1994, Asano et al. 2002). During formation of the hair shaft, the hair is exposed to elements of endogenous origin secreted from

sebaceous, apocrine, and eccrine glands (Asano et al. 2005a). The chemical composition of hair may vary by nutrition level, level of environmental pollutants, and season of the year, as well as the individual animal's breed, sex, age, physiological state, and overall state of health (Filistowicz et al. 2012).

Analysis of heavy metals in hair is a useful indicator of potential toxicities and other physiological issues in horses. For example, elevated concentrations of Ca, Sr, and Zn in mane hair of horses with an atrioventricular block suggest that concentrations of these elements can predict the susceptibility of a given horse to heart disease prior to symptoms (Asano et al. 2005a). Current literature on this topic in horses is limited. Asano et al. (2002) analyzed toxic and essential heavy metals in mane hair of horses aged 2-5 years for the purpose of establishing reference ranges for use in assessing disease states and nutritional status. They also compared these values across sexes and ages. The reference range for chromium was found to be 220 ± 160 ppb for all horses. Values for females were slightly lower than for males (200 ± 170 ppb; 230 ± 150 ppb). Chromium content also correlated with age: younger horses had higher values than older horses (300 ± 260 ppb for 2-year-olds; 160 ± 70 ppb for 5-year-olds).

Chromium values in horse hair from recent studies are shown in Table 4, though very few studies have reported ranges of chromium content in horse hair. Dobrazanski et al. (2005) reported no differences in chromium between region or season. Biricik et al. (2005) also found no effect of season on hair mineral concentrations, though chromium was not measured. Topczewska (2012) reported higher concentrations of chromium in horse hair in the winter than in the spring, and that chromium was positively correlated with concentrations of silicon. It is thought that the chromium content in horse hair is relatively

stable over time if diet and environmental conditions remain relatively stable; Krusič et al. (1990) reported that chromium content in mane hair of exercising horses did not change significantly over a period of 88 d.

It is of interest to note that minerals have different patterns of accumulation in horse hair. Armelin et al. (2003) investigated the effect of feeding a supplement containing Cu, Fe, K, Mg, and Zn chelated with glycine on horse hair. Fe, K, and Zn were different after the supplementation period, suggesting that absorption and bioaccumulation of these minerals was increased by increased oral intake, while Cu, Mg, and Mn were not affected.

It has been established that hair color greatly influences the content of various minerals in human and animal hair. For example, blond human hair contains the lowest concentration of most elements (Nowak 1998). In a study by Asano et al. (2005b) in horses, gray hair contained significantly higher levels of Cu, Ti, and Zn, and lower levels of Br, Ca, Se, and Sr. The difference in chromium levels was not significant, but tended to be higher for horses with gray hair. Chromium ranged from 380 ± 310 ppb to 700 ± 370 ppb, with an average of 430 ± 330 ppb. Therefore, hair color should be taken into consideration when possible when evaluating horse hair for mineral status.

It is of interest to note that the rate of mane hair growth in horses is linear. When studied over a 12-month period, the rate of growth varied from month to month, but was not correlated with climatic changes and the relationship between time and growth was overall linear during this period. Hair near the poll had a higher rate of growth than hair near the withers, and there was no effect of age or gender (Dunnett 2005).

Prior to analysis, hair is washed according to modified methods of the International Atomic Energy Agency (Puchyr et al. 1998). There has been some concern about the

potential of excessive washing to leach endogenous chromium out of hair samples; however, if all samples are subjected to the same washing procedure, then the assay is valid for comparison purposes (Kumpulainen et al. 1982). After washing, hair is prepared for ashing by microwave digestion, and chromium content in the resultant liquid measured through use of a graphite furnace on an atomic absorption spectrophotometer.

Objectives

Insulin resistance is condition in horses that is often correlated with obesity and laminitis. While it is now recognized that changes in dietary management such as reduction of simple sugars and starch in the diet may decrease incidence and severity of this condition, insulin resistance remains a persistent problem. Chromium supplementation in humans has been shown to reduce symptoms of type 2 diabetes, which is physiologically very similar to insulin resistance in horses. However, research on chromium supplementation in horses is limited and results are variable. Therefore, it is of interest to further investigate the effect of chromium supplementation in horses.

Furthermore, no research has been done to the author's knowledge to investigate the effect of chromium supplementation on plasma C-peptide concentrations in horses. Plasma C-peptide concentrations are a much more accurate indicator of pancreatic efficiency than serum insulin concentrations. The primary objective of the first experiment was to investigate the effect of chromium supplementation on pancreatic efficiency as indicated by plasma C-peptide concentrations following a meal or intravenous glucose administration (Chapter 2). The secondary objective was to evaluate the prevalence and extent of chromium bioaccumulation in horse mane hair during a period of chromium supplementation, and to determine whether horse mane hair could be used as an accurate bioindicator of chromium status (Chapter 3). The hypothesis was that chromium would acculumate in horse mane hair in dose-dependent amounts, thereby serving as a useful bioindicator of chromium status.

A second study was conducted to examine differences in insulin, glucose, and C-peptide kinetics during an IVGTT in horses when a low dose vs. high dose of dextrose was administered. Insulin is known to exhibit an acute first-phase peak, followed by a second

more gradual peak, in response to IV glucose administration. In the first study, it was suspected that plasma C-peptide would follow a similar pattern as it had in previous research when a larger glucose dose was administered (Tóth et al. 2010), but this was not observed. It is important to understand normal C-peptide kinetics in horses in order for C-peptide to be a useful indicator of pancreatic efficiency.

The experimental protocols in these studies were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Table 1: Chromium Picolinate, Nicotinate, Methionine, Yeast, and Chloride in Non-Horse Species

Author, year	Animal	Physiological state	Type of Chromium	Type of challenge (IV, oral, feeding), dose	General results
Striffler et al. 1998	rats	insulin resistant	picolinate	IV, 1250 mg/kg	dec. fasting plasma insulin inc. glucose clearance rate dec. insulin AUC
Amoikon et al. 1995	pigs	normal, growing	picolinate	IV, 500 mg/kg	dec. fasting plasma insulin inc. glucose clearance rate dec. glucose half life dec. insulin AUC trend
Appleton et al. 2002	cats	normal	picolinate	IV, 500 mg/kg	dec. glucose response dec. glucose AUC dec. fasting glucose
Bunting et al. 1994	cows	normal, growing	picolinate	IV, 500 mg/kg	inc. glucose clearance rate no change in insulin
Kim et al. 2002	rats	receiving dexamethasone	picolinate/nicotinate	IV, 200 mg/kg	dec. glucose AUC dec. insulin AUC dec. fasting insulin
Woodworth et al. 2007	pigs	normal, gestating	picolinate	IV; feeding	dec. insulin response dec. C-peptide response dec. glucose response
Lien et al. 1999	chickens	normal	picolinate	no challenge	dec. fasting glucose dec. fasting insulin
Wang et al. 2006	rats	insulin resistant	picolinate	no challenge (muscle biopsy)	improved glucose clearance increase in insulin signaling
Schachter et al. 2001	dogs	diabetic	picolinate	no challenge	no effects
Kegley et al. 2000	cows	normal, growing	methionine	IV, 500 mg/kg	dec. glucose response inc. glucose clearance rate

Table 1 Continued

Guan et al. 2000	pigs	normal	yeast	IV, 500 mg/kg	dec. glucose response inc. plasma insulin dec. glucose AUC (30 min) inc. glucose, insulin, C-peptide clearance rate dec. glucose, insulin, C-peptide half life
Kumar et al. 2013	buffalo	growing	chloride	no challenge	dec. fasting insulin

Table 2: Chromium Propionate in Non-horse Species

Spears et al. 2012	cows	normal	propionate	IV, 450 mg/kg	dec. glucose AUC dec. insulin response
Sumner et al. 2007	dairy heifers	growing	propionate	IV, 450 mg/kg	inc. fasting glucose dec. fasting insulin inc. glucose clearance rate
Rajalekshmi et al. 2014	chickens	normal	propionate	no challenge	dec. plasma glucose
Król et al. 2014	rats	hyperinsulinemic	propionate	no challenge	no effects

Table 3: Chromium in Horses

Author, year	Type of Chromium	Physiological State	Type of challenge (IV, oral, feeding), dose	General results
Uyanik et al. 2008	picolinate	normal mares	no challenge	no effect on glucose
Gentry et al. 1999	picolinate	normal mares	IV, 200 mg/kg	no effect on glucose inc. insulin following GTT
Ott and Kivipelto 1999	picolinate	normal yearlings	IV, 200 mg/kg	inc. glucose clearance rate dec. glucose half-life
Frape 1998	yeast	normal	NS	dec. glucose response dec. insulin response
Pagan et al. 1995	yeast	normal, exercising	feeding test w/exercise	dec. insulin response
Vervuert et al. 2006	yeast	normal, exercising	exercise (no challenge)	no effects
Chameroy et al. 2011	chromium yeast + magnesium	obese, laminitic	IV, dose NS	no effects
Cartmill et al. 2004	propionate	hyperinsulemic	IV, 200 mg/kg	no effects

Table 4: Current Reported Ranges of Chromium in Horse Mane Hair

Authors, Year	Low CR (ppb)	High CR (ppb)	Ave. CR (ppb)
Asano et al., 2002	160 ± 70	300 ± 260	220 ± 160
Asano et al., 2005	380 ± 310	700 ± 370	430 ± 330
Dobrazanski et al., 2005	300 ± 340	520 ± 320	450 ± 430
Topczewska 2012	130 ± 70	480 ± 190	220 ± 320

Literature Cited

Ahrén, B., M. S. Winzell, and G. Pacini. 2008. The augmenting effect on insulin secretion by oral versus intravenous glucose is exaggerated by high-fat diet in mice. J. Endocrinol. 197(1): 181-187.

Akers, M. R., and D.M. Denbow. 2013. Anatomy and physiology of domestic animals. 2nd ed. Ames (IA): Wiley Blackwell.

Amato, P., A. J. Morales, and S. S. Yen. Effects of chromium picolinate supplementation on insulin sensitivity, serum lipids, and body composition in healthy, nonobese, older men and women. J. Gerontol. (A Biol. Sci. Med. Sci.). 55(5): M260-M263.

Amoikon, E. K., J. M. Fernandez, L. L. Southern, D. L. Thompson, T. L. Ward, and B. M. Olcott. 1995. Effect of chromium tripicolinate on growth, glucose tolerance, insulin sensitivity, plasma metabolites, and growth hormone in pigs. J. Anim. Sci. 73(4): 1123-1130.

Anderson, R. A. 1989. Essentiality of chromium in humans. Sci. Total. Environ. 86(1): 75-81.

Anderson, R. A., N. Cheng, N. A. Bryden, M. M. Polansky, N. Cheng, J. Chi, and J. Feng. 1997. Elevated intakes of supplemental chromium improve glucose and insulin variables in individuals with type 2 diabetes. Diabetes. 46(11): 1786-1791.

Anderson, R. A. 1998. Chromium, glucose intolerance and diabetes. J. Am. Coll. Nutr. 17(6): 548.

Appleton, D. J., J. S. Rand, G. D. Sunvold, and J. Priest. 2002. Dietary chromium tripicolinate supplementation reduces glucose concentrations and improves glucose tolerance in normal-weight cats. J. Feline Med. Surg. 4(1): 13-25.

Armelin, M. J. A., R. L. Ávila, R. M. Piasentin, and M. Saiki. 2003. Effect of chelated mineral supplementation on the absorption of Cu, Fe, K, Mn and Zn in horse hair. J. Radioanal. Nucl. Ch. 258(2): 449-451.

Asano, R., K. Suzuki, T. Otsuka, M. Otsuka, and H. Sakurai. 2002. Concentrations of toxic metals and essential minerals in the mane hair of healthy racing horses and their relation to age. J. Vet. Med. Sci. 64(7): 607-610.

Asano, K., K. Suzuki, M. Chiba, K. Sera, T. Matsumoto, R. Asano, and T. Sakai. 2005a. Correlation between 25 element contents in mane hair in riding horses and atrioventricular block. Biol. Trace Elem. Res. 108(1-3): 127-135.

Asano, K., K. Suzuki, M. Chiba, K. Sera, T. Matsumoto, R. Asano, and T. Sakai. 2005b. Influence of the coat color on the trace elemental status measured by particle-induced X-ray emission in horse hair. Biol. Trace Elem. Res. 103(2): 169-176.

Biricik, H., N. Ocal, A. I. Gucus, B. Ediz, and M. Uzman. 2005. Seasonal changes of some mineral status in mares. J. Equine Vet. Sci. 25(8): 346-348.

Bonser, A. M., and P. Garcia-Webb. 1984. C-peptide measurement: methods and clinical utility. Crit. Rev. Clin. Lab. Sci. 19(4): 297-352.

Bunting, L. D., J. M. Fernandez, D. L. Thompson, and L. L. Southern. 1994. Influence of chromium picolinate on glucose usage and metabolic criteria in growing Holstein calves. J. Anim. Sci. 72(6): 1591-1599.

Burger, J., M. Marquez, and M. Gochfeld. 1994. Heavy metals in the hair of opossum from Palo Verde, Costa Rica. Arch. Environ. Con. Tox. 27(4): 472-476.

Carter, R. A., K. H. Treiber, R. J. Geor, L. Douglass, and P. A. Harris. 2009. Prediction of incipient pasture-associated laminitis from hyperinsulinaemia, hyperleptinaemia and generalised and localised obesity in a cohort of ponies. Equine Vet. J., 41(2): 171-178.

Cartmill, J. A. 2004. Leptin in horses: influences of body condition, gender, insulin insensitivity, feeding, and dexamethasone. PhD dissertation. Kansas State University.

Caumo, A., and L. Luzi. 2004. First-phase insulin secretion: does it exist in real life? Considerations on shape and function. Am. J. Physiol-Endoc. M. 287(3): E371-E385.

Cefalu, W. T., A. D. Bell-Farrow, J. Stegner, Z. Q. Wang, T. King, T. Morgan, and J. G. Terry. 1999. Effect of chromium picolinate on insulin sensitivity in vivo (abstract). J. Trace. Elem. Exp. Med. 12(2): 71-83.

Chameroy, K. A., N. Frank, S. B. Elliott, and R. C. Boston. 2011. Effects of a supplement containing chromium and magnesium on morphometric measurements, resting glucose, insulin concentrations and insulin sensitivity in laminitic obese horses. Equine vet. J. 43(4): 494-499.

Chyla, M. A., and W. Żyrnicki. 2000. Determination of metal concentrations in animal hair by the ICP method. Biol. Trace Elem. Res. 75(1-3): 187-194.

Davis, C. M., and J. B. Vincent. 1997. Chromium oligopeptide activates insulin receptor tyrosine kinase activity. Biochemistry. 36(15): 4382-4385.

Deems, R. O., R. W. Deacon, T. Ramlal, A. Volchuk, A. Klip, and D. A. Young. 1994. Insulin action on whole body glucose utilization and on muscle glucose transporter translocation in mice. Biochem. Biophys. Res. Commun. 199(2): 662-670.

De Laat, M. A., C. M. McGowan, M. N. Sillence, and C. C. Pollitt. 2010. Equine laminitis: Induced by 48 h hyperinsulineamia in Standardbred horses. Equine Vet. J. 42(2): 129-135.

Dobrzanski, Z., D. Jankowska, W. Dobicki, and R. Kupczynski. 2005. The influence of different factors on the concentration of elements in hair of horses. Animals and environment. 2. Proceedings of the XIIth ISAH Congress on Animal Hygiene, Warsaw, Poland, 4-8 September.

Duckworth, W. C., R. G. Bennett, and F. G. Hamel. 1998. Insulin degradation: progress and potential 1. Endocr. Rev. 19(5): 608-624.

Ducros, V. 1992. Chromium metabolism. Biol. Trace Elem. Res. 32(1-3): 65-77.

Dunnett, M. 2005. The diagnostic potential of equine hair: a comparative review of hair analysis for assessing nutritional status, environmental poisoning, and drug use and abuse. Advances in equine nutrition, III. Pagan, J. and Geor, R.J., eds. Kentucky Equine Research, Kentucky: 85-106.

Evans, G. W. and T. D. Bowman. 1992. Chromium picolinate increases membrane fluidity and rate of insulin internalization. J. Inorg. Biochem. 46(4): 243-250.

Field, J.B. 1973. Extraction of insulin by liver. Annu. Rev. Med. 24:309-314.

Filistowicz, A., P. Przysiecki, S. Nowicki, A. Filistowicz, and M. Durkalec. 2012. Concentration of heavy metals in hair and skin of silver and red foxes (Vulpes vulpes). Pol. J. Environ. Stud. 21(4): 865-869.

Frank, N., S. B. Elliott, L. E. Brandt, and D. H. Keisler. 2006. Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. JAVMA. 228(9): 1383-1390.

Frank, N., R. J. Geor, S. R. Bailey, A. E. Durham, and P. J. Johnson. 2010. Equine metabolic syndrome. J. Vet. Intern. Med. 24(3): 467-475.

Frape, D. Equine nutrition and feeding, 2nd. ed. 1998. Blackwell Science Ltd.

Gentry, L. R., D. L. Thompson, J. M. Fernandez, L. A. Smith, D. W. Horohov, and B. S. Leise. 1999. Effects of chromium tripicolinate supplementation on plasma hormone and metabolite concentrations and immune function in adult mares. J. Equine Vet. Sci. 19(4): 259-265.

Gentry, L. R., D. L. Thompson, G. T. Gentry, R. P. Del Vecchio, K. A. Davis, and P. M. Del Vecchio. 2004. The relationship between body condition score and ultrasonic fat

measurements in mares of high versus low body condition. Journal of Equine Veterinary Science. 24(5): 198-203.

Geor, R. J., L. Stewart-Hunt, and L. J. McCutcheon. 2010. Effects of prior exercise on insulin-mediated and noninsulin-mediated glucose uptake in horses during a hyperglycaemic clamp. Equine Vet. J. 42(s38): 129-134.

Geor, R. J., and P. A. Harris. 2013a. Chapter 27: Laminitis. In: Geor, R.J., Harris, P.A., and M. Coenen, editors. Equine applied and clinical nutrition: health, welfare and performance. Philadelphia (PA): Elsevier Health Sciences: 469-486.

Geor, R. J., and P. A. Harris. 2013b. Chapter 28: Obesity. In: Geor, R.J., Harris, P.A., and M. Coenen, editors. Equine applied and clinical nutrition: health, welfare and performance. Philadelphia (PA): Elsevier Health Sciences: 487-502.

Ghosh, D., B. Bhattacharya, B. Mukherjee, B. Manna, M. Sinha, J. Chowdhury, and S. Chowdhury. 2002. Role of chromium supplementation in Indians with type 2 diabetes mellitus. J. Nutr. Biochem. 13(11): 690-697.

Giraudet, A., K. W. Hinchcliff, C. W. Kohn, and K. H. McKeever. 1994. Early insulin response to an intravenous glucose tolerance test in horses (abstract). Am. J. Vet. Res. 55: 379–381.

Guan, X., J. J. Matte, P. K. Ku, J. L. Snow, J. L. Burton, and N. L. Trottier. 2000. High chromium yeast supplementation improves glucose tolerance in pigs by decreasing hepatic extraction of insulin. J. Nutr. 130(5): 1274-1279.

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

Henquin, J. C., N. Ishiyama, M. Nenquin, M. A. Ravier, and J. C. Jonas. 2002. Signals and pools underlying biphasic insulin secretion. Diabetes. 51(Suppl. 1): S60-S67.

Hoffman, R. M., R. C. Boston, D. Stefanovski, D. S. Kronfeld, and P. A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. J. Anim. Sci. 81: 2333-2342.

Hoffman, R. M., H. Filer, C. A. Crawford, J. C. Haffner, and K. A. Fecteau. 2009. Nonstructural carbohydrate and glycemic response of feeds: how low is "low starch?" J. Equine Vet. Sci. 29(5): 379-380.

Hoffman, R. M. 2013. Carbohydrates. In: Geor, R.J., Harris, P.A., and M. Coenen, editors. Equine applied and clinical nutrition: health, welfare and performance. Philadelphia (PA): Elsevier Health Sciences: 156-167.

Hummel, M., E. Standl, and O. Schnell. 2007. Chromium in metabolic and cardiovascular disease. Horm. Metab. Res. 39(10): 743-751.

Jackson, S. G. 1997. Trace minerals for the performance horse known biochemical roles and estimates of requirements. Aust. Eq. Vet. 16(3): 119-126.

Janiszewska, J., and A. Cieśla. 2002. Concentration of cadmium and lead in horse blood serum and hair in relation to season and environment. Electronic Journal of Polish Agricultural Universities. 5(1).

Jeejeebhoy, K. N. 1999. The role of chromium in nutrition and therapeutics and as a potential toxin. Nutr. Res. 57(11): 329-335.

Johnson, P. J., N. C. Scotty, C. Wiedmeyer, N. T. Messer, and J. M. Kreeger. 2005. Diabetes mellitus in a domesticated Spanish mustang. J. Am. Vet. Med. Assoc. 226: 584-588.

Kegley, E. B., D. L. Galloway, and T. M. Fakler. 2000. Effect of dietary chromium-L-methionine on glucose metabolism of beef steers. J. Anim. Sci. 78(12): 3177-3183.

Kim, D. S., T. W. Kim, I. K. Park, J. S. Kang, and A. S. Om. 2002. Effects of chromium picolinate supplementation on insulin sensitivity, serum lipids, and body weight in dexamethasone-treated rats. Metabolism. 51(5): 589-594.

Kjems, L. L, A. Vølund, and S. Madsbad. 2001. Quantification of beta-cell function during IVGTT in type II and non-diabetic subjects: assessment of insulin secretion by mathematical methods. Diabetologia. 44: 1339-1348.

Kobla, H. V., and S. L. Volpe. 2000. Chromium, exercise, and body composition. Crit. Rev. Food Sci. Nutr. 40(4): 291-308.

Król, E., Z. Krejpcio, and K. Iwanik. 2014. Supplementary chromium (III) propionate complex does not protect against insulin resistance in high-fat-fed rats. Biol. Trace Elem. Res. 157(2): 147-155.

Krusič, L. I., M. S. Haready, P. Schramel, and J. Dolinar. 1990. Urine and hair analysis as potential indicators of some macro and microelements in performing horses. Acta Veterinaria (Beograd). 40(2-3): 65-75.

Kumar, M., H. Kaur, A. K. Tyagi, N. J. Kewalramani, V. Mani, R. S. Deka, V. K. Sharma, G. Chandra, and A. K. Dang. 2013. Effect of feeding inorganic chromium on growth performance, endocrine variables, and energy metabolites in winter-exposed buffalo calves (Bubalus bubalis). Biol. Trace Elem. Res. 155(3): 352-360.

Kumpulainen, J., S. Salmela, E. Vuori, and J. Lehto. 1982. Effects of various washing procedures on the chromium content of human scalp hair. Analytica Chimica Acta. 138: 361-364.

Lien, T. F., Y. M. Horng, and K. H. Yang. 1999. Performance, serum characteristics, carcase traits and lipid metabolism of broilers as affected by supplement of chromium picolinate. Poult. Sci. 40(3): 357-363.

Lloyd, K. E., V. Fellner, S. J. McLeod, R. S. Fry, K. Krafka, A. Lamptey, and J. W. Spears. 2010. Effects of supplementing dairy cows with chromium propionate on milk and tissue chromium concentrations. J. Dairy Sci. 93(10): 4774-4780.

Ma, W., Y. Gu, J. Lu, L. Yuan, and R. Zhao. 2014. Effects of Chromium Propionate on Egg Production, Egg Quality, Plasma Biochemical Parameters, and Egg Chromium Deposition in Late-Phase Laying Hens. Biol. Trace Elem. Res. 157(2): 113-119.

Martin, J., Z. Q. Wang, X. H. Zhang, D. Wachtel, J. Volaufova, D. E. Matthews, and W. T. Cefalu. 2006. Chromium picolinate supplementation attenuates body weight gain and increases insulin sensitivity in subjects with type 2 diabetes. Diabetes care. 29(8): 1826-1832.

Masharani, U., C. Gjerde, S. McCoy, B. A. Maddux, D. Hessler, I. D. Goldfine, and J. F. Youngren. 2012. Chromium supplementation in non-obese non-diabetic subjects is associated with a decline in insulin sensitivity. BMC Endocr. Disord. 12(1): 31.

Mlinar, B., J. Marc, A. Janez, and M. Pfeifer. 2007. Molecular mechanisms of insulin resistance and associated diseases. Clinica Chemica Acta. 375(1): 20-35.

Moller, D. E., and J. S. Flier. 1991. Insulin resistance—mechanisms, syndromes, and implications. N. Engl. J. Med. 325(13): 938-948.

Nowak, B. 1998. Contents and relationship of elements in human hair for a non-industrialised population in Poland. Sci. Total Environ. 209(1): 59-68.

Ohtomo, Y., T. Bergman, B. J. Johansson, H. Jörnvall, and J. Wahren, 1998. Differential effects of proinsulin C-peptide fragments on Na^+, K^+-ATPase activity of renal tubule segments. Diabetologia. 41(3): 287–291.

Ott, E. A., and J. Kivipelto. 1999. Influence of chromium tripicolinate on growth and glucose metabolism in yearling horses. J. Anim. Sci. 77(11): 3022-3030.

Pagan, J. D., S. G. Jackson, and S. E. Duren. 1995. The effect of chromium supplementation on metabolic response to exercise in thoroughbred horses. Equine Nutrition and Physiology Society. 14: 96-101.

Pattar, G. R., L. Tackett, P. Liu, and J. S. Elmendorf. 2006. Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions. Mutat. Res. 610(1): 93-100.

Polonsky, K. S., J. Licinio-Paixao, B. D. Given, W. Pugh, P. Rue, J. Galloway, T. Karrison, and B. Frank. 1986. Use of biosynthetic human C-peptide in the measurement of insulin secretion rates in normal volunteers and type I diabetic patients. J. Clin. Invest. 77: 98–105.

Powell, D. M., S. E. Reedy, D. R. Sessions, and B. P. Fitzgerald. 2002. Effect of short-term exercise training on insulin sensitivity in obese and lean mares. Equine Vet. J. 34(S34): 81-84.

Pratt, S. E., R. J. Geor, and L. J. McCutcheon. 2005. Repeatability of 2 methods for assessment of insulin sensitivity and glucose dynamics in horses. J. Vet. Intern. Med. 19: 883-888.

Racek, J., C. D. Sindberg, S. Moesgaard, J. Mainz, J. Fabry, L. Müller, and K. Rácová. 2013. Effect of chromium-enriched yeast on fasting plasma glucose, glycated haemoglobin and serum lipid levels in patients with type 2 diabetes mellitus treated with insulin. Biol. Trace Elem. Res. 155(1): 1-4.

Rajalekshmi, M., C. Sugumar, H. Chirakkal, and S. V. Ramarao. 2014. Influence of chromium propionate on the carcass characteristics and immune response of commercial broiler birds under normal rearing conditions. Poult. Sci. 93(3): 574-580.

Ralston, S. L. 2002. Insulin and glucose regulation. Vet. Clin. N. Am-Equine. 18(2): 295-304.

Rigler, R., A. Pramanik, P. Jonasson, G. Kratz, O. T. Jansson, P. A. Nygren, S. Ståhl, K. Ekberg, B. L. Johansson, S. Uhlén, M. Uhlén, H. Jörnvall, and J. Wahren. 1999. Specific binding of proinsulin C-peptide to human cell membranes. PNAS. 96(23): 13318-13323.

Saner, G., V. Yüzbasiyan, and S. Cigdem. 1984. Hair chromium concentration and chromium excretion in tannery workers. Br. J. Ind. Med. 41(2): 263-266.

Schachter, S., R. W. Nelson, and C. A. Kirk. 2001. Oral chromium picolinate and control of glycemia in insulin-treated diabetic dogs. J. Vet. Intern. Med. 15(4): 379-384.

Schinner, S., W. A. Scherbaum, S. R. Bornstein, and A. Barthel. 2005. Molecular mechanisms of insulin resistance. Diabetic Med. 22(6): 674-682.

Shapiro, E. T., H. Tillil, M. A. Miller, B. H. Frank, J. A. Galloway, A. H. Rubenstein, and K. S. Polonsky. 1987. Insulin secretion and clearance: comparison after oral and intravenous glucose. Diabetes. 36(12): 1365-1371.

Spears, J. W., C. S. Whisnant, G. B. Huntington, K. E. Lloyd, R. S. Fry, K. Krafka, A. Lamptey, and J. Hyda. 2012. Chromium propionate enhances insulin sensitivity in growing cattle. J. Dairy Sci. 95(4): 2037-2045.

Staniek, H., Z. Krejpcio, and K. Iwanik. 2010. Evaluation of the acute oral toxicity class of tricentric chromium (III) propionate complex in rat. Food Chem. Toxicol. 48(3): 859-864.

Stearns, D. M. 2000. Is chromium a trace essential metal? Biofactors. 11(3): 149-162.

Steiner, D. F. 2004. The proinsulin C-peptide—a multirole model. Exp. Diabetes Res. 5: 7-14.

Striffler, J. S., M. M. Polansky, and R. A. Anderson. 1998. Dietary chromium decreases insulin resistance in rats fed a high-fat, mineral-imbalanced diet. Metabolism. 47(4): 396-400.

Sumner, J. M., F. Valdez, and J. P. McNamara. 2007. Effects of chromium propionate on response to an intravenous glucose tolerance test in growing Holstein heifers. J. Dairy Sci. 90(7): 3467-3474.

Topczewska, J. 2012. Effects of seasons on the concentration of selected trace elements in horse hair. J. Cent. Eur. Agric. 13(4).

Tóth, F., N. Frank, T. Martin-Jimenez, S. B. Elliott, R. J. Geor, and R. C. Boston. 2010. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. Equine Vet. J. 42(2): 149-155.

Uusitupa, M. I., L. Mykkänen, O. Siitonen, M. Laakso, H. Sarlund, P. Kolehmainen, T. Räsänen, J. Kumpulainen, and K. Pyörälä. 1992. Chromium supplementation in impaired glucose tolerance of elderly: effects on blood glucose, plasma insulin, C-peptide and lipid levels. Br. J. Nutr. 68(1): 209-216.

Uyanik, F., B. K. Güçlü, T. Bekyürek, M. Ün, M. Abay, E. Canoodlu, O., Demiral, O. Erdem, K. Güvenç, K. Gürbulak, and A. Sayal. 2008. The effect of chromium supplementation on body weight, serum glucose, proteins, lipids, minerals, and ovarian follicular activity in working horses. JAVA. 7(6): 771-776.

Van Weyenberg, S., M. Hesta, J. Buyse, G. A. Papadopoulos, and G. P. Janssens. 2008. Short-term effects of energy changes on plasma leptin concentrations and glucose tolerance in healthy ponies. Vet. J. 178(2): 233-237.

Verchere, C., M. Paoletta, M. Neerman-Arbez, K. Rose, J. Irminger, R. Gingerich, S. Kahn, and P. Halban,. 1996. C-peptide: A novel secretory product of the rat pancreatic beta cell produced by truncation of proinsulin connecting peptide in secretory granules. J. Biol. Chem. 271: 27475–27481.

Vervuert, I., D. Cuddeford, and M. Coenen. 2006. Effects of chromium supplementation on selected metabolic responses in resting and exercising horses. Equine Comp. Exerc. Physiol. 3(1): 19-27.

Vervuert, I., K. Voigt, T. Hollands, D. Cuddeford, and M. Coenen. 2009. Effect of feeding increasing quantities of starch on glycaemic and insulinaemic responses in healthy horses. Vet. J. 182: 67-72.

Vick, M. M., A. A. Adams, B. A. Murphy, D. R. Sessions, D. W. Horohov, R. F. Cook, B. J. Shelton, and B. P. Fitzgerald. 2007. Relationships among inflammatory cytokinase, obesity, and insulin sensitivity in the horse. J. Anim. Sci. 85(5): 1144-1155.

Vincent, J. B. 2000. Quest for the molecular mechanism of chromium action and its relationship to diabetes. Nutr. Res. 58(3): 67-72.

Wang, Z. Q., X. H. Zhang, J. C. Russell, M. Hulver, and W. T. Cefalu. 2006. Chromium picolinate enhances skeletal muscle cellular insulin signaling in vivo in obese, insulin-resistant JCR: LA-cp rats. J. Nutr. 136(2): 415-420.

Wilcox, G. 2005. Insulin and insulin resistance. Clin. Biochem. Rev. 26(2):19-39.

Wilson, B. E., and A. Gondy. 1995. Effects of chromium supplementation on fasting insulin levels and lipid parameters in healthy, non-obese young subjects. Diabetes Res. Clin. Pract. 28(3): 179-184.

Woodworth, J. C., M. D. Tokach, J. L. Nelssen, R. D. Goodband, S. S. Dritz, S. I. Koo, J. E. Minton, and K. Q. Owen. 2007. Influence of dietary-carnitine and chromium picolinate on blood hormones and metabolites of gestating sows fed one meal per day. J. Anim. Sci. 85(10): 2524-2537.

CHAPTER II. EXPERIMENT 1A

2.1 Introduction

Several serious conditions are associated with insulin resistance in horses, including obesity and chronic laminitis. Though understanding of the causes and management of these conditions is increasing, they remain a serious problem for horse owners. Insulin resistance is a condition where normal circulating concentrations of insulin do not elicit a sufficient physiologic response in target tissues (Hoffman et al. 2003). Obesity is characterized by excess adipose tissue adversely affecting overall horse health, and generally results from excess caloric intake. Obesity is defined by high body condition (8 or 9 BCS, Henneke et al. 1983) which is highly correlated with insulin resistance such that insulin sensitivity was 80% lower in obese horses than those of healthy weight (Hoffman et al. 2003). Laminitis can be induced by prolonged hyperinsulinemia, which often results from overfeeding simple carbohydrates (De Laat et al. 2010).

Insulin is synthesized within the pancreatic β-cell by the removal of C-peptide from its precursor molecule, proinsulin. Insulin and C-peptide are secreted from the pancreas in equimolar amounts upon stimulation by elevated blood glucose concentrations (Steiner 2004). Insulin has a relatively short half life of approximately 5 minutes, and a large percentage of insulin (50-90%) is rapidly cleared during its first pass through the liver in humans. C-peptide, however, has a relatively long half life of approximately 30 minutes, and is cleared by the liver at low efficiency (Duckworth et al. 1998). Therefore, circulating plasma C-peptide is a much more accurate measure of pancreatic secretion of both insulin and C-peptide, and overall pancreatic β-cell function, than are circulating insulin concentrations.

Insulin response patterns vary with mode of glucose administration. When glucose is administered intravenously, insulin typically exhibits an acute peak, followed by a second more gradual peak. When glucose is administered as a meal, insulin increases more gradually and does not exhibit a biphasic pattern. This difference is likely due to a number of factors, including incretin which upregulates insulin secretion in response to food, and gastrointestinal signaling (Caumo et al. 2004). Because insulin and C-peptide are secreted in equimolar amounts from the pancreas, it is of interest to evaluate whether C-peptide dynamics follow a similar pattern.

Chromium has been used in human medicine for over 50 years to alleviate symptoms of type 2 diabetes. It is theorized that chromium interacts with the insulin receptor, thereby upregulating downstream signaling and GLUT4 translocation to the cell membrane, thus increasing the rate of glucose clearance (Vincent 2000). Due to physiologic similarities between type 2 diabetes and insulin resistance in horses, it is of interest to determine whether chromium supplementation in horses can increase insulin sensitivity and improve overall pancreatic function.

Therefore, this experiment was conducted in order to evaluate the effects of chromium propionate supplementation on plasma C-peptide concentrations following both IV glucose and a large grain-mix concentrate meal. Should this study find that chromium propionate supplementation decreases plasma C-peptide response to IV glucose and/or a feeding challenge, that finding would suggest that chromium supplementation has applications in alleviating symptoms of insulin resistance in horses.

2.2 Materials and Methods

2.2 a. Animals and Animal Care

This experimental protocol was approved by the North Carolina State University Institutional Animal Care and Use committee. This experiment took place from March to August 2014 at the North Carolina State University Equine Research Unit in Raleigh, NC. Thirty-six quarter horse geldings (mean ± SD; BW 471.56 ± 39.14 kg; BCS 4.51 ± 0.81; age 5.08 ± 1.12 years) were fed a proprietary chromium propionate supplement for 42 d. The study was conducted in three separate periods as three identical experiments due to housing limitations at the research facility. Horses were examined by a veterinarian upon arrival and determined to be healthy and sound. Horses were housed in a dry lot with shelter and free access to hay for approximately 2 weeks and were then brought in to individual pens 7 d prior to d-0. Pens had automatic water troughs, which were cleaned once per week, and individual feed buckets and hay bins. Pens were cleaned twice daily. Horses were maintained on a preventative health protocol, including hoof care, dental exams and teeth floating, and deworming, which were performed within 7 d prior to d-0 of each trial period. All veterinary and farrier visits were documented. Horse health was checked daily, and horses were groomed and their feet picked once per week. Horses in period 3 were treated daily with fly spray repellent manufactured for use on horses. Horses were fed mixed-grass hay at approximately 1.75% of BW and 0.2 kg/100 kg BW oats per d, with hay intake adjusted to maintain body weight, and both were split equally by weight into two meals per d. Body weight and body condition scores were recorded every 14 d. Body condition scores were determined by two experienced scorers and scores were averaged.

2.2 b. Experimental Design

This experiment was a complete block design. Treatments were blocked first by age within trial period (age group) and second by glucose response to a feeding challenge on d-0. Horses were assigned to one of three age groups based on actual age in years, such that the youngest horses were in age group 1 and the oldest horses were in age group 3. Within each period, horses were assigned one of 4 treatment groups: 0 mg/d (CON; n=3), 2 mg/d (CR2; n=3), 4 mg/d (CR4; n=3), and 8 mg/d (CR8; n=3) of supplemental chromium. Supplements were mixed with 50g ground corn and fed each AM with the oats.

2.2 c. Blood Sample Collection and Analysis

On d-0 and 28 of each period, feeding challenges were administered. Feed and hay were withheld overnight and horses were fed their full daily ration of oats (0.2 kg/100 kg BW) at 7 a.m. Blood samples were collected by venipuncture immediately prior to feeding (time 0 h), and at 2 h and 4 h post feeding.

On d-42, IV glucose tolerance tests (IVGTT) were administered using 100 mg/kg BW dextrose. Horses were not fed concentrate but did receive a small allotment of hay. Blood samples were collected starting 1 hour after successful placement of the catheter. Patency of the catheter was maintained by flushing with sodium citrate after each sample was collected. Samples were collected at -10, 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min after feeding, and the average of the -10 and 0 samples was used as a baseline ("0") value.

Blood samples for plasma C-peptide analysis were collected into 4 ml EDTA Vacuette tubes (Greiner Bio-one EDTA K2 #454209). Samples were placed on ice in a cooler until spun (about 5 minutes). Tubes were spun in a centrifuge at 4° C at 500 relative

centrifugal force (RCF) for 10 minutes. Plasma was pipetted into plastic tubes and stored at -80° C until analysis.

C-peptide concentration in plasma was measured by radioimmunoassay technique, which was first employed in 1974 (Bonser and Garcia-Webb 1983). Radioimmunoassay is a method which allows radiolabeled antigen and its unlabeled counterpart to compete for binding with a limited amount of antibody. In this study, the radiolabeled antigen was radioactive C-peptide labeled with I^{125}, and the antibody was referred to as Ab. The reaction was allowed to reach equilibrium, reflecting the following equation:

$$\text{C-peptide-I}^{125} + \text{C-peptide} + 2\text{Ab} \leftrightarrows \text{C-peptide-I}^{125}\text{*Ab} + \text{C-peptide*Ab}$$

A commercially available C-peptide human double antibody radioimmunoassay kit was validated for use in horses (EMD Millipore Corporation Cat. #HCP-20K) (Appendix A). The assay was run successfully only when charcoal stripped horse serum was added to the tubes containing kit standards to correct for matrix imbalances.

2.2 d. RIA Procedure

EDTA plasma was analyzed for C-peptide using a commercially available radioimmunoassay kit validated for horses (EMD Millipore Corporation Cat. #HCP-20K). Plasma was analyzed in accordance with instructions from the manufacturer, and modified by addition of stripped horse serum to tubes containing kit standards. This matrix adjustment allowed for the accurate comparison of plasma C-peptide values with a standard line representative of horse physiology. This adjustment was necessary to correct for species-unique immunological determinants (Bonser and Garcia-Webb 1983). Samples were assayed in duplicate and the average of the two values used to calculate final C-peptide content.

Intra-assay coefficients of variation of less than 10% were required for acceptance of C-peptide assay results and sample results that did not meet this criteria were re-assayed. The relationship between total reactivity and total unlabeled C-peptide per sample is inverse: a relatively low count of the C-peptide-I^{125} complex indicates that more unlabeled C-peptide has bound to the antibody, and a relatively high count of C-peptide-I^{125} complex indicates that less unlabeled C-peptide has bound to the antibody (Bailey 1984).

2.2 e. Statistical Analysis

Plasma C-peptide values for d-28 and 42 were analyzed as a repeated measures design using the PROC MIXED procedure of SAS, and plasma C-peptide area under the curve (AUC) for d-42 were analyzed using analysis of variance for complete block designs using the PROC MIXED procedure of SAS (version 9.3, SAS Inst. Inc., Cary, NC). Each individual horse was considered the experimental unit. Factors in the model included TRT, Time, Trial period, Age group, and any significant interactions. Backwards model selection was used to determine the best fitting model. For d-28, d-0 C-peptide values for each horse at each time point were included as a covariate, but were found to be non-significant and were not included in the final model. Horse age (years) was included as a covariate. For d-42, plasma C-peptide AUC was analyzed, and d-0 C-peptide values and horse age were included as covariates. Results were considered significant at $p<0.05$, and considered to have a tendency at $p\leq0.10$. Results are expressed as least squares means ± standard error (LSmeans ± SE).

2.3 Results

2.3 a. Day 28

On d-28, age as a covariate significantly adjusted the LSmeans ($p=0.0022$). No main effects of TRT ($p=0.3104$) or Trial were observed ($p=0.3111$). A main effect of Time ($p<0.0001$) was observed such that C-peptide values increased from baseline to 2 h, and decreased from 2 h to 4 h, but did not return to baseline by 4 h (Figure 1). Mean plasma C-peptide was 1.160, 1.349, and 1.283 ± 0.041 ng/ml for Time 0 h, 2 h, and 4 h, respectively.

A tendency of Age group ($p=0.1$) was observed such that overall plasma C-peptide for age group 1 tended to be less than for groups 2 and 3. Mean plasma C-peptide was 1.132 ± 0.067, 1.353 ± 0.072, and 1.306 ± 0.065 for Age groups 1, 2, and 3, respectively (Figure 2).

2.3 b. Day 42

On d-42, age and d-0 C-peptide values as covariates tended to adjust the LSmeans ($p=0.0581$, $p=0.0710$) and were included in the model. No main effect of Trial ($p=0.7116$) was observed. No main effect of TRT ($p=0.1756$) was observed; however, a contrast of CR2 and CR4 TRT groups had a p-value of 0.04. Main plasma C-peptide AUC was 253.950 ± 22.179, 234.230 ± 14.925, 157.520 ± 21.407, and 190.820 ± 11.788 ng*min/ml for CON, CR2, CR4, and CR8, respectively. C-peptide AUC for the CR2 group was higher than for the CR4 group; tended to be higher for the CON group than the CR4 and CR8 groups; and tended to be higher for the CR2 group than the CR8 group (Figure 3).

A tendency of Age group was observed such that group 3 was higher than group 1 and group 2, and group 2 tended to be higher than group 1. Mean C-peptide AUC was 153.190 ± 25.046, 214.740 ± 10.827, and 259.460 ± 19.939 ng/ml for Age groups 1, 2, and 3, respectively (Figure 4).

Plasma C-peptide values were analyzed for d-42 with repeated measures in order to evaluate C-peptide response over time. There was a main effect of Time ($p=0.0246$) such that concentrations before time 90 were greater than after time 90. Plasma C-peptide increased numerically following IV glucose administration, but this increase was not significant. Mean plasma C-peptide for each time point were 1.207, 1.237, 1.180, 1.223, 1.218, 1.258, 1.250, 1.216, 1.090, 1.088, 1.144, and 1.103 ± 0.044 ng/ml for times 0, 5, 10, 150, 20, 30, 45, 60, 90, 120, 150, and 180 min, respectively (Figure 5).

2.4 Discussion

This study found that plasma C-peptide concentrations increased significantly after an oat-based concentrate meal, and peaked at 2 h post-feeding on d-28. In contrast, plasma C-peptide concentrations did not increase after IV glucose infusion during the IVGTT on d-42. This is in contrast to findings by Tóth et al. (2010) that plasma C-peptide did increase following IV glucose infusion, though a larger dextrose dose of 300 mg/kg BW was used in that study. Plasma C-peptide concentrations after IV glucose administration on d-42 also did not appear to follow the biphasic pattern characteristic of insulin dynamics.

The finding that C-peptide concentrations increased after a meal, but did not increase following IV glucose administration, confirms the hypothesis that factors other than elevated blood glucose stimulate plasma C-peptide (and serum insulin) to increase. Ahrén et al. (2008) also observed greater C-peptide response to oral vs. IV glucose administration. They hypothesized that this effect was due in part to hormones that upregulate secretion of insulin in response to oral glucose, such as incretin, as well as neural responses to ingestion of nutrients. It is likely that gastrointestinal signaling takes place in response to ingestion of a

meal, resulting in an increase in insulin that is not observed when insulin is administered intraveneously. IVGTT is often used as a measure of pancreatic insulin response to elevated blood glucose rather than feeding challenges, due to confounding factors in feeding challenges such as gastric emptying, glucose absorption, gastrointestinal motility, hormones such as incretin, and neural input which modify the insulin response (Caumo et al. 2004). However, feeding challenges are advantageous because they more accurately represent pancreatic response to the horse's actual feeding behavior than IVGTT. The present study suggests that feeding challenges may be more applicable than IV glucose administration for the measurement of changes in C-peptide in equine plasma.

During the IVGTT on d-42, plasma C-peptide numerically increased following IV glucose administration, but this increase was not statistically significant. It is logical to assume that in response to elevated blood glucose, C-peptide is secreted in equimolar amounts with insulin and therefore should increase in plasma; however, a statistically significant increase was not observed. It is possible that the dose of glucose administered was not sufficient to elicit a response that could be observed to be significant.

This study found that younger horses appear to exhibit a lower C-peptide response than older horses. Horses were selected for this study to be of a relatively narrow age range (2.3-7.1 years), and therefore age was not expected to have an effect on C-peptide response. However, the tendencies observed between age groups, as well as the fact that age served as a significant covariate, suggest that age does significantly affect C-peptide response. This may have applications in feeding and management, but definitive conclusions cannot be drawn from C-peptide alone. To determine how pancreatic efficiency and function vary between age groups, insulin and glucose data must also be reported. There exists a large

potential in future research for examination of differences in C-peptide response in horses of more widely varying age groups.

Based on the results reported in this study, chromium propionate supplementation did not appear to affect C-peptide response at 28 d. At 42 d, treatment remained non-significant, but was approaching a tendency, and contrasts between treatments were somewhat suggestive that the supplement may have lowered C-peptide response, particularly at the level of 4 mg/d. Conclusions are particularly difficult to determine due to the complete lack of existing studies which could be compared. While several studies have observed some effect of chromium supplementation on insulin and glucose dynamics, none have examined the effect on C-peptide concentrations. C-peptide remains an important area of study, because it serves as a much more accurate method to evaluate pancreatic efficacy than circulating insulin.

Due to beneficial effects of chromium supplementation in humans, it is of great interest to perform a similar study to further evaluate the effect of supplementation at 4 mg/d on C-peptide response. Such a study should include a larger sample size and feed the supplement for a longer period of time. The results of this study are suggestive that chromium propionate supplementation in horses may lower C-peptide response, especially when fed at 4 mg/d, but further research is needed to confirm this conclusion.

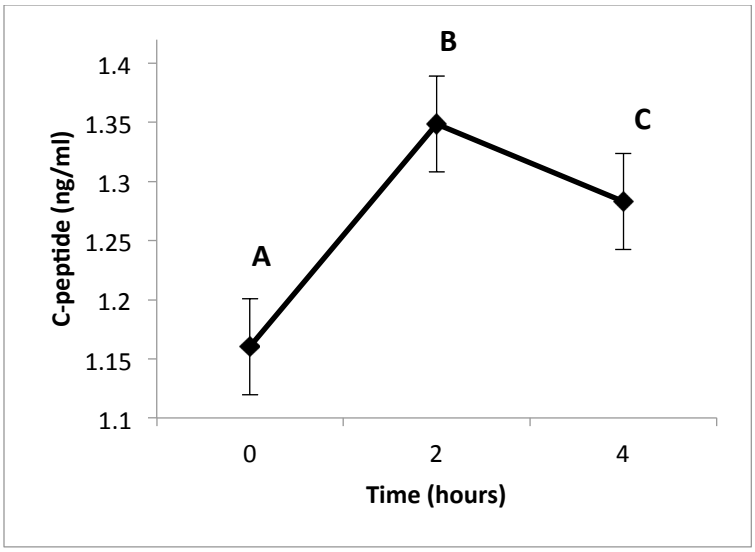


Figure 1. Day 28 plasma C-peptide during the feeding challenge increased at 2 h and decreased at 4 h, but did not return to baseline by 4 h. Plasma C-peptide for times 2 h^{B} and 4 h^{C} were greater than 0 h^{A} ($p<0.0001$), and Time 4 h was less than Time 2 h ($p=0.0119$).

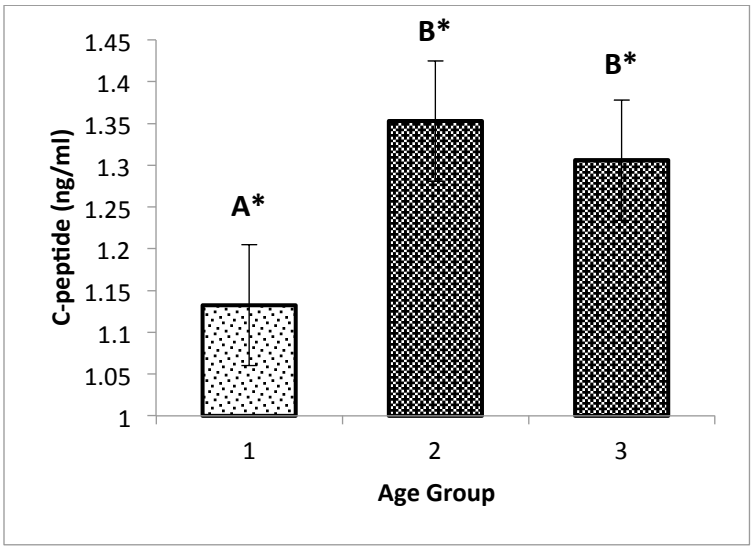


Figure 2. Day 28 plasma C-peptide tended to be lower for age group 1^{A*} than age groups 2^{B*} ($p=0.05$) and 3^{B*} ($p=0.1$).

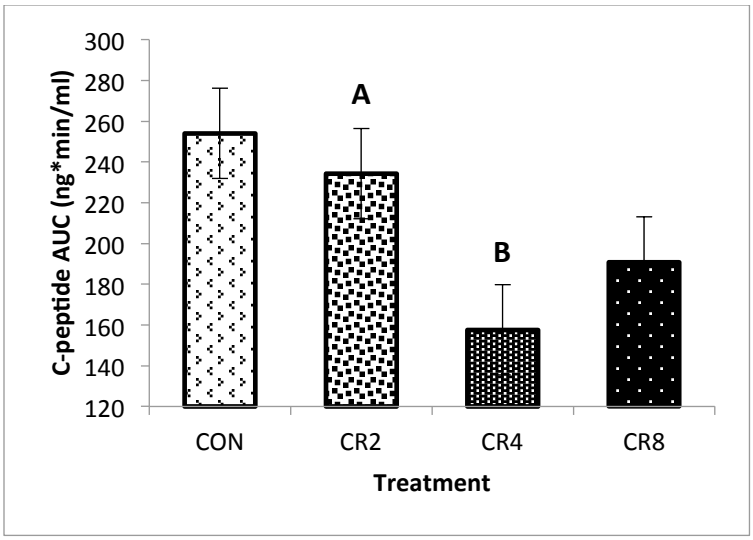


Figure 3. Day 42 C-peptide AUC was higher for the $CR2^{A}$ group than the $CR4^{B}$ group (p=0.0447). Other tendencies between TRT groups (not shown) include: higher for the CON group than the CR4 (p=0.0554) and CR8 groups (p=0.0665); tended to be higher for the CR2 group than the CR8 group (p=0.0800).

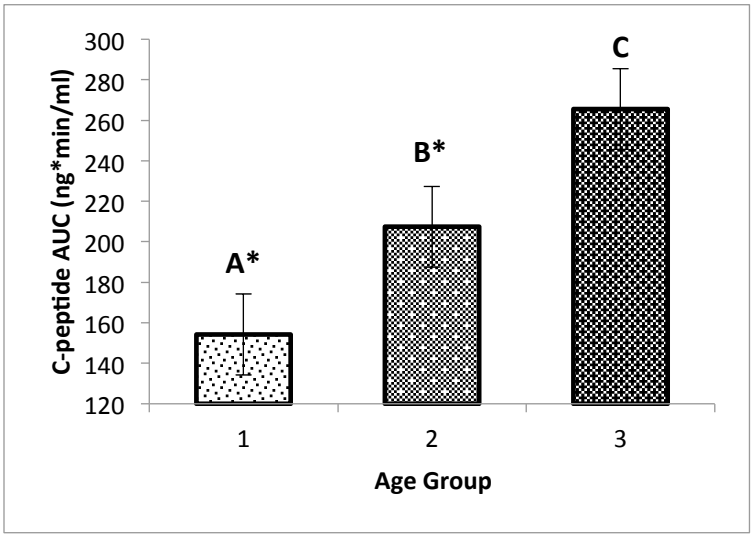


Figure 4. Day 42 C-peptide AUC tended higher for age group 3^{C} than age group 1^{A*} (p=0.0468) and 2^{B*} (p=0.0482), and for age group 2 than age group 1 (p=0.0976).

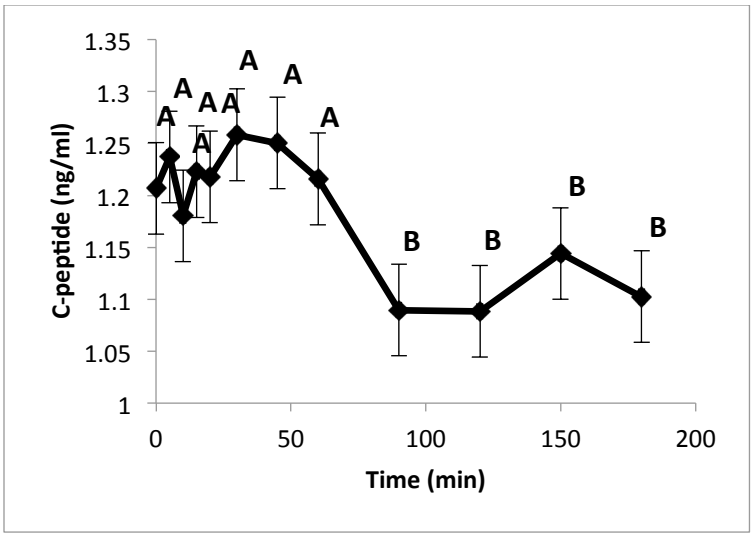


Figure 5. Day 42 plasma C-peptide over time. C-peptide numerically increases before a sharp decrease at 90 min, but this initial increase is not statistically significant. Significant differences are shown by superscripts A and B.

Literature Cited

Caumo, A., and L. Luzi. 2004. First-phase insulin secretion: does it exist in real life? Considerations on shape and function. Am. J. Physiol-Endoc. M. 287(3): E371-E385.

De Laat, M. A., C. M. McGowan, M. N. Sillence, and C. C. Pollitt. 2010. Equine laminitis: Induced by 48 h hyperinsulineamia in Standardbred horses. Equine Vet. J. 42(2): 129-135.

Duckworth, W. C., R. G. Bennett, and F. G. Hamel. 1998. Insulin degradation: progress and potential 1. Endocr. Rev. 19(5): 608-624.

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

Hoffman, R. M., R. C. Boston, D. Stefanovski, D. S. Kronfeld, and P. A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. J. Anim. Sci. 81: 2333-2342.

Steiner, D. F. 2004. The proinsulin C-peptide—a multirole model. Exp. Diabetes Res. 5: 7-14.

Vincent, J. B. 2000. Quest for the molecular mechanism of chromium action and its relationship to diabetes. Nutr. Res. 58(3): 67-72.

CHAPTER III. EXPERIMENT 1B

3.1 Introduction

Hair has been considered a useful bioindicator of nutritional and heavy metal status in animals for several decades, and modern analytical technologies now allow for detection of a wide variety of elements. Many serious conditions can result from nutritional imbalances and heavy metal toxicities in horses. Chromium deficiency results in hyperinsulinemia and disorders of the nervous system and brain (Hummel et al. 2007). Though it does not typically accumulate in toxic amounts, high levels may result in increased oxidative stress and chromosomal damage, and inhibit iron uptake (Kobla 2000).

Chromium is stored in the liver, spleen, kidney, soft tissue, and bone of mammals, as well as very small amounts in hair, sweat, and bile (Hummel et al. 2007). It follows that samples of any of these tissues should serve as bioindicators of chromium status. Hair is advantageous in comparison to the other tissues containing chromium due to noninvasive collection, and its ability to be thoroughly washed without compromising its structure. Because horse mane hair growth is linear, hair that is pulled from the mane can be extrapolated and cut to represent the length of growth during a given trial period (Dunnett 2005).

Previous research determining the concentration of chromium in horse hair is very limited. In this study, horses were fed known amounts of chromium. Therefore, it was of interest to evaluate whether chromium would bioaccumulate in mane hair in dose-dependent amounts. Should the present study find that chromium does accumulate in dose dependent amounts, the following theories would be confirmed: 1) chromium content can be reliably measured in horse mane hair; 2) chromium content in mane hair is not significantly

influenced by temperature, season, horse age, or hair color; and 3) chromium content in mane hair is representative of chromium intake.

3.2 Materials and Methods

3.2 a. Sample Collection

This experiment utilized the same study design as EXPT1A, with an additional period of horses (n=12) included. On d-0, 28, and 42, hair samples were pulled from the mane in 3 regions: near the poll, in the mid-section of the mane, and near the withers. These samples were pooled to make 1 hair sample per horse. Hair samples were sealed in individual ziploc bags and stored in a cool, dry cabinet at room temperature.

Hair samples were prepared for analysis by microwave digestion and graphite furnace on an atomic absorption spectrophotometer (Shimadzu, Japan Model #AA-6701F). Samples were prepared by a method similar to that used by Asano et al. 2002. Hair was first washed according to modified methods of the International Atomic Energy Agency. Hair samples were aligned by the roots and cut to a length of approximately 0.3 cm, an estimate representative of new hair growth during the period of chromium supplementation. Based on a linear relationship between millimeters of hair growth and time in months established by Dunnett (2005), hair growth during the 42 d period of chromium supplementation was estimated to be 0.3 cm. Each sample was washed and dried to prepare for ashing.

3.2 b. Washing Procedure

Hair samples were placed in 14 ml conical tubes. Each sample was washed within its tube four times with a 1:200 dilution of Triton X-100 and rinsed with deionized water three times after each washing. Each washing was done by holding the conical tube on the vortex

for 1 min. Hair samples were rinsed in two, two minute inversions with isopropyl alcohol, then with acetone in the same manner, and allowed to air dry with caps placed loosely on the tubes to prevent contamination. Hair samples were mixed by treatment per each day to allow a representative subsampling of that pool.

3.2 c. Ashing Procedure

Representative aliquots of 0.6-1.0 g of the pooled hair samples were weighed into 50 ml disposable polypropylene tubes. Ten ml of concentrated nitric acid was added, and caps with a small hole were placed; tubes were allowed to pre-digest overnight. For microwave digestion, tubes were placed into the outermost ring of a turntable and transferred to the microwave. A fiber optic temperature probe was placed in one of the samples containing a medium weight of hair predigesta. Samples were digested in the microwave. Samples were ramped to 110° C over a period of 20 min, then held at that temperature for 20 min. Samples were allowed to cool in the microwave to 50° C for 10 minutes.

After digestion, samples were removed from the microwave and contents transferred to 25 ml Erlenmeyer flasks. A pipet and rinsing procedure was used to maximize sample recovery from the cap and residual on the sides of the tube. Samples were allowed to cool to room temperature, after which deionized water was added to bring the final volume of each sample to 25 ml. Samples were then analyzed by graphite furnace analysis for chromium. Standard methods of addition was employed; a standard line was produced for each sample by preparation of 4 dilutions of the sample with nitric acid, allowing each sample to serve as its own standard.

3.2 d. Statistical Analysis

Chromium content in horse mane for d-0, 28, and 42 was analyzed by ANOVA using the PROC MIXED procedure of SAS (version 9.3, SAS Inst. Inc., Cary, NC). Each pooled sample (TRT per day) was considered the experimental unit. Factors in the model included TRT, Day, Trial, and any significant interactions. Backwards model selection was used to determine the best fitting model. Results were considered significant at $p<0.05$, and considered to have a tendency at $p\leq0.10$. Results are expressed as least squares means ± standard error (LSmeans ± SE).

3.3 Results

Raw data for chromium content in hair ranged from 124 to 362 ppb, and mean ± SD was 208 ± 55 ppb. Main effects of TRT ($p=0.0461$) and Day ($p=0.0383$) were observed. While no significant TRT*Day interaction was observed, the p-value was approaching tendency at $p=0.14$. Overall means ± SE for TRT were 205, 238, 176, and 212 ± 14 parts per billion (ppb) for CON, CR2, CR4, and CR8 TRT groups, respectively. For the CON TRT group, chromium content on d-42 was lower than on d-0, and tended to be lower than d-0 on d-28. For the CR2 TRT group, chromium content was lower at d-28, but increased at d-42. Chromium content for the CR8 TRT group increased numerically over time but was not significantly different between d-0 and 42 (Figure 6). Overall chromium content decreased from d-0 to 28, and increased from d-28 to 42, such that chromium content on d-0 and 42 were not different. Overall means ± SE for Day were 226, 180, and 218 ± 12 ppb for d-0, 28, and 42, respectively (Figure 7). Chromium content for Trial period 1 tended less than for Trial period 3 ($p=0.0793$).

3.4 Discussion

Chromium content in horse mane hair was analyzed in this study due to the potential of hair to be a noninvasive bioindicator of nutritional status and/or heavy metal toxicity. Because this study was conducted specifically to evaluate whether chromium deposition in hair was related to chromium in the diet, and to ease difficulty of laboratory testing, samples were pooled by treatment per day. For this reason, factors such as animal age and hair color could not be included in the statistical model.

The concentrations of chromium found in this study were similar to those in the very limited previous research available. Raw data for chromium content in hair ranged from 124 to 362 ppb, and mean ± SD was 208 ± 55 ppb. This value is very similar to the reference value established by Asano et al. (2002) of 220 ± 160 ppb for all horses aged 2-5 years. The standard deviation in this study is considerably smaller than that in previous studies (Asano et al. 2002, Asano et al. 2005, Dobrazanski et al. 2005, Topczewska 2012).

The findings of this study are in contrast with many of the current assumptions regarding the usefulness of hair analysis for the determination of nutritional status and/or heavy metal toxicity. Because there was no effect of trial period, this study is in agreement with previous research that chromium content in horse hair is fairly stable throughout changes in weather and season (Dobrazanski et al. 2005, Krusič et al. 1990). However, this study found that chromium content was not stable over time within each trial period. Chromium content in mane hair decreased at 28 d and increased at 42 d. While chromium exposure prior to d-0 is not known for these horses, it is not logical to conclude that the increase between d-28 and 42 is due to chromium supplementation, but rather to some other

factors. Because hair was not analyzed by horse age or hair color, it may be that these factors play a role in chromium content.

Hair was processed according to a very strict and thorough protocol based on recommendations by the IAEA; therefore, it is unlikely that sample contamination at significant levels occurred, though this always remains possible. Based on this study, chromium content of mane hair does fluctuate significantly over a period of 42 d and is not solely dependent on chromium intake. This finding brings into question the usefulness of hair analysis for the determination of nutritional status and/or heavy metal toxicity.

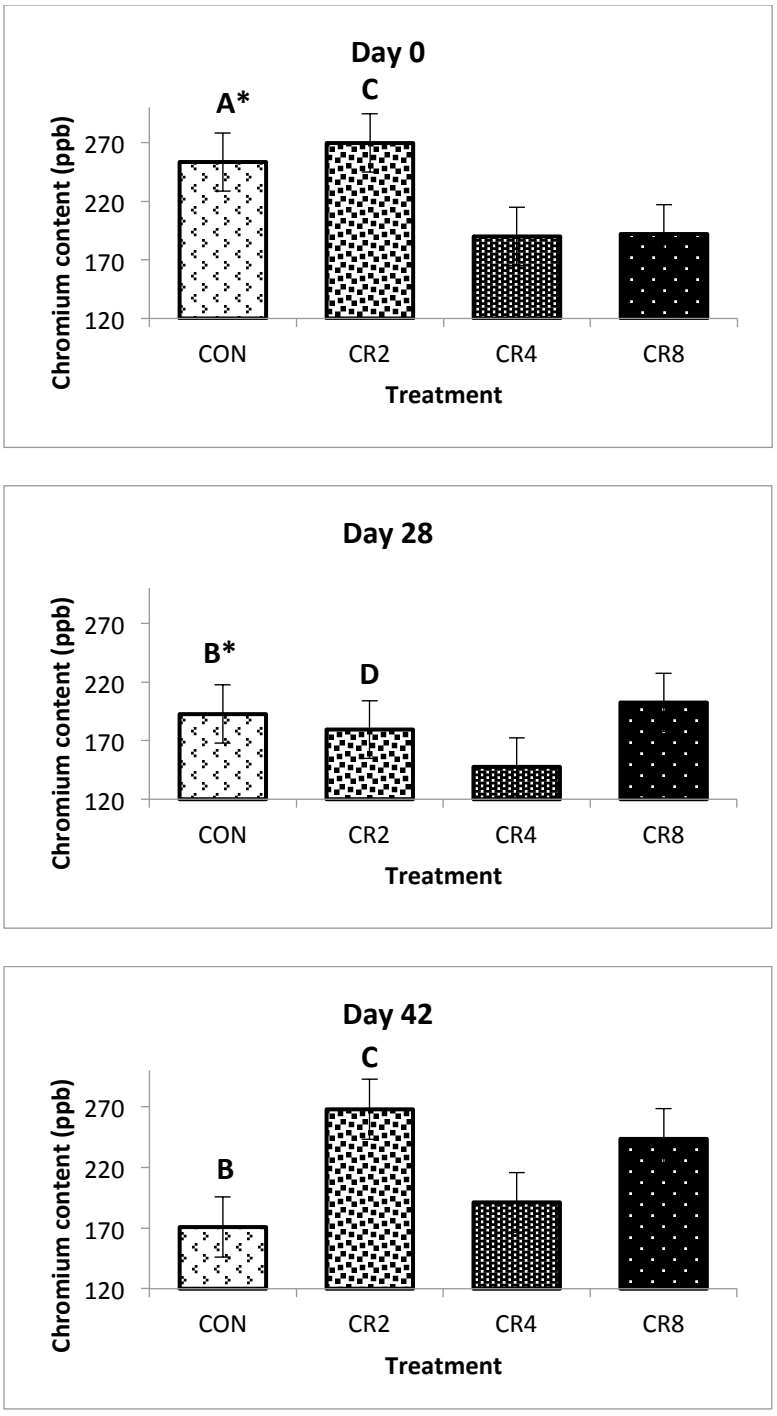


Figure 6. Chromium content for the CON group was lower than d-0^{A*} on d-42^{B} (p=0.0300) and tended lower than d-0 on d-28^{B*} (p=0.0995). CR content for the CR2 group decreased from d-0^{C} at d-28^{D} (p=0.0192) and increased at d-42^{C} (p=0.0212). Chromium content for the CR8 group increased numerically over time but was not significantly different between d-0 and 42 (p=0.1587).

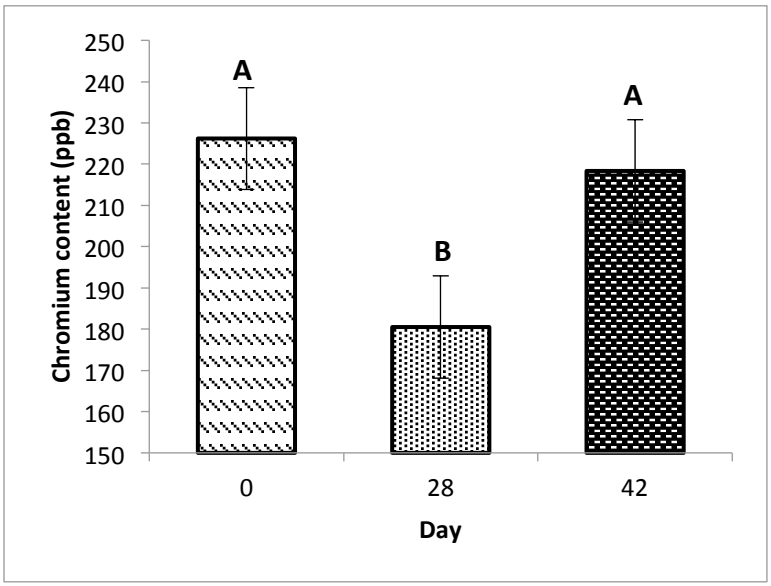


Figure 7. Chromium content in hair decreased from d-0^{A} at d-28^{B} (p=0.0177) and increased at d-42^{A}(p=0.0444).

Literature Cited

Dunnett, M. 2005. The diagnostic potential of equine hair: a comparative review of hair analysis for assessing nutritional status, environmental poisoning, and drug use and abuse. Advances in equine nutrition, III. Pagan, J. and Geor, R.J., eds. Kentucky Equine Research, Kentucky: 85-106.

Hummel, M., E. Standl, and O. Schnell. 2007. Chromium in metabolic and cardiovascular disease. Horm. Metab. Res. 39(10): 743-751.

Kobla, H. V., and S. L. Volpe. 2000. Chromium, exercise, and body composition. Crit. Rev. Food Sci. Nutr. 40(4): 291-308.

CHAPTER IV: EXPERIMENT 2

4.1 Introduction

Insulin and C-peptide are secreted from the pancreatic β-cell in response to elevated blood glucose concentrations. As glucose begins to transport into the cell by facilitated diffusion by GLUT4 proteins, a change in cell membrane polarization induces an influx of calcium. This stimulates insulin and C-peptide to be released from the pancreatic β-cells (Akers and Denbow 2013). Insulin binds to its receptor, increasing downstream signaling and translocation of GLUT4 proteins to the cell membrane, allowing glucose to be cleared from circulation at a faster rate.

Insulin response to IV glucose administration in horses is typically a biphasic pattern, the first phase being an acute peak, and the second a gradual, flatter curve. It is theorized that the first peak represents secretion of stored insulin from the pancreas, while the second peak represents both stored and newly produced insulin (Wilcox 2005). Previous studies administering IVGTTs in horses have used glucose doses ranging from 100-500 mg/kg.

In EXPT1A, plasma C-peptide concentrations did not increase following IV glucose administration at a dose of 100 mg/kg. This finding was in contrast to the 2010 study by Tóth et al. which did observe an increase in plasma C-peptide following IV glucose administration, but used a dose of 300 mg/kg. The present study was conducted to determine whether IV glucose administration would stimulate plasma C-peptide concentrations, and if concentrations would follow a biphasic pattern similar to that of insulin, if glucose were infused at a higher dose of 300 mg/kg.

4.2 Materials and Methods

4.2 a. Animals and Experimental Design

This experiment took place on February 24 and March 3, 2015 at the North Carolina State University Equine Research Unit in Raleigh, NC. Prior to the experiment, horses were at pasture and maintained on a preventative health maintenance protocol. The experiment utilized a crossover design. On d-1, four geldings (mean ± SD; BW 518.30 ± 40.40 kg; BCS 4.50 ± 0.41) were randomly assigned to one of two glucose dose treatments: low dose (100 mg/kg BW), and high dose (300 mg/kg BW). Seven days later (d-2), the treatments were reversed and the experiment was repeated, so that all horses received both treatments.

4.2 b. Blood Sample Collection and Analysis

Feed was withheld overnight prior to the IVGTT. Jugular catheters were placed; IVGTT were administered and blood samples were collected at intervals of 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min after glucose infusion. Patency of the catheter was maintained by flushing with saline with heparin additive after each sample was collected. Blood samples for plasma C-peptide and glucose analysis were collected into 4 ml EDTA Vacuette tubes (Greiner Bio-one EDTA K2 #454209). Samples were placed on ice in a cooler until spun (approximately 5 minutes). Tubes were spun in a centrifuge at 4° C at 500 RCF for 10 minutes. Plasma was pipetted into plastic tubes and stored at -80° C until analysis.

C-peptide concentrations in plasma were measured by modified radioimmunoassay technique utilized in EXPT1 using a commercially available radioimmunoassay kit validated for horses (EMD Millipore Corporation Cat. #HCP-20K). Samples were assayed in duplicate and the average of the two values used to calculate final C-peptide content.

Insulin concentrations in serum were measured by radioimmunoassay technique in accordance with instructions from the manufacturer (EMD Millipore Corporation Cat. #HI-14K). Samples were assayed in duplicate and the average of the two values used to calculate final insulin content.

Glucose concentrations in plasma were measured by colorimetric assay in accordance with instructions from the manufacturer (Sigma GHAK#20). Samples were assayed in duplicate and the average of the two values used to calculate final glucose content.

4.2 c. Statistical Analysis

Plasma C-peptide, serum insulin, and plasma glucose values were run as a repeated measures crossover design using in the PROC MIXED procedure of SAS. Each individual horse was considered the experimental unit. Factors in the model included Sequence, Day, Dose level, Time, and any significant interactions. Backwards model selection was used to determine the best fitting model. No covariates were included in the model. Results were considered significant at $p<0.05$, and considered to have a tendency at $p\leq0.10$. Results are expressed as least squares means ± standard error (LSmeans ± SE).

4.3 Results

4.3 a. Plasma C-peptide

Main effects of Dose ($p=0.0182$) and Time ($p=0.0010$) were observed. There was no effect of Day on plasma C-peptide values, and no significant interaction effects were observed. Overall means for Dose ± SE were 1.4260 and 1.4985 ± 0.05117 ng/ml for low dose and high dose, respectively (Figure 8).

Overall means for Time ± SE were: 1.394, 1.485, 1.453, 1.460, 1.475, 1.486, 1.453, 1.489, 1.484, 1.486, 1.463, 1.433, and 1.416 ± 0.056 ng/ml for times 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min, respectively. Plasma C-peptide increased visually following IV glucose administration, but this initial increase was not significant. A slight peak at time 3 was observed, with times 3 tending greater than time 6 ($p=0.06$).

4.3 b. Serum Insulin

Main effects of Dose ($p<0.0001$), Time ($p<0.0001$), and Day ($p=0.0324$) were observed, and no significant interaction effects were observed. Overall means for Dose ± SE were 18.802 and 25.179 ± 2.365 μU/ml for low dose and high dose, respectively (Figure 9).

Overall means for Time ± SE were: 13.164, 26.804, 21.576, 21.273, 21.648, 23.615, 26.520, 27.479, 26.733, 24.885, 22.888, 19.231, 16.340, and 15.713 ± 2.690 μU/ml for times 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min, respectively. Serum insulin had an acute peak at 3 min, decreased until 9 min, and was followed by a gradual second peak at 30 min before decreasing and returning to baseline at 150 min (Figure 10). Overall means for Day ± SE were 23.119 and 20.862 ± 2.365 for d-1 and 2, respectively, and serum insulin on d-1 was greater than on d-2 (Figure 11).

4.3 c. Insulin to C-peptide Ratio

Main effects of Time ($p<0.0001$) and Day ($p=0.0025$) were observed. Overall I:C-peptide ratio for the low dose also tended to be greater than the high dose (Figure 12). No interaction effects were observed. Overall means for Dose ± SE were 15.912 and 14.172 ± 1.417 μU/ng for low dose and high dose, respectively (Figure 12).

Overall means for Time ± SE were: 9.478, 18.123, 14.911, 14.695, 14.723, 16.014, 18.361, 18.430, 17.800, 16.708, 15.353, 13.160, 11.460, and 11.176 ± 1.743 μU/ng for times

0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min, respectively. I:C-peptide ratio increased acutely at 3 min and decreased after, followed by an equal but more gradual peak until returning to baseline at 150 min (Figure 13). Overall means for Day ± SE were 13.276 and 16.808 ± 1.417 μU/ng for d-1 and 2, respectively, and I:C-peptide ratio for d-1 was less than for d-2 (Figure 14).

4.3 d. Plasma Glucose

Main effects of Time ($p<0.0001$) and Day ($p=0.0006$) were observed, as well as an interaction effect of Day*Time ($p=0.0024$). No main effect of Dose was observed, but peak glucose was lower for the low dose than for the high dose ($p=0.0356$).

Overall means for Time ± SE were: 1.128, 3.093, 2.824, 2.764, 2.638, 2.544, 2.484, 2.383, 2.240, 2.028, 1.629, 1.384, 1.204, and 1.160 ± 0.258 mg/mL for times 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min, respectively (Figure 15). Overall means for Day ± SE were 1.755 and 2.460 ± 0.223 mg/mL for d-1 and d-2, respectively. Plasma glucose and peak glucose (at 3 min) were lower on d-1 than on d-2 (Figure 16).

After administration of the high dose of glucose, muscle fasciculations were observed in each horse beginning approximately at 3 min and ending approximately at time 20 min.

4.3 e. Insulin to Glucose Ratio

Main effects of Dose ($p=0.0298$), Time ($p=0.0038$), Day ($p=0.0004$), and Dose*Time ($p=0.0227$) were observed. Overall means ± SE for Dose were 10.435 and 13.726 ± 1.164 μU/mg for low and high dose, respectively, and I:G ratio for low dose was less than for high dose ($p=0.0298$) (Figures 17-19). Overall means ± SE for Day were 15.010 and 9.152 ± 1.105 μU/mg for d-1 and d-2, respectively, and I:G ratio on d-1 was greater than on d-2 (Figure 20).

4.4 Discussion

The findings of this study contribute to the overall existing knowledge of insulin, glucose, and C-peptide dynamics following IV glucose administration in horses. C-peptide is of interest due to the extreme lack of current research on C-peptide concentrations in horses following glucose administration. Insulin is of interest due to current conflicting conclusions regarding the existence of biphasic insulin concentrations. The present study finds that insulin concentrations do appear to exhibit biphasic characteristics, while C-peptide concentrations do not. However, C-peptide dynamics are inconsistent even between this experiment and EXPT1, so further research is needed to determine clear conclusions.

Kjems et al. (2001) reported that C-peptide is secreted in a biphasic pattern in humans. However, C-peptide has not appeared to follow this pattern in horses in the very few studies conducted on the topic. Tóth et al. 2010 reported a single increase in plasma C-peptide following glucose administration of 300 mg/kg BW, equal to the high dose used in this study. However, this pattern was not observed at either dose administered in the present study. Visually, C-peptide appears to peak at 3 min after administration of the low dose of glucose (100 mg/kg BW), and may exhibit a secondary peak at 90 min. C-peptide after administration of the high dose of glucose (300 mg/kg BW) appears to increase and remain high throughout the IVGTT; however, these fluctuations are visual only and are not statistically significant, and no Dose*Time interaction effect was observed. Overall C-peptide with both doses pooled exhibited a peak at 3 min, followed by a tending decrease to 6 min. This pattern is in contrast to the dramatic increase in plasma C-peptide following IV glucose administration observed in the study by Tóth et al. (2010), though both studies used a similar number of horses (5 vs. 4) and the same glucose dose in the IVGTT.

C-peptide response to the low dose in the present study also does not correspond with C-peptide response from EXPT1A, which used the same glucose dose. In EXPT1A, plasma C-peptide appeared to increase (though this increase was not statistically significant), and decreased at 90 min; whereas, in the present study, C-peptide appeared to peak at 90 min and did not change significantly over time. Considering the extremely limited amount of research on C-peptide kinetics in horses, all of these findings strongly indicate that C-peptide in horses merits further research to investigate both the presence of an increase following IV glucose administration and the existence of a second peak. However, examination of the existing studies' methodologies finds that both this experiment and that by Tóth et al. (2010) used less than 10 horses, while EXPT1A used 24 horses. Therefore, EXPT1A is likely a more accurate representation of typical C-peptide response following IV glucose infusion; however, in EXPT1A a relatively low glucose dose of 100 mg/kg was administered, which may account for the difference between that observed shape and that in the previous study.

In contrast to unclear C-peptide dynamics, insulin dynamics in this study were clearly dose-dependent. Overall insulin response and peak insulin for the low dose was less than for the high dose. Interestingly, the shape of the response also differed. When examined visually, after glucose administration of the low dose, there was a peak at 3 min, followed by a much lower, more gradual secondary peak between 15 and 60 min which gradually decreased. After glucose administration of the high dose, there appeared to be peaks at the same time intervals, but the second peak was much greater (Figure 9).

This study contributes to the theory of biphasic serum insulin concentrations in horses. When serum insulin concentrations were plotted over time for both doses pooled to represent the main effect of time, insulin followed a pattern similar to that seen in previous

studies which administered IVGTTs in horses (Figure 10). The shape of the overall insulin response was characterized by an acute peak at 3 min, followed by a more gradual peak of equal magnitude between 15 and 90 min, with the highest point occurring at 30 min. Pratt et al. (2005) observed a very similar shape following glucose administration of 500 mg/kg BW: an acute peak at 2 min following infusion, and a more gradual secondary peak reaching its highest point at 60 min. This finding may serve to answer questions regarding the existence of second-phase insulin secretion in horses. However, it is important to emphasize that circulating insulin concentrations are a result of a combination of pancreatic secretion and hepatic extraction, so the term "biphasic insulin secretion" is inaccurate and should not be used.

It is necessary to discuss differences in insulin dynamics between d-1 and d-2. Firstly, overall insulin response were higher on d-1 than on d-2. This is likely due to a dramatic difference in temperature on these two days. On d-1, (February 25, 2015) the low temperature in Raleigh according to National Weather Service was 18° F and snowfall was 1.9 in. On d-2 (March 3, 2015), only 7 days after d-1, the low temperature was 35° F and snowfall was 0 in. It is possible that d-1 values are different either due to much colder temperatures, subsequent retardation in glucose administration rate, or a combination of these factors. The hypothesis that colder temperatures would result in increased insulin could result from increased stress and thermoregulation energy expenditure, and potentially from changes in other hormones such as cortisol. The higher insulin:glucose ratio observed on d-1 represents that more insulin is needed per unit of glucose on d-1 than on d-2. This could be explained by stress-induced cortisol on d-1 and the compensatory action of insulin to maintain blood glucose homeostasis. In future research, it would be of interest to examine

the correlation between insulin and cortisol at high and low temperatures or other stressful situations.

The shape of the insulin response also varied between days. Insulin on d-1 exhibited a secondary peak greater than the initial acute peak, while insulin on d-2 exhibited a secondary peak lower than the initial acute peak. It follows that I:C-peptide ratio was higher for the low dose than the high dose, since C-peptide was not different between doses. It was also observed that glucose exhibited a lower peak on d-1 than on d-2. This effect is likely due to a retardation in rate of glucose administration due to extremely cold temperatures of dextrose solution and technicians. This theory cannot be confirmed, as rate of glucose administration was not recorded during the procedure, but it is highly likely that glucose was administered more slowly on d-1. The differences in insulin response shape could be explained by this observation in glucose dynamics. If the lower glucose peak on d-1 was a result of a retardation in glucose administration rate, it follows that serum insulin response would be augmented and clearance would occur at a slower rate. Overall glucose was lower on d-1 than on d-2, an effect likely due to the absence of an acute peak on d-1. It is likely, then, that the higher insulin response on the much colder d-1 is due to increased concentrations of cortisol due to such temperatures.

No effect of dose on plasma glucose was observed, which can be attributed to effective maintenance of glucose homeostasis by increased insulin at the high dose. This hypothesis is confirmed by differences between doses in I:G ratio (Figures 17-19). For both doses, I:G ratio decreased from baseline following IV glucose administration, and increased after. This increase was significantly greater for the high dose, was tending higher at time points 30 and 45 min, and was statistically higher then the low dose at time points 60 and

above. Because glucose was not different between doses, this difference in I:G ratio can be entirely attributed to a higher requirement for insulin per each unit of glucose after administration of the high dose. This is the expected response in healthy horses due to the need to maintain glucose homeostasis; more insulin is required to counteract a more extreme level of hyperglycemia which would be induced by the high dose of glucose administered.

Overall, findings of the present study may serve to confirm certain observations and contribute to overall knowledge of expected behavior of response variables during an IVGTT in horses. C-peptide shows potential as a useful indicator of pancreatic efficiency in horses, but due to the lack of consistency in response curves between the present study, EXPT1A, and previous studies (Tóth et al. 2010), further research is necessary in order to determine characteristic plasma C-peptide dynamics following IV glucose administration. The shape of the insulin response curve in this study was very clearly biphasic, consisting of an acute peak followed by a more gradual curve, which is in agreement with some previous studies and contributes to the overall understanding of insulin behavior in horses following IV glucose administration. The present study also introduces questions regarding differences in the magnitude and shape of insulin response on days of different temperature and potentially stress level. In general, this study contributes to existing knowledge and theories concerning dynamics of C-peptide, insulin, and glucose following IV glucose administration, and creates new questions for further research.

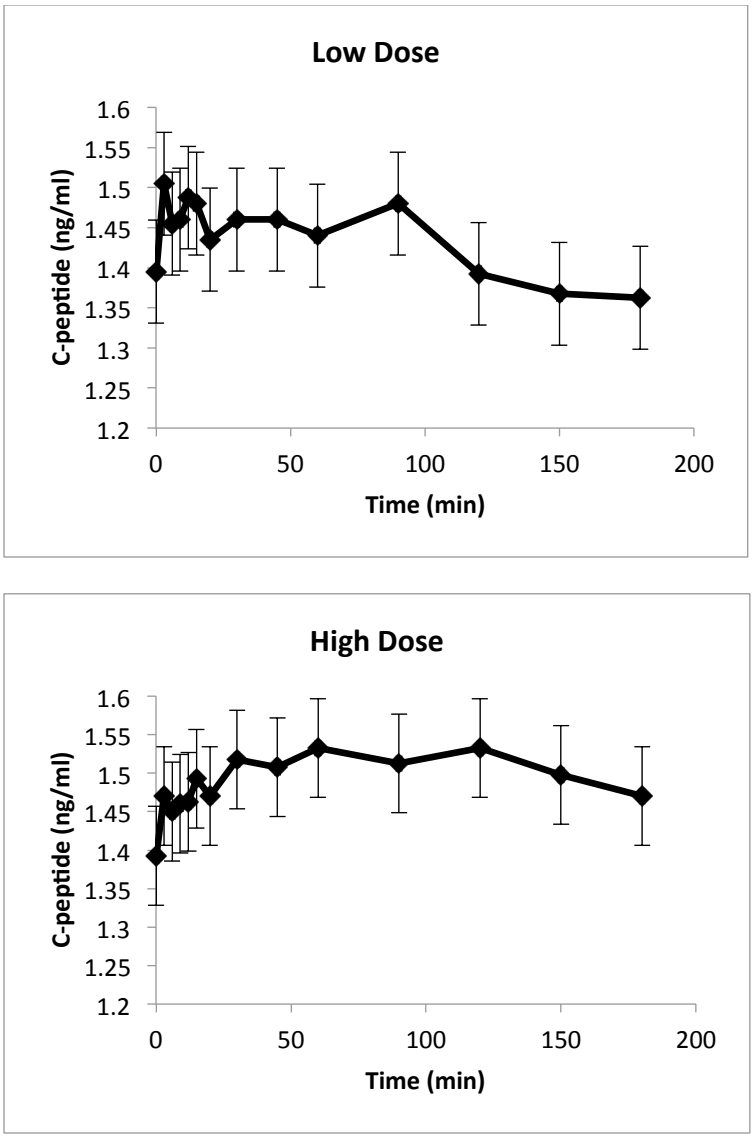


Figure 8. Plasma C-peptide concentrations over time for the low and high dose. Overall C-peptide response was less for the low dose than for the high dose (p=0.0182).

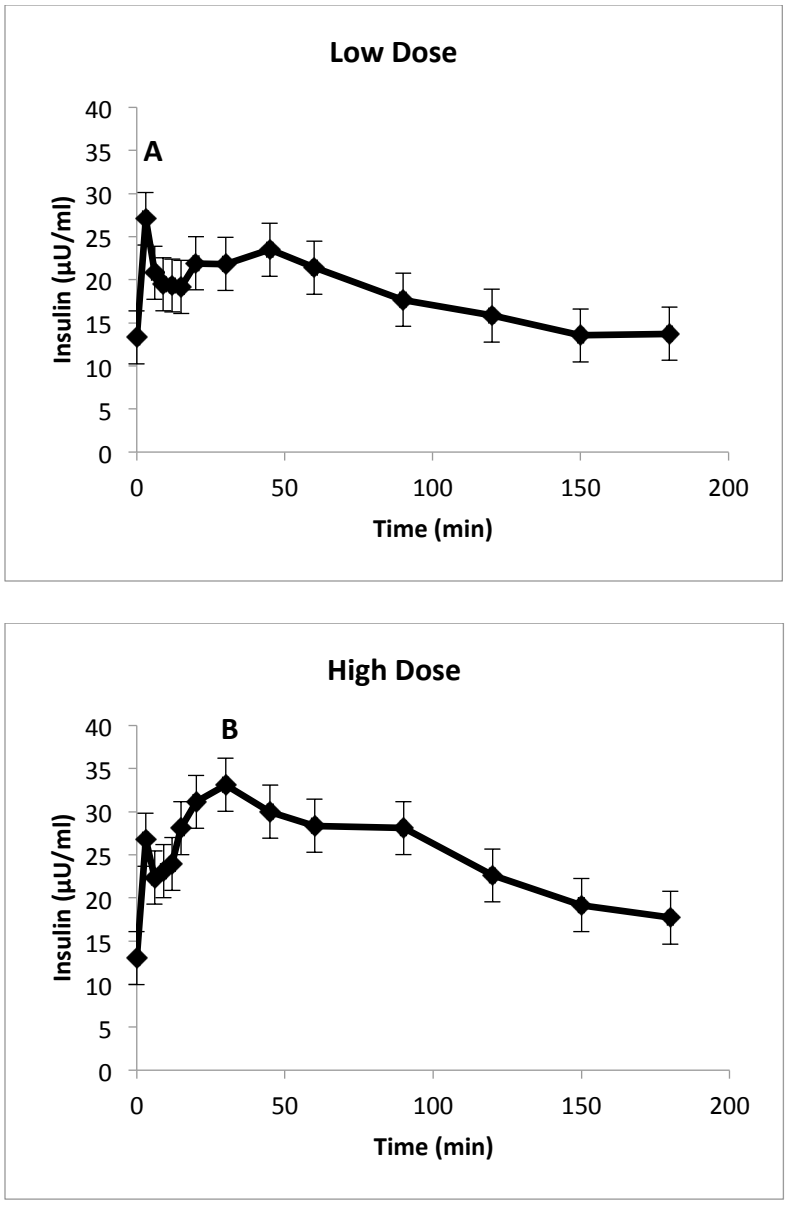


Figure 9. Serum insulin concentrations over time for the low and high dose. Overall insulin response for the low dose was lower than for the high dose ($p<0.0001$). Peak insulin tended to be less for the low dose^{A} than for the high dose^{B} ($p=0.0937$).

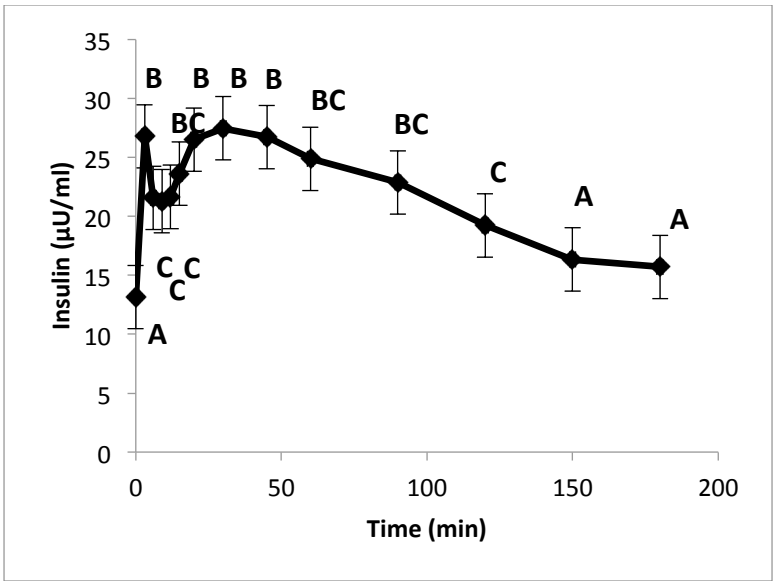


Figure 10. Serum insulin concentrations over time (low and high doses pooled). Serum insulin had an acute peak at 3 min, followed by a second more gradual peak at 30 min, and returned to baseline at 150 min.

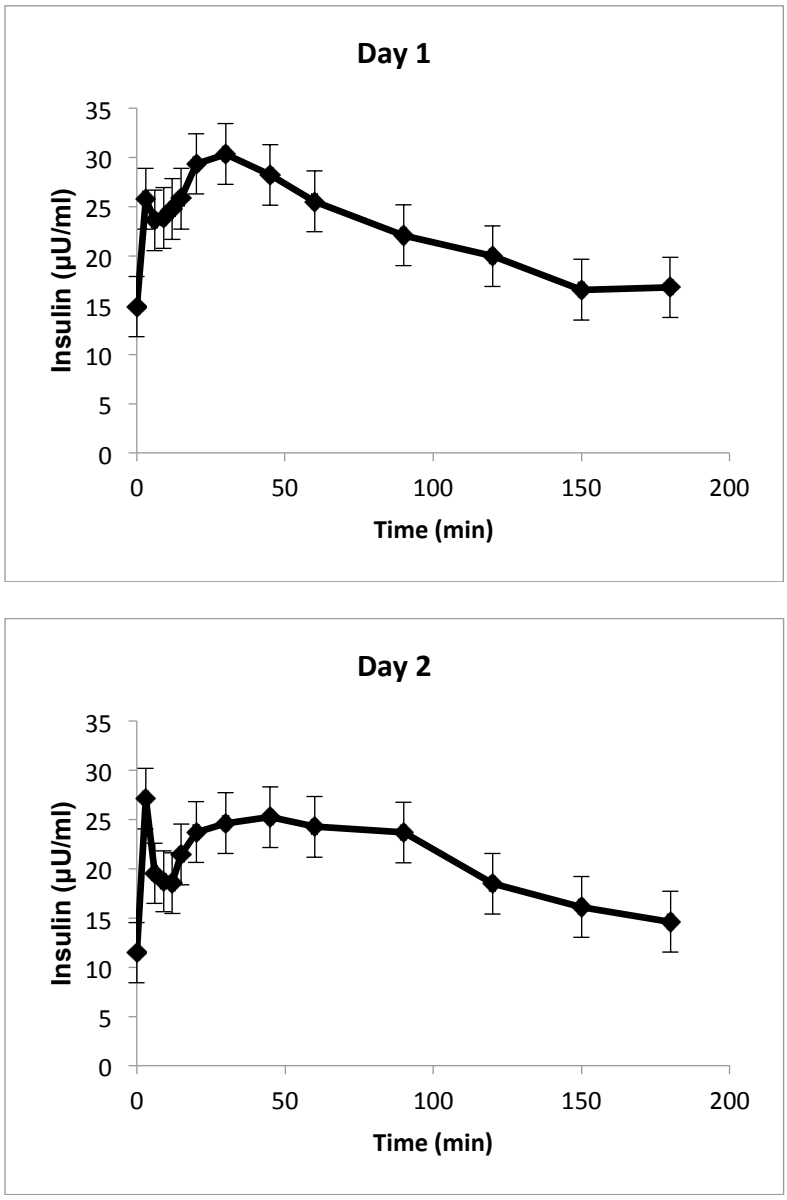


Figure 11. Serum insulin concentrations over time for d-1 and d-2. Overall insulin concentrations were higher on d-1 than on d-2 (p=0.0324). The shape of the insulin response also varies between the two days.

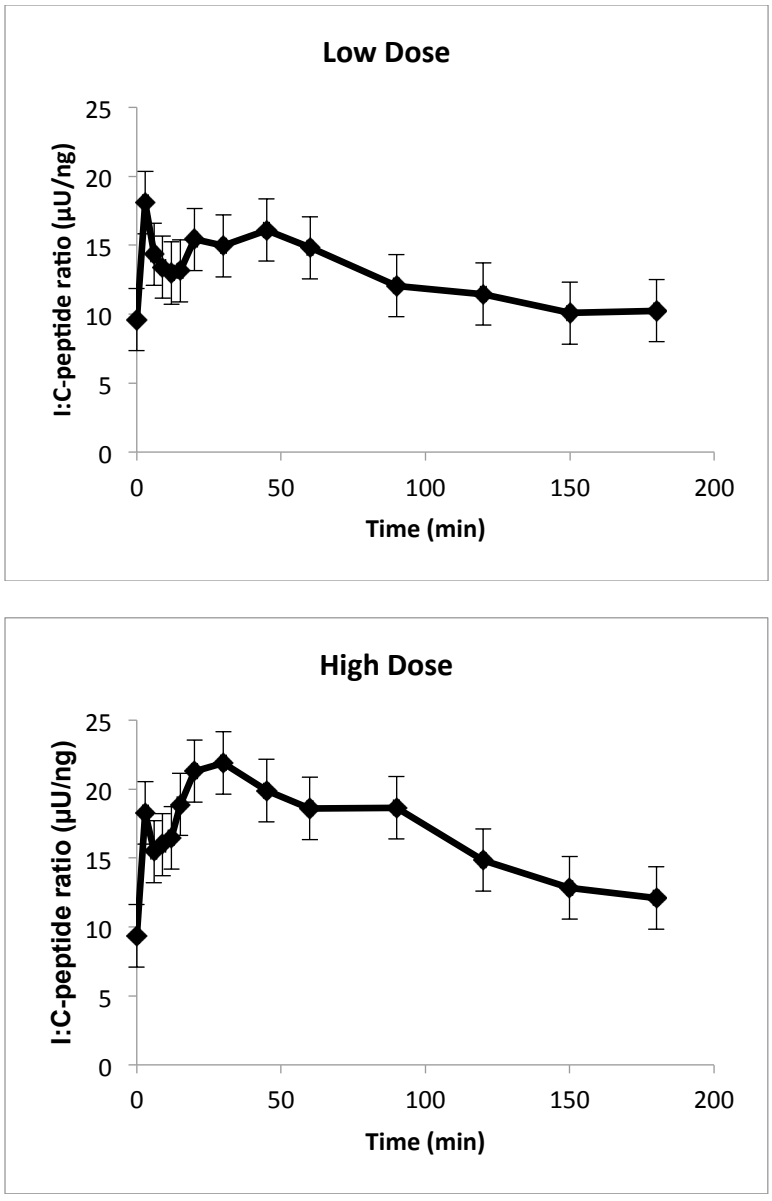


Figure 12. Insulin:C-peptide ratio over time for low and high dose. Overall I:C-peptide ratio was lower for the low dose than for the high dose ($p<0.0001$).

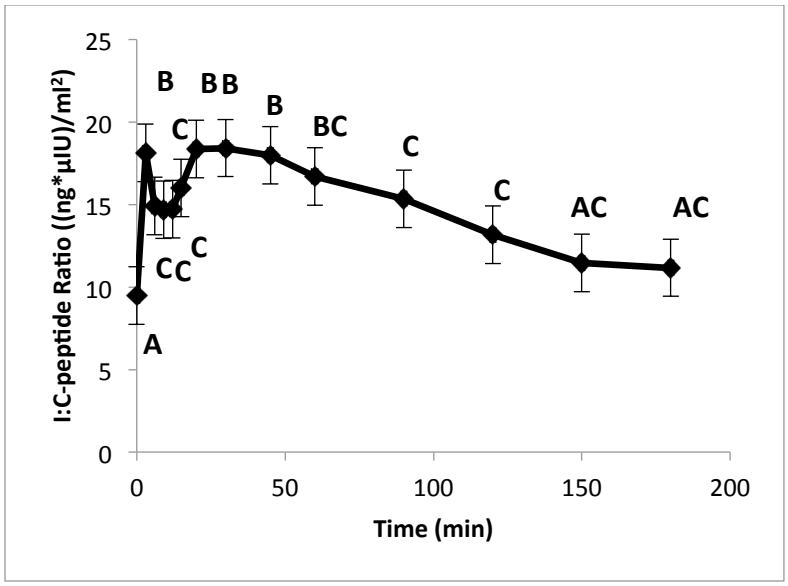


Figure 13. Insulin:C-peptide ratio over time. I:C-peptide ratio increased acutely at 3 min and decreased after, followed by an equal but more gradual peak until returning to baseline at 150 min.

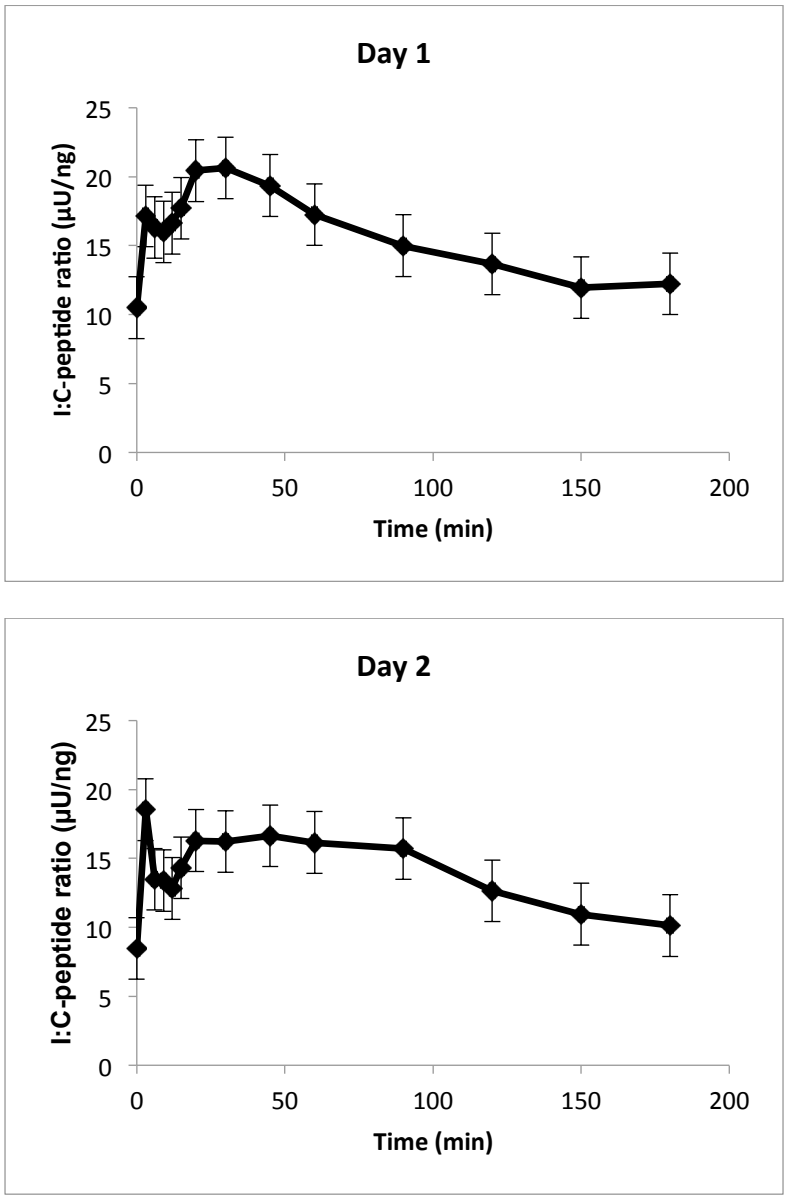


Figure 14. Insulin:C-peptide ratio over time for d-1 and d-2. Overall I:C-peptide ratio for d-1 was greater than for d-2 (p=0.0036).

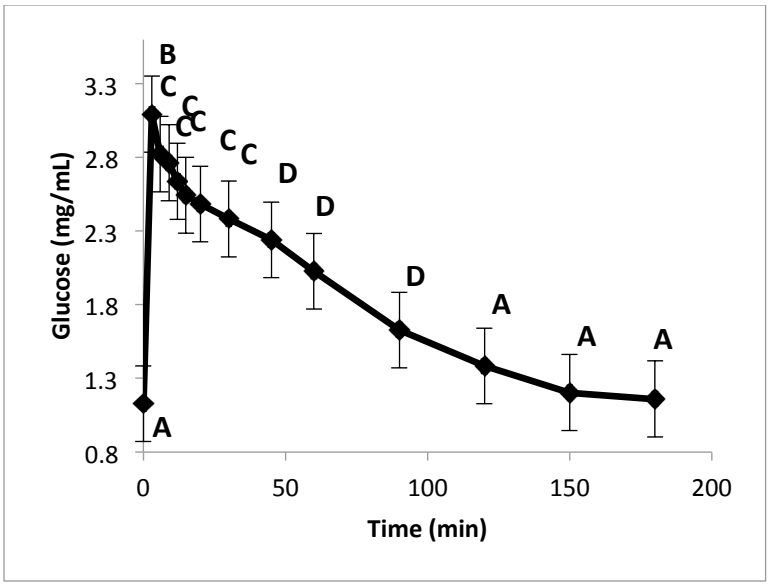


Figure 15. Plasma glucose concentrations over time. Because there was no effect of Dose, low and high doses are pooled in this figure. Plasma glucose peaked at 3 min and gradually decreased until returning to baseline at 120 min.

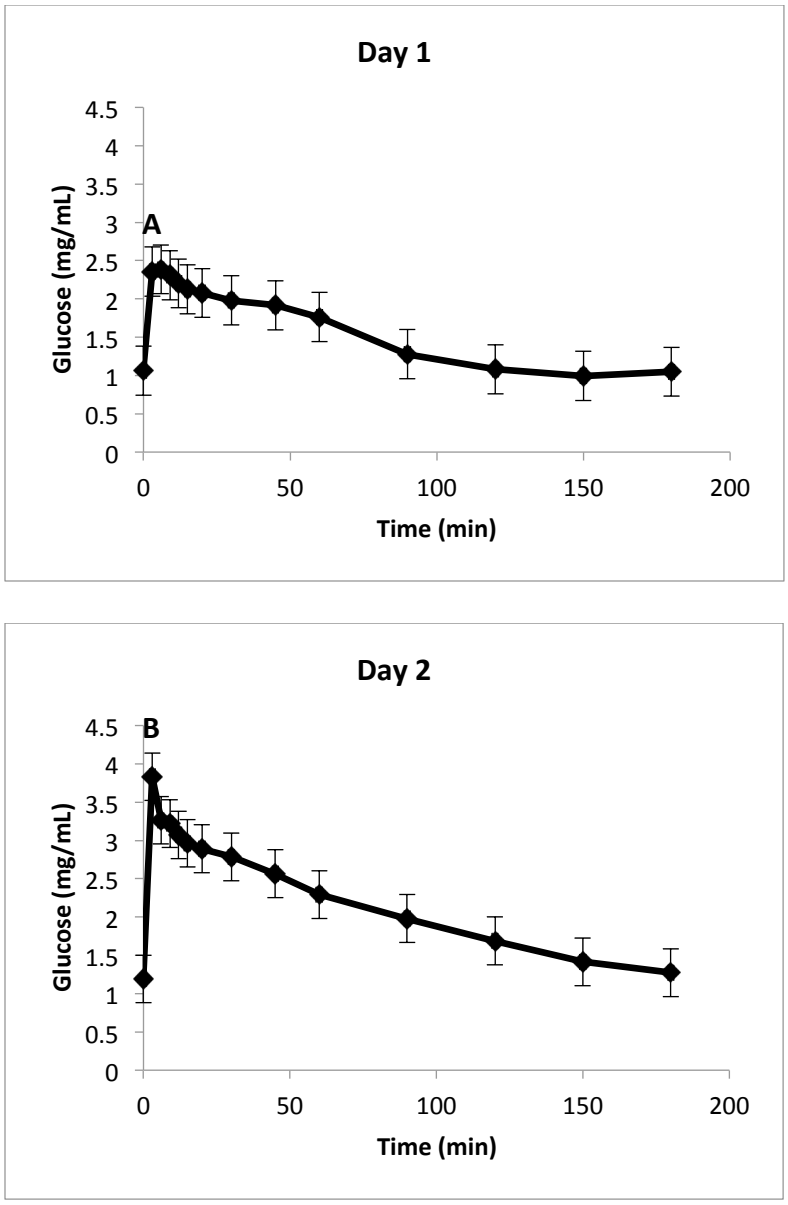
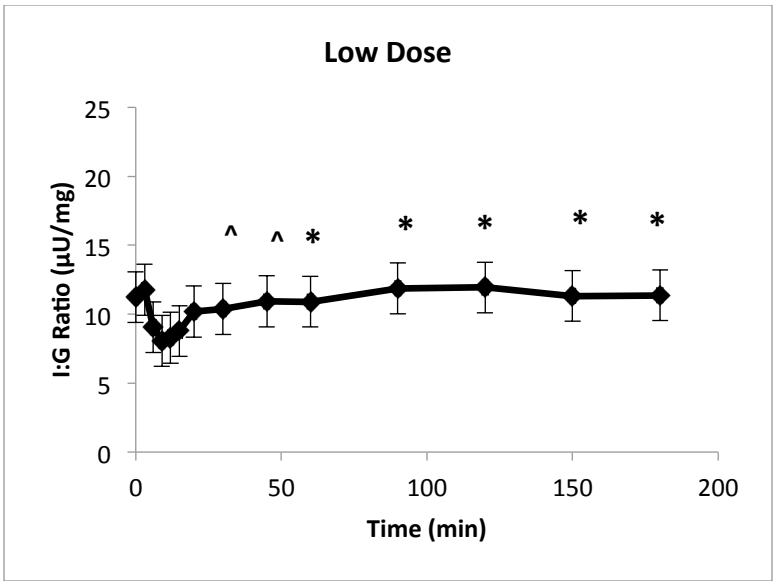


Figure 16. Plasma glucose over time for d-1 and d-2. Overall plasma glucose was lower on d-1 than on d-2 ($p=0.0006$). Peak glucose at 3 min on d-1^{A} was lower than on d-2^{B} ($p=0.0356$).



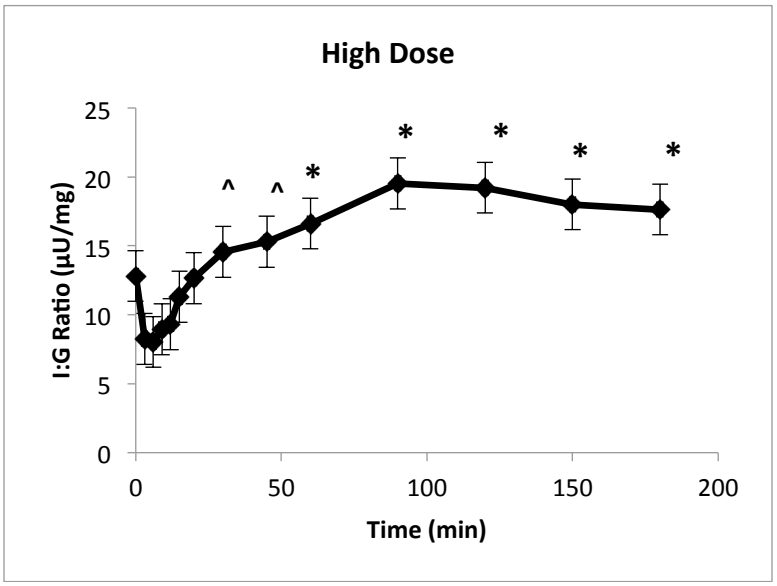


Figure 17. Insulin:glucose ratio over time is depicted. I:G ratio tended different at times 30 and 45 min ($p=0.0949$^, $p=0.0821$^), and different at time points 60 through 180 min ($p>0.05$*).

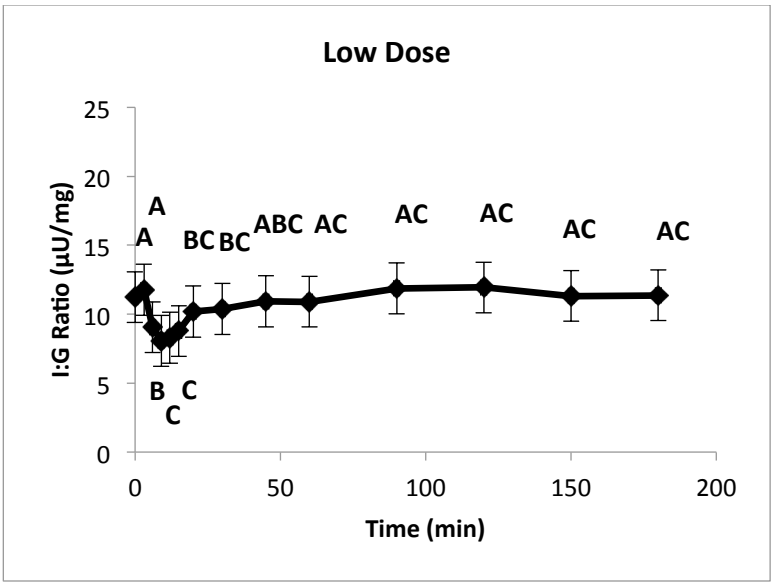


Figure 18. Insulin:glucose ratio over time for the low dose. I:G ratio decreased from baseline until 9 min, then gradually increased until reaching baseline ratio at 45 min.

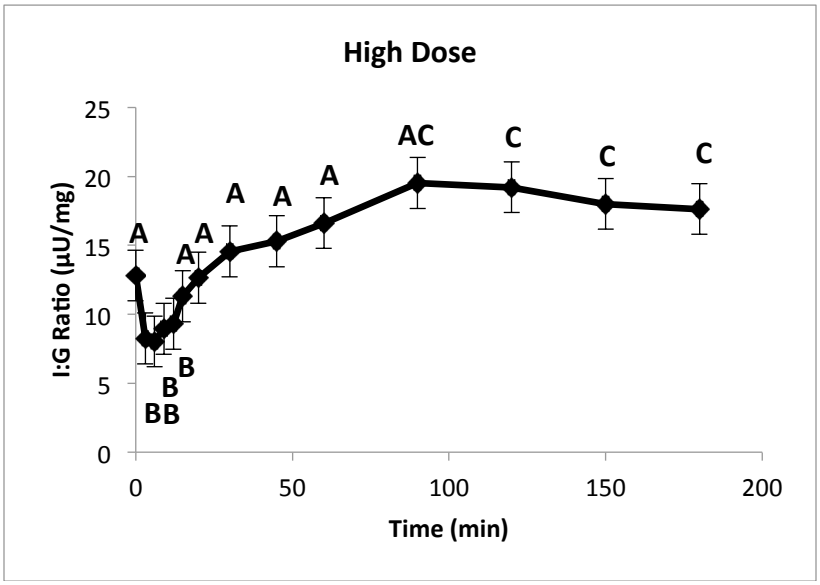


Figure 19. Insulin:glucose ratio over time for the high dose. I:G ratio decreased until 6 min, then gradually increased and reached baseline values at 15 min. I:G ratio continued to increase until reaching above baseline at 120 min and did not return to baseline by 180 min.

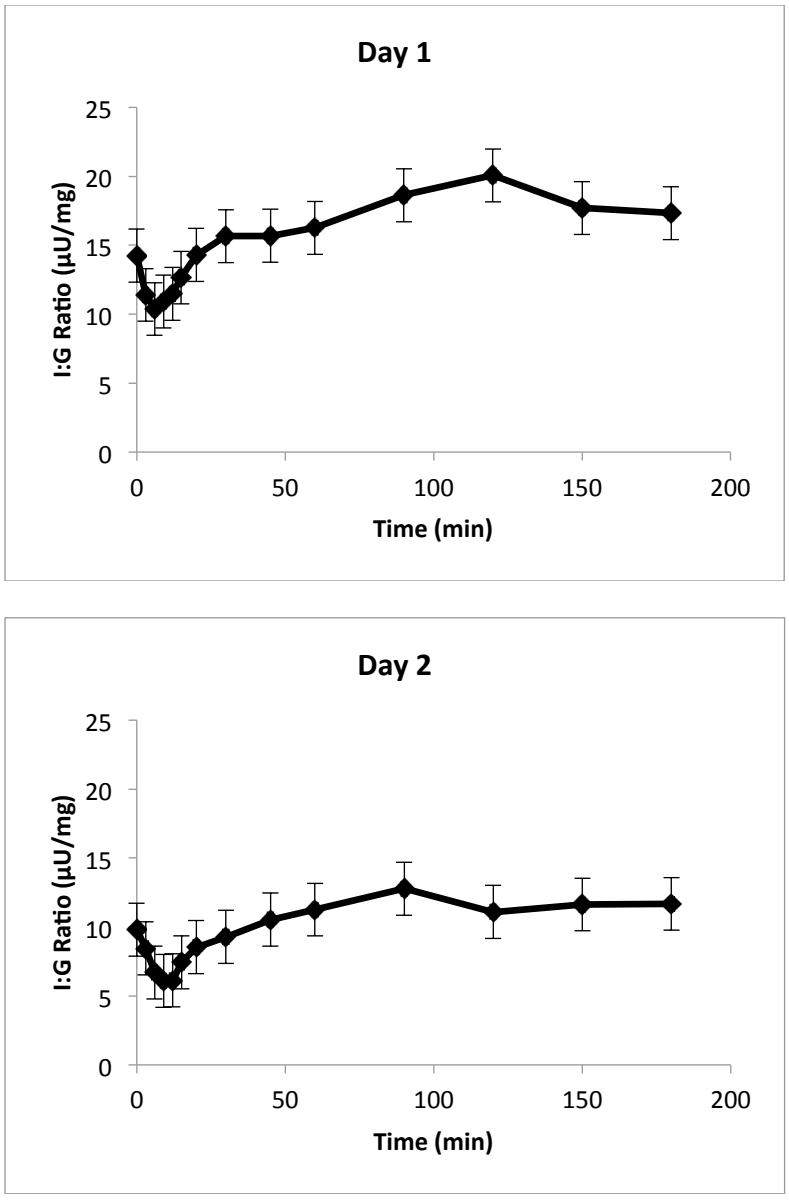


Figure 20. Insulin:glucose ratio over time for d-1 and d-2. Overall I:G ratio on d-1 was greater than on d-2 (p=0.0004).

Literature Cited

Akers, M. R., and D.M. Denbow. 2013. Anatomy and physiology of domestic animals. 2nd ed. Ames (IA): Wiley Blackwell.

Kjems, L. L, A. Vølund, and S. Madsbad. 2001. Quantification of beta-cell function during IVGTT in type II and non-diabetic subjects: assessment of insulin secretion by mathematical methods. Diabetologia. 44: 1339-1348.

Pratt, S. E., R. J. Geor, and L. J. McCutcheon. 2005. Repeatability of 2 methods for assessment of insulin sensitivity and glucose dynamics in horses. J. Vet. Intern. Med. 19: 883-888.

Tóth, F., N. Frank, T. Martin-Jimenez, S. B. Elliott, R. J. Geor, and R. C. Boston. 2010. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. Equine Vet. J. 42(2): 149-155.

Wilcox, G. 2005. Insulin and insulin resistance. Clin. Biochem. Rev. 26(2):19-39.

GENERAL CONCLUSIONS

Insulin resistance is a common problem in horses and is highly associated with obesity and laminitis, conditions which can have serious negative health consequences. Due to its use to treat symptoms of type 2 diabetes in humans, which is physiologically similar to insulin resistance in horses, supplementation with chromium is of interest in horses to improve pancreatic efficiency and increase insulin sensitivity. EXPT1A was conducted to evaluate the effect of chromium propionate supplementation on plasma C-peptide concentrations, as an indicator of pancreatic efficiency. This was especially of interest due to the lack of research on C-peptide concentrations in horses. It was concluded that chromium supplementation did not have a significant effect on C-peptide concentrations following IV glucose administration. However, significant treatment contrasts indicated that the supplement may potentially affect C-peptide concentrations, and further research is needed to definitively confirm its effect on pancreatic efficiency. However, while plasma C-peptide is a promising tool for assessment of pancreatic function, glucose and insulin should also be reported and considered in order to fully evaluate the horse's response to glucose tolerance tests.

The purpose of EXPT1B was to evaluate changes in hair chromium content in horses fed a chromium propionate supplement for 42 d. While some treatment effects were observed, chromium content in mane hair fluctuated throughout the 42 d treatment period in inconsistent patterns, decreasing at 28 d of supplementation and increasing at 42 d. While chromium content in hair does appear to be a useful indicator of overall status, it does not seem to be consistently representative of dietary intake. Therefore, hair as a bioindicator of nutritional status or toxicity level should be evaluated with great caution.

The objective of the second study (EXPT2) was to evaluate plasma C-peptide response following IV glucose administration in horses at a low and high dose of glucose infusion. Overall plasma C-peptide response was dose-dependent in correspondence with the dose administered; C-peptide was lower when the low dose of glucose was administered compared to the high dose. However, no consistent shape of C-peptide response has been observed across current studies. Between EXPT2, EXPT1A, and the study by Tóth et al. (2010), all observed C-peptide response curves have been different from one another. Again, due to the potential usefulness of C-peptide as an indicator of pancreatic efficiency, determination of typical dynamics merits further investigation.

The intent of these studies was to determine the effect of chromium supplementation on plasma C-peptide concentrations and bioaccumulation in mane hair in healthy horses, and to evaluate plasma C-peptide response to IV glucose infusion at two doses. Previous studies have produced conflicting results concerning each of these objectives. While the work presented in this thesis does not confirm previous findings absolutely, it serves to contribute to the overall knowledge base concerning these topics and asks new questions which are useful to future research and investigation, and to the overall understanding of C-peptide dynamics in horses and the affect of chromium supplementation on C-peptide concentrations.

Literature Cited

Tóth, F., N. Frank, T. Martin-Jimenez, S. B. Elliott, R. J. Geor, and R. C. Boston. 2010. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. Equine Vet. J. 42(2): 149-155.

APPENDIX

Appendix

RIA Validation Procedure

A commercially available C-peptide radioimmunoassay kit was validated for use in horses (EMD Millipore Corporation Cat. #HCP-20K). A pooled plasma sample was prepared by taking blood samples in 4 ml EDTA Vacuette tubes (Greiner Bio-one EDTA K2 #454209) from 3 quarter horses and 2 draft cross horses approximately 15 min post-feeding a pelleted concentrate ration. Tubes were spun in a centrifuge at 4° C at 500 RCF for 10 minutes. Plasma from all samples was pooled and mixed thoroughly, then pipetted into centrifuge tubes and stored at -80° C until the validation procedure was conducted. Samples were assayed in duplicate. The areas of validity tested were recovery, parallelism, and linearity. The validation was run successfully only when charcoal stripped horse serum was used as a matrix modifier in preparation of serial dilutions of horse plasma and added to the standard line (r=0.986).

Recovery was assessed by preparation of a high and low spike, and comparison of observed vs. expected C-peptide values were assessed by percent difference (Table 5). Low spike and high spike recovery were 100.80% and 100.00%, respectively.

Table 5: Recovery Assessment of High and Low Spikes

Spike	**Preparation**	**Expected C-peptide (ng/ml)**	**Observed C-peptide (ng/ml)**	**% Recovered**
High	25% 5 ng/ml std. 75% pooled sample	2.13	2.13	100.00%
Low	9% 5 ng/ml std. 91% pooled sample	1.29	1.30	100.80%

Linearity was assessed by the generation of a standard line using different ratios of a low pool and high spike pool, thus generating 7 points of calibration (Table 6).

Table 6: Linearity Assessment of Varying Proportions of Low Pool and High Spike

Preparation	**Expected C-peptide (ng/ml)**	**Observed C-peptide (ng/ml)**	**% Difference**
100% Low	0.85	0.83	-1.83%
83.2% Low 16.8% High	0.959	0.95	-0.94%
66.8% Low 33.2% High	1.08	1.02	-5.90%
50% Low 50% High	1.21	1.22	0.75%
33.2% Low 66.8% High	1.34	1.27	-5.01%
16.8% Low 83.2% High	1.46	1.44	-1.12%
100% High	1.59	1.59	0.00%

C-peptide values in serial dilutions of equine serum ranged from 0.18 to 1.34 ng/ml. The highest C-peptide value measured by this assay was the high spike of 2.13 ng/ml. The assay was used to measure equine C-peptide values ranging from 0.6 ng/ml to 1.93 ng/ml. This validation found that this commercial human double antibody RIA kit is applicable for the measurement of C-peptide in equine plasma when charcoal stripped equine serum is added to kit standards to correct for matrix imbalances.

RIA Sample Analysis Procedure

Standards A-F were made by serial dilutions of the kit standard with assay buffer. Assay buffer was added to all assay tubes containing samples. Tubes 1-2 were total count tubes, to which only radioactive C-peptide-I^{125} complex was added. Tubes 3-4 were Non-

Specific Binding tubes, to which 200 µl of assay buffer only were added. Tubes 5-6 contained 100 µl of assay buffer and 100 µl of first antibody. Tubes 7-18 contained 100 µl each of standards A-F. All samples for each individual horse were assayed in the same grouping, with samples blocked between horses by treatment to help ensure accurate results. Samples were added to each tube in volumes of 100 µl. Pooled plasma and kit QC #2 were used as quality controls after standards and before samples, and after samples in each run. Stripped horse serum was added in volumes of 100 µl per tube to tubes 3-18. Assay buffer was added in volumes of 100 µl per tube to tubes 19-end. First antibody was added in volumes of 100 µl to all tubes except non-specific binding tubes. Runs consisted of one day of one period for d-0 and 28 (e.g. d-0 period 2), and one-third of d-42. Radioactive C-peptide-I^{125} complex was added in volumes of 100 µl to all tubes. Tubes were vortexed at medium speed for 10 seconds and incubated for 22 hours at 4° C.

After incubation, polyethyleneglycol (PEG) was added to all tubes except Total Count tubes as a precipitating reagent of the radiolabeled antibody molecules. PEG acts to remove the hydration shell of these molecules (Bailey 1984). Tubes were incubated at 4° C for 20 min. Tubes were then spun in a centrifuge at 4° C at 2500 RCF for 20 min. Liquid supernatant was decanted, leaving a pellet of radiolabeled C-peptide complex bound to antibodies remaining in the tube. Radioactivity of this pellet was measured using a gamma counter (Perck and Elmer). The amount of unlabeled C-peptide was then determined from this value by a mathematical calculation. Exact C-peptide concentrations were determined by a logarithmic equation utilizing the slope and y-intercept of the standard line for each assay run.