MECHANISM OF INTESTINAL AND SKELETAL MUSCLE GLUCOSE AND AMINO ACID UPTAKE IN PITUITARY PARS INTERMEDIA DYSFUNCTION

By

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ABSTRACT

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Pituitary pars intermedia dysfunction (PPID) is characterized by a progressing loss of dopaminergic inhibition of the pituitary pars intermedia and commonly causes poor physical appearance, hirsutism, and muscle loss in the geriatric horse. We hypothesized that horses with PPID have reduced tolerance to glucose and sensitivity to insulin, reduced mRNA abundance of *SLC2A4*, *INSR*, and *SLC38A2*, and reduced protein expression of GLUT4 and INSR in muscle tissue, reduced intestinal morphology, and reduced D-glucose and L- Gln transport capacity across the jejunal mucosa, and mRNA abundance of *SLC5A1* and *SLC38A2* compared to horses with no PPID. Horses were maintained on hay and senior feed for a period of 5 d while being monitored for medical stability. Presence and grade of the PPID condition was determined based on endocrine testing and post mortem histopathological evaluation of the pituitary gland. Intestinal and gluteal muscle samples were harvested and processed to assess glucose and glutamine tissue nutrient utilization. The results of this study show that muscle wasting and poor physical appearance associated with the pathology of the PPID condition is not due to a reduced transporter abundance at the intestinal and systemic level, but may be due in part to a reduced intestinal morphology. Geriatric horses and those with PPID in particular should be carefully managed with feeding diets containing low concentration of soluble carbohydrates in an effort to decrease the risk of laminitis.

This thesis is dedicated to my loving fiancé and life partner, Ashley Brown. Without her unwavering support, encouragement, patience and most of all love, the completion of this work would not have been possible. I look forward to spending the rest of my life supporting and loving her in return. I love you, Ashley.

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KEY TO ABBREVIATIONS

ACTB	β-actin
ACTH	adrenocorticotropic hormone
AIRg	acute response of insulin to glucose
AUC_{Gluc}	area under the glucose curve
AUC_{Ins}	area under the insulin curve
BCS	body condition score
CS	Cushing's Syndrome
dGLUC	difference between peak glucose concentration at time twenty min and baseline glucose concentration at time zero min
dINS	difference between peak insulin level at time twenty min and baseline insulin level at time zero min
DI	disposition index
FSIGTT	frequently sampled intravenous glucose tolerance test
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GB	baseline plasma glucose concentration
GLUT4	glucose transporter *SLC2A4*
HS	histological score of the pituitary gland
IB	baseline serum insulin
INSR	insulin receptor
IR	insulin resistance
ODST	overnight dexamethasone suppression test
OGTT	oral glucose tolerance test
POMC	pro-opiomelanocortin peptides

PPID	pituitary pars intermedia dysfunction
Sg	glucose sensitivity
SI	insulin sensitivity
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SNAT2	neutral AA transporter *SLC38A2*
TBS	tris-buffered saline
TBST	tris-buffered saline with 0.1% tween-20

CHAPTER ONE: INTRODUCTION

Pituitary pars intermedia dysfunction (PPID), commonly referred to as "Equine Cushing's Disease" or "Equine Cushing's Syndrome", is an endocrine disorder affecting mainly the aged horse (Schott, 2006). It is estimated that 15 to 30% of horses older than 15 years of age are affected (McGowan et al., 2013; Brosnahan and Paradis, 2003), with an overwhelming 50% of the affected horses euthanized due to the deteriorating physical appearance and laminitis. Geriatric horses play a key role in the equestrian industry where they serve as experienced schoolmasters for less experienced riders. The safety of many parents' children depends on aged horses prospering until their maximum life expectancy. Although two drugs, pergolide mesylate and cyproheptadine, are commonly used to treat the PPID condition, they are very costly and their effectiveness decreases over time. In addition, the daily management of horses with PPID is tedious.

There appears to be no specific breed (McGowan, 2008) or sex (Schott et al., 2001) more prone to developing PPID. The known physiological mechanisms behind the pathology is represented in Figure 1.1.

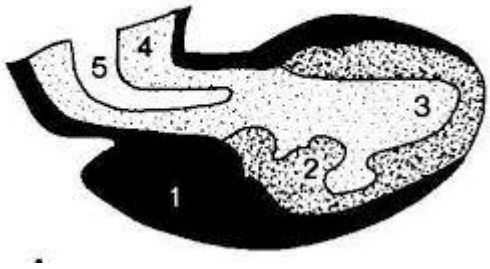

Figure 1.1. The anatomical structure of the equine pituitary. The pars distalis (1), pars intermedia (2), pars nervosa (3), hypophyseal stalk (4), and recess of third ventricle (5). From Dyce K.M, Sack W.O, and Wensing C.J. Textbook of Veterinary Anatomy. 2nd ed., Philadelphia, PA; WB Saunders, 1987.

The condition is progressive and characterized by hypertrophy, hyperplasia, adenoma and loss of dopaminergic inhibition of the pars intermedia of the pituitary gland resulting in increased circulating concentration of pro-opiomelanocortin peptides (POMC) as illustrated in Figure 1.2 and Figure 1.3, respectively. Horses with PPID are reported to have as much as one-eighth the serum concentration of dopamine (Millington et al., 1988) and one-sixth the dopaminergic nerve terminals (McFarlane et al., 2005). The increase in POMC from corticotropes and melanotropes increases the production of ACTH from the pars intermedia and β-lipotropin from the pars distalis by stimulating the activity of prohormone convertase 1 (PC1) (Hillyer et al., 1992; Love, 1993; van der Kolk et al., 1993; Dybdal et al., 1994; van der Kolk et al., 1995; Eiler et al., 1997; SM Reed, 1998). In non-PPID horses, prohormone convertase 2 (PC2) cleaves ACTH into α-melanocyte-stimulating hormone (α –MSH) and corticotropin-like intermediate lobe peptide (CLIP), and β-lipotropin into β-endorphin (β –END), lipotrophin and other small peptides (McFarlane, 2011). Horses without PPID almost exclusively produced ACTH in the pars distalis however horses affected with PPID produce increased concentrations of ACTH from the pars intermedia at a rate faster than its conversion into α –MSH and CLIP. Horses diagnosed with PPID characteristically have increased blood concentration of cortisol, although the mechanism remains largely not understood (Cartmill et al., 2003).

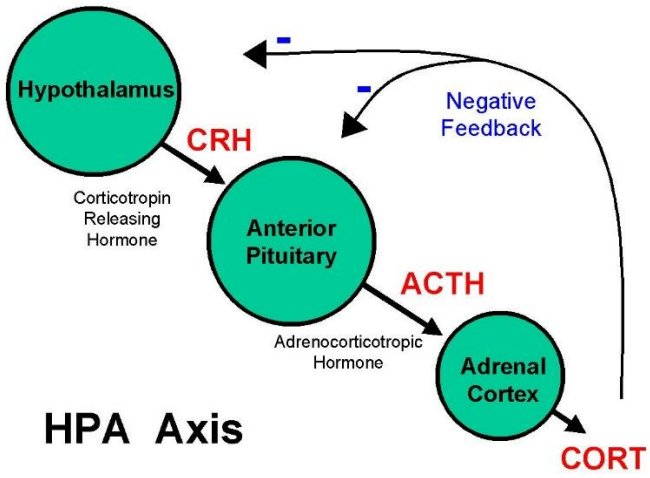

Figure 1.2. Hypothalamus-pituitary-adrenal axis. Hypothalamic release of corticotrophin releasing hormone (CRH) stimulates release of ACTH from the pituitary. Adrenocorticotropic hormone acts on the adrenal cortex to secrete cortisol, a glucocorticoid which acts on the hypothalamus and pituitary to inhibit release of CRH and ACTH, respectively. From www.biology.ucr.edu.

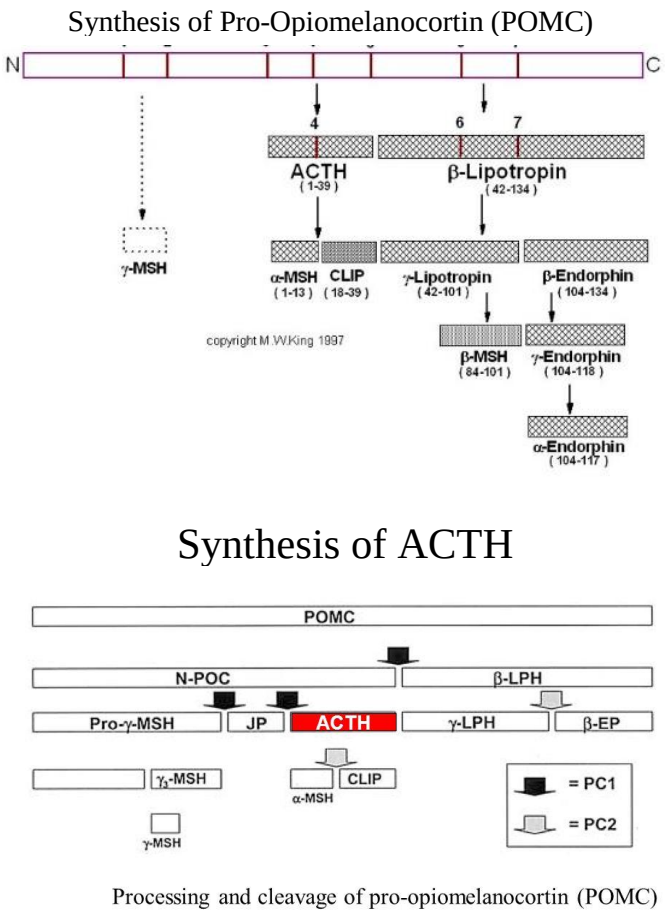

Figure 1.3. Proopiomelanocortin prohormone produced in the pars intermedia is cleaved by PC-1 to produce ACTH. Adrenocorticotropic hormone is cleaved by PC-2 to produce α-MSH and CLIP. From www.themedicalbiochemistrypage.org and http://slideplayer.com/slide/1496700/.

Horses diagnosed with PPID exhibit a variety of clinical signs, most commonly including poor body condition, appearance of muscle wasting, abnormal fat deposits, hirsutism, hyperhidrosis, abnormal fat deposits, and a heightened risk for secondary infections (Innerá et al., 2013; Klinkhamer et al., 2011; McFarlane et al., 2010; Aleman et al., 2006; Donaldson et al., 2004). Hirsutism is the incomplete, delayed, and lightening of the coat and the most obvious clinical sign of PPID with a 90% prediction for the disease (Frank et al., 2006). It has been proposed that hirsutism results from the chronically high blood concentration of α –MSH (Van

der Kolk et al., 1990-1992). Hyperhidrosis or excessive sweating was originally thought to be a result of hirsutism, but recent studies have shown PPID horses to excessively sweat in cool conditions and after being body clipped (Schott, 2002). Muscle wasting of epaxial and gluteal muscles in horses with PPID possibly could be attributed to the increased glucocorticoid production, most notably cortisol, which is known in other species to be a contributing factor to type 2 muscle fiber atrophy, sarcoplasmic lipid accumulation, greater myofiber size variation, and increased proteolysis (Aleman and Nieto, 2010; Aleman et al., 2006). Inhibition of lipolysis due to hyperinsulinemia has been postulated as a reason why horses with PPID develop abnormal fat deposits over the neck, tail head, and eyes (Schott, 2002). Human studies have shown visceral adiposity stores to be highly correlated with higher plasma leptin concentrations (Davis et al., 2009). The concentration of leptin in many species, including hamsters (Dodson and Steiner, 1998) and sheep (Wilcox, 2005), has also shown to fluctuate with season (Fazio et al., 2008; McFarlane, 2007; Frank et al., 2006; Couetil et al., 1996; McFarlane et al., 2010; Beech et al, 2009; Fazio et al, 2008; Dybdal et al, 1994; McFarlane et al., 2012; Donaldson et al., 2004; Davis et al., 2009; Van Camp et al., 2004). Leptin regulates CRH, ACTH, and α-MSH secretion in both the pars distalis and the pars intermedia (Fazio et al., 2008). Leptin levels are lowest in horses during the day and peak at night, with a greater effect of season in the spring and summer (Pørksen et al., 2002). The effect on the endocrine system due to seasonal changes is another characteristic feature of the PPID condition. Aged horses with PPID were shown to exhibit seasonal changes in the circadian peripheral plasma concentrations of α –MSH serotonin, dopamine, and cortisol (Jeffcot et al., 1986). Dopamine release from the pars intermedia neurons inhibits POMC-derived peptide secretion (Schmidt et al., 1992).

Despite their poor physical appearance, horses with PPID have characteristically high appetite (Mountjoy, 2010). Thus poor nutrient absorption and peripheral nutrient utilization may contribute to the pathophysiology of the condition. The overarching goal of this thesis was to assess nutrient absorption and peripheral muscle utilization in PPID and age-matched non PPID horses.

In the first chapter of this thesis, insulin sensitivity and glucose tolerance was measured in a total of 14 horses donated for this study. The aim was to classify horses into 3 groups: aged control horses without PPID, aged PPID horses without insulin resistance, and aged PPID horses with insulin resistance. This goal was based on the available literature with an estimated insulin resistance rate of at least 50% in the PPID population. In 57-94% (Keen et al., 2004; van der Kolk et al., 1995; Hillyer et al., 1992) and 45 to 90% of the PPID-afflicted horse population (Keen et al., 2004; van der Kolk et al., 1995), abnormalities in glucose homeostasis or glucose intolerance and insulin resistance have shown to persist. Also, horses with PPID have been reported to have both hyperinsulinemia at rest and a reduced hypoglycaemic response to exogenous insulin (Freestone et al., 1992; Garcia and Beech, 1986; Jeffcott et al., 1986). Studies have shown differing results in regards to whether insulin resistance is directly (Keen et al., 2004; van der Kolk et al., 1995; Donaldson et al., 2004) or indirectly (Mastro et al., 2015; van den Berg et al., 2013; Vick et al., 2007) associated with the PPID condition. Insulin resistance and central fat deposit in men is associated with excess blood cortisol concentration and cortisol production rates (Purnell et al., 2009; Phillips et al., 1998; Filipovsky et al., 1996; Stolk et al., 1996). Pituitary pars intermedia dysfunction has also been associated with an increased risk for laminitis (Donaldson et al., 2004; Carter et al., 2009; Geor, 2009) and laminitis in PPID horses has been thought to be a result of high serum insulin concentrations (Treiber et al., 2006; Asplin

et al., 2007). Many of the studies reported above however lacked in adequate control subjects that were age-matched control, or included ponies. In Keen et al. (2004) cited above, the majority of test subjects were ponies who are known to be more susceptible to insulin resistance than horses (Schott, 2002; van der Kolk, 1997). Also, in Klinkhamer et al. (2011) and Garcia and Beech (1986), the increased insulin resistance status of horses diagnosed with PPID was made in comparison to younger non-PPID horses. Older horses however have been shown to be more prone to insulin resistance than younger horses (Malinowski et al., 2002), with similar observations in humans (Barbieri et al., 2001; Paolisso et al., 1999). In a recent study by Mastro et al. (2015), PPID-affected horses compared to age-matched controls did not differ in insulin sensitivity or glucose tolerance when assessed using the reciprocal of the square root of insulin (RISQI), the modified insulin to glucose ratio (MIRG), and isoglycemic hyperinsulinemic clamp approaches. Therefore, in the first chapter of this thesis, geriatric horses were evaluated for PPID based on both endocrinological testing and post-mortem examination of the PG, and assessed for insulin resistance and glucose intolerance using oral glucose tolerance and frequently sampled intravenous glucose tests.

In the second chapter, molecular entities involved in the regulation of intracellular muscle glucose and glutamine utilization were determined to further confirm the insulin status determined in this cohort of horses. Thus, transcript abundance of *SLC2A4*, *INSR* and *SLC38A2*, respectively encoding GLUT4, insulin receptor and SNAT2, and protein abundance of GLUT4 and insulin receptor were measured in muscle tissue. In adipocytes and muscle cells, insulin stimulates the translocation of GLUT4 to the plasma membrane (Huang, 2007). The first determinant of insulin-dependent peripheral glucose uptake is the availability on the cell membrane of the GLUT4 transporter (Huang, 2007). With respect to AA transport, insulin

activation involves in part the recruitment of the Na^+-coupled neutral AA transporter 2 (SNAT2) from an endosomal compartment (Evans et al., 2008). Molecules associated with recruitment and fusion of SNAT2-containing vesicles to the surface membrane are also implicated in the regulation of GLUT4 translocation (Evans et al., 2008). Insulin-mediated neutral AA uptake via SNAT2 is down-regulated in metabolic acidosis and insulin resistance, causing proteolysis and muscle wasting in humans (Evans et al., 2008). Therefore, characterizing the abundance of SNAT2 in muscle tissue of PPID and non-PPID horses provided additional insight into the possible impact of PPID on insulin-dependent nutrient transport.

The second part of the thesis is focused on the link between PPID and intestinal function based on the notion that gastrointestinal health status is a key determinant of nutrient availability. It is proposed that reduced nutrient absorptive capacity by the small intestinal mucosa contribute to the apparent poor nutritional status of geriatric PPID horses. First, nutrient absorptive capacity was assessed by histomorphometry analysis of the small intestinal mucosa in Chapter 3. Histomorphological changes to the small intestinal mucosa such as villus atrophy and increased crypt depth are associated with poor nutrient absorption and malnutrition in animals (Stevenson et al., 2013) and humans (Dewar and Ciclitira, 2005; and Ventura et al., 2013). To date, no study has addressed whether PPID is associated with histomorphometric changes of the small intestinal mucosa. Second, in Chapter 4, glucose and Gln transport across brush border membrane of the jejunal mucosa was measured ex vivo, along with the molecular entities responsible for their transport. Brush border membrane vesicles (BBMV) preparation from small intestinal mucosa is a useful ex vivo tool to precisely characterize transport kinetics of nutrients in response to changes in physiological or dietary processes. Dietary AA are a major fuel for the small intestinal mucosa and are necessary for maintaining the intestinal mucosal morphology and

function in neonatal piglets (Wu, 1998). Glutamine is a preferred nutrient of intestinal enterocytes for mucosal growth and function (Ganapathy et al, 1994; Salloum et al., 1994; Windmueller and Spaeth, 1980). Glutamine is transported by SNAT2, which is encoded by *SLC38A2* (solute carrier family 38, member 2). In horses, glucose is transported across the apical membrane of small intestinal epithelial cells (Dyer et al., 2002) by the high-affinity, Na^+-glucose cotransporter SGLT1, encoded by *SLC5A1* (solute carrier family 5, member 1). In chapter 4, it is hypothesized that the transport capacity (V_{max}) of D-glucose and L-Gln across the apical membrane is reduced in PPID compared to non-PPID horses, and that reduced V_{max} is related to lower mRNA abundance of *SLC38A2* and *SLC5A1*.

The overarching hypothesis of this thesis is that glucose and glutamine utilization by geriatric horses suffering from PPID is reduced in the intestine and peripheral muscle tissue. The objectives of this study were to: (1) measure glucose tolerance and insulin sensitivity in horses with PPID and horses with no PPID (control); (2) quantify mRNA abundance of *SLC2A4*, *INSR* and *SLC38A2*, and quantify protein abundance of INSR and GLUT4 in muscle tissue of PPID and non-PPID horses (control); (3) evaluate the morphology of the mucosa in the proximal, mid and distal regions of the small intestine in horses clinically confirmed with PPID and no PPID; (4) measure transport capacity of D-glucose and L-Gln across jejunal BBMV of PPID and non-PPID horses, and quantify the mRNA abundance of *SLC5A1* and *SLC38A2* in duodenal, jejunal and ileal mucosa of PPID and non-PPID horses.

The following chapters are organized into individual manuscripts which respectively address the above objectives. Chapter 5 presents a summary of these results and overall conclusions.

CHAPTER TWO: Evaluation of glucose tolerance and insulin sensitivity in horses with pituitary pars intermedia dysfunction

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Abstract

Pituitary pars intermedia dysfunction (PPID) is characterized by a progressing loss of dopaminergic inhibition of the pituitary pars intermedia. It commonly causes lethargy, poor physical appearance, abnormal sweating, hirsutism and muscle loss in the geriatric horse, and therefore is of welfare concern. We hypothesized that horses with PPID have reduced tolerance to glucose and sensitivity to insulin compared to horses with no PPID. The objective was to measure glucose tolerance and insulin sensitivity in horses with PPID and horses with no PPID (control). Eight PPID and 4 age-matched control geriatric horses (> 19 yr) were donated for this study. Grade of PPID was determined based on an overnight dexamethasone suppression test (DST), ACTH testing and histopathology evaluation of the pituitary gland (PG). Glucose tolerance and insulin sensitivity were measured based on an oral glucose tolerance test (OGTT) and a frequently sampled intravenous glucose tolerance test (FSGITT). Compared to control horses, PG grade and ACTH concentration of PPID horses were greater ($P \leq 0.001$) and tended to be greater ($P = 0.079$), respectively. Glucose concentration of PPID horses tended to be greater than that of control at 90 min ($P = 0.099$). Compared to control, insulin concentration of PPID horses was greater or tended to be greater at times 3 ($P = 0.091$), 23 ($P = 0.083$), 24 ($P = 0.056$), 25 ($P = 0.027$) and 27 min ($P = 0.048$) following i.v. infusion of insulin during FSIGTT. The OGTT parameters IB_{OGTT}, Peak Ins_{OGTT}, GB_{OGTT}, Peak $Gluc_{OGTT}$, $dGLUC_{OGTT}$, and $dINS_{OGTT}$ did not differ between control and PPID horses. Compared to control horses, the OGTT parameter $dGLUC_{OGTT}$ tended to be greater ($P = 0.078$) in PPID horses. The FSIGTT parameters AIRg, DI, SI, Sg, IB_{FSGITT}, GB_{FSGITT}, $dGLUC_{FSIGTT}$, $dINS_{FSIGTT}$, $FSIGTTAUC_I$, and $FSIGTTAUC_G$ did not differ between control and PPID horses. The disposition index (DI) correlated with ACTH concentration ($P = 0.04$). In this study, geriatric horses with PPID did not

have lower insulin sensitivity and glucose tolerance compared to non PPID, geriatric horses. The positive correlation between DI and ACTH concentration is indicative of some degree of insulin resistance related to PPID, and a tendency for greater $FSIGTTAUC_I$ in PPID horses may be indicative of some degree of hyperinsulinemia. Neither PPID nor control horses however could be classified as insulin resistant based on the estimated SI parameter. During the OGTT, there was an equal number of horses in both PPID and control cohorts that exhibited signs of glucose intolerance. Geriatric horses and those with PPID in particular should be carefully managed with feeding diets containing low concentration of soluble carbohydrates in an effort to reduce the risk for laminitis.

Introduction

Pituitary pars intermedia dysfunction (PPID) also known as Cushing's disease is a progressive endocrine disorder affecting the aging horse population (Schott, 2002; van der Kolk, 1997). The disorder is characterized by hypertrophy, hyperplasia, adenoma and loss of dopaminergic inhibition of the pars intermedia of the pituitary gland resulting in increased circulating concentration of pro-opiomelanocortin peptides (POMC), including ACTH (Reed, 1998; Eiler et al., 1997; van der Kolk et al., 1995; Dybdal et al., 1994; Love, 1993; van der Kolk et al., 1993; Hillyer et al., 1992). Horses diagnosed with PPID exhibit a variety of clinical signs, most commonly including poor body condition, appearance of muscle wasting, abnormal fat deposits, hirsutism, and heightened risk for secondary infections (Innerá et al., 2013; Klinkhamer et al., 2011; McFarlane et al., 2010; Aleman et al., 2006; Donaldson et al., 2004). Pituitary pars intermedia dysfunction has also been associated with an increased risk for laminitis (Carter et al., 2009; Geor, 2009; Donaldson et al., 2004).

Earlier studies reported horses with PPID to have resting hyperinsulinemia and reduced hypoglycaemic response to exogenous insulin post glucose tolerance test (Freestone et al., 1992; Garcia and Beech, 1986; Jeffcott et al., 1986). Abnormalities in glucose homeostasis or glucose intolerance have been reported in 57 to 94% (Keen et al., 2004; van der Kolk et al., 1995; Hillyer et al., 1992) and insulin resistance in 45 to 90% of the PPID-afflicted horse population (Keen et al., 2004; van der Kolk et al., 1995). In the work reported by Keen et al. (2004) and van der Kolk et al. (1995) however half to 100% of the candidates were ponies, which are typically at higher risk for insulin resistance (Donaldson et al., 2004). Conversely, several more recent studies have reported absence of insulin resistance in horses with PPID (Mastro et al., 2015; van den Berg et al., 2013; Vick et al., 2007). Reeves et al. (2001) and more recently McGowan et al. (2013)

suggested that insulin insensitivity may not be specific to PPID *per se* and Malinowski et al. (2002) proposed that insulin resistance in horses with PPID is related to aging. So far, only one of the studies cited above (Mastro et al., 2015) included matched-age controls. Furthermore, there have been no studies examining the incidence of insulin resistance and glucose intolerance in horses confirmed with PPID based on both endocrinological testing and post-mortem examination of the PG. Continued assessment of glucose metabolism in the aging, PPID horse is critical in order to design dietary and management strategies targeted to this segment of the equine population and maintain their welfare. We hypothesized that geriatric horses with PPID have a lower sensitivity to insulin and tolerance to glucose compared to geriatric horses without PPID. The objective of this study was to determine insulin sensitivity and glucose tolerance in geriatric horses with PPID and without PPID condition.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University (03-12-064-001).

Animals and Housing

Fourteen horses, 10 presenting clinical symptoms of PPID and 4 presenting no clinical symptoms of PPID were donated for this study between September and December of 2012 following owners' consent. The premise for recruiting a larger sample size of PPID than non PPID horses was to obtain three experimental groups including non PPID horses, PPID horses with insulin resistance and glucose intolerance, and PPID horses with no insulin resistance and glucose intolerance. This specific study was part of a larger study aimed as assessing intestinal and peripheral muscle nutrient utilization of PPID horses with and without insulin insensitivity

and glucose intolerance. Horses were of multiple breeds and genders (mare and gelding) ranging from 19 to 39 years of age. No ponies were admitted. Average age between control (25.3 ± 2.1 years) and PPID (28.6 ± 1.5 years) did not differ (Table 1). Following admission, all horses were housed indoor in individual stalls (3.05 m × 3.05 m or 3.66 m × 3.66 m) and the ambient temperature maintained at 21˚C. Horses were fed second cutting mixed orchardgrass (*Dactylis glomerata L.*) and bromegrass hay (*Bromus mollis L.),* and a commercial grain concentrate[a] containing 14% crude protein, 1.8% fat and 18% crude fiber, at 1.8 and 0.5% of their body weight, respectively.

Clinical Evaluation

Horses were monitored for medical stability for a period of 5 days prior to being assessed for PPID. Horses were subjected to ACTH testing as described in Beech et al. (2009) and an overnight dexamethasone suppression test (ODST) as described by Dybdal et al. (1994). Plasma ACTH concentration was measured using a commercially available radioimmunoassay kit according to the manufacturer's instructions, and radioactivity counted with a gamma counter[c]. Serum cortisol concentration was measured using a commercially available kit according to the manufacturer's instructions, and radioactivity counted with a gamma counter. Information including history of clinical signs associated with PPID, treatments and diet was collected for each horse. Body condition scoring (BCS) was performed based on the Henneke scoring method (Henneke et al., 1983) and body weight (BW) recorded.

Glucose and Insulin Challenges

Three days post ODST, horses were fitted with jugular catheters in the evening as described in Marc et al. (2000). The following morning (i.e., day 4), prior to feeding, horses

were subjected to an oral glucose tolerance test (OGTT) per Pratt et al. (2006). Briefly, following a baseline blood sample at time 0 min, Karo Syrup Light[b] was administered orally, with blood samples taken at 60, 75, 90 and 120 min after administration.

On day 5, prior to feeding, a frequently sampled intravenous glucose tolerance test (FSGITT) was performed as described in Tóth et al. (2009) by administering 100 mg/kg BW dextrose (50% wt/vol) at time zero min and 20 mU/kg BW insulin[1] at time 20 min. Blood samples were collected for determination of plasma glucose and serum insulin concentrations at the following times: -15, -1, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min. Horses were loosely restrained in a stall during the entire procedure. Blood was transferred to evacuated tubes containing EDTA[c] or no additive[d] for separation of plasma and serum, respectively. Tubes containing EDTA were placed on ice and centrifuged (1,500 × *g* for 15 min at 4˚C) within 30 min of collection for plasma and or allowed to clot at room temperature for 2 h prior to centrifugation for separation of serum. Plasma and serum aliquots were harvested and stored at -20˚C until analyses.

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[a] (Equine Senior, Purina, St. Louis, MO)
[b] (Karo Syrup Light, ACH Food Companies Inc., Summit, IL)
[c] (BD Vacutainer®, Franklin Lakes, NJ)
[d] (BD Vacutainer®, Franklin Lakes, NJ)
[e] (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI)
[f] (Pureview® Hematoxylin and Eosin Stain System, Cancer Diagnostics Inc. Durham, NC)
[g] (Wako Autokit Glucose,Wako Life Sciences, Inc., Mountain View, CA)
[h] (Spectramax Plus 384 Microplate Reader, Molecular Devices LLC, Sunnyvale, CA)
[i] (Insulin Coat-a-Count®, Siemens, Munich, BAV)
[j] (Cobra II Auto Gamma, Perkin-Elmer Inc., Waltham, MA)
[k] (WinSAAM, Raymond Boston, University of Pennsylvania, Kennet Square, PA and SEM, Stata Corp., College Station, TX)
[l] (SAS version 9.3, SAS Institute Inc., Cary, NC)

Pituitary Gland Collection

Horses were euthanized on the following day (i.e., day 6) with pentobarbital[e] (95 mg/kg BW) administered IV using the jugular catheter already in place. Following confirmation of death, the skull was opened on the dorsal aspect to excise the brain and access the pituitary gland (PG). The PG was gently dissected out and the weight, length, width, and height were recorded. The gland was cut in half, and one half was flash-frozen in liquid N and stored at −80 ˚C and the other half transferred into 10% formalin solution (10 mL formalin/100 mL H_20) for subsequent preparation for hematoxylin and eosin[f] staining and grading as described in Miller et al. (2008). The following numerical grading system was used: 1 (within normal limits), 2 (focal or multifocal PI hypertrophy or hyperplasia), 3 (diffuse PI adenomatous hyperplasia), 4 (PI adenomatous hyperplasia with microadenomas [1- to 5-mm diameter]), or 5 (adenoma [>5-mm diameter] in PI or pars anterior). Other tissues were also harvested, including muscle, tail fat, and the small intestinal mucosa. These tissues were flash frozen and stored at −80 ˚C for later analyses as part of a subsequent study. The liver was removed and weighed.

Analysis of Plasma Glucose and Serum Insulin Concentration

Glucose concentration in plasma was measured using a colorimetric assay kit[g] according to the manufacturer's instructions and absorbance determined at 505 ηm with a microplate reader[h]. Serum insulin concentrations were measured using a commercially available radioimmunoassay kit[i] according to the manufacturer's instructions, and radioactivity counted with a gamma counter[j]. Use of this kit for horses has been previously validated (Tinworth et al., 2011; Freestone et al., 1991). Both assays were performed in duplicate. The assay was repeated for any duplicate samples with values differing by more than 5% for glucose and 10% for

insulin. The intra assay coefficient of variation was 4.12 and 8.14%, and inter assay coefficient of variation was 7.79 and 12.61%, for glucose and insulin, respectively.

Kinetic Analysis of Glucose and Insulin

Glucose and insulin values from FSIGTT were analyzed based on the minimal model developed by Bergman et al. (1981) with a commercially available software[k] as described by Hoffman et al. (2003). The following parameters were estimated for each horse: acute insulin response to glucose (AIRg), insulin sensitivity (SI), disposition index (DI), glucose-dependent glucose disposal (SG), baseline insulin (IB_{FSIGTT}), baseline glucose (GB_{FSIGTT}), area under the glucose curve ($FSIGTTAUC_G$) and area under the insulin curve ($FSIGTTAUC_I$). Disposition index was determined according to the following equation:

$$DI = AIRg \times SI$$

The difference between peak glucose or insulin level and baseline glucose or insulin level at time zero min ($dGLUC_{OGTT}$ and $dINS_{OGTT}$, respectively) were calculated from the OGTT. The reference ranges for glucose and insulin values used to interpret the OGTT were based upon criteria established by Frank (2013) as followed: plasma glucose concentration values following oral administration of glucose higher than 125 mg/dL were considered as indicative of an excessive glucose response and glucose intolerance; insulin concentrations values less than 45 µIU/mL were considered as normal insulin responses, values ranging from 45 to 60 µIU/mL as equivocal and values exceeding 60 mIU/mL as indicative of an exaggerated response to insulin characteristic of insulin resistance.

Statistical Analysis

Commercially available statistical software[l] was used to perform analysis of data using the mixed procedure with significance at $P \leq 0.05$ and statistical trends at $0.05 < P \leq 0.1$. Glucose tolerance and insulin sensitivity were determined using a one-way analysis of variance with the fixed effect of treatment (PPID and control) and the random effect of horse nested within treatment. The Shapiro-Wilk test was used to test the ANOVA assumption of normally distributed errors using the studentized residuals in the mixed procedure, and the Levene's F test was used to test the assumption of homogeniety of variance. Differences between treatments were assessed using Student's *t*-test for all data collected: cortisol concentration, ACTH concentration, BCS, body weight, and pituitary gland grade, weight, length, and diameter, OGTT parameters (IB_{OGTT}, Peak Ins_{OGTT}, $dINS_{OGTT}$, GB_{OGTT}, Peak $Gluc_{OGTT}$, and $dGLUC_{OGTT}$), and FSIGTT parameters (AIRg, DI, SI, Sg, IB_{FSIGTT}, GB_{FSIGTT}, $dGLUC_{FSIGTT}$, $dINS_{FSIGTT}$ $FSIGTTAUC_G$ and $FSIGTTAUC_I$). Spearman correlation coefficients were determined between the PG grade, PG weight and ACTH concentration and each of the glucose metabolism parameters from the OGTT, i.e., IB_{OGTT}, Peak Ins_{OGTT}, $dINS_{OGTT}$, GB_{OGTT}, Peak $Gluc_{OGTT}$, and $dGluc_{OGTT}$, and from the FSGITT, i.e., AIRg, DI, SI, Sg, IB_{FSIGTT} and GB_{FSIGTT}. Spearman correlation coefficients were also determined between IB_{FSIGTT} and SI, and between IB_{OGTT} and Peak Ins_{OGTT}.

Results

PPID Assessment

Of the ten horses presenting clinical signs associated with PPID and thus admitted as possible PPID candidates, eight were confirmed with PPID (horses 7-14 in Table 2). Criteria for confirming presence and assessing severity of PPID included the ODST results combined with the PG histologic scores and weights. The other 2 potential PPID horse candidates (horses 1 and 2 in Table 2) could not be clearly classified as PPID and thus were excluded from the study. The four horses admitted as potential non-PPID candidates, i.e., control group (horses 3-6 in Table 2), clearly suppressed the oral dexamethasone administration, and had PG weighing less than 3 g and histological grades of 3 and less.

Variables BCS and BW, PG grade, PG_L, PG_W, and PG_H, plasma ACTH concentration and cortisol concentrations pre and post-dexamethasone are presented in Table 1. The BCS and BW did not differ between control and PPID. Compared to control, the PG of PPID horses was heavier (P = 0.012) and greater in length ($P = 0.035$), width and height ($P = 0.002$ and $P = 0.001$, respectively). Histological PG grades were greater ($P < 0.001$) for horses with PPID compared to control horses. Compared to control horses, ACTH concentration tended to be greater ($P = 0.079$) and post-dexamethasone plasma cortisol concentration was greater ($P = 0.008$) in PPID afflicted horses. Results from the ODST (Table 2) revealed complete suppression in all 4 control horses (horses 3-6), and partial (horses 7 and 13) and failed suppression (horses 8, 9, 10, 11, 12 and 14) in the 8 PPID afflicted horses.

OGTT and FSIGTT

Glucose and insulin concentrations during the OGTT are presented in Figures 1 A and B, respectively. Glucose and insulin concentrations did not differ between control and PPID horses

at any time points of the OGTT. Four horses out of the PPID group and two of the control group had glucose values > 125 mg/dL at times 60, 75 and 90 min (data not shown). Plasma glucose and serum insulin concentrations during the FSIGTT are presented in Figure 2 A though D. Glucose concentration of PPID horses tended to be greater than that of control at 90 min (P = 0.099) (Figure 2 A and B). Insulin concentration tended to be greater at 3 min (P = 0.091), was greater at 8 min (P = 0.068), tended to be greater at 23 (P = 0.083) and 24 min (P = 0.056), and greater at 25 (P = 0.027) and 27 min (P = 0.048). At one time point (16 min) prior to insulin infusion, insulin concentration of PPID horses was lower (P = 0.023) than that of control. Glucose and insulin concentrations during FSIGTT did not differ between PPID and control horses at any other ime points. Table 3 presents calculated and estimated parameters derived from glucose and insulin responses to the OGTT and FSIGTT. Baseline $insulin_{OGTT}$, Peak Ins_{OGTT}, GB_{OGTT}, Peak $Gluc_{OGTT}$, and $dINS_{OGTT}$ did not differ between PPID and control horses, and $dGLUC_{OGTT}$ tended to be lower (P = 0.078) in PPID horses. The FSIGTT estimated parameters including AIRg, DI, SI, Sg, IB_{FSIGTT}, GB_{FSIGTT}, $dGLUC_{FSIGTT}$, $dINS_{FSIGTT}$, $FSIGTTAUC_I$, or $FSIGTTAUC_G$ did not differ between PPID and control horses.

Correlation coefficients between the PG grade, PG weight and ACTH concentration and each of the OGTT and FSGITT parameters are presented in Table 4. The PG grade and weight were poorly associated with most of the OGTT and FSIGTT parameters ($0 < r < 0.5$; $P > 0.1$). The PG weight correlated with Peak $Gluc_{OGTT}$ ($r = -0.77$, $P < 0.01$) and the PG grade and weight correlated with $dGluc_{OGTT}$ ($r = -0.50$, $P = 0.10$ and $r = -0.66$, $P = 0.02$, respectively). The ACTH concentration correlated with DI ($r = 0.60$, $P = 0.04$), Peak $Gluc_{OGTT}$ ($r = -0.77$, $P < 0.01$) and $dGLUC_{OGTT}$ ($r = -0.68$, $P = 0.02$). The SI correlated with IB_{FSIGTT} ($r = -0.60$, $P < 0.04$) and $PeakIns_{OGTT}$ correlated with IB_{OGTT} ($r = -0.72$, $P = 0.01$). The IB_{OGTT} correlated with IB_{FSIGTT} (r

= 0.64, *P* =0.03), whereas, GB_{OGTT} did not correlate with GB_{FSIGTT} (r = 0.25, *P* = 0.44).

Table 2.1. Body condition score, body weight, pituitary gland characteristics, serum ACTH and cortisol concentrations of horses with PPID and horses without PPID.

Variable	Control	PPID	P-value
BCS	4.75 ± 0.80	5.13 ± 0.57	0.710
BW	463.3 ± 21.6	433.8 ± 15.3	0.291
Liver Wt	5.236 ± 0.495	5.020 ± 0.350	0.730
Liver Wt/BW (%)	1.10 ± 0.10	1.20 ± 0.10	0.679
PG			
Grade	2.50 ± 0.268	4.38 ± 0.19	<0.001
Wt	2.28 ± 0.767	5.18 ± 0.54	0.012
L	8.50 ± 1.63	13.40 ± 1.56	0.035
W	19.50 ± 1.23	25.75 ± 0.87	0.002
H	19.50 ± 0.879	24.38 ± 0.62	0.001
ACTH	23.00 ± 26.12	85.63 ± 18.47	0.079
Cortisol			
Pre-dex	158.0 ± 36.7	156.0 ± 25.9	0.972
Post-dex	10.0 ± 21.9	99.3 ± 14.5	0.008

BCS, body condition score; BW, body weight; PG, pituitary gland; Wt, weight (g); L, length (µm); W, width (µm); H, height (µm); ACTH, adrenocorticotropic hormone (pg/mL); Cortisol, cortisol concentration samples (nmol/L pre and post dexamethasone administration; Pre-dex, pre-dexamethasone plasma cortisol concentration sample (nmol/L); Post-dex, post-dexamethasone plasma cortisol concentration sample (nmol/L) .

Two groups were included in this study: horses with PPID and non-PPID horses of > 19 years of age.

Table 2.2 Demographics, History, Clinical Signs, and ODST of horses with PPID and horses without PPID.

Horse	Treatment	Breed	Sex	Age	ODST
1	None	QH	M	32	Partial
2	None	Pinto	G	27	Fail
3	Control	TWH	M	26	Suppressed
4	Control	TB	G	27	Suppressed
5	Control	TB	G	29	Suppressed
6	Control	Morgan	G	19	Suppressed
7	PPID	Pinto	G	30	Partial
8	PPID	Arabian	M	31	Fail
9	PPID	TB	M	29	Fail
10	PPID	Arabian	M	31	Fail
11	PPID	Arabian	M	19	Fail
12	PPID	Arabian	M	30	Fail
13	PPID	QH	M	32	Partial
14	PPID	QH	G	27	Fail

QH, quarter horse; TWH, tennessee walking horse; TB, thoroughbred; G, gelding; M, mare; ODST, overnight dexamethasone suppression test.

Two groups were included in this study: horses with PPID and non-PPID horses of > 19 years of age.

Table 2.3 OGTT and FSIGTT parameters of horses with PPID and horses without PPID.

Variable	Control	PPID	P-value
OGTT			
IB	8.45 ± 5.33	13.56 ±3.77	0.452
Peak Ins	24.68 ± 10.49	27.93 ± 7.42	0.805
GB	96.20 ± 5.56	94.43 ± 3.93	0.800
Peak Gluc	134.48 ± 8.62	120.19 ± 6.09	0.206
dGLUC	39.47 ± 6.14	24.73 ± 4.34	0.078
dINS	15.55 ± 6.42	12.16 ± 4.54	0.676
FSIGTT			
AIRg	185.9 ± 85.7	333.3 ± 60.6	0.190
DI	290 ± 278	805 ± 196	0.161
SI	2.86 ± 0.97	2.55 ± 0.69	0.803
Sg	1.66 ± 0.35	1.76 ± 0.25	0.821
IB	9.24 ± 3.63	12.09 ± 2.57	0.536
GB	86.65 ± 3.83	89.18 ± 2.71	0.602
dGLUC	47.08 ± 7.13	45.58 ± 5.04	0.867
dINS	186 ± 23.0	203 ± 16.2	0.552
AUC_I	508 ± 140	795 ± 98.9	0.124
AUC_G	2515 ± 131	2306 ± 95.6	0.221

OGTT, oral glucose test; IB, baseline insulin (mU/L); Peak Ins, peak insulin (mU/L); GB, baseline glucose (mg/dL); Peak Gluc, peak glucose (mg/dL); OGT dGLUC, difference between peak glucose concentration at time sixty-five min and baseline glucose concentration at time zero min (mg/dL); OGT dINS, difference between peak insulin concentration at time sixty-five min and baseline insulin concentration at time zero min (mU/L); FSIGTT, frequently sampled intravenous glucose tolerance test; AIRg, acute insulin response to glucose ($mU \bullet min \bullet L^{-1}$); DI, disposition index ($\times 10^{-2}$); SI, insulin sensitivity ($\times 10^{-4}$ mU/min/L); Sg, glucose effectiveness ($\% \bullet min^{-1}$); FSIGTT dGLUC, difference between peak glucose concentration and baseline glucose concentration at time zero min (mg/dL); FSIGTT dINS, difference between peak insulin concentration and baseline insulin concentration at time zero min (mU/L); AUC_{Gluc}, area under the glucose curve (mmol/L/180 min); AUC_{Ins}, area under the insulin curve (mmol/L/180 min).

Two groups were included in this study: horses with PPID and non-PPID horses of > 19 years of age.

Table 2.4. Pearson correlation coefficients (R) between pituitary gland grade, pituitary gland weight and ACTH concentration and each of the OGTT and FSIGTT parameters.

FSIGTT and OGTT parameters	Pituitary gland grade		Pituitary gland weight		ACTH	
	R	P-value	R	P-value	R	P-value
OGTT						
IB	0.13	0.69	0.17	0.59	0.14	0.65
Peak Ins	0.00	0.99	−0.01	0.97	−0.07	0.83
dINS	−0.11	0.74	−0.17	0.60	−0.24	0.46
GB	0.05	0.87	−0.19	0.56	−0.48	0.11
Peak Gluc	−0.32	0.31	−0.58	0.05	−0.77	< 0.01
dGLUC	−0.50	0.10	−0.66	0.02	−0.68	0.02
FSIGTT						
AIRg	0.32	0.31	0.37	0.23	0.40	0.20
DI	0.46	0.13	0.47	0.12	0.60	0.04
SI	0.03	0.93	0.12	0.71	0.31	0.32
Sg	0.10	0.76	0.13	0.69	0.14	0.67
IB	0.17	0.59	0.05	0.87	−0.10	0.77
GB	−0.01	0.98	−0.12	0.70	−0.36	0.26

OGTT, oral glucose test; IB, baseline insulin (mU/L); Peak Ins, peak insulin (mU/L); dINS, difference between peak insulin level and baseline insulin level at time zero min (mU/L); GB, baseline glucose (mg/dL); Peak Gluc, peak glucose (mg/dL); dGLUC, difference between peak glucose concentration at time sixty-five min and baseline glucose concentration at time zero min (mg/dL); FSIGTT, frequently sampled intravenous glucose tolerance test; OGTT, oral glucose test; AIRg, acute insulin response to glucose ($mU \bullet min \bullet L^{-1}$); DI, disposition index ($\times 10^{-2}$); SI, insulin sensitivity ($\times 10^{-4}$ mU/min/L); Sg, glucose effectiveness ($\% \bullet min^{-1}$); AUC_{Gluc}, area under the glucose curve (mmol/L/180 min); AUC_{Ins}, area under the insulin curve (mmol/L/180 min).

Two groups were included in this study: horses with PPID and non-PPID horses of > 19 years of age.

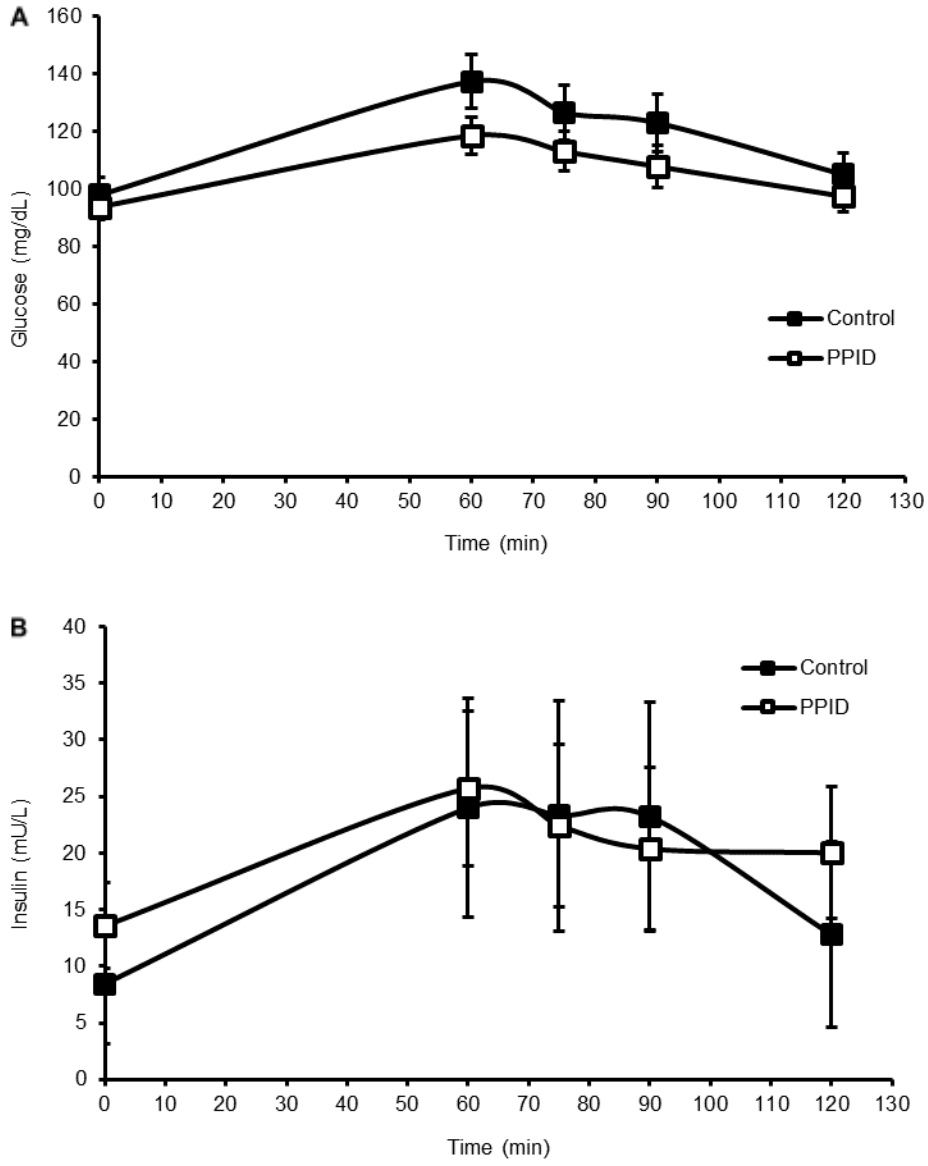


Figure 2.1. Plasma glucose (A) and serum insulin (B) concentrations during the OGTT.

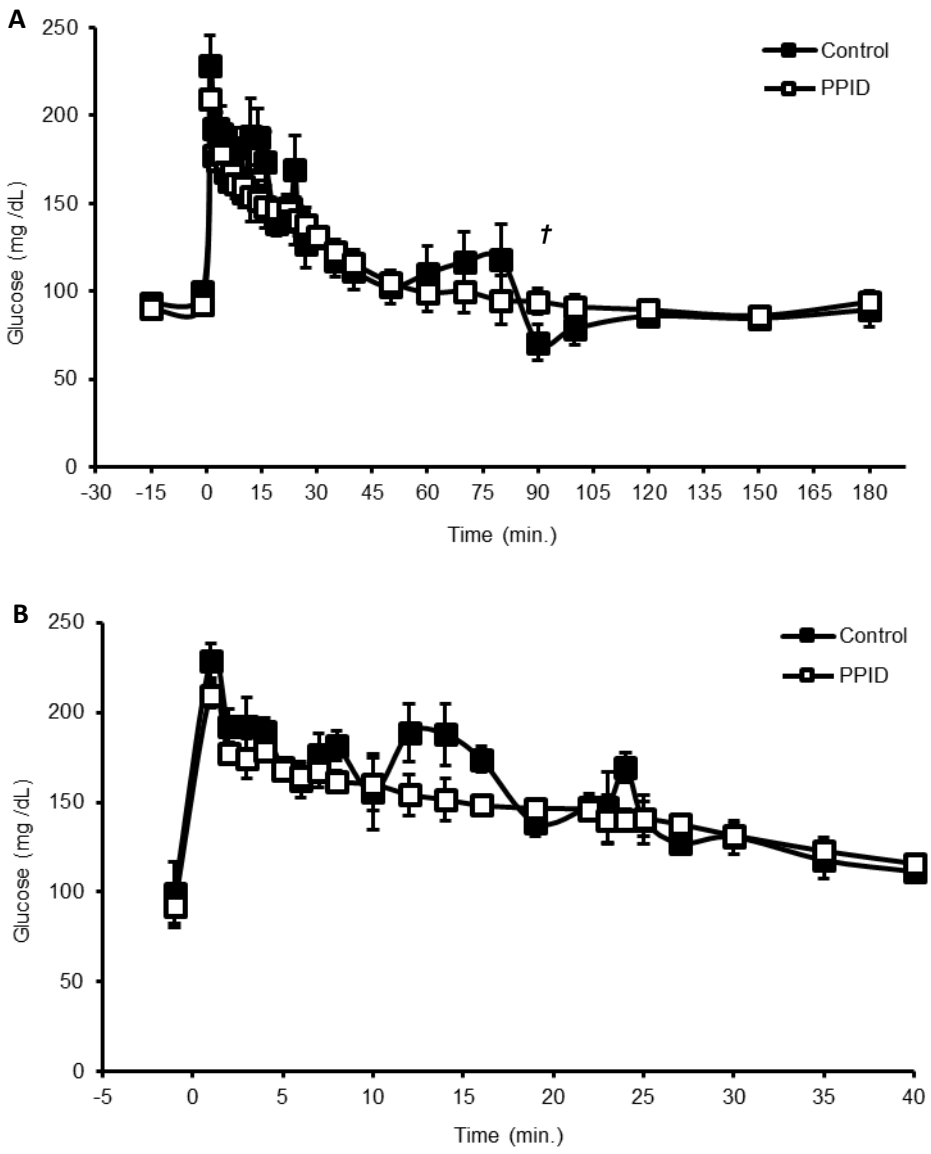
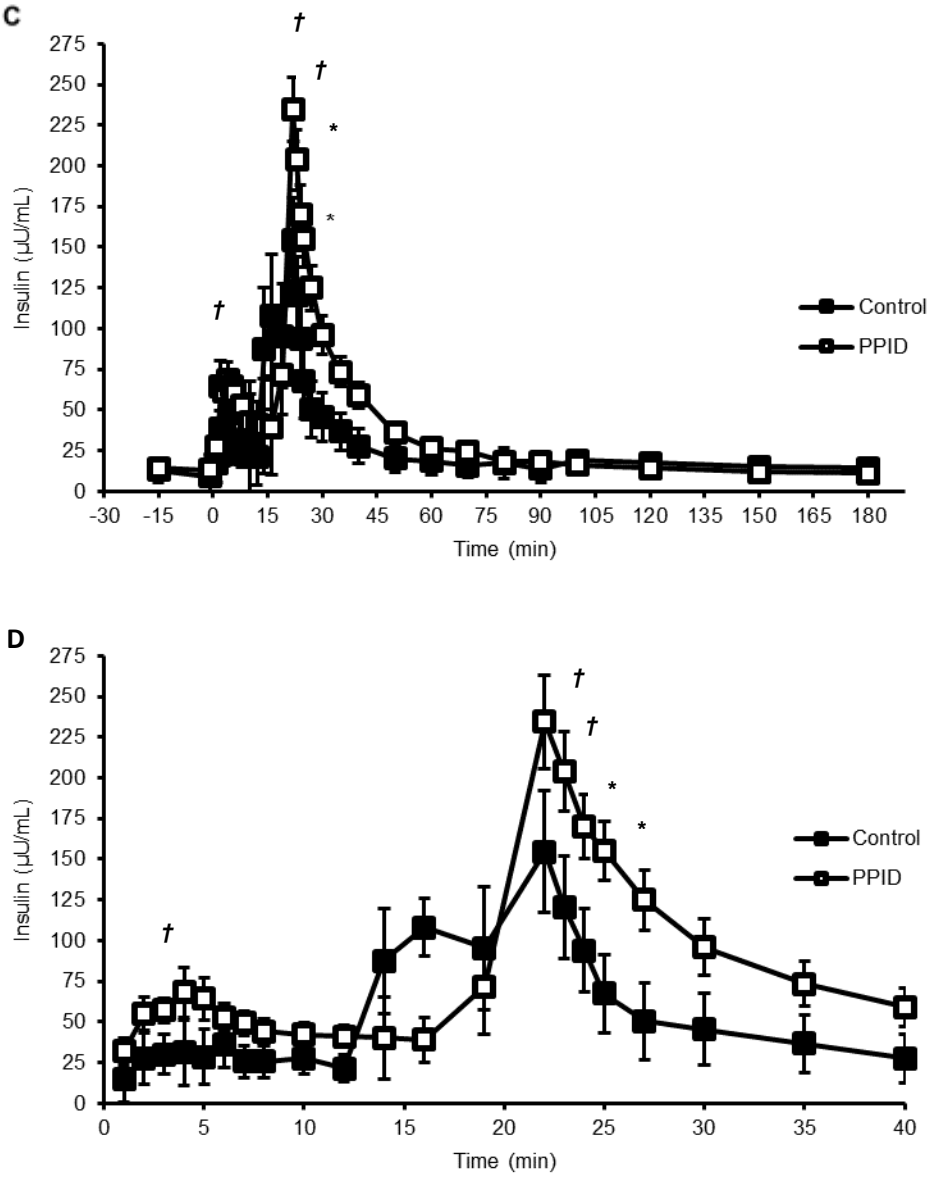


Figure 2.2. Plasma glucose during full FSIGTT and first 40 min (A and B) and serum insulin during full FSIGTT and first 40 min (C and D) concentrations during the FSIGTT.

Figure 2.2. (cont'd)



Discussion

The concentration of basal plasma ACTH and dexamethasone suppression testing are accepted as the most sensitive and specific diagnostic tests for PPID (Perkins et al., 2002; Couëtil et al., 1996; van der Kolk et al, 1995; Dybdal et al., 1994), assuming careful interpretation given the seasonal variation in cortisol and ACTH concentrations (Donaldson et al., 2005; McFarlane et al., 2004). In this study, all samples were collected between September and December of the same year. Only a handful of published studies however have confirmed the sensitivity and specificity of the disorder histopathologically in addition to endocrine testing (Klinkhamer et al., 2011). McFarlane et al. (2005) reported a 79% agreement between post- and ante-mortem assessment. In our study, of the horses clearly classified as PPID following post-mortem examination of the PG, 75 % failed and 25 % partially failed to suppress the dexamethasone. The PG of these horses was one-fifth the size (H, L, and W) and weight relative to the horses classified as controls who had clearly all suppressed dexamethasone.

This study reports on the relationship between PPID condition and insulin and glucose dynamics. Insulin resistance and central fat deposit in men has been well established to be associated with excess blood cortisol concentration and cortisol production rates (Purnell et al., 2009; Phillips et al., 1998; Filipovsky et al., 1996; Stolk et al., 1996) however the mechanisms of action remains largely not understood (Rosmond, 2005) and poorly documented in horses. Our study is the first to use both the OGTT and FSIGTT to assess insulin sensitivity and glucose tolerance in PPID horses. The PPID and control horses had comparable body weights and condition scores, thus any difference in glucose metabolism parameters would not be attributed to body fatness per se.

Our mean AIRg values fell within the reported range from 206 ± 88 to 973 ± 393 by Carter et al. (2009) and 67 to 805 mU•min•L^{-1} by Bamford et al. (2014). However despite a near two-fold greater AIRg and $FSIGTTAUC_i$ for the PPID group relative to control (333.25 ± 60.58 vs. 185.85 ± 85.67 mU•min•L^{-1} and 908 ± 136 vs. 518 ± 192 mU•min•L^{-1}, respectively), these values did not differ, presumably due to the large variation. This variation was more attributed to the control group in part because of the smaller sample size but also because this was a more heterogeneous group by nature. The AIRg represents the pancreatic insulin secretion in response to an acute intravenous dose of glucose (Huffman et al., 2009) and thus greater AIRg reflects an increased pancreatic response to glucose challenge (Treiber et al., 2005). The latter may be related to reduced insulin tissue sensitivity (Tiley et al., 2007), interference from other hormones (Huffman et al., 2009) or delayed insulin clearance by the liver (Mittleman and Bergman, 2000).

Both PPID and non-PPID horses in our study did not differ in their ability to effectively use glucose based on the estimated Sg values, which closely aligned with those of Pratt et al. (2005) (i.e.,1.77 ± 0.90 % • min^{-1}). Similarly, parameters of glucose dynamics from the OGTT did not differ either between PPID and control horses. Half of the horses out of the PPID cohort and control group however presented some degree of glucose intolerance when basing glucose results on the reference values suggested by Frank et al. (2013). Frank et al. (2013) proposed that glucose concentration values exceeding 125 mg/dL during the OGTT are indicative of excessive glucose responses. When based on the SI values, neither PPID nor control horses appeared to be insulin resistant, with values falling in the reported range of 0.471 to 5.43 ± 0.94 ($\times 10^{-4}$ mU/min/L) (Pratt et al., 2005; Bamford et al., 2014). There was a noticeable 4-fold greater DI for PPID relative to control horses despite a lack of statistical significance. The DI provides an indication of whether pancreatic insulin response is compensating for any degree of insulin

resistance (Boston et al., 2013). Thus this raises an important question as to whether our sample size limited our ability to clearly rule out an association between PPID and altered insulin metabolism. A similar remark can be made regarding the AIRg and in particular the $FSIGTTAUC_I$. Indeed, the relatively strong positive correlation in particular between DI and ACTH concentration, and the higher insulin concentration following insulin administration may be further indicative of some degree of altered glucose metabolism in the PPID group. The results from the OGTT however indicated that neither PPID nor control horses exhibited hyperinsulinemia, since insulin concentrations remained well below 45 mU/ mL (Frank et al., 2013). On the other hand, the FSIGTT represent an acute response to intravenous glucose and is more sensitive compared to the traditional OGTT (Pratt et al., 2005).

Prior studies have reported on the increased insulin resistance status of horses diagnosed with PPID in comparison to younger non-PPID controls (Klinkhamer et al., 2011; Garcia and Beech, 1986). Another study by Keen et al. (2004) reported insulin resistance in the PPID horse population as well, yet the majority of the test subjects in these studies were ponies who are known to be more susceptible to PPID than horses (Schott, 2002; van der Kolk, 1997). The inclusion of ponies (Couëtil et al., 1996; van der Kolk et al., 1993; Hillyer et al., 1992; Schott et al., 1992) is common in part due to the difficulty with recruitment of horses with PPID. Older horses have been shown to be more prone to insulin resistance than younger horses (Malinowski et al., 2002), with similar observations in humans (Barbieri et al., 2001; Paolisso et al., 1999). In a recent study by Mastro et al. (2015), PPID affected horses (no ponies) compared to age-matched controls did not differ in insulin sensitivity or glucose tolerance when assessed by the reciprocal of the square root of insulin (RISQI), the modified insulin to glucose ratio (MIRG), and isoglycemic hyperinsulinemic clamp approaches. Finally, PG weight and PG grade

correlated poorly with the majority of parameters of glucose metabolism from the OGTT and FSIGTT. The negative correlation between ACTH and Peak $Gluc_{OGTT}$, and ACTH and $dGluc_{OGTT}$ is puzzling. However, we have found that PPID horses have a reduced uptake of glucose across the small intestinal mucosa as well as shorter villi (Chapter 4) and therefore may have reduced capacity for glucose absorption. This might also explain the lower glucose response to the OGTT in the PPID horses compared to the controls. Consequently, the FSIGTT instead of the OGGT may likely be a better tool to assess glucose metabolism in PPID horses. Due to the lack of difference in insulin response in the OGTT and FSGITT between treatments, we were unable to classify any PPID horses as IR. One limitation of this study was the recruitment of geriatric horses that were otherwise healthy. Despite a clear homogeneity of variance across both treatment groups and normal distribution of all of the data, additional control horses would have decreased variability, in particular that associated with DI, AIRg and $FSIGTTAUC_I$.

In conclusion, no difference in insulin sensitivity and glucose tolerance were found during either OGTT or FSGITT when comparing horses with PPID to their age-matched non-PPID control. Both PPID and control horses in this study had comparable body weight and condition score. Equal number of PPID and non-PPID horses exhibited signs of glucose intolerance during OGTT when evaluated with reference values established by Frank et al. (2013). Although the DI was noticeably greater in PPID than control horses, the large variation in particular in the control group precluded detecting a statistical difference. Nonetheless, the positive and relatively strong correlation between ACTH concentration and the DI, and the sustained higher insulin concentration following the i.v. insulin administration is indicative that there was compensation to some degree of insulin resistance in the PPID group. Again however

neither PPID nor control horses however could be classified as insulin resistant based on the estimated SI values alone. Nonetheless, our results suggest that geriatric horses and those in particular with PPID should be carefully managed with feeding diets containing low concentration of soluble carbohydrates.

CHAPTER THREE: Skeletal muscle expression of GLUT4, INSR, and SNAT2 in horses with pituitary pars intermedia dysfunction

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Abstract

Pituitary pars intermedia dysfunction (PPID) is the most common endocrinopathy of the aging horse. Loss of dopaminergic inhibition of the pituitary pars intermedia results in an increase in blood concentration of adrenocorticotropic hormone (ACTH) and cortisol resulting in a range of clinical symptoms. Horses affected with PPID exhibit a range of clinical symptoms that may include lethargy, abnormal sweating, hirsutism, and muscle loss. Peripheral nutrient utilization appears to be limiting based upon these clinical symptoms. Expression level of glucose and amino acid transporter and the insulin receptor in the gluteal muscle of horses with PPID were quantified and plasma amino acid profile was measured. We hypothesized that horses with PPID have a reduced mRNA abundance of *SLC2A4*, *INSR*, and *SLC38A2* encoding GLUT4, INSR, and SNAT2, as well as reduced protein expression of GLUT4 and insulin receptor (INSR) in muscle tissue compared to non-PPID horses (control). The objectives were to 1) quantify mRNA abundance of *SLC2A4*, *INSR* and *SLC38A2* and 2) quantify protein abundance of INSR and GLUT4 in muscle tissue of PPID and non-PPID horses (control). Twelve horses of multiple breeds and genders, and of similar ages (28.6 ± 1.5 and 25.3 ± 2.1 years for PPID and control, respectively) were donated for this study. Following admission to the study, horses were maintained on second cutting mixed orchardgrass and bromegrass hay and senior feed at 1.8 and 0.5% of their BW, respectively for a period of 5 d while being monitored for medical stability. Presence and grade of PPID was determined based on basal ACTH concentration, overnight dexamethasone suppression test (ODST) and post mortem histopathological evaluation of the pituitary gland. Plasma samples were collected to measure the amino acid profile. Gluteal muscle samples were collected and flash frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated and assessed for purity followed by cDNA synthesis. Primers for target genes

SLC2A4, *INSR*, and *SLC38A2* (respectively encoding GLUT4, INSR, and SNAT2), and for the reference genes GAPDH, ACTB, and SDHA (respectively encoding glyceraldehyde-3-phosphate dehydrogenase, β-actin and succinate dehydrogenase complex, subunit A, flavoprotein) were designed using equine gene sequences from GenBank. Quantification was done using qRT-PCR and abundance of each target gene expressed as fold change relative to control. The content of glucose transporter (GLUT4) and insulin receptor (INSR) was determined in muscle biopsies using Western blot analysis. Differences between treatments were determined using a mixed model in SAS, including treatment as a fixed effect and horse nested within treatment as the random term. Significant differences were evaluated at $P \leq 0.05$ and tendency at $P \leq 0.1$. Plasma aspartic acid was greater ($P = 0.01$) and ornithine tended to be greater ($P=0.07$) in PPID compared to control horses. Plasma threonine ($P = 0.07$) and carnosine ($P = 0.08$) tended to be lower, and glutamine ($P = 0.05$), methionine ($P = 0.04$), 3 methyl histidine ($P = 0.02$) were lower in PPID compared to control horses. Compared to control horses, mRNA abundance of *SLC2A4*, *INSR*, and *SLC38A2*, and protein abundance of GLUT4 and INSR in the gluteal muscle of PPID did not differ. The results of this study show that the muscle wasting associated with the pathology of the PPID condition is not due to a reduced utilization of glucose and amino acid at the systemic level in regards to *SLC2A4*, *INSR*, and *SLC38A2* abundance and content.

Introduction

Equine Cushing's disease or pituitary pars intermedia dysfunction (PPID) is a progressive endocrine disorder affecting the aging horse population (van der Kolk., 1997; Schott, 2002). The disorder is characterized by hypertrophy, hyperplasia, and adenoma of the pars intermedia of the pituitary gland resulting in increased circulating concentration of pro-opiomelanocortin peptides (POMC) including ACTH (Hillyer et al., 1992; van der Kolk et al., 1993; Love, 1993; Dybdal et al., 1994; van der Kolk et al., 1995; Eiler et al., 1997; Reed, 1998). Horses diagnosed with PPID exhibit a variety of clinical signs most commonly including poor body condition, appearance of muscle wasting, abnormal fat deposits, hirsutism, and heightened risk for secondary infections (Donaldson et al., 2004; Aleman et al., 2006; McFarlane, 2011; Klinkhamer et al., 2011; Innerå et al., 2013). Despite their poor nutritional status, PPID-affected horses demonstrate increased appetite indicating that changes in intermediary metabolism are present and that peripheral nutrient utilization may be compromised. Abnormalities in glucose homeostasis or glucose intolerance have been reported in 57 to 94% (Hiller et al., 1992; van der Kolk et al., 1995; Keen et al., 2004) and insulin resistance in 45 to 90% of the PPID-afflicted horse population (van der Kolk et al., 1995; Keen et al., 2004). In the work presented by Keen et al. (2004) and van der Kolk et al. (1995) however, half to 100% of the candidates were ponies, which are typically at higher risk for insulin resistance (Donaldson et al., 2004). Conversely, more recent studies have reported absence of insulin resistance in horses with PPID (Vick et al., 2007; van den Berg et al., 2013; Mastro et al., 2015). Reeves et al. (2001) and more recently McGowan et al. (2013) suggested that insulin insensitivity may not be specific to PPID *per se* and Malinowski et al. (2002) proposed that insulin resistance in horses with PPID may be related to aging. So far, only one of the studies cited above (Mastro et al., 2015) included matched-age controls. We have also

shown (Chapter 1) that horses with PPID were not insulin resistant nor glucose intolerant compared to age-matched non-PPID horses based on an oral glucose tolerance test (OGTT) or frequently sampled intravenous glucose tolerance test (FSIGTT). The data was indicative nonetheless of compensation to some degree of insulin resistance in the PPID group based on a relatively strong positive correlation between ACTH concentration and insulin disposition index, and a sustained higher insulin concentration following the intravenous insulin administration.

In adipocytes and muscle cells, insulin stimulates the translocation of GLUT4 to the plasma membrane (Huang and Czech, 2007). The first determinant of insulin-dependent peripheral glucose uptake is the availability on the cell membrane of the GLUT4 transporter (Huang and Czech, 2007). With respect to AA transport, insulin activation involves in part recruitment of the Na^+-coupled neutral AA transporter 2 (SNAT2) from an endosomal compartment (Dyer et al., 2009). Molecules associated with recruitment and fusion of SNAT2-containing vesicles to the surface membrane are also implicated in the regulation of GLUT4 translocation (Dyer et al., 2009). Insulin-mediated neutral AA uptake via SNAT2 is down-regulated in metabolic acidosis and insulin resistance, causing proteolysis and muscle wasting in humans (Evans et al., 2008).

Based on these notions, the objectives of this study were to 1) quantify the mRNA abundance of *SLC2A4* and *INSR*, and protein abundance of GLUT4 and INSR in the gluteal muscle tissue to further evaluate the insulin and glycemic status in the cohort of PPID and non PPID horses available for this study; 2) quantify the mRNA abundance of *SLC38A2* in gluteal muscle tissue to assess whether neutral amino acid transport is compromised in PPID horses; and 3) determine the basal plasma amino acid profile to assess muscle protein metabolism.

We hypothesized that geriatric horses with PPID have reduced mRNA abundance of *SLC2A4, SLC38A2,* and *INSR,* and reduced protein abundance of INSR and GLUT4 compared to geriatric non-PPID horses.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University (03-12-064-001).

Animals and Housing

Eight horses confirmed with PPID and 4 non PPID (control) horses were donated for this study following owners' consent and between the months of September and December as described earlier (Chapter 1). Horses were of multiple breeds and genders (mare and gelding) across groups with an average age of 25.3 ± 2.11 years for control and 28.6 ± 1.49 years for PPID horse. Following admission for the study, all horses were housed indoor, in a temperature controlled environment (21˚C) and individual stalls (3.05m × 3.05m or 3.66m × 3.66m). All horses were fed second cutting mixed orchardgrass (*Dactylis glomerata L.*) and brome-grass hay (*Bromus mollis L.)*, and a commercial grain concentrate (Equine Senior, Purina) containing 14% crude protein, 1.8% fat and 18% crude fiber at 1.8 and 0.5% of their body weight, respectively.

PPID and Insulin Status Assessment

Horses were evaluated for presence and grade of PPID, and insulin status as described elsewhere (Chapter 1). In brief, horses were subjected to ACTH testing (Beech et al., 2009) and an overnight dexamethasone suppression test (ODST) (Innerå et al., 2003). Insulin sensitivity and glucose tolerance was assessed following an OGTT (Pratt et al., 2006) and a FSGITT (Tóth et al., 2009).

Collection of Tissue

Horses were euthanized on the day following the frequently sampled intravenous glucose tolerance test (FSGITT) (i.e., day 6) with pentobarbital (Fatal-Plus, Vortech Pharmaceuticals) (95 mg/kg BW) administered intravenously using the jugular catheter already in place. Immediately following confirmation of death, samples from various tissues were collected and archived. Middle gluteal muscle tissue was sampled and any fat and fascia was removed. The muscle sample was cut into smaller pieces, transferred to conical tubes (~5 g) and immediately flash frozen in liquid nitrogen, and stored at −80°C.

Amino Acid Profile Analysis of Plasma

Plasma was collected and shipped to Agricultural Experiment Station Chemical Laboratories on dry ice (University of Missouri-Columbia, Columbia, MO) for free AA analysis according to Spackman et al. (1958) and Deyl et al. (1986). Plasma samples were analyzed for analysis of complete free AA profile concentration according to AOAC method (AOAC Official Method, Agricultural Experiment Station Chemical Laboratories, University of Missouri-Columbia College of Agriculture, Food and Natural Resources).

Isolation of RNA and Preparation of cDNA

Total RNA was isolated from the gluteal muscle according to the manufacturer's instructions using the *mir*Vana™ miRNA Isolation Kit (Ambion®, LifeTechnologies Inc.™). Approximately 0.1 mg of tissue was weighed out and homogenized (Kinematica™ Polytron™) on ice for 3-s intervals in 10 volumes of lysis/binding buffer per tissue mass. The isolated RNA concentration was measured using a spectrophotometer (Nanodrop 1000, Thermo Scientific) and further assessed for both purity and confirmation of concentration (Agilent Bioanyalyzer 2100).

Complimentary DNA was manufactured using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies Inc.™), diluted to 10 ng/μL^{-1}, and stored at -20°C.

Measurement of SLC2A4, SLC38A2 and INSR Relative mRNA Abundance by Quantitative Reverse Transcription PCR

Primers for target genes *SLC2A4*, *SLC38A2*, and *INSR* and for the reference genes *GAPDH*, *ACTB*, and *SDHA* (respectively encoding glyceraldehyde-3-phosphate dehydrogenase, β-actin and succinate dehydrogenase complex, subunit A, flavoprotein were designed using equine gene sequences from GenBank (National Center for Biotechnology Information, United States National Library of Medicine) (Table 1). Gene specific assays for *SLC2A4* and *SLC5A1*, and for all three reference genes were designed using Taqman® Gene Expression Assays kit (Applied Biosystems™, Life Sciences Technologies Inc.™). Custom Taqman® Gene Expression assays were designed for *SLC38A2* and *INSR* on human equivalent sequences referenced in GenBank (National Center for Biotechnology Information, United States National Library of Medicine) blasted against equine sequences (e!Ensembl, EMBL-EBI).

Quantification was assessed using reverse transcription quantitative PCR in 96-well plates (MicroAmp® Fast Optical Plate, Life Technologies Inc.™). Plates were briefly centrifuged at 400 × *g* for 1 min. at 15°C, loaded into the ABI Prism 7000 Sequence Detection System (Applied Biosystems™, Life Technologies Inc.™), and analyzed with 7000 RQ Sequence Detection Systems Software (version 2.2.1, Applied Biosystems™, Life Technologies Inc.™). Abundance of each target gene was calculated according to the following equations (Livak and Schmittgen, 2001; Pfaffl, 2001):

$$\Delta C_T = C_T \text{ target gene} - \text{Average of } C_T \text{ reference genes.}$$

$$\Delta\Delta C_T = \Delta C_{TPPID} - \Delta C_{TControl}$$

$$\text{Fold Change (FC)} = 2^{-\Delta\Delta C_T}$$

Western Blotting Analysis

The western blot analysis was performed according to Annandale et al. (2004) with the following modifications. The antibody against GLUT4 (rabbit polyclonal GLUT4 antibody) was graciously donated by Dr. Samuel Cushman (Diabetes Centre, Lund University) and has been used previously in horse tissues (Annandale et al., 2004). The insulin receptor antibody was purchased from Millipore (Anti-Insulin Receptor (β-Subunit), clone CT-3) and has been previously used in equine tissues (Kullman et al., 2015). The secondary antibody was a goat anti-rabbit HRP-conjugate from Pierce (Goat Anti-Rabbit IgG (H+L). Crude membrane fractions were prepared from gluteal muscle by homogenizing 1 g of muscle tissue in 8 mL buffer [(25 mM HEPES, 0.5 mM PMSF, 4 mM EDTA, and 5 µL protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, EMD Chemicals Inc.) at pH 7.4]. Samples were centrifuged at 100,000 × *g* for 1 h at 4°C, the pellet re-suspended in 1 mL of the original buffer + 1% Triton-X-100, incubated for 1.5 h on ice, and centrifuged for 500 x *g* for 10 min at 4°C. The supernatant was analyzed for protein content with a Pierce BCA kit (ThermoFisher Scientific). SDS-PAGE gels were run with 25 µg of each sample separated on 8-20% polyacrylamide gels (Criterion TGX Stain-Free Precast Gels, Bio Rad) and transferred to PVDF membrane (Trans-blot Turbo transfer pack, Bio-Rad). The membrane was blocked for 1h with a commercially available blocking reagent (StartingBlock (TBS) Blocking Buffer, Thermo Scientific) and 0.1% non-ionic detergent (Tween-20, Sigma Aldrich) and blocked with the primary antibody (1:4000 for GLUT4; 1:1000 for INSR) overnight at 4°C. The membrane was washed 5 times with 0.1% (100 mL Tween 20/ 10 µL Tris-Buffered Saline) TBST and secondary antibody (1:10,000 for GLUT4; 1:5000 for

INSR) and incubated for 1 hr at 4°C. Images were immune-detected with a chemiluminescent kit (West Pico Chemiluminescent Substrate, Thermo Scientific).

Statistical Analysis

All data was normally distributed based on Gaussian distribution analysis in SAS (version 9.3, SAS Institute Inc., Cary, NC). Analysis of data was performed using the PROC MIXED procedure (SAS version 9.3, SAS Institute Inc.) with treatment as fixed effect and individual horse nested within treatment as random effect. The response variables were ΔC_T for mRNA and arbitrary units for protein expression. Pairwise differences between treatments were tested based on the student's *t*-test. Treatment effects were represented as fold change by back transforming the estimated mean difference in C_T ($^{\Delta\Delta C_T}$) between control and PPID for each transporter of interest using the expression FC= $2^{-\Delta\Delta C_T}$ according to Livak and Schmittgen (2001). Following Steibel et al. (2009), 95% confidence intervals of mean differences were also back transformed to provide confidence interval for the estimated fold change. Fold changes with transformed confidence intervals spanning across the value of 1.0 were considered not significant at $\alpha = 5\%$.

Results

Amino Acid Profile in Plasma

The amino acid profile in plasma is presented in Table 1. The majority of amino acids (alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, taurine, tryptophan, tyrosine, valine, urea, and citrulline) did not differ between PPID and control horses. Two amino acids, aspartic acid and ornithine, were greater (P = 0.01) and tended to be greater (P=0.07) in PPID horses when compared to

controls. Threonine (P = 0.07) tended to be lower, and glutamine (P = 0.05), methionine (P = 0.04), 3-methylhistidine (P = 0.02) were lower in PPID horses than that of control horses.

SLC2A4, INSR, and *SLC38A2 mRNA Abundance in Gluteal Muscle*

Transcript abundance of *SLC2A4*, *INSR*, and *SLC38A2* in gluteal muscle of PPID (n=8) relative to control horses (n=4) is presented in Figure 1. The mRNA abundance of *SLC2A4*, *INSR*, and *SLC38A2* was not different in PPID horses relative to control horses.

GLUT4 and INSR Content in Gluteal Muscle

In equine skeletal muscle lysates, the GLUT4 and INSR antibody detected a single immunoreactive band that migrated with an apparent molecular mass of approximately 45 kDa (Figure 3 A and B) and 95 kDa, respectively (Figure 4 A and B). Densitometric quantification of Western blot band intensities revealed no differences in protein abundance of GLUT4 or INSR between PPID and control horses (Figure 3A and 4A).

Table 3.1. Amino acid profile in the plasma of PPID and non-PPID horses (µg/mL).

Amino Acid	Control	PPID	*P*-value
Alanine	13.52 ± 2.15	14.77 ± 1.52	0.64
Arginine	14.14 ± 1.41	13.06 ± 1.00	0.55
Asparagine	2.44 ±0.75	1.87 ± 0.53	0.55
Aspartic Acid	0.93 ± 0.20	1.74 ± 0.14	0.01
Cysteine	0.00 ± 0.00	0.00 ± 0.00	1.00
Glutamic Acid	4.44 ± 1.75	7.66 ±1.24	0.16
Glutamine	45.24 ± 3.99	34.16 ± 2.82	0.05
Glycine	40.65 ± 3.86	38.17 ± 2.73	0.62
Histidine	11.16 ± 0.65	10.38 ± 0.46	0.35
Isoleucine	6.55 ± 0.68	6.19 ± 0.48	0.68
Leucine	12.65 ± 0.90	11.48 ± 0.64	0.31
Lysine	15.07 ± 1.74	13.31 ± 1.23	0.43
Methionine	3.70 ± 0.30	2.85 ± 0.21	0.04
Phenylalanine	10.38 ± 0.58	9.62 ± 0.41	0.31
Proline	6.4 ± 0.56	6.73 ± 0.39	0.64
Serine	20.68 ± 2.29	23.52 ± 1.62	0.34
Taurine	4.58 ± 0.46	4.07 ± 0.33	0.38
Threonine	15.68 ± 1.66	11.50 ±1.18	0.07
Tryptophan	12.48 ± 1.54	12.35 ± 1.09	0.95
Tyrosine	10.93 ± 1.05	11.20 ± 0.74	0.83
Valine	20.87 ± 1.37	19.89 ± 0.97	0.57
Urea	298.17 ± 27.58	291.92 ± 19.51	0.86
3-methylhistidine	4.09 ± 0.63	2.04 ± 0.44	0.02
Carnosine	4.63 ± 0.53	3.35 ± 0.38	0.08

Table 3.1. (cont'd)

Citrulline	9.99 ± 1.86	11.18 ± 1.31	0.61
Ornithine	6.16 ± 0.73	7.99 ± 0.52	0.07

Table 3.2. Reverse transcription quantitative real-time (RT-qPCR) assay primer information in the gluteal muscle of PPID and non-PPID horses.

Gene Name	Symbol	Protein	Taqman Assay/Accession number[1]
Solute carrier family 38, member 2	*SLC38A2*	SNAT2	NC_009149
Solute carrier family 2 (facilitated glucose transporter), member 4	*SLC2A4*	GLUT4	NM_001081866.1(10-11)
Insulin receptor	*INSR*	INSR	XM_001496584.3
glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	GAPDH	NM_001163856
actin, beta	*ACTB*	β-actin	NM_001081838
Succinate dehydrogenase complex subunit A	*SDHA*	SDHA	DQ402987

1- Applied Biosystems TaqMan® assay product number or GenBank accession number of sequence submitted for Custom TaqMan® Gene Expression Assay design

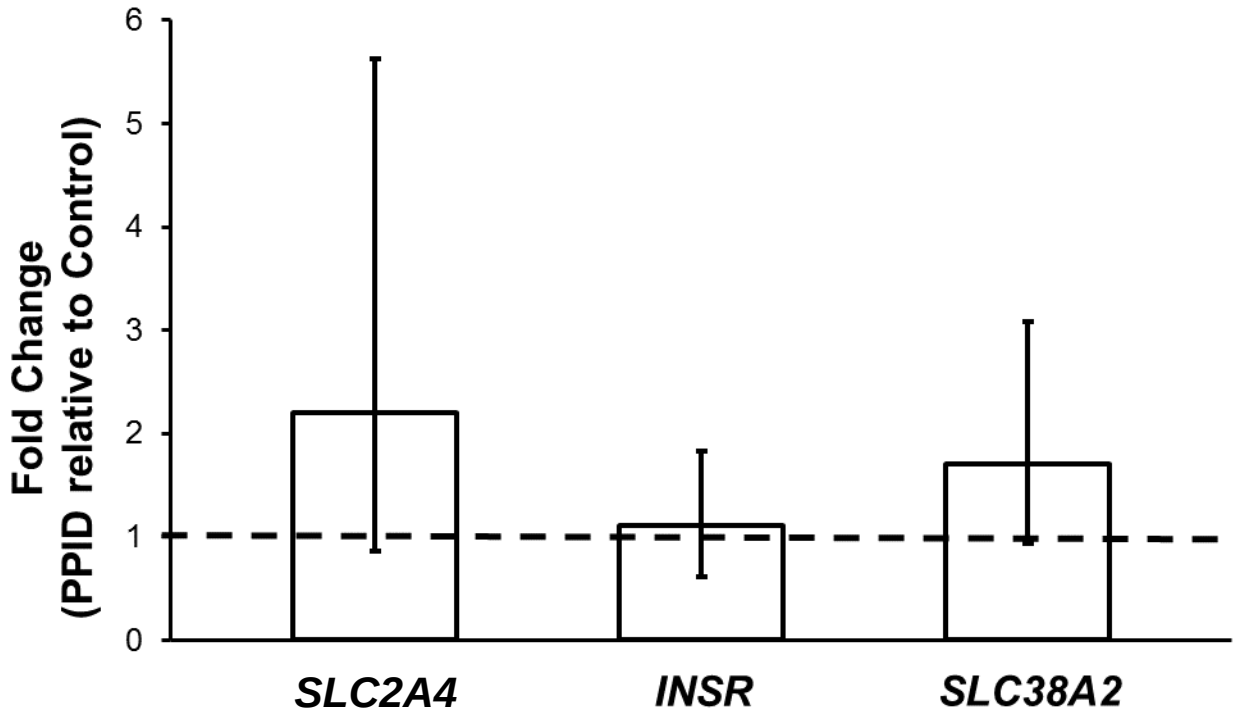

Figure 3.1. Fold change in mRNA abundance of *SLC2A4*, *INSR*, and *SLC38A2* in the gluteal muscle for PPID (n=8) relative to control (n=4) horses.

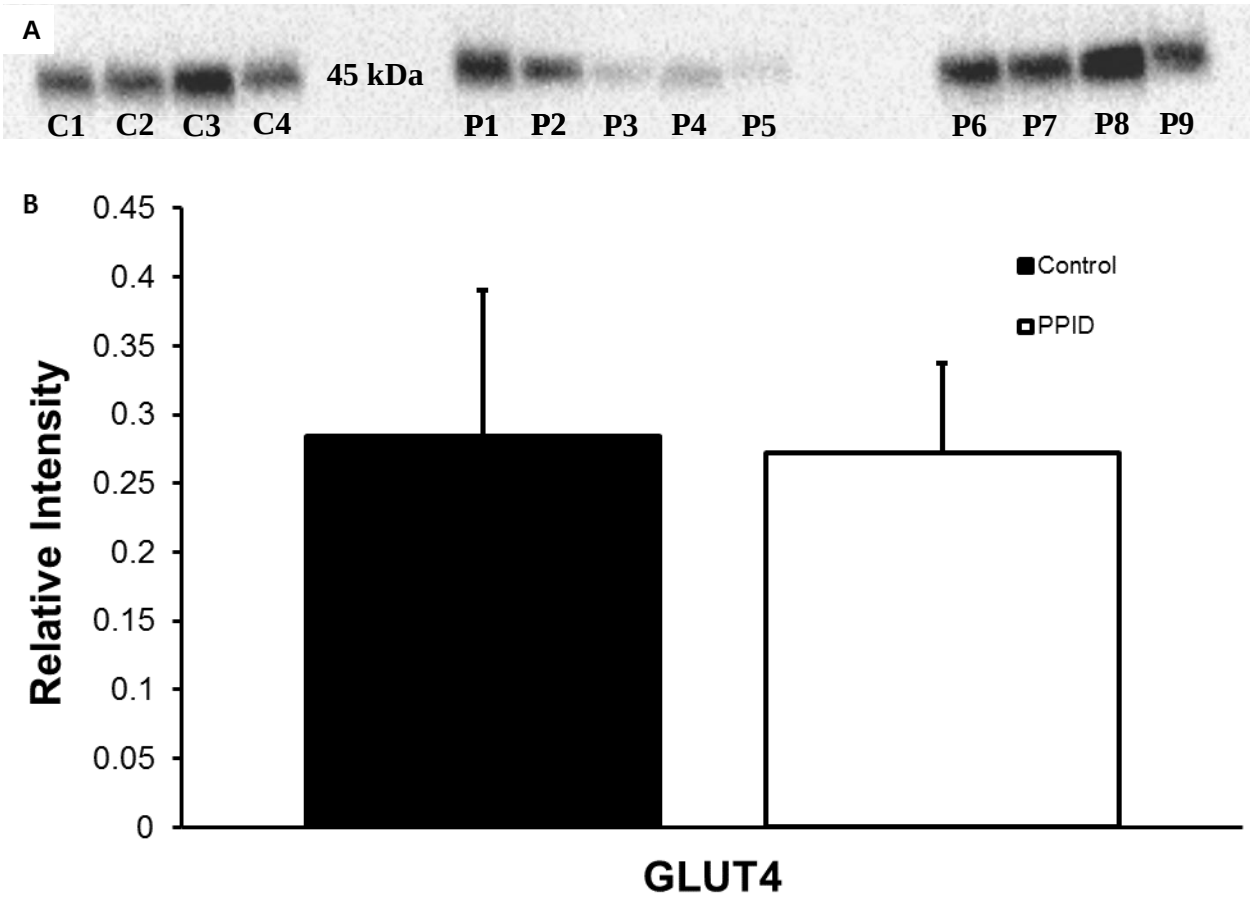

Figure 3.2. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies for PPID (n=8), represented as P1-P9, and control (n=4), represented as C1-C4, horses treated with antibodies against GLUT4 depicted by (A) Western blotting showing protein expression level and (B) a graph indicating the relative expression level. Intensities of bands were estimated by using the program "ImageLab" from BioRad.

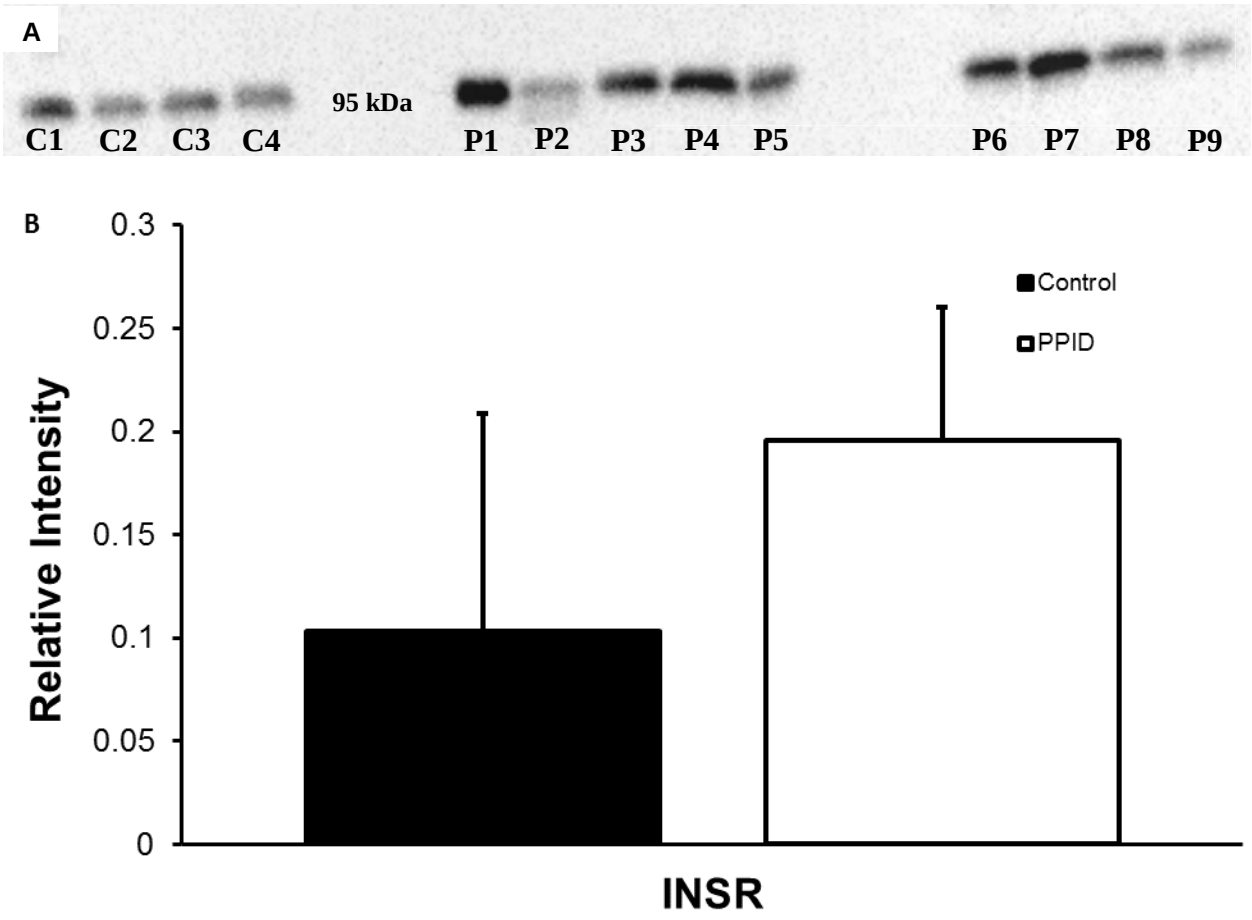

Figure 3.3. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies for PPID (n=8), represented as P1-P9, and control (n=4), represented as C1-C4, horses treated with antibodies against INSR depicted by (A) Western blotting showing protein expression level and (B) a graph indicating the relative expression level. Intensities of bands were estimated by using the program "ImageLab" from BioRad.

Discussion

Horses suffering from PPID demonstrate poor physical appearance and muscle wasting while maintaining a high appetite (Donaldson et al., 2004; Aleman et al., 2006; McFarlane, 2011; Klinkhamer et al., 2011; Inneråa et al., 2013). The condition is characterized by chronically elevated concentrations of glucocorticoids, namely ACTH and cortisol (Hillyer et al., 1992; van der Kolk et al., 1993; Love, 1993; Dybdal et al., 1994; van der Kolk et al., 1995; Eiler et al., 1997; Reed, 1998). We reported earlier (Chapter 1) that horses with PPID in this study had significantly higher serum concentrations of ACTH and cortisol compared to non-PPID horses (Chapter 1). Basal ACTH serum concentrations were 85.6 ± 18.5 and 23.0 ± 26.1 pg/mL in PPID and non-PPID horses (Chapter 1). Basal and post-dexamethasone cortisol concentrations were 158.0 ± 36.7 and 10.0 ± 21.9 nmol/L for control horses and were 156.0 ± 25.9 and 99.3 ± 14.5 nmol/L, respectively, for PPID horses. Glucocorticoids play a role in accelerating muscle proteolysis in rats with sepsis (Löfberg et al., 2002). High doses of prednisolone lead to a sharp increase in net protein catabolism in human patients (Ferrando et al., 1996). In humans patients diagnosed with Cushing's disease, muscle atrophy is attributed to a decrease in the rate of whole-body protein synthesis and an increase in the rate of protein breakdown (Burt et al., 2007) suggested muscle atrophy to be a result of high circulating concentrations of glucocorticoids. The high levels of glucocorticoids in horses diagnosed with the PPID condition are proposed to be responsible for the decrease in skeletal muscle tissue. In human patients diagnosed with Cushing's disease, muscle atrophy is attributed to a decrease in the rate of whole-body protein synthesis and an increase in the rate of protein breakdown (Aleman et al., 2010). The loss of epaxial and gluteal muscles seen in PPID horses (McFarlane, 2011) results from atrophy in types 1A and 2B muscle fibers, and loss of type 2B myofibers (Coderre et al., 1995). However, the

mechanism responsible for the muscle wasting characteristic to PPID horses remains largely unknown.

Cortisol reduces GLUT4 translocation to the cell surface in rat skeletal muscle (Weinstein et al., 1995; Dimitriadis et al., 1997; Ekstrand et al., 1996) and increases lipolysis in human fat tissue (Kennedy et al. 1999, Filipovsky et al., 1996). Insulin resistance and central fat deposit in men has been well established to be associated with excess blood cortisol concentration and cortisol production rates (Stolk et all., 1996; Phillips et al., 1998). In this study, horses with PPID had significantly higher serum concentrations of ACTH and cortisol compared to non-PPID horses (Chapter 1). Basal ACTH serum concentrations were 85.6 ± 18.5 and 23.0 ± 26.1 pg/mL in PPID and non-PPID horses (Chapter 1). Basal and post-dexamethasone cortisol concentrations were 158.0 ± 36.7 and 10.0 ± 21.9 nmol/L for control horses and were 156.0 ± 25.9 and 99.3 ± 14.5 nmol/L, respectively, for PPID horses. Similarly, horses with PPID have abnormal fat deposits across the crest of the neck, tail head, and abdominal region and several studies have reported insulin resistance in horses with PPID (Schott, 2002; Garcia and Beech, 1986). It has been suggested that the major mechanism of insulin resistance may be related to a defect in the events post-receptor versus a defect at the INSR level (Glass, 2003). Protein accretion and breakdown signaling pathways are affected by insulin (Glass et al., 2010; Suagee et al., 2011) therefore a decrease in INSR protein abundance could explain muscle wasting in horses diagnosed with PPID. Suagee et al. (2011) reported reduction in GLUT4 and INSR in horses with elevated insulin levels. Studies in mice and humans indicate reductions in peripheral tissue responsiveness to insulin may be due to hyperinsulinemia (Nankervis et al., 1895; Marban and Roth, 1996; Barr et al., 1997). The first determinant of insulin-dependent peripheral glucose uptake is the availability on the cell membrane of the GLUT4 transporter (Andrew and Walker,

1999). The effect of glucocorticoids on insulin receptors has been examined with limited consensus among studies. Some studies have shown insulin resistance in PPID horses when compared to non-PPID horses (Schott, 2002; Garcia and Beech, 1986). However, horses were not age matched and it is possible that the decrease in insulin sensitivity seen was a function of age rather than the PPID status (Vick et al., 2007). The INSR and GLUT4 mRNA abundance and protein expression was not different in PPID horses relative to control horses in our study. This is further confirmed in our previous work with no difference being found in either insulin sensitivity or glucose tolerance during either OGTT or FSGITT when comparing horses with PPID to their age-matched non-PPID control (Chapter 1). Although the DI was noticeably greater in PPID than control horses, the large variation in particular in the control group precluded detecting a statistical difference. Nonetheless, the positive and relatively strong correlation between ACTH concentration and the DI, and the sustained higher insulin concentration following the i.v. insulin administration is indicative that there was compensation to some degree of insulin resistance in the PPID group. Hyperinsulinemia is associated with insulin resistance (Carter et al., 2009; Frank et al., 2011). Whether the indications of insulin sensitivity reported here and elsewhere (Schott, 2002; Garcia and Beech, 1986) are due to the severity of the PPID condition or age alone remains inconclusive.

Metabolic acidosis in humans with chronic renal failure has been shown to cause wasting of lean tissue (Mitch et al., 1994), similar to the muscle wasting seen in horses with PPID. Muscle wasting in these patients has been attributed to low pH causing inhibition of L-Gln transport via SNAT2 (Evans et al., 2007). Our study reported no effect of PPID status on the expression of SNAT2 in the gluteal muscle. Further, we sought to evaluate the plasma amino acid profile differences in PPID horses when compared to non-PPID control horses. Studies have

shown that SNAT2 inhibition indirectly depleted other amino acids whose intracellular concentrations are maintained by the L-Gln gradient across the plasma membrane (Evans et al., 2007). In this respect, SNAT2 may determine intracellular anabolic amino acid levels thus regulating the signaling that affects protein mass, nucleotide/nucleic acid metabolism, and cell growth (Evans et al., 2007). A study by Bergstrom et al. (1990) has shown the depletion of intramuscular free amino acids to be an early step in muscle wasting of human uremia patients. Interestingly, the correction of acidosis reverses intramuscular free amino acid depletion (Löfberg et al., 1997). This depletion is sensed through the protein synthesis stimulating mammalian target of rapamycin (mTOR) (Proud, 2004). As shown in Mastro et al. (2014), a numerical reduction in mTOR signaling was indicated in PPID horses when compared to control horses, thus potentially making it difficult for horses afflicted with the PPID condition to detect a depletion in free amino acids. A study in geriatric humans with trauma by Jeevanandam et al. (1990) demonstrated a reduction in lean body mass to be related to a reduced protein metabolic response to trauma, namely due to changes in free plasma amino acid concentrations. The PPID horses of our study showed a similar decrease in methionine and an increase in ornithine concentration, suggesting that the change in free amino acid concentrations seen in PPID horses could be related to the stress of the PPID condition. Further, the urinary excretion of 3-methylhistidine (3-MH) has been used widely in humans and animals as a biological marker for skeletal protein breakdown (Young et al., 1972; Long et al., 1988). In our study, horses with PPID had a reduced concentration of 3-MH in their plasma compared to control horses, indicating that they had a reduced product of protein degradation. This is contrary to what would be expected. However, in times of prolonged starvation in human patients in a study by Long et al. (1977), 3-MH excretion in urine decreases. This decrease in 3-MH is an adaptive attenuation

of muscle breakdown and a reduction in lean body mass (Long et al., 1977). Horses with PPID are widely characterized by a poor physical appearance and muscle wasting, mimicking the effect of a body during times of prolonged starvation (Donaldson et al., 2004; Aleman et al., 2006; McFarlane, 2011; Klinkhamer et al., 2011; Innera et al., 2013). Human patients with severe abdominal sepsis, display a decreased plasma concentration of glutamine, indicating intracellular glutamine formation in muscle tissue may be compromised (Roth et al., 1982). In addition, reduced plasma glutamine concentration has been reported in patients with untreated diabetes mellitus, diet-induced metabolic acidosis, and in the recovery period following high intensity intermittent exercise (Walsh et al., 1998). Common among the above mentioned states of stress are the rises in plasma cortisol and glucagon concentrations and an increased tissue requirement for glutamine for gluconeogenesis (Walsh et al., 1998). Together, this suggests that intracellular glutamine formation in the muscle tissue plays a role in the characteristic muscle wasting of PPID horses.

Aleman et al. (2010) reported that muscle wasting in PPID horses was not caused by reduced expression of major proteolytic systems and growth regulators. However, the numerical reduction in mTOR signaling in PPID horses when compared to non-PPID control horses could potentially explain, in part, some of the mechanism behind muscle wasting (Aleman et al., 2010). Mastro et al. (2015) reported that muscle fractional protein breakdown rate was not affected by PPID status when compare to control horses without PPID. Additionally, in this same study, there was no impact of PPID status relative to control horses on the abundance or activation of positive and negative regulators of protein synthesis and positive regulators of protein breakdown. Other factors must be at work to account for the muscle wasting typically seen in the PPID condition. Early reports by Ralston et al. (1989) reported a decrease in apparent crude

protein digestion in aged horses in relation to younger control animals, but were later followed by showing no decrease (Ralston et al., 2001). A study in rats found the splanchnic extraction of amino acids to be reduced by 20-30% in aged rats when compared to younger controls (Jourdan et al., 2011). Human studies have found a similar trend in whole body muscle protein synthesis declining with age (Short et al., 2004). Future studies in PPID horses should be compared not only to aged non-PPID horses, but younger non-PPID and PPID affected horses in an effort to better understand those characteristics which are a function of age and which are related to the PPID condition.

In summation, whether a decrease in utilization of glucose and amino acid at the systemic level contributes to the pathology of PPID remains largely unknown. Additionally, whether the indications of insulin sensitivity reported here and elsewhere (Schott, 2002; Garcia and Beech, 1986) are due to the severity of the PPID condition or age alone remains inconclusive. Although the gene expression of *SLC2A4*, *INSR*, and *SLC38A2* was not different in PPID horses compared to controls, additional factors related to muscle atrophy such as posttranscriptional and posttranslational mechanism that affect protein production, activation or inactivation, and function may be responsible and should receive further attention. The changes in free amino acid concentrations in the PPID horses of our study could be related to the stress of the PPID condition. Specifically, the role intracellular glutamine formation plays in the muscle tissue plays is indicative of muscle wasting of PPID horses. We have investigated the role of the SI in reduced nutrient absorption and utilization of glucose and glutamine in regards to the structural and functional integrity of the promixal and mid small intestinal mucosa and found lower morphological changes in villi and cryptal layers, and decreased glucose absorption across the jejunal brush border membrane in PPID horses compared to controls (Chapter 3). Future studies

should focus on distinguishing the effects of age versus the PPID condition alone. The results of this study show that the muscle wasting associated with the pathology of the PPID condition is not due to a reduced utilization of glucose and amino acid at the systemic level in regards to *SLC2A4*, *INSR*, and *SLC38A2* abundance and content. Many factors affect nutrient absorption and muscle wasting and thus diligent research should be continued in PPID horses to determine the mechanisms behind the condition. In conclusion, our results suggest that geriatric horses and those in particular with PPID should be carefully managed with feeding diets containing low concentration of soluble carbohydrates in order to reduce the risk of laminitis.

CHAPTER FOUR: Morphology of the small intestinal mucosa in horses with pituitary pars intermedia dysfunction

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Abstract

The objective was to evaluate the morphology of the small intestinal mucosa of horses with PPID and without PPID. Eight PPID and 4 non PPID (control) of multiple breeds and genders, and over 19 years of age were donated for this study. Horses were maintained on second cutting grass timothy hay and senior feed at 1.8 and 0.5% of their BW, respectively for a period of 5 d. Horses were euthanized, the entire gastrointestinal tract removed and samples collected from the duodenum at 25 and 50 cm distal to the pyloric sphincter, the mid-jejunum and the mid-ileum. Compared to control horses, villus height proximal average of PPID horses was lower ($P < 0.05$) and did not differ in duodenum at 25 cm or 50 cm, mid-jejunum or ileum. Villus width did not differ between PPID and control horses across any segment or proximal average. Compared to control horses, villus crypt depth of PPID horses tended to be greater in mid-jejunum ($P < 0.10$), and did not differ in duodenum at 25 or 50 cm, mid-jejunum, or the ileum. Compared to control horses, VH:CD of PPID horses was lower in mid-jejunum ($P < 0.05$), and did not differ between PPID and control horses in duodenum at 25 or 50cm, ileum, or proximal average. In conclusion, PPID in horses is associated with small intestinal villi atrophy and greater VH:CD in proximal to mid regions of the small intestine.

Keywords: histology; Cushing's disease; small intestine; pituitary pars intermedia dysfunction, horse

Introduction

Pituitary pars intermedia dysfunction (PPID) is an endocrine disorder primarily affecting the geriatric horse (Schott, 2006). It is a progressive condition characterized by hypertrophy, hyperplasia, adenoma and loss of dopaminergic inhibition of the pars intermedia of the pituitary gland resulting in increased circulating concentration of pro-opiomelanocortin peptides (POMC), including ACTH (Hillyer et al., 1992; S Love, 1993; van der Kolk et al., 1993; Dybdal et al., 1994; van der Kolk et al., 1995; Eiler et al., 1997; SM Reed, 1998). Horses diagnosed with PPID exhibit a variety of clinical signs, most commonly including poor body condition, appearance of muscle wasting, abnormal fat deposits, hirsutism, and heightened risk for secondary infections (Donaldson et al., 2004; Aleman et al., 2006; McFarlane et al., 2010; Klinkhamer et al., 2011; Innerá et al., 2013). Pituitary pars intermedia dysfunction has also been associated with an increased risk for laminitis (Donaldson et al., 2004; Carter et al., 2009; Geor, 2009). Despite a characteristically high appetite (K.G., Mountjoy, 2010), physical appearance of horses with PPID indicate that poor nutrient absorption may play a role in the pathophysiology of the condition.

A variety of ailments targeting the small intestinal mucosa have been shown to cause malabsorption, including lesions (Platt, 1986), chronic inflammatory and lymphoproliferative disorders of the small intestine (Pass and Bolton, 1982), alimentary lymphomas (Platt, 1986) and mycobacterial infections (Merritt et al., 1975). Changes in the histomorphology of the small intestinal mucosa, including villi atrophy and increased cryptal layer, are related to poor nutrient absorption and malnutrition in animals (Stevenson et al., 2013) and humans (Dewar and Ciclitira, 2005; and Ventura et al., 2013). The clinical and gross pathological features of these conditions are not readily distinguishable and thus histopathological examination of intestinal biopsy or

post-mortem specimens for diagnosis are necessary (Platt, 1986). Histomorphometric analysis of the small intestinal mucosa in horses afflicted with PPID has not been previously documented.

We hypothesized that mucosal villus height and crypt depth of the small intestine is lower and greater, respectively in horses with PPID. The objective was to evaluate the morphology of the mucosa in the proximal, mid and distal regions of the small intestine in horses clinically confirmed with PPID and no PPID.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University (03-12-064-001).

Animals and Housing

Eight horses confirmed with PPID and 4 non PPID (control) were donated for this study following owners' consent all within the fall of 2012 as described in Chapter 2. Horses were of multiple breeds and genders (mare and gelding) across groups ranging from 19 to 39 years with an average age of 25.3 ± 2.11 years for control and 28.6 ± 1.49 years for PPID horse. Following admission for the study, all horses were housed indoor, in a temperature controlled environment (21˚C) and individual stalls (3.05m × 3.05m or 3.66m × 3.66m) at the Michigan State University College of Veterinary Medicine Research and Instructional Building. All horses were fed second cutting mixed orchardgrass (*Dactylis glomerata L.*) and brome-grass hay (*Bromus mollis L.)*, and a commercial grain concentrate (Equine Senior, Purina, St. Louis, MO) containing 14% crude protein, 1.8% fat and 18% crude fiber at 1.8 and 0.5% of their body weight, respectively.

Clinical Evaluation

Clinical evaluation has been described in detailed elsewhere (Chapter 2). In brief, horses were subjected to ACTH testing as described in Beech et al. (2009), an overnight dexamethasone suppression test (ODST) as described by Innera et al. (2013), an oral glucose tolerance test (OGTT) per Pratt et al. (2006), and a frequently sampled intravenous glucose tolerance test (FSGITT) was performed as described in Tóth et al. (2009).

Collection of Tissue

Horses were euthanized on the day following FSGITT (i.e., day 6) with pentobarbital (120 mL Fatal Plus at 392 mg/mL) administered intravenously using the jugular catheter already in place. Following confirmation of death, the skull was opened on the dorsal aspect to excise the brain and access the pituitary. The pituitary gland was gently dissected out, weighed, and measured for length and diameter. The gland was cut in half with one half flash-frozen in liquid N for tissue archive and the other half transferred into 10% formalin solution (10mL Formalin/100mL H20) and submitted to the Histopathology lab for histopathological examination. Slides were stained with hematoxylin and eosin (HE) and graded as described in Miller et al. (2008).

Immediately following confirmation of death, horses were positioned in either the right or left lateral recumbent position and an incision was made along the ventral midline of the abdominal cavity to expose and remove the entire gastrointestinal tract. Samples (25-cm long segment) were collected from the duodenum at 25 cm (Duo25) and 50 cm (Duo50) distal to the pyloric sphincter, the mid-jejunum (Jej), and the ileum (Ile) at 15 cm proximal to the cecum.

Approximately 5-cm long transversal cut of each collected segments were kept intact and gently rinsed in ice-cold NaCl solution (0.9 g NaCl/100 mL H_2O), and transferred in formalin-distilled water solution (10% vol/vol) for fixation over a 24hour period. Following fixation, segments were transferred to an ethanol-distilled water solution (30% vol/vol) until histological processing.

Histological Examination of the Small Intestine

For histological visualization and morphometric analysis of the intestinal mucosa, each segment was cut longitudinally to expose the mucosa, with two transverse sections (approximately 0.4-cm thick) at the proximal and dorsal end and placed in histological cassettes. Cassettes were sent to the Michigan State University Histopathology Laboratory for processing. Tissue samples were vacuum infiltrated with paraffin (Excelsior tissue processor, ThermoFisher Scientific Inc., Waltham, MA) and embedded (HistoCentre III, ThermoFisher Scientific Inc., Waltham, MA). The resulting blocks were cut using a microtome (Reichert Jung 2030 rotary microtome, Rankin Biomedical Corporation, Holly, MI) in fine sections at 4-5 microns and mounted via incubation at 56 °C for 2-24 hrs. Slides were stained with Hematoxylin and Eosin (Pureview® Hematoxylin and Eosin Stain System, Cancer Diagnostics Inc. Durham, NC) according to Porter and Joseph (2015).

Histological visualization of the intestinal mucosa was performed at 200 × magnification (Nikon Eclipse Ti Inverted Microscope) and measurements performed using a microscope imaging software (NIS Elements Microscope Imaging Software) at 100× magnification. For each segment, a minimum of 10 well-defined villi and associated crypts were measured for villus

height (VH), villus width (VW), and crypt depth (CD). The ratio of height to crypt depth (VH:CD) and average or proximal segments (Duo25, Duo50 and Jejunum) was calculated.

Statistical Analysis

Normal distribution of data was assessed using the Gaussian distribution analysis in SAS (version 9.3, SAS Institute Inc., Cary, NC). Analysis of data was performed using PROC MIXED procedure of SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). The model included segment and treatment as fixed effects and horse (treatment) as the random term. Segment was included as a repeated measure. Difference in VH, VW, CD and V:C between treatments were determined using the Student's *t*-test with significant differences evaluated at $P \leq 0.05$ and tendency at $P \leq 0.1$.

Results

Compared to control horses, villus height of PPID horses was lower in the proximal average of segments and did not differ in the duodenum at 25 cm or 50 cm, mid-jejunum or ileum. Villus width did not differ between control and PPID horses across any segment or the average of proximal segments. Compared to control horses, villus crypt depth of PPID horses tended to be greater in the mid-jejunum ($P < 0.10$), and did not differ in the duodenum at 25 or 50 cm, ileum, or the average of proximal segments. Compared to control, V:C of PPID horses was lower in the mid-jejunum ($P < 0.05$) and did not differ in the duodenum at 25 cm or 50 cm, Ileum, or the average of proximal segments. The proximal average did not differ between control and PPID horses in VH ($P = 0.181$), VW ($P = 0.803$), CD ($P = 0.334$), or V:C ($P = 0.244$). The proximal average of villus height in control horses was 518 ± 56.7 and 418 ± 40.1 in PPID

horses. Villus width proximal average in control horses was 183 ± 9.07 and 185 ± 6.42 in PPID horses. The proximal average of crypt depth in control horses was determined to be 189 ± 13.1 in non-PPID horses and 173 ± 9.23 in PPID horses. The V:C proximal average in control horses was 2.80 ± 0.26 and 2.41 ± 0.18 in PPID horses.

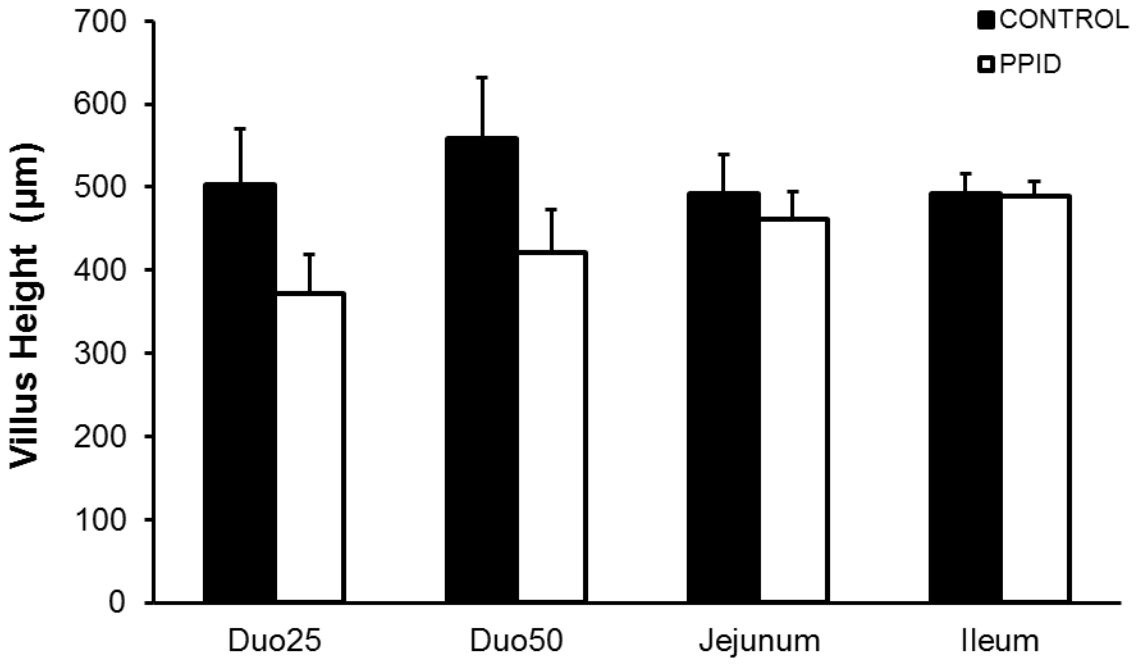

Figure 4.1. Villus height of the small intestine in PPID (n=8) and control (n=4) horses. Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

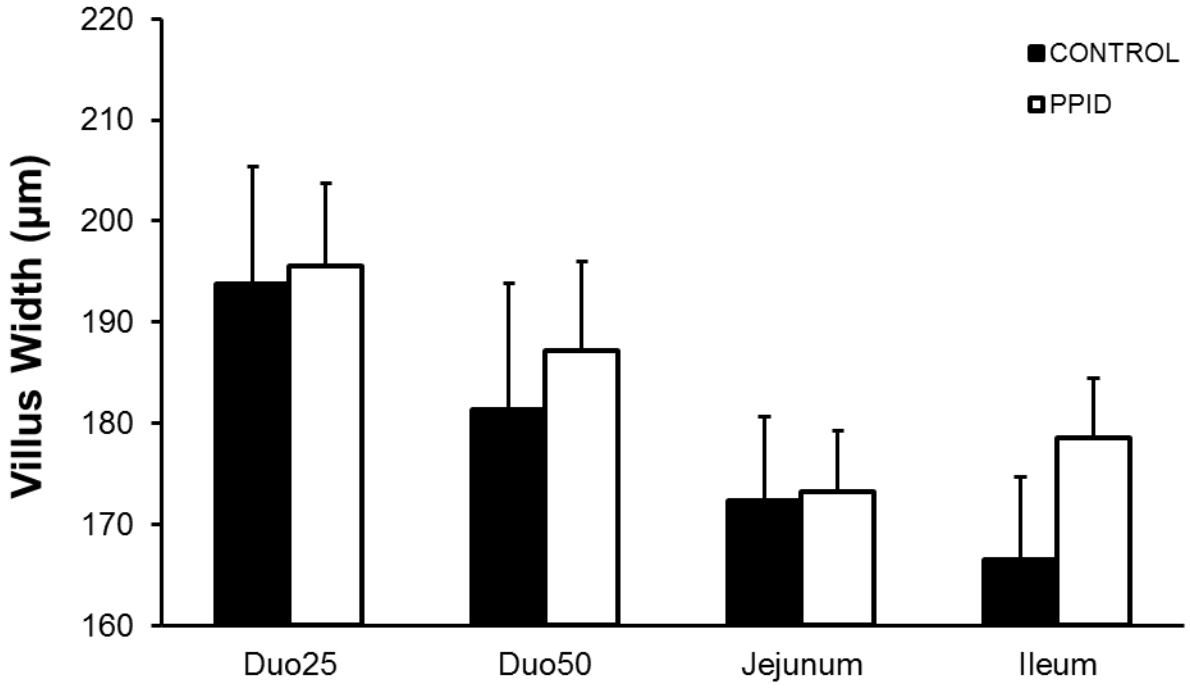

Figure 4.2. Villus width of the small intestine in PPID (n=8) and control (n=4) horses. Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

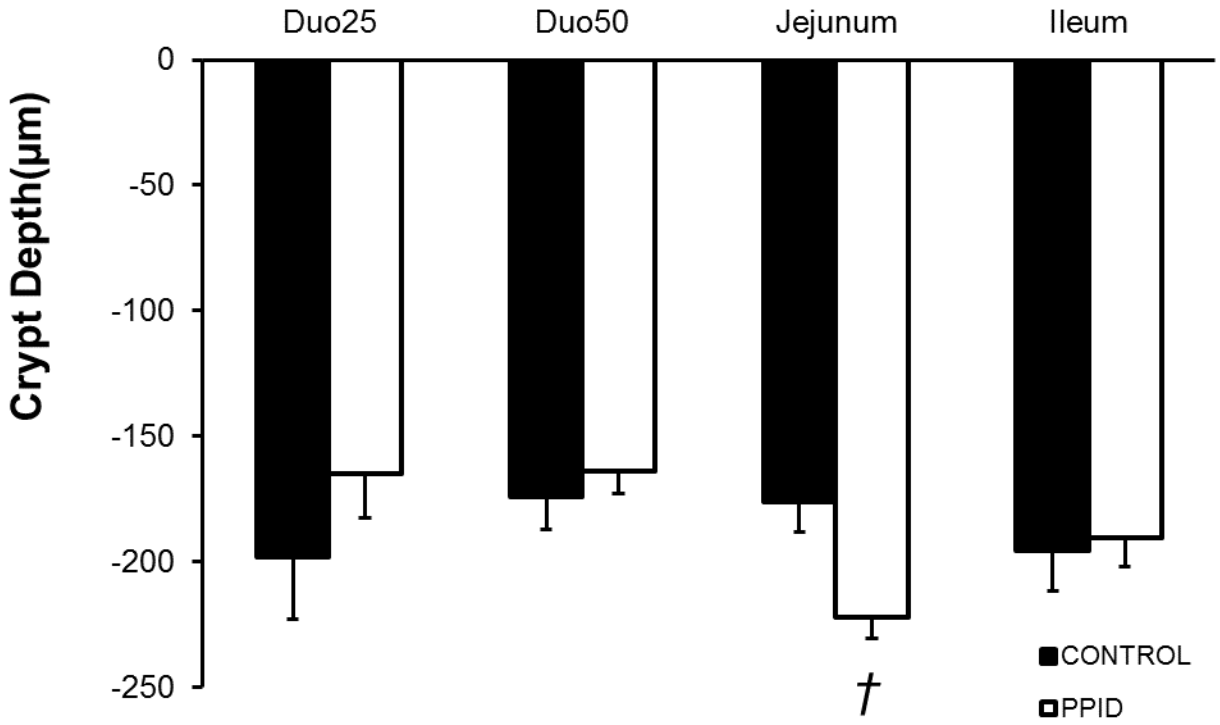

Figure 4.3. Villus crypt depth of the small intestine in PPID (n=8) and control (n=4) horses. † Trending difference from control horses ($P \leq 0.05$). Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

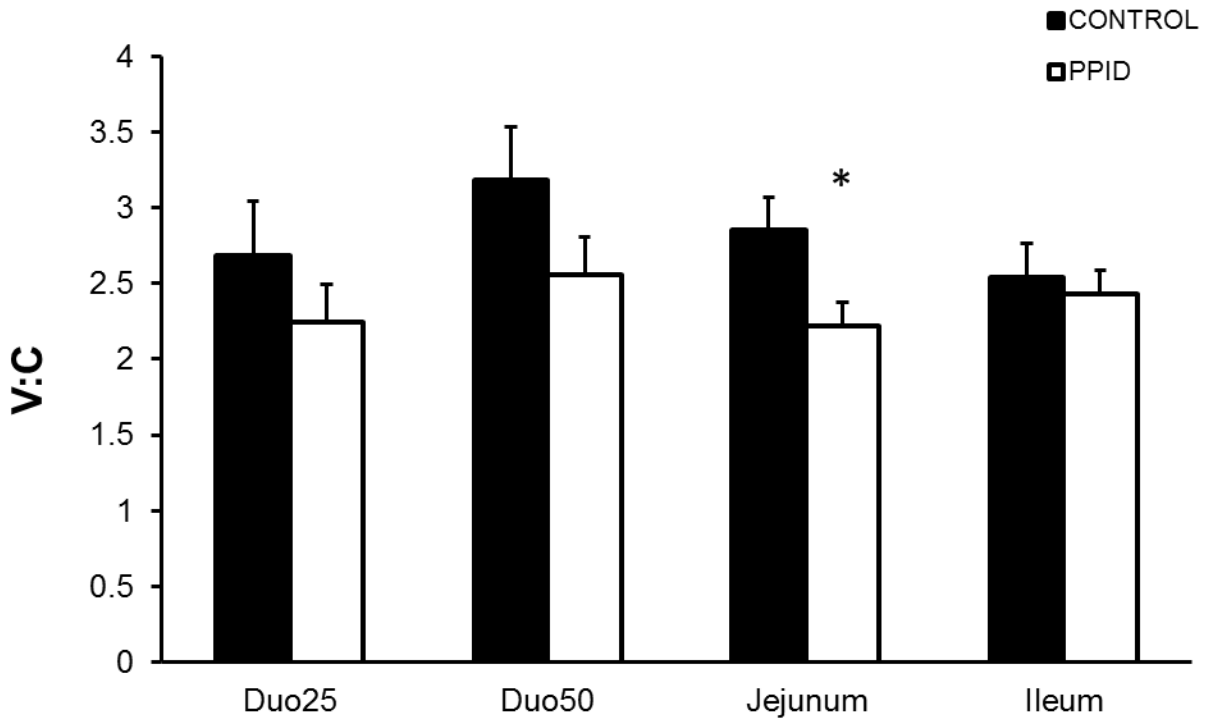

Figure 4.4. Villus height:Crypt Depth (V:C) of the small intestine in PPID (n=8) and control (n=4) horses. *Significantly different from control horses ($P \leq 0.05$). Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

Discussion

Clinical manifestations of PPID in horses include poor body condition, abnormal fat deposits, muscle atrophy, lethargy and hirsutism (Donaldson et al., 2004; Aleman et al., 2006; McFarlane et al., 2010, Klinkhamer et al., 2011; Innerá et al., 2013). The PPID condition is characterized by excess ACTH production, resulting in high concentration of cortisol. Sustained high levels of cortisol are associated with suppression of the immune and inflammatory response systems in humans (Reiche et al., 2004). Humans with PPID have chronic elevated cortisol concentration and suffer from degeneration of muscle, bone, and neuronal tissue (Melzack and Katz, 2004), impaired digestion (Jones and Quinn, 2005) and a weakened immune system (Cohen and Williamson, 1991; Andersen, et al., 1994). High doses of corticosteroids have also been shown to cause mucosal lesions on the duodenum in humans (Kiziltas et al., 1998), which decreases the protective barrier function of the mucosa (Travis and Menzies, 1992). Insulin resistance and central fat deposit in men has been well established to be associated with excess blood cortisol concentration and cortisol production rates (Filipovsky et al., 1996; Stolk et al., 1996; Phillips et al., 1998; Purnell et al., 2009) however the mechanisms of action remains largely misunderstood (Rosmond, 2005) and poorly documented in horses.

While the etiology of PPID is multifaceted, horses suffering from this condition demonstrate poor physical appearance suggesting that optimum nutrient utilization and (or) absorption may be compromised. Intestinal morphology is of vital importance in dictating nutrient absorption (Dewar and Ciclitira, 2005; Stevenson et al., 2013; Ventura et al., 2013) and gut health (Xu et al., 2003). Gut health is compromised in the face of toxins, resulting in rapid changes of the intestinal mucosa morphology as seen by a reduction in villus height and increase in crypt depth of broiler chickens (Yason et al, 1987). In humans affected by Celiac disease,

inflammation of the small intestinal mucosa induces a malabsorption syndrome and intestinal lesions including reduced villus height, increased villus width, and increased cryptal layer (Murray, 1999). There is very little information on the intestinal mucosa histomorphometry of horses and to date, the impact of PPID on intestinal mucosa morphology has not been reported.

In this study, horses suffering from PPID displayed alterations in their mucosal architecture including reduced villi height, greater crypt depth, and lower villus height to crypt depth ratio in the proximal and mid regions of the small intestine. The VH values along the small intestinal mucosa ranged from 491 ± 24.8 to 559 ± 73.1 µm in control and 372 ± 47.6 to 490 ± 17.5 µm in PPID horses. Values for the control horses nearly fall within range to some degree with those reported by Lindberg and Karlson (1985), i.e., 600-860 µm. This discrepancy may be attributed to the age difference between the horses in this study,i.e., > 19 years and those of Lindberg and Karlson (1985), i.e., > 4 years). Villus height decreased with age in rabbit jejunal mucosa when comparing young and weanling rabbits (Keelan et al., 1985). Studies in male rats have shown a similar trend of decreased villus height when moving from the proximal to distal region of the small intestine seen in the horses of our study (Hohn et al., 1978). Villus width in our study ranged from 167 ± 8.22 to 193 ± 11.6 µm in control and 173 ± 5.93 to 196 ± 8.18 µm in PPID horses, which fall within previous range of 150 to 250 µm reported by Lindberg and Karlson (1985). The ratio of villus height: crypt depth (V:C) is considered to be a better indicator of mucosal integrity and nutrient absorption than villus height alone (Montagne et al., 2003). The V:C increased from Duo25 to Duo50 and decreased from Duo50 to the jejunum in both PPID and control horses, and increased in control and decreased in PPID from jejunum to ileum. In pigs, V:C also decreased from the proximal to distal regions moving from the duodenum to jejunum (Guay et al., 2006). The V:C ranged from 2.54 ± 0.23 to 3.18 ± 0.25 in control and 2.22

± 0.15 to 2.55 ± 0.25 in PPID horses and are comparable to those in pigs ranging from 1.5 ± 0.11 to 3.29 ± 0.25 (Spreeuwenberg et al., 2001; Guay et al., 2006), and those of humans ranging from 1.59 ± 0.12 to 2.50 ± 0.28 (Pironi et al., 1994). Greater villus height and shallower crypt depth are related to increased nutrient absorption (Xu et al., 2003). Increased villus extrusion in the duodenum and ileum is a common response to infection (Domeneghini et al., 2006). In an effort to maintain functional integrity, the intestine compensates for infection by greater activity in the crypts causing them to become deeper (Domeneghini et al., 2006). The mechanisms by which PPID may potentially compromises intestinal function are unknown, however, morphological changes in intestinal mucosa of horses with PPID are similar to those seen in horses and other animal species exposed to immunological challenges. For instance, horses with equine granulomatous enteritis (EGE) display villous atrophy and pronounced lengthening of the crypts (Lindberg, 1984). Equine granulomatous enteritis is associated with carbohydrate malabsorption and excessive protein loss into the gut (Meuten et al., 1978). Other species exhibit similar morphological changes as seen in this study. In humans, celiac disease is an inflammatory bowel disease characterized by inflamed and flattened villi, resulting in impaired absorption function (Murray et al., 1999; Dewar et al., 2005). Severe villi atrophy in all segments of the small intestine is characteristic of porcine epidemic diarrhea virus resulting in malabsorption (Stevenson, 2013). The absorptive capacity of humans with Giardiasis is decreased due to villi shortening (Ventura et al., 2013).

Intestinal mucosa development and maintenance is achieved through cell renewal and extrusion (Yamauchi et al., 1996). A balance between these events ensures constant turnover of old to new cells and thus maintenance of villus height (Yamauchi et al., 1996). For instance, long periods of fasting are associated with large lysosomal autophagic vacuoles causing cell death

which leads to an increased rate of extrusion and reduction in villus size (Yamauchi et al., 1996). Crypt depth in our study ranged from 174 ± 12.9 to 198 ± 24.7 μm in control horses and 164 ± 9.13 to 222 ± 8.64 μm in PPID horses, which compare to previous reported values in pigs ranging from 102 ± 16.4 to 169 ± 14.4 μm (Pluske et al., 1996). The crypts are a source of replacement cells for the entire villus; a greater crypt depth, as seen in the jejunum of PPID horses when compared to controls in our study, indicates a high demand for new tissue and thus a greater rate of tissue turnover (Yason et al., 1987). This increased turnover requires a greater energy and protein demand from diet in order to sustain gut morphology (Luk et al., 1980; Alican and Kubes, 1996; Burrin and Reeds, 1997; Reeds et al., 1997). Overall, if the increased energy and protein demand from diet is not adequately met, a change in intestinal morphology can result reducing amino acid availability for maintenance of muscle mass.

The inflammatory response in the small intestine has been extensively studied for many disorders. Our study did not address this area, but additional studies should seek to address this aspect based on the following available literature. Celiac disease is characterized by increased numbers of lymphocytes, plasma cells, and macrophages in the lamina propria and increased lymphocytes numbers in the surface layer of the epithelium (Murray, 2014). Enterocytes of Celiac-affected individuals are decreased in height, increased in width, and deeper in crypt depth, and more poorly nucleated in comparison to enterocytes of healthy individuals (Murray, 2014). Additionally, the intestinal damage as seen in Celiac disease is mediated largely by cellular immunity. Studies by MacDonald (1990) have shown in Celiac disease that the inflammatory response likely affects both the structural support and microcirculation of the villus resulting in villus collapse. It is unknown whether immunological factors play any roles in the pathology of PPID and the associated mucosal morphological change. Human studies in patients with

Parkinson's disease show inflammation playing a role in the advancement of progressive loss of dopaminergic neurons by activated macrophage glial cells (Dauer et al., 2003) which secrete inflammatory cytokines and free radicals (McGeer et al., 1988). Glucocorticoid levels higher in horses affected by the PPID condition (Wilson et al., 1982; Horowitz et al., 2003), and as such they may be immunologically compromised. However, McFarlane and Holbrook (2008), reported that horses with PPID exhibit decreased pro-inflammatory cytokine expression when compared to healthy aged horses, which may be related to their high plasma concentration of anti-inflammatory hormones. These same authors reported increased expression of IL-8 and suggested that horses with PPID have a decreased ability to respond to pathogen challenge.

Specific nutritional guidelines for PPID horses have yet to be established. However, digestion is improved and incidence of GI diseases reduced when smaller, more frequent meals are fed, access to water increased, and starch (corn) intake reduced in geriatric horses (Ralston, 1990; Ralston, 1999; Pugh, 2000). Based on our previous results and the ones presented herein, we suggest that geriatric horses and those in particular with PPID should be carefully managed with feeding diets containing low concentration of soluble carbohydrates. Currently, there is no cure for the PPID condition. Serotonin antagonists (cyproheptadine) and dopamine agonists (pergolide mesylate or Prascend®) are drugs commonly used to treat the PPID condition (van der Kolk, 1997; Schott, 2002). The use of cyproheptadine has been discouraged (cited in Schott, 2006) based on an improvement in the condition with management alone. While pergolide is considered the drug of choice, no studies have been performed in horses to verify its safety nor bioavailability and metabolism. Additionally, there is no evidence that early medical intervention has an effect on long-term outcome. Therapies targeted at improving GI function of the PPID affected horse are needed. Probiotics and prebiotics in mice reduce intestinal turnover and

modulate the immune system (Bi et al., 2004). Santos Rocha et al. (2012) reported that supplemental feeding of threonine or mannan oligosaccharides in *Salmonella* birds increased villus height, decrease the crypt depth, and subsequently reduces V:C when compared to healthy birds. The results of this study show that the structural integrity of the proximal to mid small intestinal mucosa is compromised in horses suffering from PPID. Based on the notion that structural mucosa integrity is associated with nutrient absorption, we propose that the poor nutritional status of PPID horses is related, in part, to the functional morphology of their small intestinal mucosa.

CHAPTER FIVE

Jejunal brush border membrane uptake of D-glucose and L-glutamine and small intestinal mucosa SLC5A1 and SLC38A2 mRNA abundance in horses with pituitary pars intermedia dysfunction

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Abstract

The goal of this study was to test the hypothesis that D-glucose and L- Gln transport capacity across the apical membrane of the jejunal mucosa, and mRNA abundance of *SLC5A1* and *SLC38A2* throughout the small intestinal mucosa are lower in horses with pituitary pars intermedia dysfunction (PPID) compared to horses without PPID. The objectives were 1) to measure transport capacity (V_{max}) of D-glucose and L-Gln across brush border membrane (BBM) of the jejunal mucosa and 2) to determine mRNA abundance of *SLC5A1* and *SLC38A2* in the duodenal, jejunal and ileal mucosa of PPID and non-PPID horses (control). Twelve horses of multiple breeds and genders, and of similar age (28.6 ± 1.5 and 25.3 ± 2.1 yr for PPID and control, respectively) were donated for this study. Horses were subjected to overnight dexamethasone suppression test and histopathological evaluation of the pituitary gland, and clearly classified as PPID (n=8) or non-PPID (control; n=4). Following euthanasia, mucosal scrapings were collected from the duodenum at 25 and 50 cm distal to the pyloric sphincter, the mid-jejunum and the mid-ileum. Mucosal scrapings were flash frozen in liquid N and stored at -80°C. Maximal rate (V_{max}) and affinity (K_m) of D-glucose and L-Gln transport across the jejunal apical membrane was determined in vitro using brush border membrane vesicles. Messenger RNA abundance was assessed by quantitative Reverse Transcription PCR and results expressed as fold change relative to control. Compared to control horses, D-glucose uptake across BBM of PPID horses was lower at 5 m*M* (P = 0.045) and 7.5 m*M* (P = 0.016). L-Glutamine uptake did not differ between PPID and control horses at any L-Gln concentrations. Compared to control, PPID Glucose V_{max} was lower (P = 0.021) and K_m did not differ. L-Glutamine V_{max} and K_m did not differ between PPID and control horses. The mRNA abundance of *SLC5A1* and *SLC38A2* was not different in PPID horses relative to control horses in any segment. In conclusion, the

poor physical appearance, muscle wasting, and laminitis associated with PPID horses is not due to lack of nutrient transporter abundance in the small intestinal mucosa compared to healthy horses of similar ages, but may in part be related to a function of age or glucose intolerance.

Introduction

Pituitary pars intermedia dysfunction (PPID) is a progressive endocrine disorder that primarily affects the geriatric horse (Schott, 2006). The condition is characterized by hypertrophy, hyperplasia, adenoma and loss of dopaminergic inhibition of the pituitary gland pars intermedia. This loss of inhibitory control results in increased circulating concentration of pro-opiomelanocortin peptides (POMC), including ACTH (Eiler et al., 1997; van der Kolk et al., 1995). Horses with PPID exhibit a variety of clinical signs, generally including poor body condition, appearance of muscle wasting, abnormal fat deposits, hirsutism, and heightened risk for secondary infections (Innerá et al., 2013; Klinkhamer et al., 2011; McFarlane et al., 2010). Similarly, humans with PPID have chronic elevated cortisol concentration and suffer from degeneration of muscle, bone, and neuronal tissue (Melzack and Katz, 2004), and a weakened immune system (Cohen and Williamson, 1991; Andersen, et al., 1994). Despite characteristically high appetite, clinical signs associated with PPID in horses are typical of poor nutritional status. Impaired digestion is reported in human subjects with PPID (Jones and Quinn, 2005). Peripheral dexamethasone administration to broiler chickens causes dose response jejunal morphological changes including increased crypt depth, and decreased in villus height, absorptive surface area and villus height/crypt depth ratio (Li et al., 2009). We have shown hitherto (Chapter 3) a degree of histomorphological changes in the small intestinal mucosa of PPID horses that would suggest some impairment in nutrient absorption. The impact of PPID on intestinal nutrient transport in horses has not previously been investigated. Brush border membrane vesicles (BBMV) preparation from small intestinal mucosa is a useful ex vivo tool to precisely characterize transport kinetics of nutrients in response to changes in physiological or dietary processes. Dietary AA are a major fuel for the small intestinal mucosa and are necessary for maintaining the

intestinal mucosal morphology and function in neonatal piglets (Wu, 1998). Glutamine is a preferred nutrient of intestinal enterocytes for mucosal growth and function (Ganapathy et al, 1994; Salloum et al., 1994; Windmueller and Spaeth, 1980). Glutamine is transported by the sodium-dependent neutral amino acid transporter 2 (SNAT2), which is encoded by *SLC38A2* (solute carrier family 38, member 2). In horses, glucose is transported across the apical membrane of small intestinal epithelial cells (Dyer et al., 2002) by the high-affinity, Na^+-glucose cotransporter SGLT1, encoded by *SLC5A1* (solute carrier family 5, member 1). We hypothesized that the transport capacity (V_{max}) of D-glucose and L-Gln across the apical membrane is reduced in PPID compared to non-PPID horses, and that reduced V_{max} is related to lower mRNA abundance of *SLC38A2* and *SLC5A1*. The objectives of this study were to 1) measure transport capacity of D-glucose and L-Gln across jejunal BBMV of PPID and non-PPID horses; and 2) quantify the mRNA abundance of *SLC5A1* and *SLC38A2* in duodenal, jejunal and ileal mucosa of PPID and non-PPID horses.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University (03-12-064-001).

Animals and Housing

Twelve horses, 8 confirmed with PPID and 4 confirmed free of PPID (control) were donated for this study between the months of September 2012 and December 2012 following owners' consent. Horses were of multiple breeds and genders (mare and gelding) ranging from 19 to 39 yr of age (25.3 ± 2.11 yr for control and 28.6 ± 1.49 yr for PPID horses) (Table 1). No ponies were admittedfor this study. All horses were housed indoor in individual stalls (3.05 m × 3.05 m or 3.66 m × 3.66 m) with an ambient temperature maintained at 21 °C. All horses were

fed second cutting mixed orchardgrass (*Dactylis glomerata L.*) and bromegrass hay (*Bromus mollis L.)*, and a commercial grain concentrate (Equine Senior, Purina, St. Louis, MO) containing 14% CP, 1.8% fat and 18% crude fiber. Hay and concentrate were fed at 1.8 and 0.5% of body weight, respectively. Horses were monitored for medical stability for a period of 5 d prior to being tested for presence and severity grade of PPID. Briefly, horses were subjected to ACTH testing as described in Beech et al. (2009) (Chapter 1) and an overnight dexamethasone suppression test (ODST) as described by Innera et al. (2013) (Chapter 1). Body condition scoring (BCS) was performed based on the Henneke scoring method (Henneke et al., 1983) and body weight (BW) recorded on the day of admission.

Collection of Tissue

Horses were euthanized and intestinal segments (approximately 24-cm long samples) including the proximal and distal duodenum, jejunum and ileum were removed. Segments were rinsed in ice-cold NaCl solution (0.9 g NaCl/100mL H_20), cut and opened longitudinally to expose the mucosal lining, and rinsed again in fresh ice-cold NaCl solution. The mucosal layer was scraped from the serosal layer using a glass microscope slide. Scrapings were transferred to 15-mL plastic conical tubes (Corning, Sigma-Aldrich Corp., St. Louis, MO), immediately flash frozen in liquid nitrogen and stored at −80°C. Each tube contained approximately 3 g for a total of approximately 12 g of mucosal scraping collected from each segment.

Preparation of Brush Border Membrane Vesicles

Preparation of brush border membrane vesicles (BBMV) was based on methods described by Fan et al. (1998, 2004) using jejunal mucosal scrapings (Woodward et al. 2012). Approximately 2 g of tissue was weighed into homogenization buffer kept on ice (52 mL; 50 m*M* D-Mannitol, 10 m*M* HEPES, 0.2 m*M* phenylmethanesulfonylfluoride; pH = 7.4) and

homogenized (Ultra-Turrax T25, Janke & Kunkel, IKA®-Labortechnik, Staufen, DE). A portion of this crude homogenate was set aside and kept on ice for measurement of protein concentration and alkaline phosphatase activity. The remaining crude homogenate was further processed for purification of the BBM. It was first centrifuged at 2000 × *g* for 15 min at 4°C, the supernatant recovered and the pellet discarded. To the recovered supernatant, a final volume of 10 m*M* $MgCl_2$ was achieved by adding 200 m*M* $MgCl_2$, gently shaken on ice for 15 min, and centrifuging at 2,400 × *g* for 15 min at 4°C. The supernatant was collected and the pellet discarded. The supernatant was transferred to ultracentrifuge tubes for centrifugation at 19,000 × *g* for 30 min at 4°C. The supernatant was discarded and the pellet (representing the crude BBM) was suspended using a Pasteur pipette in vesicle pre-loading buffer kept on ice (150 m*M* potassium thiocyanate, 10 m*M* D-Mannitol, 5 m*M* HEPES; pH = 7.4). Final centrifugation was completed at 39,000 × *g* for 30 min at 4°C, the supernatant discarded and the pellet re-suspended with vesicle pre-loading buffer. A sample of purified BBMV homogenate was saved and kept on ice for measurement of protein concentration and alkaline phosphatase activity. The remaining BBMV homogenate was kept on ice until use for the measurement of D-glucose and L-Gln uptake as described later.

Measurement of Protein Concentration and Alkaline Phosphatase Enzyme Activity

Protein concentration of the crude homogenate sample and the BBMV suspension was determined with a Lowry DC™ Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA) following manufacturer's instructions. The standard curve was based on different concentrations of BSA at 1.5 m*M*, 1 m*M*, 0.5 m*M*, 0.25 m*M* and 0.125 m*M*. The purity of the BBMV was verified by measuring the specific activity of alkaline phosphatase (EC 3.1.3.1) (AP) in both the crude mucosal homogenate and the BBVM suspension. The AP was measured according to

EngstÖm (1964) with modifications as described by Fan et al (1998) using P-nitrophenyl phosphate as the substrate.

Measurement of D-Glucose and L-Gln Uptake into Jejunal Brush Border Membrane Vesicles

Fresh BBMV suspensions were diluted to approximately 10 mg protein·mL^{-1} with vesicle preloading buffer. Uptake of D-Glucose and L-Gln was determined using the rapid filtration technique as described by Fan et al. (1998, 2004) using fresh uptake buffer (50 µL; 10 m*M* D-glucose or 10 m*M* l-Gln, 5 m*M* HEPES, 150 m*M* sodium thiocyanate; pH = 7.4). Nitrocellulose membrane filters (0.22 µm) (Millipore, Billerica, MA) were soaked in the uptake buffer prior to the initiation of each uptake experiment. Uptake buffer kept at room temperature and containing either ^{3}H-D-glucose or ^{3}H- L-Gln and varying concentrations of D-glucose or L-Gln, respectively (0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 7.5 m*M*), was dispensed (50 µL) directly into the bottom of polystyrene tubes (brand, company). Next, two 5 µL BBMV droplets were added to the side of the tube and allowed to warm to room temperature (22-24°C) for approximately 5 s. Uptake was initiated with a foot-switch activated vibromixer connected to an electronic timer (Timer model 545, GraLab Corporation, Centerville, OH) for 3 s for D-glucose (Woodward et al, 2012) and 15 s for L-Gln (Salloum, 1993). Uptake was terminated with addition of ice-cold stop and wash solution (1.2 mL; 150 m*M* potassium thiocyanate, 10 m*M* mannitol, 10 mM HEPES, 0.1 m*M* $HgCl_2$; pH = 7.4). The uptake solution was transferred onto filters mounted on a vacuum connected Manifold Filtration Unit (Millipore, Billerica, MA), washed with the ice-cold stop and wash buffer, and the filters were transferred to scintillation vials (Research Products International Corps., Mount Prospect, IL) containing 10 mL scintillation fluid. Filters were allowed to equilibrate for a minimum of 30 min and radioactivity determined by liquid

scintillation counting (LS6500, Beckman, Brea, CA). The remaining uptake solutions at each concentration was pooled and 10 µL used to determine the average initial radioactivity.

At the beginning and end of each uptake experiment, non-specific binding was determined by adding the stop and wash solution to uptake buffer (without BBMV sample). All uptake experiments were performed in triplicate. Jejunal samples from two horses were processed per experiment, from opposing treatment groups when possible.

Estimation of D-Glucose and L-Gln Kinetics

Uptake at different concentrations of D-glucose or L-Gln was determined using the following equation:

$$J = [((R_F - R_B) \times S) / R_I] / (W \times T), \quad [1]$$

Where J is the initial rate of D-glucose or L-Gln uptake into BBMV (J, ρmol·mg protein^{-1}·s^{-1}); Rf is the radioactivity in disintegration per minutes on filters (DPM/ Filter); RB is the radioactivity for non-specific binding to filters (DPM/ filter); S is the extravesicular concentration of D-glucose of L-Gln (m*M*), R_I is the radioactivity in the uptake media (DPM/ µL); W is the amount of membrane protein provided for the incubation (mg of protein) and T is the time of incubation for initial uptake (s).

The kinetic parameters V_{max} and K_m for D-glucose and L-Gln transport across the BBMV were determined using a multi-parameter curve fitting program (Curve Fitter, MathSol-Mathematical Solutions Development, Institute of Mathematics and Statistics, Helsinki, FI) based on the following equation:

$$J = \frac{V_{max}[S]}{K_m[S]} + D[S] \quad [2]$$

Where *J* is the initial rate of uptake into BBMV (J, ρmol·mg protein^{-1}·s^{-1}); V_{max} is the maximal transport rate (ρmol • mg protein^{-1} • s^{-1}); *S* is the extravesicular D-glucose or L-Gln concentrations (m*M*); K_m is the Michaelis constant (50% saturation; m*M*); and *D* is the diffusion component (ρmol·mg protein^{-1}·s^{-1}).

Real-time quantitative PCR (qRT-PCR) analysis

Messenger RNA was isolated from the intestinal mucosa scrapings (proximal and distal duodenum, jejunum and ileum) according to the manufacturer's instructions using the *mir*Vana™ miRNA Isolation Kit (Ambion®, LifeTechnologies Inc.™, Carlsbad, CA). Approximately 0.1 mg of tissue was weighed and homogenized (Kinematica™ Polytron™, Luzern, CH) on ice for 3-s intervals in 10 volumes of lysis/binding buffer per tissue mass. The purity of the isolated mRNA concentration was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific, Waltham, MA; Agilent Bioanyalyzer 2100, Palo Alto, CA). Complementary DNA was prepared using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies Inc.™, Foster City, CA), diluted to 10 ng/µL and stored at -20°C.

Microarray results were confirmed through quantitative real-time reverse transcription PCR (qRT-PCR) in 96-well plates (MicroAmp® Fast Optical Plate, Life Technologies Inc.™, Carlsbard, CA) using TaqMan® technology (Applied Biosystems). Each assay consisted of two unlabeled PCR primers and a FAM ™ dye-labelled TaqMan® MGB (minor groove binder) probe. Three of the primer-probe sets (*SLC5A1*, *GAPDH*, and *ACTB*) were commercially available from Applied Biosystems and the fourth primer-probe set (*SLC38A2*) was custom designed by Applied Biosystems for this project (Custom TaqMan® Gene Expression Assay). TaqMan® primer-probe information is provided in Table 1. Plates (MicroAmp® Fast Optical

Plate, Life Technologies Inc.^{TM}, Carlsbard, CA) were briefly centrifuged at 400 × *g* for 1 min and 15°C, loaded into a real time PCR System (Step One Plus ^{TM} Real-Time PCR Systems, Thermo Fisher Scientific Inc., Waltham, MA) and analyzed with the associated software (Step One^{TM} Software, Thermo Fisher Scientific Inc., Waltham, MA). Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s and 60 °C for 1 min. Transcription levels for *SLC38A2* and *SLC5A1* were normalized using both genes *GAPDH* and *ACTB*. Abundance of each target gene was calculated according to the following equations (Pfaffl, 2001 and Livak and Schmittgen, 2001):

$$\Delta C_T = C_T \text{ target gene} - \text{Average of } C_T \text{ reference genes} \quad [3]$$

$$\Delta\Delta C_T = \Delta C_{T(PPID)} - \Delta C_{T(Control)} \quad [4]$$

$$\text{Fold Change (FC)} = 2^{-\Delta\Delta C_T} \quad [5]$$

Statistical Analysis

All data was found to be normally distributed based on the Gaussian distribution analysis in SAS (version 9.3, SAS Institute Inc., Cary, NC). Analysis of data (uptake kinetic parameters and mRNA quantification) was performed using the PROC MIXED procedure (SAS version 9.3, SAS Institute Inc.) with treatment as fixed effects and the random effect of each individual horse nested within treatment. The response variable was cycles to threshold (C_T) for mRNA expression. Pairwise differences between treatments were tested based on the student's *t*-test. Fold change were calculated by back transforming the estimated mean difference in C_T ($^{\Delta\Delta C_T}$) between control and PPID for each gene of interest using the expression FC= $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001). Following Steibel et al. (2009), 95% confidence intervals of mean differences were also back transformed to provide the confidence interval for the estimated fold change.

Fold change means with transformed confidence interval spanning across the value 1.0 were considered not significant at $\alpha = 5\%$.

Results

Membrane Purity

Alkaline phosphatase activity in the mucosal scrapping crude homogenate of PPID and control horses did not differ and was 58.08 and 58.75 ± 14.52 U for control and PPID horses, respectively. In the purified BBVM of PPID and control horses, AP did not differ and was 152.2 and 171.4 ± 30.26 U, respectively. The average enrichment factor, i.e., AP BBMV/AP crude homogenate, was 2.83 ± 0.28 fold, indicating that the BBMV preparations were enriched in brush border membrane.

Initial Time Course of D-Glucose and L-Gln Uptake into Brush Border Membrane Vesicles

Extravesicular D-glucose and L-Gln concentrations of 0.00, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 7.50 m*M* were based on Woodward et al. (2013) for D-glucose and L-Lys uptake in equine BBMV preparations. Incubation time of 3 s for D-glucose was based on studies by Dyer et al. (2002), Wolfram et al. (1986) and Woodward et al. (2010), and of 15 s for L-Gln was based on one study by Salloum et al. (1993) in horses and Fan et al. (1998) in pigs.

D-Glucose and L-Gln Uptake into Brush Border Membrane Vesicles

Initial uptake rates of D-glucose and L-Gln are presented in Figures 1 and 2, respectively. In all figures, the diffusion and saturable uptake are represented. Compared to control horses (Figure 1A), D-glucose uptake across BBVM of PPID horses was lower at 5 m*M* ($P = 0.045$) and 7.5 m*M* D-glucose ($P = 0.016$) (Figure 1B). L-Glutamine uptake did not differ between PPID (Figure 2B) and control horses (Figure 2A) at any L-Gln concentration.

Kinetic parameters (V_{max} and K_m) are presented in Table 2. Compared to control horses, D-glucose V_{max} of PPID horses was lower (P = 0.021), and K_m and diffusion did not differ. L-Glutamine V_{max} and K_m did not differ between PPID and control horses.

SGLT1 and SNAT2 mRNA Abundance in Intestinal Mucosa

Transcript abundance of *SLC5A1* and *SLC38A2* in the duodenal, jejunal, and ileal mucosa of PPID (n=8) relative to control horses (n=4) is presented in Figure 3 and 4, respectively. The mRNA abundance of *SLC5A1* and *SLC38A2* was not different in PPID horses relative to control horses in any segment.

Table 5.1. Reverse transcription quantitative real-time (RT-qPCR) assay primer information in the intestinal mucosa of PPID and non-PPID horse.

Gene Name	Gene Symbol	Protein	Taqman Assay/Accession Number[1]
Solute carrier family 5 (Sodium/Glucose Cotransporter, member 1	*SLC5A1*	SGLT1	NM_001081872
Solute carrier family 38, member 2	*SLC38A2*	SNAT2	NC_009149
actin, beta	*ACTB*	β-actin	NM_001081838
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	GAPDH	NM_001163856

1-Applied Biosystems Taqman® assay product number or GenBank accession number of sequence submitted for Custom Taqman® Gene Expression Assay design

Table 5.2. Kinetic parameters of D-glucose and L-Gln uptake in equine small intestinal (jejunum) brush border membrane vesicles of control and PPID horses of similar ages. Each parameter measured is representative of the LSMEAN ± SEM from four (control) and eight (PPID) horses.

Kinetic Parameter	Control	Control	PPID	*P*-value
V_{max}[1]	D-Glucose	262.4 ± 33.43	146.2 ± 25.14	0.021
	L-Gln	44.77 ± 27.62	60.28 ± 19.53	0.656
K_m[2]	D-Glucose	0.21 ± 0.16	0.27 ± 0.12	0.762
	L-Gln	0.75 ± 0.47	0.67 ± 0.33	0.883
Diffusion[3]	D-Glucose	42.43 ± 11.76	31.74 ± 7.70	0.469
	L-Gln	11.87 ± 4.88	9.84 ± 3.45	0.741

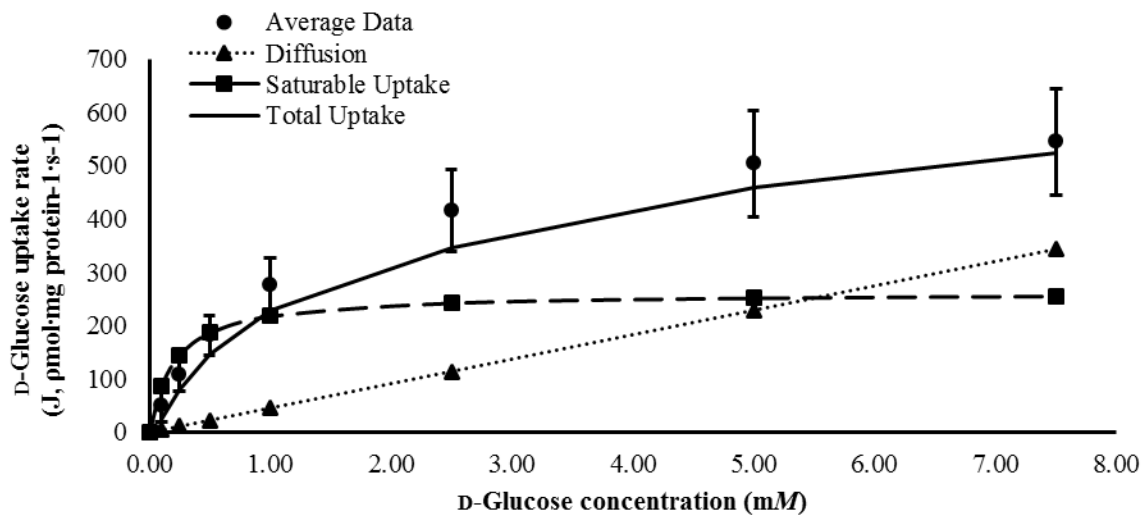
1 - (ρmol·mg protein^{-1}·s^{-1}); 2 - (m*M*); 3 - (ρmol·mg protein^{-1}·s^{-1})

Table 5.3. Kinetic parameters of D-glucose and L-Gln uptake in several mammalian and chicken jejunal brush border membrane vesicles.

Animal	Segment	Substrate	V_{max} [1]	K_m [2]	Source
Human	Jejunum	D-Glucose	931	0.86	Harig et al., 1989
Human	Jejunum	D-Glucose	805	0.75	Malo and Berteloot, 1991
Rabbit	Jejunum	D-Glucose	470	0.57	Ikeda et al., 1989
Rat	Jejunum	D-Glucose	233	0.43	Thiesen et al., 2003
Rat	Jejunum	D-Glucose	270	0.53	Boudry et al., 2007
Chicken	Jejunum	D-Glucose	392	2.40	Garriga et al., 2006
Pig	Jejunum	D-Glucose	2595	0.09	Woodward et al, 2013
Horse	Jejunum	D-Glucose	918	0.49	Dyer et al., 2002
Pony	Jejunum	D-Glucose	655	0.27	Woodward et al., 2013
Horse	Jejunum	D-Glucose	262.4	0.21	This study
Human	Jejunum	L-Gln	637	1.36	Said et al., 1989
Rabbit	Jejunum	L-Gln	4.7	0.34	Talukder et al., 2008
Rat	Jejunum	L-Gln	222	2.23	Salloum et al., 1993
Dog	Jejunum	L-Gln	393	0.97	Bulus et al., 1989
Pig	Jejunum	L-Gln	71	0.77	Fan et al., 1998
Horse	Jejunum	L-Gln	308	0.52	Salloum et al., 1993
Horse	Jejunum	L-Gln	45	0.75	This Study

1 - (ρmol·mg protein^{-1}·s^{-1}); 2 - (m*M*); 3 - (ρmol·mg protein^{-1}·s^{-1})

A.



B.

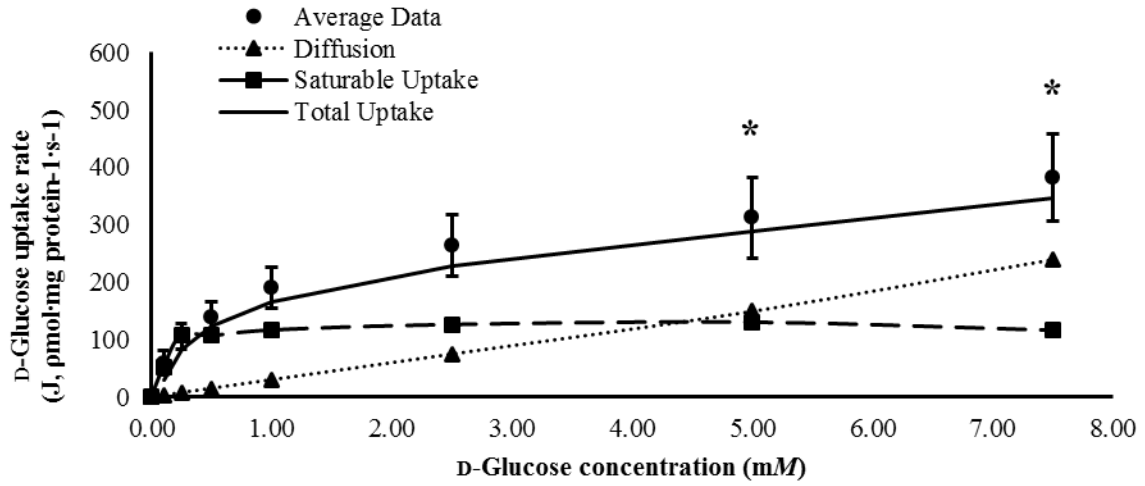
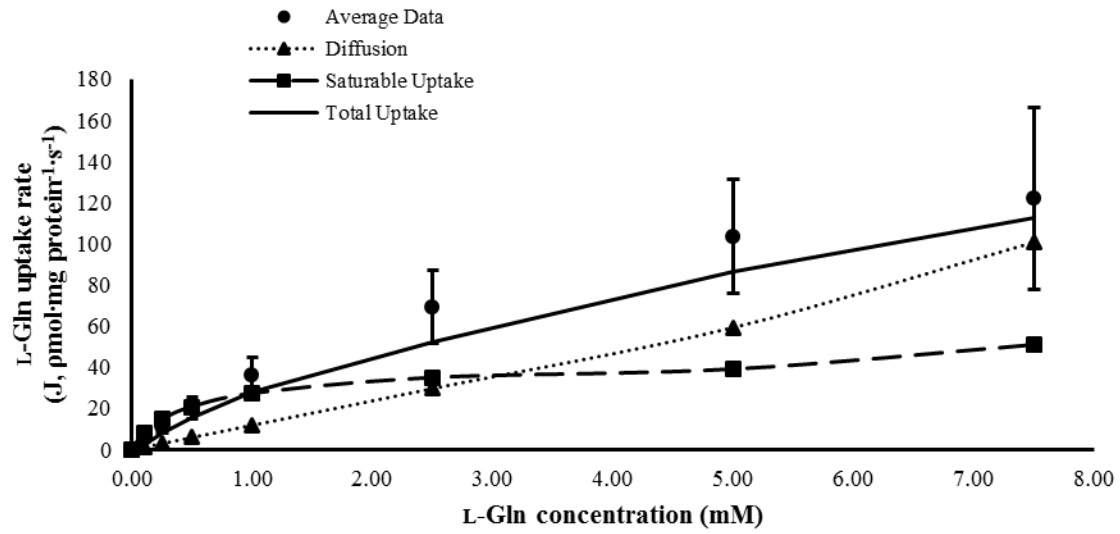


Figure 5.1. Kinetics of D-glucose uptake into equine jejunal Control (A) and PPID (B) brush border membrane vesicles. Brush border membrane vesicles were loaded with a buffer containing 150 m*M* KSCN, 10 m*M* Mannitol, 5 m*M* HEPES (pH 7.4) and incubated with another buffer containing 150 m*M* NaSCN and 5 m*M* HEPES (pH 7.4), 0.8 µM D [^{3}H]-glucose at concentrations of 0, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 7.50 m*M* D-glucose. D-Mannitol was added to maintain osmolarity. Each point is representative of the LSMEAN ± SEM from four (control, A) and eight (PPID, B) horses in triplicate uptake experiments per horse.

A.



B.

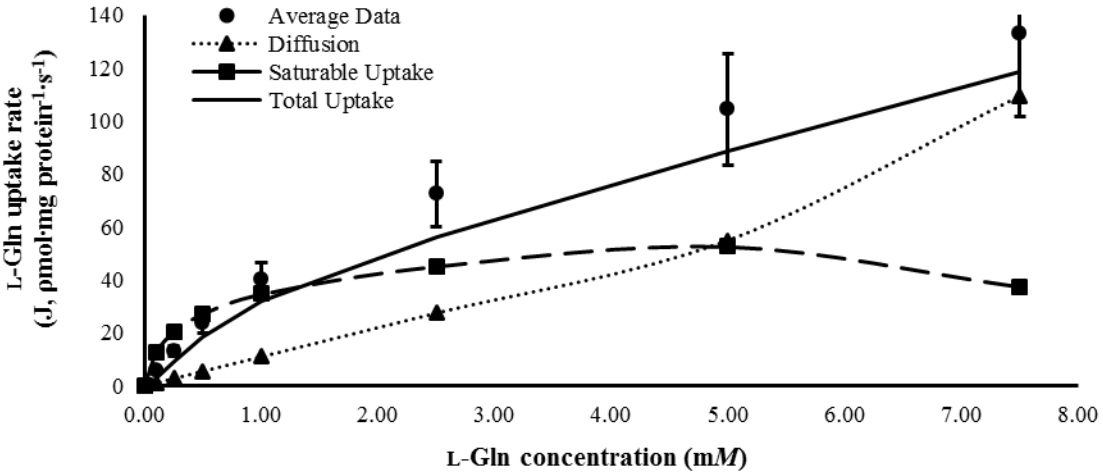


Figure 5.2. Kinetics of L-Gln uptake into equine jejunal Control (A) and PPID (B) brush border membrane vesicles. Brush border membrane vesicles were loaded with a buffer (150 *mM* KSCN, 10 m*M* Mannitol, 5 m*M* HEPES (pH 7.4) and incubated with another buffer containing 150 m*M* NaSCN and 5 m*M* HEPES (pH 7.4), 0.8 µ*M* L [^{3}H]-Glutamine at concentrations of 0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 7.5 m*M* L-Gln. D-Mannitol was added to maintain osmolarity. Each point is representative of the LSMEAN ± SEM from four (control, A) and eight (PPID, B) horses in triplicate uptake experiments per horse.

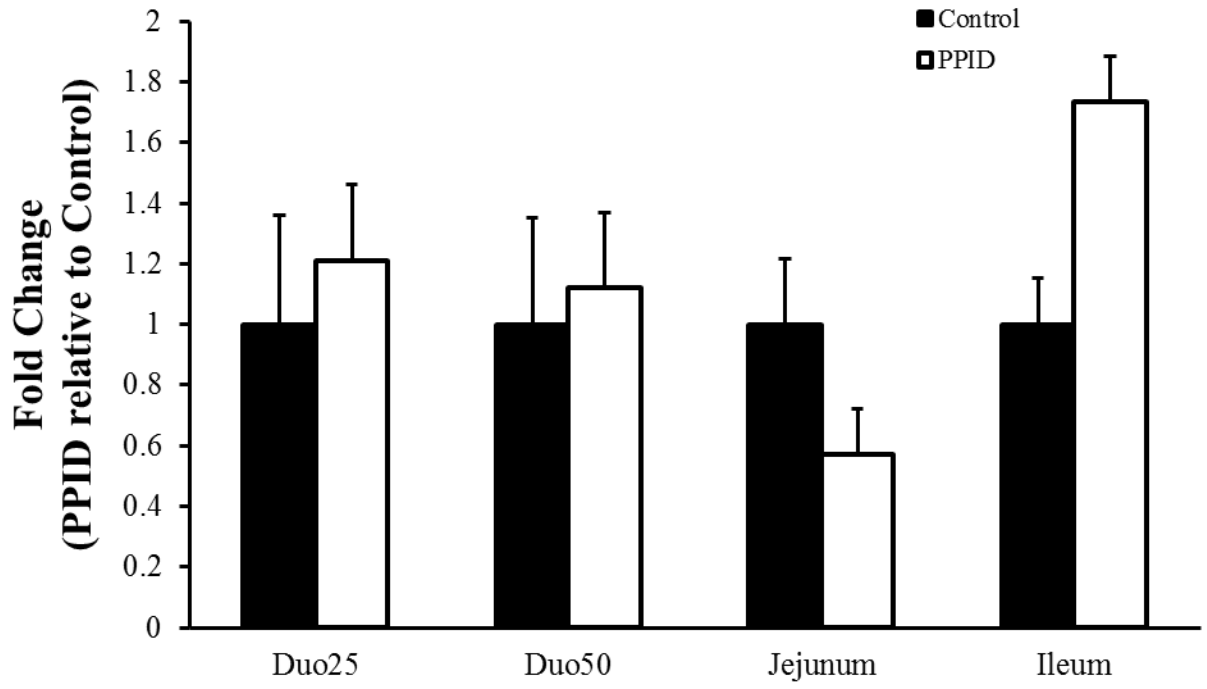


Figure 5.3. Fold change in mRNA abundance of *SLC5A1* in PPID horses relative to Control. Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

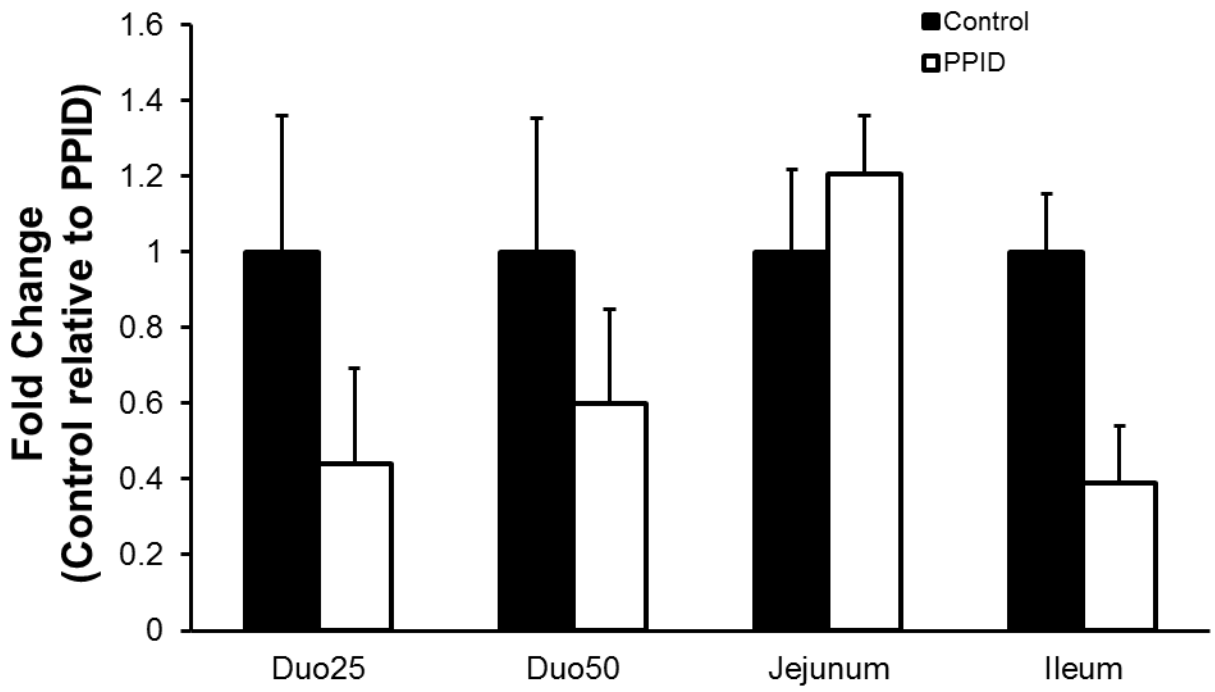


Figure 5.4. Fold change in mRNA abundance of *SLC38A2* in PPID horses relative to Control. Duo25, duodenum at 25 cm; Duo50, 50 cm distal to the pyloric sphincter; Jejunum, mid-jejunum; Ileum, mid-ileum 15 cm proximal to the cecum.

Discussion

This is the first study reported to characterize transport of D-glucose and L-Gln at the molecular and phenotypic level along the small intestinal mucosa of horses with PPID. While the etiology of PPID is multifaceted, horses suffering from this condition commonly demonstrate poor physical appearance while maintaining normal appetite, suggesting that optimum nutrient utilization and (or) absorption may be compromised. Chronic elevated concentration of cortisol have been shown to impact peripheral tissue metabolism by reduction in GLUT4 translocation to the cell surface in muscle tissue (Dimitriadis et al., 1997; Coderre et al., 1996; Oda et al., 1995; Weinstein et al., 1995; Owen et al., 1973) and increased lipolysis (Kennedy et al., 1993; Ekstrand et al., 1992; Ong et al., 1992). Insulin resistance and central fat deposit in men has been well established to be associated with excess blood cortisol concentration and cortisol production rates (Purnell et al., 2009; Phillips et al., 1998; Filipovsky et al., 1996; Stolk et al., 1996). The impact of chronic elevated cortisol concentration on nutrient absorption is less well documented and not available for horses. In this study, horses with PPID had significantly higher serum concentrations of ACTH and cortisol compared to non-PPID horses (Chapter 1). Basal ACTH serum concentrations were 85.6 ± 18.5 and 23.0 ± 26.1 pg/mL in PPID and non-PPID horses (Chapter 1). Basal and post-dexamethasone cortisol concentrations were 158.0 ± 36.7 and 10.0 ± 21.9 nmol/L for control horses and were 156.0 ± 25.9 and 99.3 ± 14.5 nmol/L, respectively, for PPID horses. These horses also presented histomorphological changes in the small intestinal mucosa of PPID horses that would suggest some impairment in nutrient absorption (Chapter 3). Gastrointestinal and hepatic conditions like inflammatory bowel diseases and chronic active hepatitis are commonly treated with high doses of oral glucocorticoids (references?).

Oral administration of glucocorticosteroids increased the rate of fructose transport across the jejunal BLM weaning and adult rats, but not of glucose (Theisen et al, 2003). (Thiesen et al., 2003). In this study, the maximal capacity for glucose uptake in PPID horses was near half that of control horses. Across both treatments, glucose V_{max} was nonetheless considerably lower than we previously reported for ponies (Woodward et al. 2013).Transport of D-glucose via the Na+-glucose co-transporter activity in ageing human subjects was reported to remarkably decrease as age increased with the "overshoot" phenomenon disappearing altogether in the oldest subjects (Vincenzini et al., 1989). Those results were comparable to that observed in intestinal membrane vesicles of young patients suffering from Crohn's disease. The relationship between age and nutrient digestion in horses is unclear. Ralston et al. (1989) reported lower apparent digestibility of crude protein when comparing geriatric horses to young horses, but failed to show similar results in later studies (Ralston et al., 1996; 1989). Variation in dentition structure and function across aging horses may render it difficult to clearly demonstrate a direct impact of aging on digestive functions per se.

The affinity for D-glucose transport in horses has been shown to be 0.49 mmol/L suggesting a high affinity for the isoform SGLT1 (Dyer et al., 2002). The SGLT1 protein is a high-affinity, Na^+-dependent transporter located in the apical membrane of small intestinal epithelial cells in many species including horse and specific for glucose and galactose (Dyer et al., 2002). The K_m for D-glucose in this study was even lower, supporting high affinity transport for glucose, and was comparable to values previously reported in ponies by Woodward et al. (2013). Dyer et al. (2009) reported an increased transport rate to be related to an enhancement in V_{max}, and not K_m. Thus, the reduced D-glucose V_{max} rate of PPID afflicted horses when compared to control horses is a strong indication that D-glucose transport capacity across the

jejunal epithelium was limited in PPID horses. We expected mRNA abundance of *SLC5A1* to be lower in horses with PPID, in particular given the Na^+-dependent D-glucose V_{max} uptake in those horses was near half that of control horses. In horses fed increasing levels of carbohydrates, D-glucose uptake across intestinal BBMV paralleled transcriptional abundance of SLC5A1 (Dyer et al., 1997). In contrast, oral prednisone in rats decreased SLC5A1 mRNA abundance without reduction in glucose Vmax transport across BBBV (Theisen et al, 2003), suggesting possible disassociation between expression of SLC5A1 protein abundance and transporter SGLT1 activity.

Glutamine is a functional amino acid with diverse physiological roles in protecting the gut from atrophy and injury under various stress conditions (Wang et al., 2014), making it a preferred nutrient of intestinal enterocytes for mucosal growth and function (Ganapathy et al, 1994; Salloum et al., 1994; Windmueller and Spaeth, 1980). Glutamine transport is facilitated by both Na^+-dependent transport mechanisms in horses (Souba et al., 1992), human (Stevens et al., 1992, 1982), dogs(Bulus et al., 1989) and rat (Said et al., 1989), and Na^+-independent transport mechanisms in horses (Salloum et al., 1993). Transporter SNAT2 is a sodium-coupled neutral amino acid co-transporter (Sugawara et al., 2000; Yao et al., 2000). Increased mRNA expression of *SLC38A2* is indicative of an increased ability to transport L-Gln across the intestine in vitro (Reimann et al., 2004). We expected horses with PPID to have lower L-Gln Vmax and transcript abundance of *SLC38A2*. Our L-Gln V_{max} values in this study compare well with those reported in pig (Fan et al., 1998) and rat jejunal BBMV (Salloum et al., 1993) but lower than reported by Salloum et al. (1993) in horse and dog jejunal BBMV (Table 3). Our K_m results are consistent with the values previously reported for L-Gln transport in rats (Salloum et al., 1993) and dogs (Bulus et al., 1989). The only other study of L-Gln transport in horses by Salloum et al. (1993)

has K_m values which differ marginally from those seen in our study (Table 3). The study by Salloum et al. (1993) however had a very limited number of horses (n = 3).

In conclusion PPID horses have a remarkably lower maximal uptake capacity of glucose by the jejunal brush border membrane compared to horses with no PPID. This reduced capacity may explain in part the biological mechanism behind increased risk of dietary induced laminitis in horses with PPID. Thus, dietary management consisting in diets containing minimal concentration of non-structural carbohydrates should be considered. Conversely, there appears to be no difference in the maximal uptake capacity of glutamine nor SNAT2 transporter abundance by the jejunal brush border membrane in PPID horses compared to horses without PPID. These findings suggest AA nutrient utilization is not limiting in PPID horses. Another mechanism is still responsible and unknown for the muscle wasting characteristically associated with the PPID condition. In conclusion, the poor physical appearance, muscle wasting, and laminitis associated with PPID horses is not due to lack of nutrient transporter abundance in the small intestinal mucosa compared to healthy horses of similar ages, but may in part be related to a function of age or glucose intolerance.

CHAPTER SIX: SUMMARY AND CONCLUSIONS

The overarching goal of this thesis was to determine the impact of PPID on nutrient utilization in the small intestine and the peripheral muscle tissue. The objectives were to (1) measure glucose tolerance and insulin sensitivity, and assess abundance of insulin-dependent molecular entities of glucose and neutral amino acid transport in skeletal muscle, and (2) evaluate the impact of PPID on small intestinal mucosa morphology and transport of glucose and glutamine.

The data presented in this thesis is novel, as the mechanisms of nutrient utilization in PPID horses has never been studied before. In the first chapter, horses were clearly classified with PPID condition based not only on clinical symptoms and endocrine testing, but also post-mortem histological examination as well. This study was originally designed to include three treatment groups: control, insulin resistant PPID, and non-insulin resistant PPID horses. The OGTT and FSIGTT results clearly demonstrated that PPID horses did not differ from non-PPID horses in parameters defining insulin or glucose kinetics. As such, PPID horses could not be classified as either insulin resistant or non-insulin resistant and comparisons were based on two treatment groups including PPID and non PPID (i.e., control). The absence of insulin resistance was further ascertained by characterizing the molecular entities of glucose and glutamine utilization. Abundance of mRNA of *SLC2A4*, *INSR* and *SLC38A2* and protein GLUT4 and INSR did not differ between PPID and non PPID horses. Our study was carefully designed to include clear, age-matched, non PPID horses, which is in contrast with most of the previously reported studies. Our data corroborate with the only study so far using age-matched control horses (Mastro et al, 2014) indicating that the incidence of insulin resistance in horses with PPID may be overestimated in the current horse population.

Mucosal intestinal morphology and glucose uptake differed between PPID and control horses. Horses with PPID had a reduced proximal average of villus height, tendency for greater crypt depth in the mid-jejunum, and a lower villus height to crypt depth ratio in the mid-jejunum. Maximal glucose transport capacity across the apical membrane of the small intestinal mucosa of PPID horses was 146.2 ± 25.14 ρmol·mg protein^{-1}·s^{-1} compared to 262.4 ± 33.43 ρmol·mg protein^{-1}·s^{-1} in control horses, which represents a 44% reduction in maximal glucose uptake. The reduction in glucose transport was not mirrored at the transcriptional level as mRNA abundance of *SLC5A1* did not differ between PPID and non-PPID horses. Neither the maximal glutamine transport capacity nor the mRNA abundance of *SLC38A2* in any of the small intestinal segments differed between PPID and non PPID horses. It is unclear whether the reduction in glucose transport was associated with the reduction in average proximal villus height and villus height:crypt depth since glutamine transport capacity was unaffected. Transport however was only characterized at the mid-jejnum for both glucose and glutamine and it is possible that glutamine transport was affected in regions either proximal or distal to that examined. It was expected that abundance of *SLC5A1* would be lower in PPID horses given the remarkable reduction in glucose transport across BBMV. Albeit not measured herein, SGLT1 abundance may have paralleled that of glucose transport capacity seen in this work. Other glucose transporters have been characterized along the horse's small intestine (Woodward et al., 2011; Freeman et al., 1993) however SGLT1 is reported to be the dominant glucose transporter in the apical membrane of the small intestine of the horse (Dyer et al., 2002). Nonetheless, the fact that neither *SLC5A1* nor *SLC38A2* differed between PPID and non PPID horses is further indicative that insulin regulation unlikely played a role in the pathology of PPID in this group of horses.

Geriatric horses play a key role in the equestrian industry where they serve as companions and experienced schoolmasters for less experienced riders. A large proportion of the aging horse population is affected by PPID hence continued research to uncover tools to improve the welfare and reduce costs to owners is essential. The work presented in this thesis showed that horses with PPID may benefit from dietary interventions tailored to the maintenance of intestinal integrity and health. Decreased glucose transport rate in the small intestine may lead to increased flow to the large intestinal compartment. Given these notions geriatric horses and those in particular with PPID should be carefully managed with nutritional strategies aiming at increased digestion and nutrient absorption, including feeding small, frequent meals, high in fiber and low in starch.

APPENDICES

APPENDIX A:

CHAPTER TWO MATERIAL

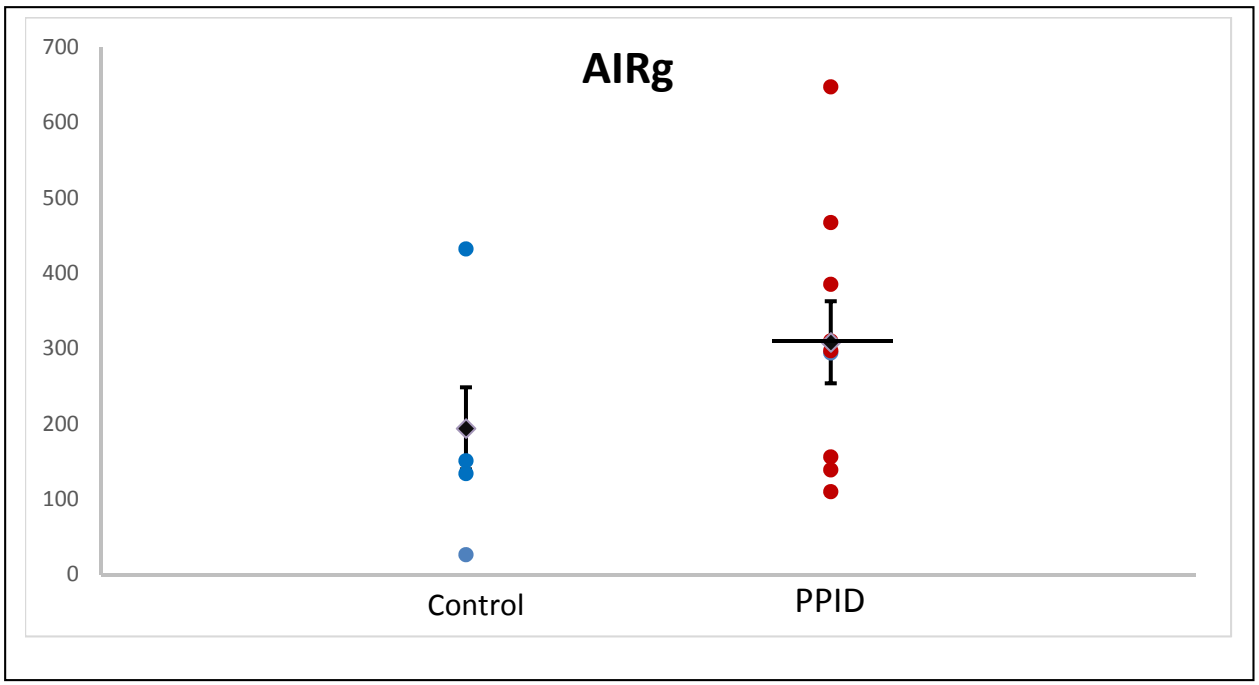


Figure A.1. Mean ± s.e acute response of insulin to glucose values derived for 8 PPID and 4 control horses following i.v. administration of 300 mg/kg bwt 50% dextrose solution followed later by the administration of 20 mu/kg bwt insulin in the FSGITT testing using the minimal model for analysis.

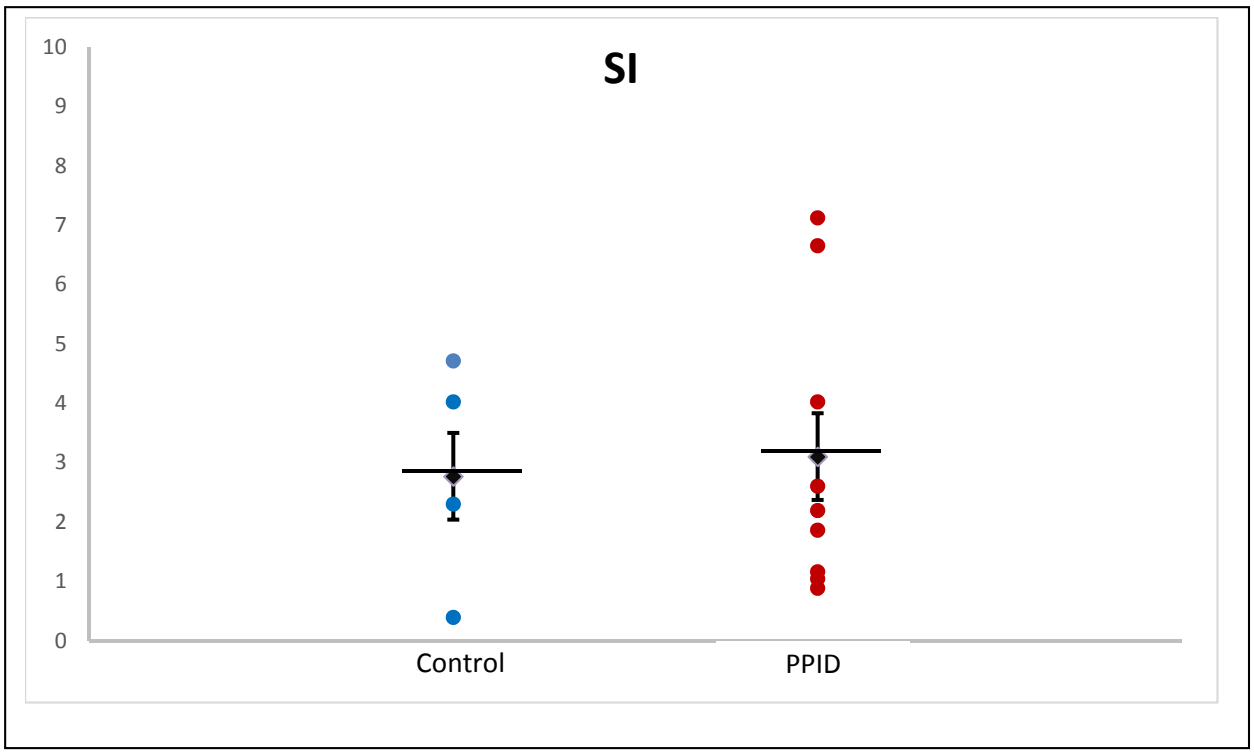


Figure A.2. Mean ± s.e insulin sensitivity values derived for 8 PPID and 4 control horses following i.v. administration of 300 mg/kg bwt 50% dextrose solution followed later by the administration of 20 mu/kg bwt insulin in the FSGITT testing using the minimal model for analysis.

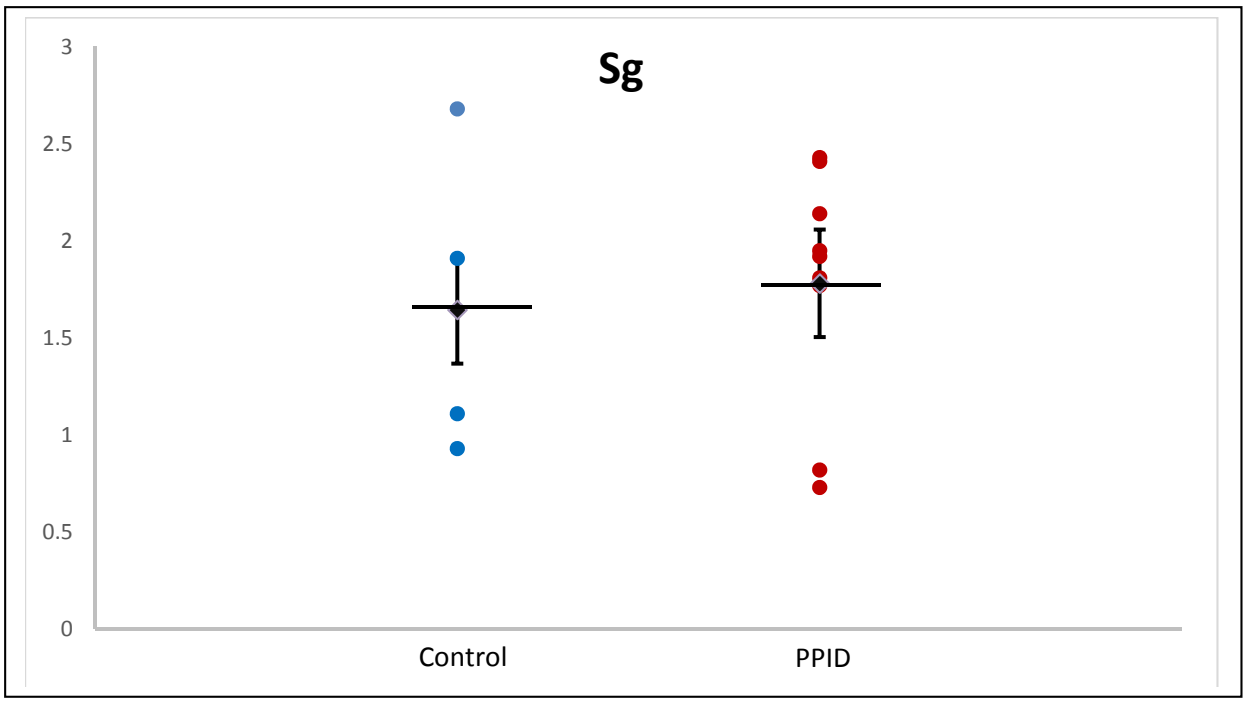


Figure A.3. Mean ± s.e glucose sensitivity values derived for 8 PPID and 4 control horses following i.v. administration of 300 mg/kg bwt 50% dextrose solution followed later by the administration of 20 mu/kg bwt insulin in the FSGITT testing using the minimal model for analysis.

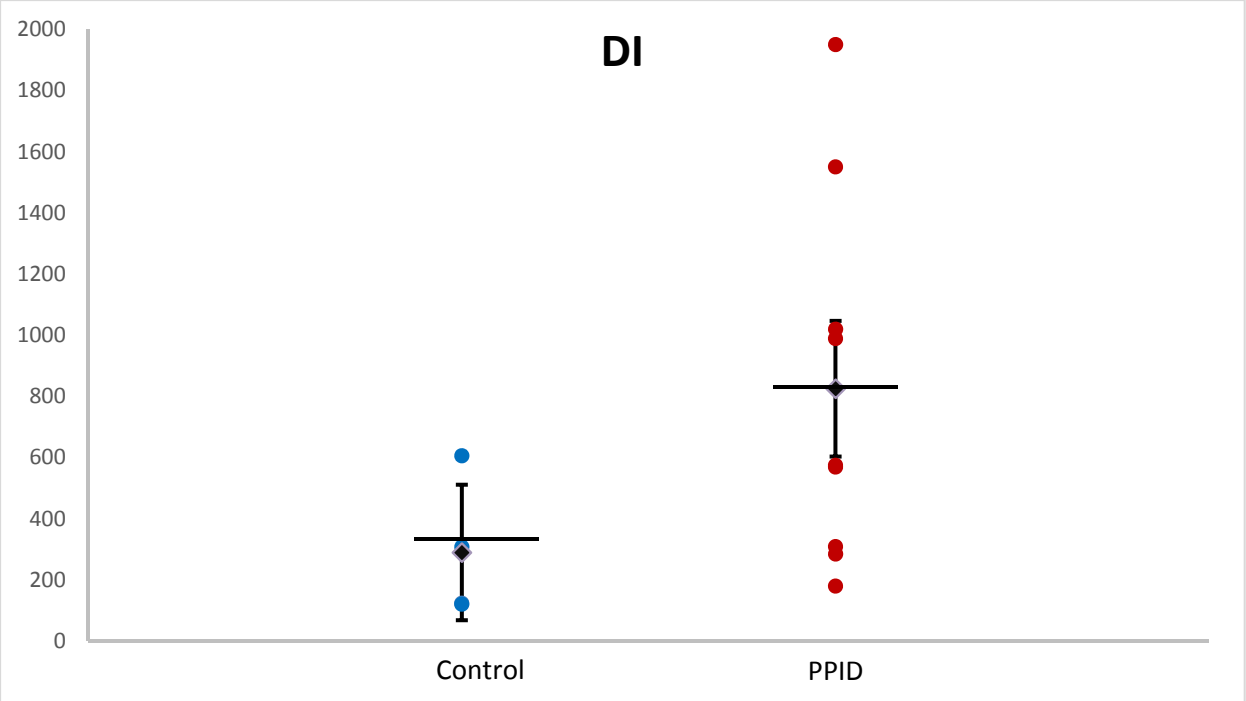


Figure A.4. Mean ± s.e disposition index values derived for 8 PPID and 4 control horses following i.v. administration of 300 mg/kg bwt 50% dextrose solution followed later by the administration of 20 mu/kg bwt insulin in the FSGITT testing using the minimal model for analysis.

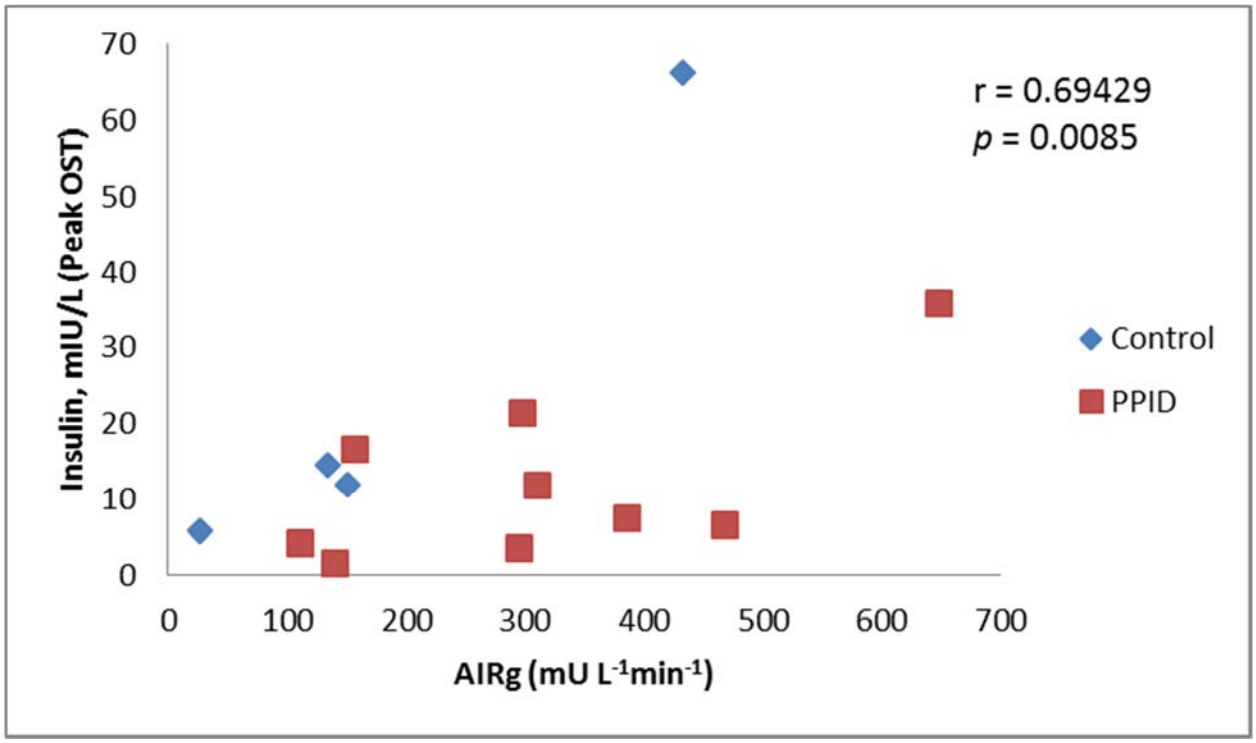


Figure A.5. Correlation of the Peak Insulin of OGT vs Acute Insulin Response to Glucose (AIRg) Correlation when comparing PPID horses to control horses of similar ages.

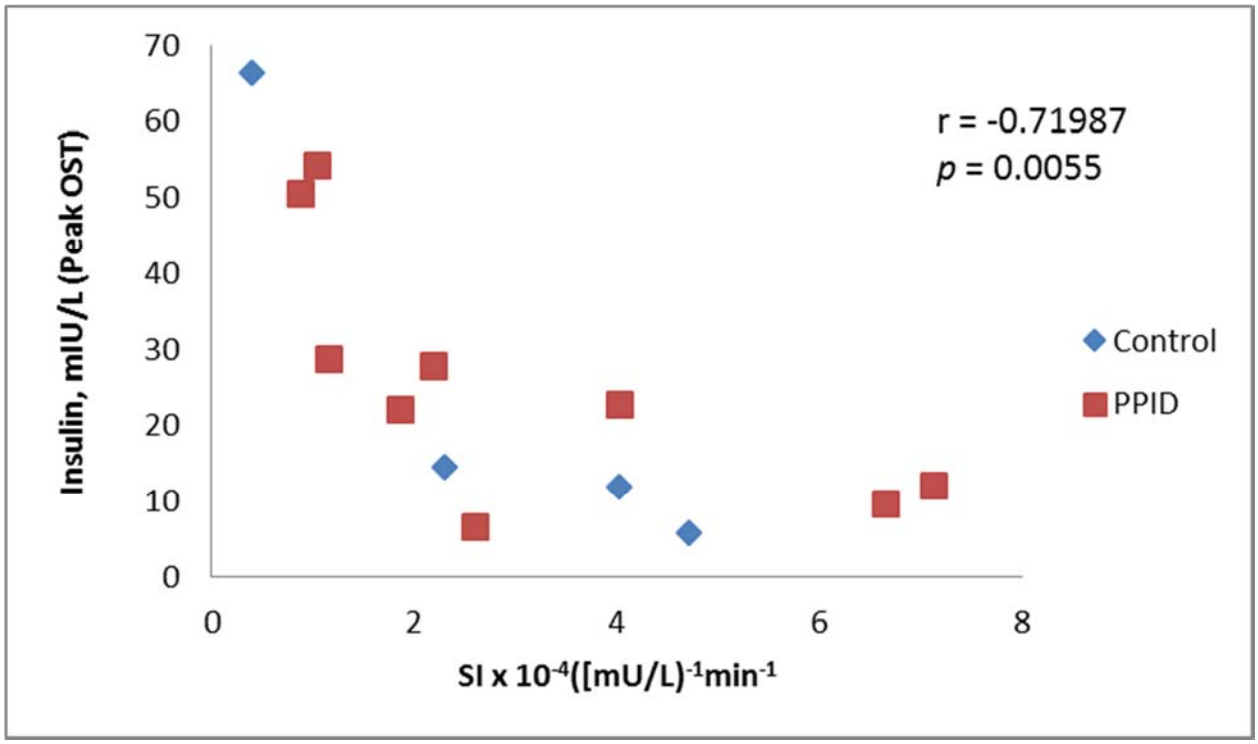


Figure A.6. Correlation of the change in insulin OGT vs Insulin Sensitivity when comparing PPID horses to control horses of similar ages.

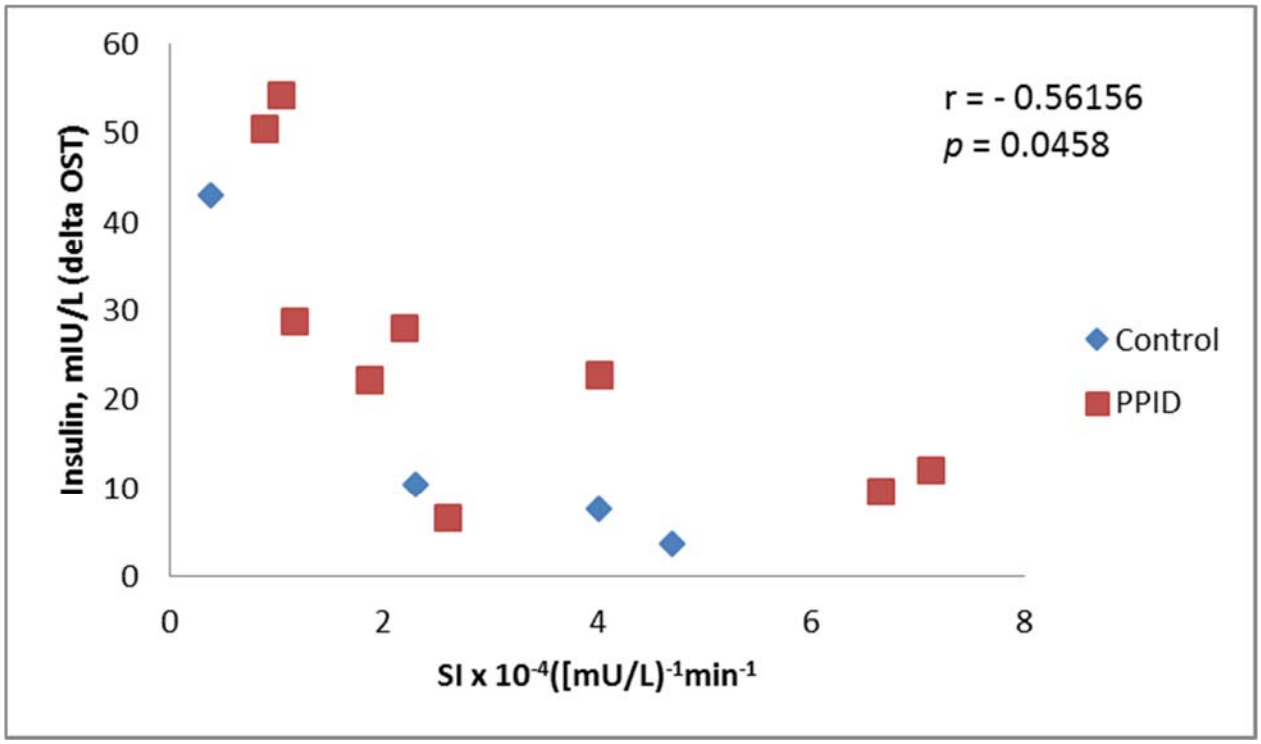


Figure A.7. Correlation of Insulin Sensitivity vs Acute Response of when comparing PPID horses to control horses of similar ages.

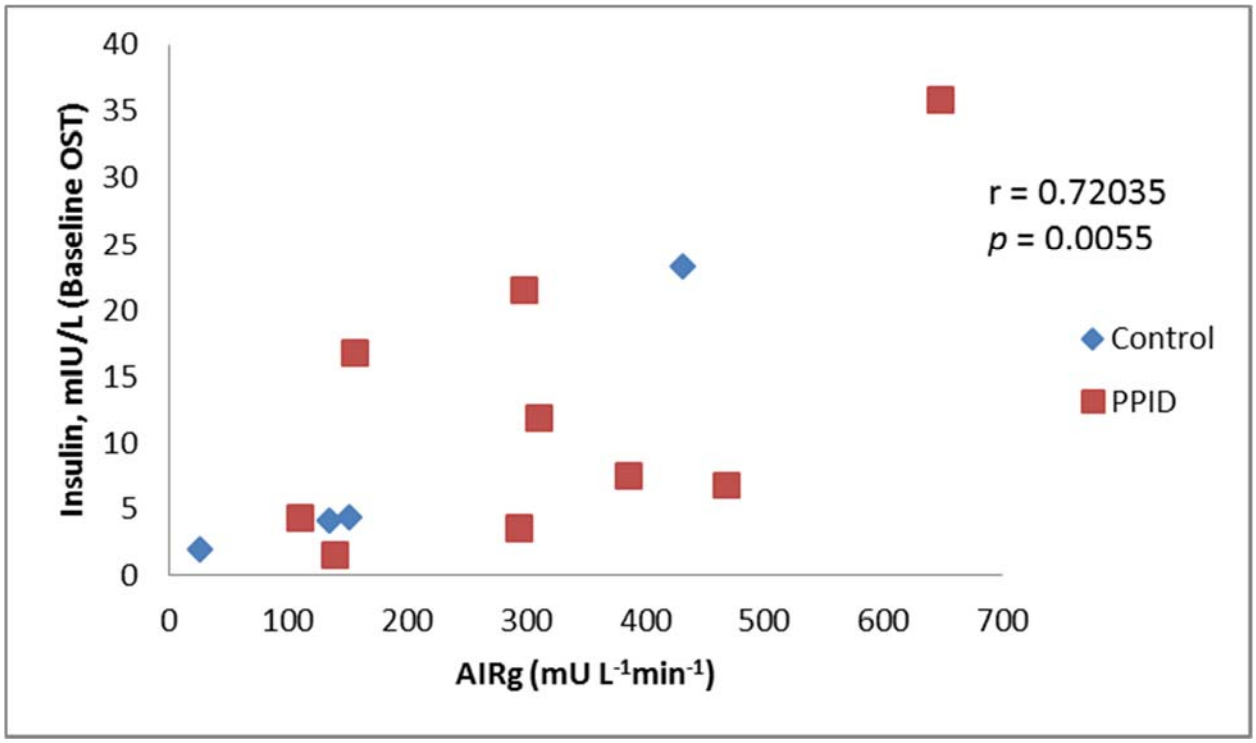


Figure A.8. Correlation of the change in insulin OGT vs Insulin Sensitivity when comparing PPID horses to control horses of similar ages.

APPENDIX B

CHAPTER THREE MATERIAL

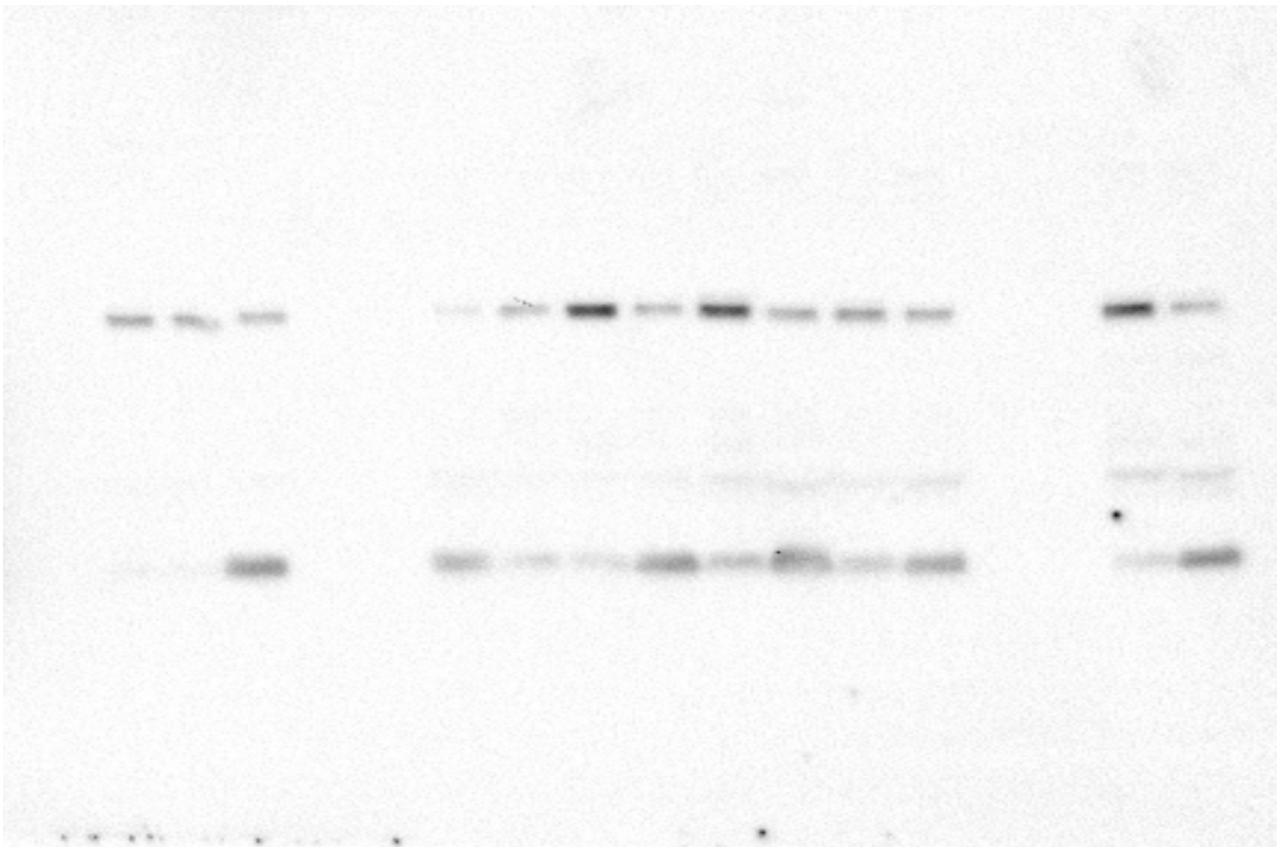


Figure B.1. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies of control horses and PPID horses and treated with antibodies against INSR showing full blot.

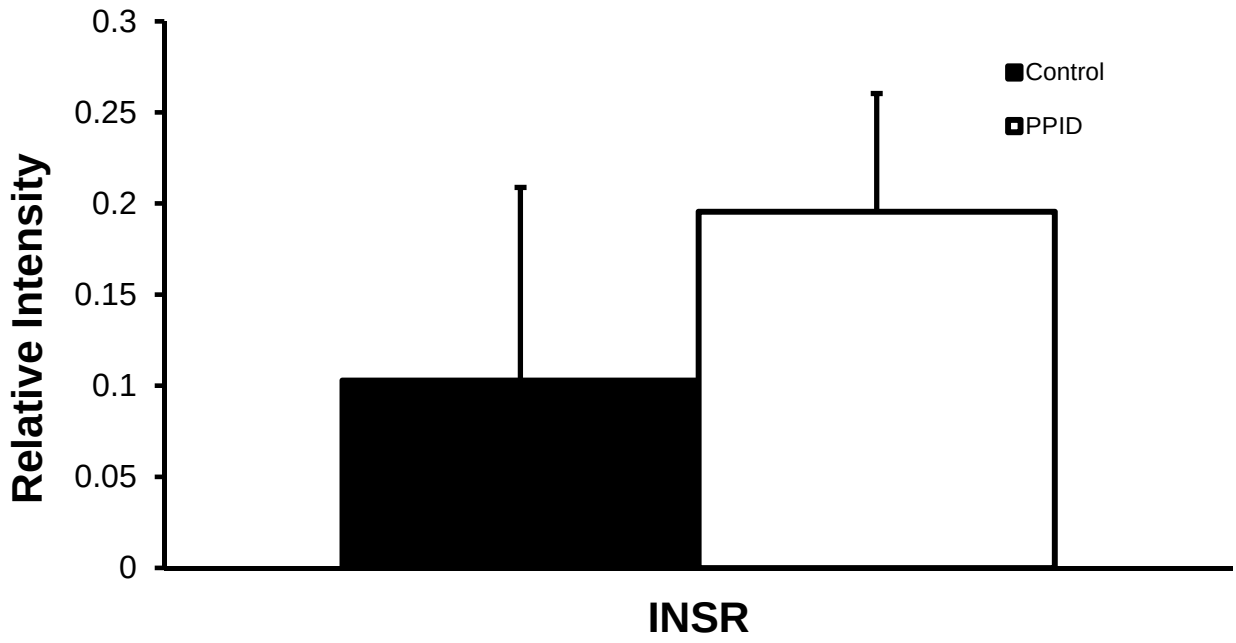


Figure B.2. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies of control horses and PPID horses and treated with antibodies against INSR depicted by a graph indicating the relative expression level. Intensities of bands were estimated by using the program "ImageLab" from BioRad. Values were normalized to the INSR control and expressed relative to the value of control horses. The data are expressed as means ± s.e. using Student's t-test.

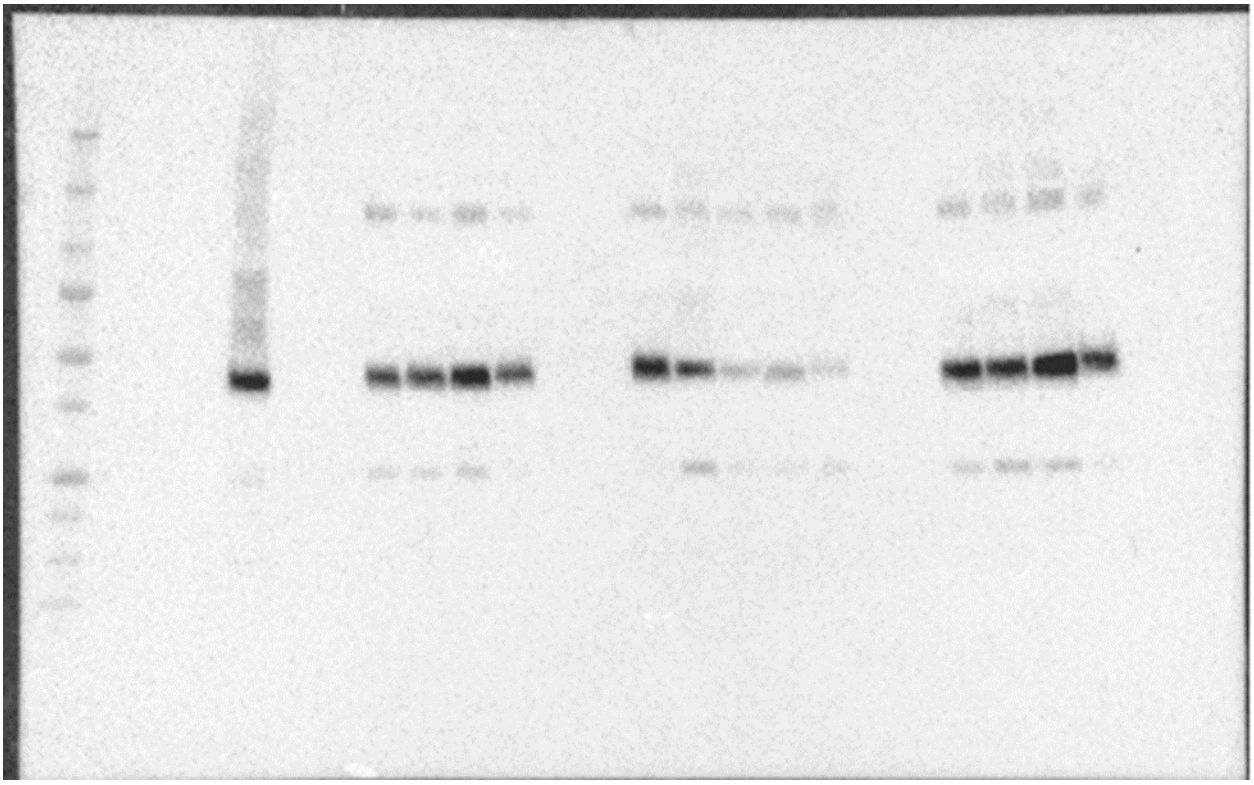


Figure B.3. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies of control horses and PPID horses and treated with antibodies against GLUT4 showing full blot.

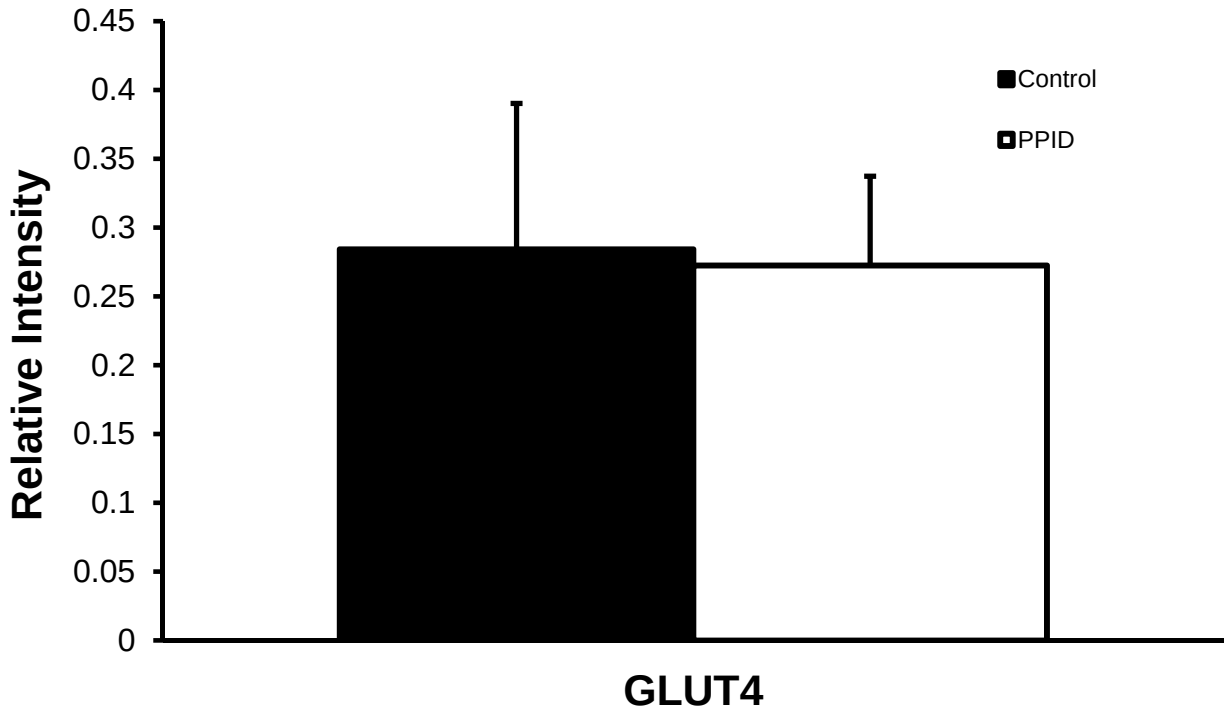


Figure B.4. Western blot analysis of crude muscle membrane extracts prepared from gluteal biopsies of control horses and PPID horses and treated with antibodies against GLUT4 depicted by a graph indicating the relative expression level. Intensities of bands were estimated by using the program "ImageLab" from BioRad. Values were normalized to the GLUT4 control and expressed relative to the value of control horses. The data are expressed as means ± s.e. using Student's t-test.

APPENDIX C

CHAPTER FIVE MATERIAL

Table C.1. Kinetics of glucose uptake rate into equine jejunal control and PPID brush border membrane vesicles. Each point is representative of the mean ± SEM from four control horse's and eight PPID horse's uptake experiments.

	Control	PPID	Control SEM	PPID SEM	p-value
0.0000	0	0	0	0	0
0.1000	76.7316	67.3569	17.5665	13.2791	0.6803
0.2500	164.64	131.9	39.0554	29.5231	0.5204
0.5000	244.4	194.28	45.6777	34.5291	0.4042
1.0000	227.3	158.22	38.7969	29.3277	0.1892
2.5000	328.63	244.1	55.7127	42.1148	0.257
5.0000	252.041385	139.485019	38.7969	29.3277	0.0449
7.5000	255.393487	141.444856	76.2794	57.6618	0.0449

Table C.2. Kinetics of Glutamine uptake rate into equine jejunal control and PPID brush border membrane vesicles. Each point is representative of the mean ± SEM from four control horse's and eight PPID horse's uptake experiments.

	Control	PPID	Control SEM	PPID SEM	p-value
0.0000	0	0	0	0	0
0.1000	8.19147907	11.0014254	2.3166	1.5166	0.1316
0.2500	14.7117332	18.203359	6.9997	4.5824	0.3421
0.5000	20.8291951	25.0321454	11.0153	7.2112	0.2637
1.0000	27.3002978	32.8576905	10.3215	6.757	0.1696
2.5000	34.8880588	44.3085268	12.3563	8.0891	0.4786
5.0000	38.993408	48.6574797	21.6667	14.1842	0.1006
7.500	40.6833948	57.9075866	36.4307	21.0333	0.904

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