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To the Graduate Council:

I am submitting herewith a thesis written by Sarah Beth Elliott entitled "Effects of pituitary pars intermedia dysfunction (PPID), season, and pasture diet on blood adrenocorticotropic hormone and metabolite concentrations in horses.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Nicholas Frank, Major Professor

We have read this thesis and recommend its acceptance:

Claudia Kirk, Naima Moustaid-Moussa, Jonathan Wall

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for

the Masters of Science Degree

The University of Tennessee, Knoxville

Sarah B. Elliott

December 2010

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ABSTRACT

Studies described in this thesis were performed to investigate associations among season, diet, pituitary pars intermedia dysfunction (PPID) and blood concentrations of adrenocorticotropic hormone (ACTH), insulin, glucose, and leptin in horses. In the first study, higher ACTH concentrations were detected in horses affected with PPID. A seasonal increase in plasma ACTH concentration was detected in the late summer and early fall, but PPID did not affect the timing or duration of this increase. Pasture grazing raised glucose and insulin concentrations with a peak in September, at the same time that horses had higher ACTH concentrations, and this convergence of risk factors may raise the risk of laminitis. All of the horses included in this study were from the same farm. The second study was performed to determine whether horses from different locations within the same region exhibited the same seasonal increase in ACTH concentrations. Results of this study indicate that the seasonal increase in plasma ACTH concentrations occurs in horses from different farms with varying management practices. The third study investigated the effects of season on plasma leptin concentrations in the horses from the first study. We hypothesized that higher leptin concentrations would be detected in advance of the seasonal increase in plasma ACTH concentrations. Results did not support our hypothesis because leptin concentrations increased after ACTH concentrations peaked in September. Our findings suggest that the seasonal increase in ACTH concentrations induced leptin resistance, which might facilitate weight gain in the autumn. Alternatively, leptin concentrations increased as a result of weight gain or change in body fat composition. In summary, season appears to signal upregulation of the hypothalamic-pituitary-adrenal axis in horses, in an effort to prepare for winter. This upregulation is retained in horses with PPID, a disorder associated with loss of dopaminergic

inhibition to the pars intermedia of the pituitary. The seasonal rise in plasma ACTH concentrations is followed by an increase in leptin concentrations, which suggests the development of leptin resistance or an increase in adiposity.

Keywords: pituitary pars intermedia dysfunction, leptin, season, adrenocorticotropic hormone

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LIST OF ABBREVATIONS

ACTH – adrenocorticotropic hormone
AgRP – agouti-related protein
AH – anterior hypothalamic nucleus
ARC – arcuate nucleus
ATP – adenosine triphosphate
α-MSH – alpha-melanocyte stimulating hormone
BCS – body condition score
BDNF – brain-derived neuronal growth factor
β-END – beta-endorphin
β-MSH – beta-melanocyte stimulating hormone
cAMP – cyclic adenosine monophosphate
CCK – cholecystokinin
CLIP – corticotrophin-like intermediate lobe peptide
CRH – corticotrophin-releasing hormone
DM – dorsomedial hypothalamic nucleus
DST – dexamethasone suppression test
ERK – extracellular signal-regulated kinase
ESC – ethanol-soluble carbohydrate
GC – glucocorticoid receptor
GLUT4 – glucose transporter 4
HPA – hypothalamic-pituitary-adrenal axis
IR – insulin resistance
MB – mammillary nuclei
MCR – melanocortin receptor
NEFA – nonesterified fatty acid
NPY – neuropeptide Y
PC – prohormone convertase
PI3K – phosphatidyl inositol 3 kinase
PN – posterior nucleus
POMC – proopiomelanocortin
PPID – pituitary pars intermedia dysfunction
PVN – paraventricular nucleus
SCN – suprachiasmatic nucleus
SI – small intestine
Sim1 – single-minded gene-1
SO – supraoptic nucleus
TRH – thyrotropin-releasing hormone
VMN – ventromedial nucleus

CHAPTER 1

INTRODUCTION

1.1 Project Summary

Pituitary pars intermedia dysfunction (PPID), or equine Cushing's disease, is becoming more common as owners allow their horses to live longer. This disorder has often been diagnosed by detecting high adrenocorticotropic hormone (ACTH) plasma concentrations or performing a dexamethasone suppression test (DST). However, test results became harder to interpret after Donaldson et al. [1] published data indicating that healthy horses exhibited positive dexamethasone suppression test and high resting ACTH concentrations (>35 pg/ml) in September. Since this observation, other research groups have corroborated these data, demonstrating high ACTH plasma concentrations in healthy horses in August [2], and positive thyrotropin releasing hormone response test in healthy horses [3] in late July.

Currently, no research has investigated the relationship of ACTH to other blood metabolites, such as glucose, insulin, and leptin. These measures might provide clinicians with an alternative method for diagnosing PPID. Season could also affect horses with PPID through pasture grazing as it does in ponies with insulin resistance (IR) [4]. Exacerbation of insulin resistance has been associated with the development of laminitis, a debilitating disease in horses. Horses suffering from PPID had been shown to have decreased insulin sensitivity [5], so the effects of pasture grazing should also be investigated in horses suffering from this dysfunction. The possibility of a convergence of risk factors should be investigated to better

provide clinicians with adequate recommendations for horses affected with PPID to prevent the onset of laminitis.

This literature review will begin with an overview of normal endocrine function, and then PPID. Publications that are relevant to season, pasture grazing, ACTH, insulin, glucose, and leptin will be reviewed.

1.2 Normal Equine Hypothalamic-Pituitary-Adrenal (HPA) Axis

1.2.1 Physiology of HPA axis

The hypothalamus lies between the thalamus and the pituitary at the base of the brain [6]. Although it constitutes less than 1% of total brain weight, it is the most important control area for homeostatic regulation of the body. The hypothalamus regulates reproduction, growth, appetite, and thermoregulation [6]. There are three regions of the hypothalamus, each having multiple nuclei. The anterior region houses the medial preoptic nucleus, supraoptic nucleus (SO), paraventricular nucleus (PVN), anterior hypothalamic nucleus (AH), suprachiasmatic nucleus (SCN), and lateral preoptic nucleus. The tuberal region contains the dorsomedial hypothalamic nucleus (DM), the ventromedial nucleus (VMN), and arcuate nucleus (ARC). Finally, the posterior region includes the mammillary nuclei (MB), and posterior nucleus (PN) [7]. The hypothalamus synthesizes and secretes neurohormones which travel through the infundibulum (a stalk containing nerve fibers and blood vessels) to the pituitary gland to stimulate or inhibit hormones produced there [6].

The pituitary gland is located at the base of the brain in close physical and functional association with the hypothalamus [6]. It can be divided into two components based on embryologic origin. The posterior pituitary, or neurohypophysis, grows downward from neural tissue. It is structurally continuous with the hypothalamus of the brain, to which it remains

attached by the hypophyseal, or pituitary, stalk. The anterior and intermediate pituitary, or adenohypophysis, originates embryologically from the oral cavity. The hypothalamus controls almost all secretions of the pituitary. The posterior lobe is controlled by nerve fibers that originate in hypothalamic neurons and the anterior lobe is controlled by releasing factors that are transported from the hypothalamus by small blood vessels [6]. In the horse, the intermediate pituitary, or pars intermedia (Figure 1.1), is located between the pars distalis (anterior pituitary) and pars nervosa (posterior pituitary) [8].

Figure 1.1

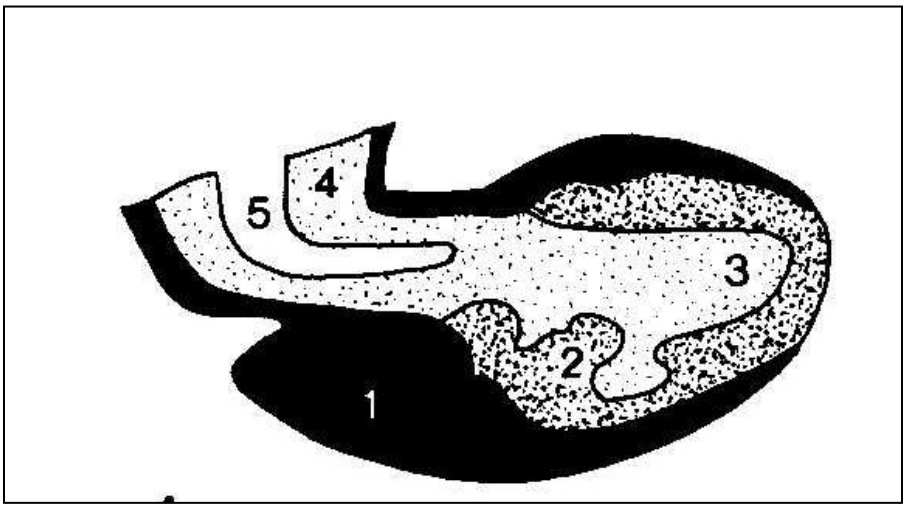

Fig 1.1 The anatomical structure of the equine pituitary. The pars distalis (1), pars intermedia (2), pars nervosa (3), hypophyseal stalk (4), and recess of third ventricle (5). From Dyce K.M, Sack W.O, and Wensing C.J. Textbook of Veterinary Anatomy. 2nd ed., Philadelphia, PA; WB Saunders, 1987.

The pars distalis gland consists of cells that produce and secrete hormones involved in the endocrine functions of the body [9]. Somatotrophic cells secrete growth hormone which stimulates all tissues in the body to grow. Gonadotrophic cells secrete follicle-stimulating hormone, and luteinizing hormone, which control the growth and reproductive activity of the gonads. Thyrotrophic cells secrete thyroid-stimulating hormone which controls the rate of thyroxine synthesis by the thyroid gland, the main regulator of body metabolic rate. Lactotrophic cells secrete prolactin which stimulates milk production. Finally, corticotrophic cells are important for the production and secretion of beta-endorphin (β-END), a pain blocker, and adrenocorticotropic hormone (ACTH). Adrenocorticotropic hormone controls the secretion of cortisol by the adrenal cortex (Figure 1.2), which affects glucose, protein, and fat metabolism [6].

The pars nervosa releases two hormones, antidiuretic hormone and oxytocin, that are synthesized by nerve cells in the hypothalamus. They are transported by nerve fibers to nerve endings in the posterior lobe, where they are released. Antidiuretic hormone alters the permeability of the kidney tubules, permitting more water to be retained by the body. Oxytocin aids in the release of milk from mammary glands and causes uterine contractions.

The pars intermedia of the horse is comprised of a single cell type, melanotropic cells that produce proopiomelanocortin (POMC) prohormone. The major peptides derived from POMC are ACTH, α-melanocyte stimulating hormone (α-MSH), and β-melanocyte stimulating hormone (β-MSH), corticotropin-like intermediate lobe peptide (CLIP), and β-END [10]. Expression of POMC is under tonic inhibitory control by dopamine, which is released locally in the pars intermedia from the nerve terminals of the periventricular dopaminergic neurons [11]. Leptin, an adipokine, also has long-form receptors on POMC-producing neurons [12].

Figure 1.2

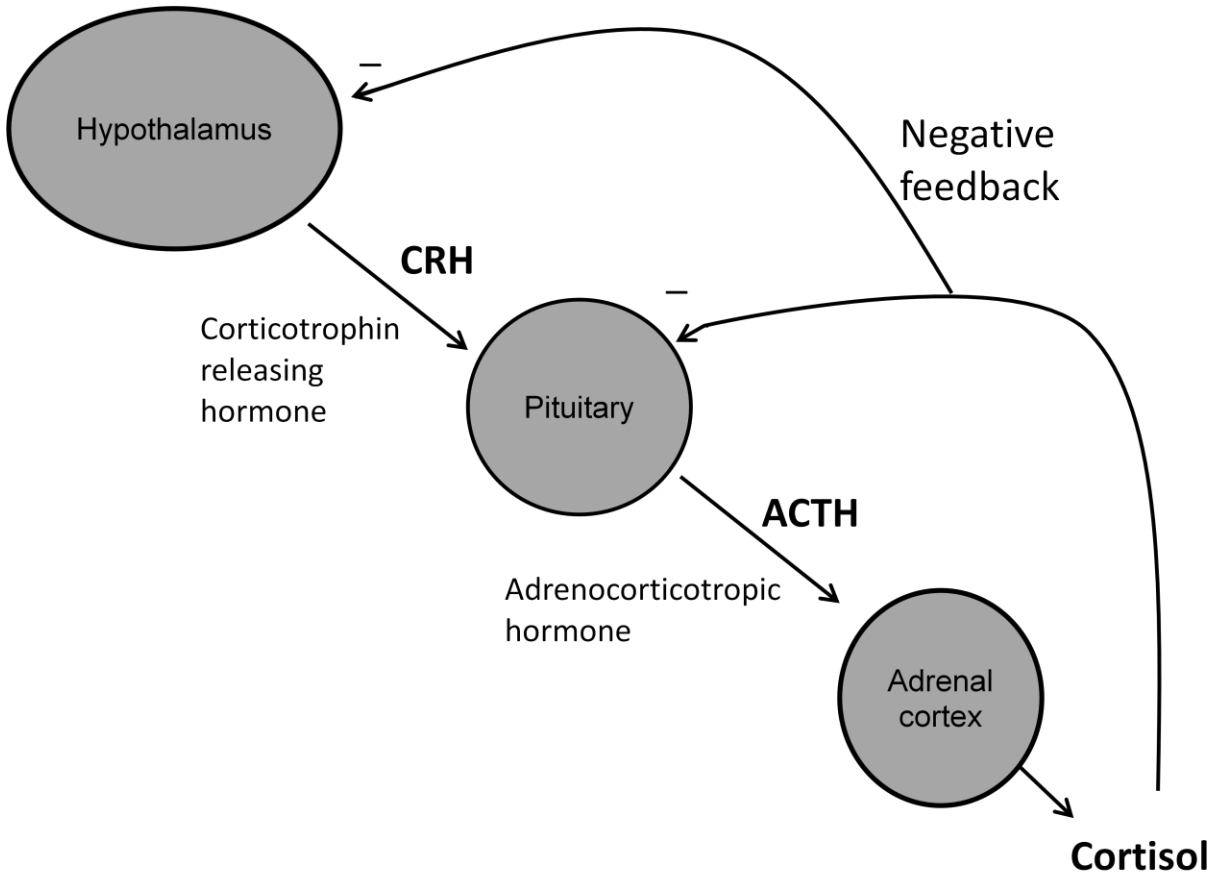

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Fig 1.2 Hypothalamus-pituitary-adrenal axis. Hypothalamic release of corticotrophin releasing hormone (CRH) stimulates release of ACTH from the pituitary. Adrenocorticotropic hormone acts on the adrenal cortex to secrete cortisol, a glucocorticoid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

The adrenal cortex produces 3 major types of steroid hormones: mineralocorticoids, glucocorticoids, and androgens. Glomerulosa cells synthesize aldosterone, a hormone involved in the regulation of sodium and potassium balance. Cortisol and the adrenal androgens are derived from the fasciculata and reticularis cells, respectively. The important control mechanism of cortisol secretion is via ACTH, which regulates adrenocortical growth, as well as the rate steroid biosynthesis occurs [13]. High levels of cortisol in circulation can interfere with energy homeostasis; increasing metabolism rates (see discussion to follow).

1.2.2 Hormones interacting with appetite and energy metabolism

Melanocortin peptides

Melanocortin peptides are produced in the arcuate nucleus of the hypothalamus, neurons in the commissural nucleus of the solitary tract of the brainstem, and the anterior and intermediate lobes of the pituitary, skin, and a wide range of peripheral tissues, including reproductive organs [14]. These peptides originate from the precursor POMC prohormone. In the corticotropic cells of the pars distalis, the major end product of the POMC pathway, by cleavage with prohormone convertase I (PC-1), is ACTH. Melanotrophic cells of the pars intermedia produce POMC prohormone as well, but prohormone convertase II (PC-2) [15] as well as PC-1 enzymes are active in this tissue (Figure 1.3). Adrenocorticotropin hormone is further cleaved by prohormone convertase II into α-MSH, β-END, and CLIP [10]. The pars intermedia does not readily secrete ACTH into the bloodstream.

Figure 1.3

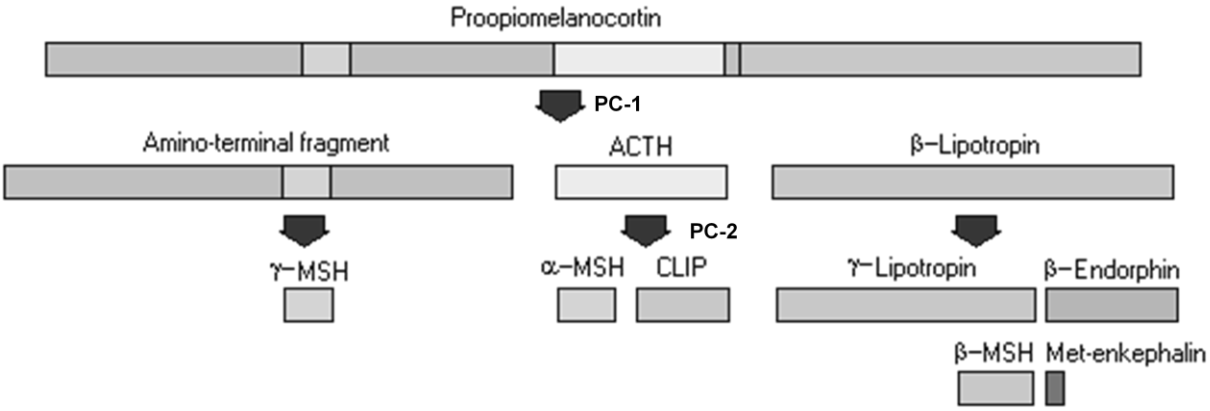

Fig 1.3 Proopiomelanocortin prohormone produced in the pars intermedia is cleaved by PC-1 to produce ACTH. Adrenocorticotropic hormone is cleaved by PC-2 to produce α-MSH and CLIP. From www.themedicalbiochemistrypage.org.

Melanocortin receptors

Melanocortin peptides released from the hypothalamus or pituitary trigger cell responses by binding melanocortin receptors, which are members of the G-protein-coupled receptor family [16]. There are currently five melanocortin receptors identified, named MC1R to MC5R [14]. Melanocortin receptors 3 and 4 are the most widely studied in humans and mice due to their role in obesity. Melanocortin 1R, MC2R, and MC5R knock-out/recessive subjects do not develop obesity [14]. Melanocortin 1 receptor is involved in pigmentation and inflammation, and MC5R stimulates exocrine function [16]. Agouti and agouti-related protein (AgRP) are antagonists of all five melanocortin receptors. Only MC2R, MC3R, and MC4R will be discussed in detail for this review.

Adrenocorticotropic hormone binds to the melanocortin-2 receptor and initiates cyclic AMP (cAMP) production [17]. Cyclic AMP activates protein kinase A, which increases phosphorylation in the cell, thus triggering cellular responses [6]. Melanocortin-2 receptors have been detected in the adrenals of mice, adipose tissue of rats, and pulmonary artery of rabbits [18-20]. In the adrenal glands, ACTH stimulates secretion of glucocorticoids, primarily cortisol [16]. In adipose tissue, Oelofsen et al. [19] found that ACTH stimulated lipolysis, which was closely correlated with binding of the MC2 receptor. When ACTH was administered to mice, MC2R mRNA levels increased in adrenal tissues, but had no significant effect on adipose tissues. In the same study, administration of dexamethasone decreased MC2R mRNA levels in the adrenal tissues, but had no significant effect on adipose tissues [17]. This finding suggests that different mechanisms exist for the action of ACTH on different tissues. The presence of MC2R on adipose tissue in horses has not been investigated, but currently MC2R mRNA has not been found in human adipose tissue [16]. The presence of MC2R in the skin cells indicates that ACTH mediates DNA synthesis and cell proliferation of keratinocytes [16].

Melanocortin-3 receptor mRNA has been found in the hypothalamus, intestine, and pancreas [14]. Receptors found in the ARC suggest that melanocortin 3 receptors play a role in POMC synthesis, thus regulating energy homeostasis. These receptors also affect insulin secretion from the pancreas. Alpha-MSH, β-MSH, and γ-MSH can all bind to MC3R and stimulate cell activity. Binding of melanocortins to the MC3R activates phosphatidyl inositol 3 kinase (PI3K) through activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway [21]. A role of MC3R in insulin secretion by the β-cells of the pancreas was shown in a study where MC4R knockout mice and MC3R knockout mice were given a melanocortin agonist. A significant reduction in hyperinsulinemia was seen in the MC4R knockout mice, but no recovery was made in the MC3R knockout mice [22]. Mice lacking the MC3R show a

disturbance in the ability to anticipate feeding time. Also, MC3R mRNA is highly abundant in the medial habenula nucleus, an additional site of melatonin synthesis [14]. Based on these observations, melanocortin-3 receptor may be involved in circadian rhythms. Alternatively, MC3R found on agouti-related protein neurons may provide negative feedback to inhibit further production of POMC derived peptides [23].

An important receptor for appetite regulation, the MC4 receptor is found in the PVN and possibly the amygdala [14]. When bound to the MC4 receptor, α-MSH induces satiety. Agouti-related protein is a potent antagonist of the MC4 receptor, promoting hunger and food intake [24]. The finding of MC4R mRNA in vagal afferent fibers suggests that cholecystokinin (CCK) may be involved in satiety signals through POMC pathways. Other mediators of the MC4 receptor are single-minded gene-1 (Sim1), brain-derived neuronal growth factor (BDNF), and oxytocin [14]. Sim1 knockout mice are obese and lack the suppression of appetite by α-MSH [25]. Similarly, dominant agouti yellow obese mice normalize food intake with over expression of Sim1 [25]. In Sim1 (+/-) mice, administration of oxytocin reduced food intake and weight gain when compared to wild type mice [26]. Phenotypes seen in BDNF (+/-) mice include obesity, hyperphagia, and insulin-resistance (IR). When BDNF(+/-) mice were restricted of their food intake, BDNF levels increased to the level of the ad-libitum fed wild-type mice [27]. Expression of BDNF is mostly found in the VMN, and administration of BDNF suppressed appetite in MC4R-knockout mice. This indicates a role for appetite suppression by BDNF in the VMH after MC4R stimulation in the PVN [28].

Hormone actions

Adrenocorticotropin hormone, α-MSH, and β-MSH inhibit food intake when administered to the brain in rodents, and also when administered peripherally [14]. When injected intravenously in sheep, ACTH increased appetite in August and decreased appetite in January [29].

In humans, a defect in N-terminal acetylation of desacetyl-α-MSH is thought to contribute to the development of obesity [14]. This acetylation reaction is regulated by leptin and dopamine, two hormones involved in energy homeostasis [14]. Corticotropin-like intermediate lobe peptide acts on the pancreatic β cells to stimulate insulin release [30]. This peptide is produced in greater quantities in horses with PPID [31], but the importance of this observation has not been investigated to date. Action of CLIP on pancreatic β cells warrants further investigation as associations among PPID, season, and pasture grazing are examined in horses.

Agouti-related protein (AgRP) and neuropeptide Y (NPY) increase appetite [14]. Agouti-related protein has been show to block MC3R and MC4R signaling, and also signals endoplasmic reticulum endocytosis of MC3/4 receptors to decrease the number of receptors on the cell surface [32], further contributing to increases in appetite. Neuropeptide Y is a neuropeptide that increases food intake in mammals by acting on the paraventricular nucleus [33].

Cortisol is the major glucocorticoid produced by the adrenal cortex, and is synthesized from cholesterol [34]. Cortisol is released in response ACTH action on the adrenal tissue and provides negative feedback to the hypothalamus and pars distalis, but not the pars intermedia. Cortisol binds to glucocorticoid receptors (GC) that are found ubiquitously throughout the body [35], signaling cell response. Glucocorticoids promote differentiation and proliferation of adipocytes, and also redistribute adiposity to central depots in humans, as well as signaling

lipolysis and release of free fatty acids [36]. In a study in obese children with or without insulin resistance, weight loss reduced cortisol levels and increase insulin sensitivity in the IR group, but had no effect on the non-IR group. Overall, humans with visceral obesity show an upregulation in the HPA axis [36]. Basal levels of cortisol stimulate gluconeogenesis and lipolysis in a fasting state [6]. High circulating levels of cortisol stimulate increased protein catabolism, gluconeogenesis, triglyceride breakdown, and decrease glucose uptake by muscle and adipose cells [6].

In a study comparing rats that were treated with cortisone acetate injections or left untreated, rats in the treated group lost weight indicating catabolic state in treated animals [37]. Increased levels of cortisone lead to decreased glucose uptake by cells via insulin-mediated methods. This occurs through a decrease in tyrosine phosphorylation of insulin receptors as well as a decrease in IRS-1 phosphorylation [37]. The decreased ability of insulin to facilitate glucose uptake leads to hyperglycemia, resulting in an increased response of the pancreas to release more insulin. Chronic hyperinsulinemia worsens the insulin resistant state.

Leptin is an adipokine that plays a major role in the negative control of feeding [38] (Figure 1.4). Leptin acts on the ARC and SCN of the hypothalamus and also the pituitary to inhibit secretion of proteins that increase appetite, and promote synthesis and secretion of proteins that suppress appetite [38]. Leptin is able to self-regulate, rodents that over express leptin have a reduced number of receptors on the hypothalamus, and prolonged hyperleptinemia over time will diminish its physiologic actions [39-40]. The neuropeptides AgRP and NPY increase food intake and lower energy consumption, as previously described.

Figure 1.4

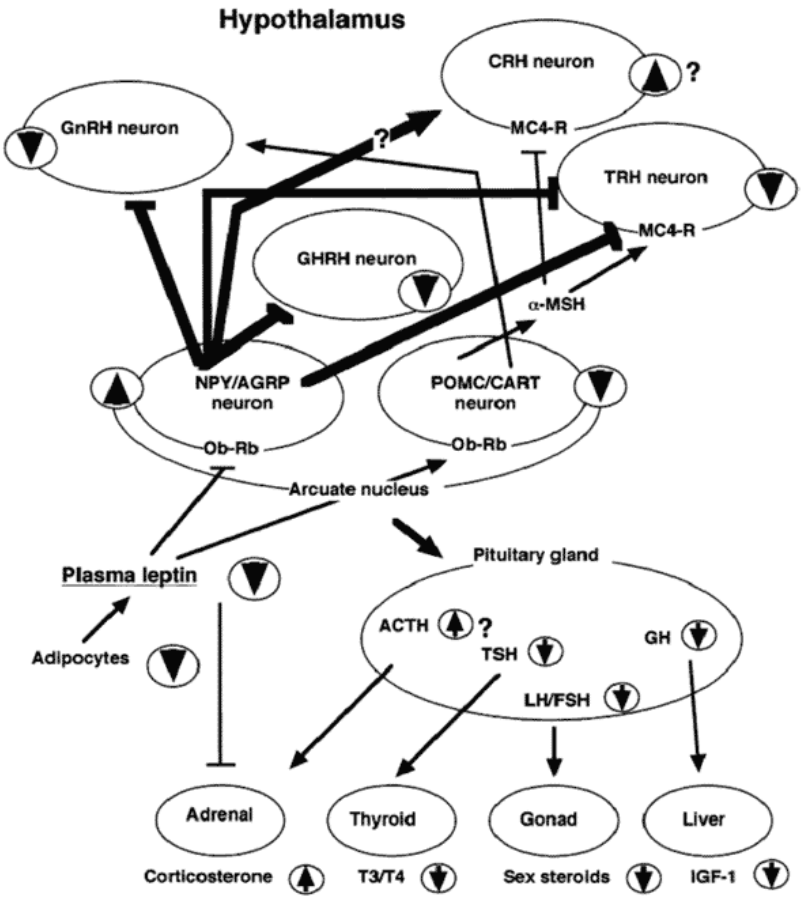

Fig 1.4 Leptin's action on the hypothalamus to suppress appetite. Leptin stimulates production of α-MSH, and suppresses secretion of NPY and AgRP. From www.innovitaresearch.org.

Leptin acts on neuropeptide Y-producing neurons and AgRP-producing neurons to inhibit NPY and AgRP secretion (Figure 1.4). Leptin resistance occurs when the body becomes unable to control appetite and energy expenditure via leptin signaling [41]. The transition of cells to develop leptin resistance may increase NPY secretion, leading to an increase in appetite.

In humans, higher plasma leptin concentrations have been highly correlated to visceral adiposity stores [42]. Hyperleptinemia has been associated with obesity in horses, and has also been shown to predict the occurrence of laminitis in ponies [4]. Leptin can affect both the pars distalis and the pars intermedia to regulate corticotrophin-releasing hormone (CRH), ACTH, and α-MSH secretion [38]. Seasonal changes in leptin have been reported in many species, with leptin resistance occurring in the autumn [38, 43-44]. Leptin resistance results from a reduction in the density or activity of leptin receptors within the hypothalamus [45], and may explain why horses gain weight in the late summer and autumn. In Siberian hamsters, leptin signaling on the ARC was suppressed during long day photoperiods [46]. In sheep, melatonin secretion was unaffected by leptin administration during long day photoperiods, but was stimulated during short day secretions [47].

Leptin shows a circadian pattern in horses with the lowest levels seen in day time, and peaking at night, and this rhythm was seen regardless of fed or fasted conditions [48]. However, Storer et al. [49] found that horses fed grass hay in a drylot had lower plasma insulin and leptin concentrations than when they were housed on pasture. In older mares (>10 yrs old), leptin fluctuated with season, with a peak in September, at the same time that body weight peaked [50]. In the same study, younger mares (<10 yrs old) did not show an autumn peak in leptin concentrations, but levels decreased in late winter [50]. Older mares gained more weight than the younger mares, which may explain the difference in leptin concentrations.

When dexamethasone has been administered to geldings and mares, each group exhibited an increase in leptin secretion [51]. In mares, the response to dexamethasone injections was almost twice as large, even though baseline leptin concentrations did not differ between the two groups [51]. Interestingly, the leptin response diminished when dexamethasone injections were continued [51]. When given ACTH injections, the horses in the

previous study also showed an increase in leptin, but it was more modest as compared to dexamethasone [51]. Glucose infusion failed to elicit a leptin response, possibly due to insufficient insulin stimulation by the low dose of glucose infused [51].

1.3 Effects of season on the endocrine system

Melatonin, an amino acid derivative synthesized from serotonin, is produced by the pineal gland. Secretion of melatonin is stimulated by sympathetic postganglionic neurons which are primarily triggered by receptors in the eyes. Darkness stimulates melatonin production and light inhibits it [6]. The circadian system regulates the pattern of melatonin secretion, and the melatonin pattern serves as a signal to convey day length to the rest of the body [52]. Melatonin secretion can also be signaled by CCK, a hormone released from neuroendocrine cells in the mucosa of the small intestine [53]. Melatonin secretion regulates androgen secretion and is a signal for reproduction [54].

Dopamine is a neurotransmitter derived from the amino acid tyrosine [55]. In sheep, dopamine concentrations within tissues, as well as the bioactivity of tyrosine hydroxylase in the median eminence are higher during long days when compared to short days [56]. Dopamine is a precursor to epinephrine and norepinephrine [6]. Dopaminergic neurons begin in the periventricular nucleus of the hypothalamus, pass through the infundibulum, and release dopamine into the pars intermedia. When dopamine is released from these neurons it inhibits the secretion of POMC-derived peptides [55]. Dopamine is also released into the pars nervosa where it acts to inhibit secretion of prolactin. Dopamine can also be synthesized in the adrenals and released into circulation in response to stress [6].

1.4 Normal glucose/insulin metabolism

1.4.1 Glucose

Glucose, a 6-carbon monosaccharide, is the major energy source for mammalian cells as well as an important substrate for protein and lipid synthesis. Glucose is the body's preferred way to store adenosine triphosphate (ATP) for energy when needed. Hypoglycemia in mammals can lead to alterations in neural activity, resulting in coma and ultimately death [34]. When ingested, carbohydrates are processed into monosaccharides such as glucose, galactose, and fructose by hydrolytic enzymes bound to the brush border of enterocytes [57]. Mammalian cells take up monosaccharides, most importantly glucose, through two families of structurally related glucose transporters. Passive transport is mediated by a family of facilitative glucose transporters, whereas sodium-dependent glucose transport is mediated by Na^+/glucose co-transporters [57].

In the fed state, the major fates of glucose are energy storage as glycogen in liver and skeletal muscle, or conversion to triacylglycerol, which is stored in adipose tissue [6]. Ingested nutrients enter the blood from the gastrointestinal tract and are delivered to the liver, adipose tissue, and muscle for processing. Glucose transporter 4 (GLUT4), a member of the facilitative glucose transporters, translocates to the cell membrane in response to insulin during fed states and rapidly increases glucose uptake across the plasma membrane. Therefore, the highest expression of GLUT4 is found in insulin-sensitive tissues including brown and white adipose tissue and skeletal and cardiac muscle [57]. In a study looking at meal type after exercise in horses, GLUT4 gene expression in skeletal increased 4.3 times 4 hours following exercise, and was still increased 2.6 times 24 hours post exercise compared to baseline. However, ingestion

of carbohydrates such as starch-rich meals did not enhance GLUT4 gene expression in muscle as expected, compared with isocaloric fiber-rich meals or fasting conditions [58].

When the cells have high concentrations of ATP and NADH, isocitrate dehydrogenase activity is inhibited in the Kreb's cycle, thereby preventing breakdown of citrate and leading to accumulation in the mitochondria. Accumulation of citrate signals the cell to promote glucose storage, e.g., by facilitating fatty acid synthesis. Citrate leaves the mitochondria and enters fatty acid synthesis, a cyclical reaction that generates palmitate and stearate. Esterification of glycerol-3-phosphate and palmitate form triacylglycerol for storage in adipose tissues [34]. Another method citrate is involved in glucose storage is by the inhibition of phosphofructokinase-1, preventing entry of fructose-6-phostate into glycolysis. Therefore, glucose-6-phosphate enters glycogenesis, storing glucose as glycogen [34].

During fasting states, glycogen stores are catabolized to provide glucose for energy. The average meal in humans requires approximately 4 hours for complete absorption [6]. In a study with 6 horses and varying diets, gastric emptying based on ^{13}C-octanoate breath tests had an average half life of 2.7 hours [59]. After this, the body enters the post-absorptive state. Glycogenolysis and lipolysis supply the liver, and kidneys, with glycerol, lactate, and pyruvate, which are precursors for gluconeogenesis. When glucose from the blood enters the cell, it is converted by glucokinase/hexokinase to glucose-6-phosphate, which can also be generated from the breakdown of glycogen. Glucose-6-phosphate enters glycolysis, which generates NADH and two pyruvate molecules that pass into the mitochondria and enter the Krebs cycle to generate $FADH_2$ and NADH. These molecules enter the respiratory chain or oxidative phosphorylation and generate ATP for energy [34].

In adipose tissue, hormone-sensitive lipase breaks the glycerol backbone away from the three attached fatty acid chains. The fatty acids enter beta-oxidation in the mitochondria, and

with each turn of the cycle, 2-carbon fragments of acetyl CoA are released that then enter the Kreb's cycle for oxidation. The glycerol backbone is phosphorylated in the liver by glycerol kinase to form glycerol-3-phosphate, a precursor for gluconeogenesis.

1.4.2 Insulin

Insulin is a dipeptide hormone secreted by the β cells of the pancreatic islets of Langerhans. Insulin is the driving force that regulates the storage of nutrients during fed states so that there are energy sources for periods of fasting [60]. This hormone maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism, and promoting cell division and growth through its mitogenic effects [61-62].

During fed periods, insulin secretion is characteristically biphasic, with an initial rapid phase of insulin secretion, followed by a less intense, but more sustained release of the hormone [62]. Following secretion of insulin into the portal venous system, 50% is subsequently removed by the liver, so portal vein insulin concentrations are almost three times higher than the peripheral circulation [63]. Insulin in peripheral circulation binds to its receptor on the major insulin responsive tissues of the body, namely skeletal muscle, liver, and adipose tissue, which account for 60-70%, 30%, and 10% of the whole body insulin mediated uptake, respectively [62], triggering GLUT4 translocation to the cell membrane. Insulin also deactivates cyclic AMP (cAMP) through protein kinase B. Deactivation of cAMP prevents activation of protein kinase A, preventing lipolysis [34].

Insulin is responsible for governing the tight control of glucose that is absorbed from the intestine, produced by the liver, and taken up and metabolized by peripheral tissues [64]. Rising blood glucose concentrations signal pancreatic beta cells to secrete insulin. After its release from the pancreas, insulin causes a 30-fold increased rate of transport of glucose into

adipocytes [34]. Insulin also inhibits hepatic glucose production. This is accomplished through coordinated regulation of enzyme synthesis and activity [64]. In muscle tissues, the presence of insulin promotes glucose uptake. Glucose undergoes glycolysis, forming lactate. Lactate is released into circulation, enters the liver, and is converted to glycogen or glucose there [34].

Circulating insulin concentrations decrease during fasting and this allows glycogenolysis, lipolysis, and gluconeogenesis to occur. Pancreatic β cells only secrete 0.25 to 1.5 units insulin per hour during fasting states in humans, which is sufficient to enable insulin-dependent glucose entry into cells [62]. This level prevents uncontrolled hydrolysis of triacylglycerides and limits gluconeogenesis, thereby maintaining fasting blood glucose concentrations within reference ranges [62]. Glucagon is a hormone released by the pancreatic α-cells under fasting conditions that acts to oppose the affects of insulin, i.e., it inhibits glycolysis in the liver by activating protein kinase A through cAMP activation [34]. An excess of plasma glucagon relative to insulin can cause hyperglycemia because of its action on the liver to activate glycogenolysis and gluconeogenesis [65].

1.5 Pituitary Pars Intermedia Dysfunction (PPID)

Equine pituitary pars intermedia dysfunction (PPID) is a naturally occurring, clinically progressive neuroendocrine disease that affects a large population of horses and ponies 15 years and older [66]. Clinical signs for PPID include hirsutism, muscle wasting, abnormal fat distribution, glucose intolerance, lethargy, infertility, polydipsia and polyuria [67]. In one epidemiological study [68], 30% of owners of aged horses (>20 years) who were surveyed about the clinical condition of their animals reported haircoat changes highly suggestive of PPID. In two recent studies, 33 to 40% of adult horses had pituitary pars intermedia microadenoma or macroadenoma identified by routine histological examination [69-70].

The mechanism of pars intermedia hypertrophy, hyperplasia and adenoma formation in PPID is unclear. It is possible that the lesion is a spontaneously occurring primary pituitary tumor. Alternatively, pars intermedia enlargement may result from loss of hypothalamic dopaminergic inhibition. McFarlane et al. [66] demonstrated that tyrosine hydroxylase, a marker of dopaminergic neurons, was reduced by 20% in PPID horses as compared to controls. This supports a loss of functional dopaminergic nerve terminals in diseased horses [66]. Orth et al. [10] found that horses affected with PPID have a marked increase in circulating concentration of POMC-derived peptides, including α-MSH, β-END, and CLIP. Adrenocorticotropic hormone is also increased, although more modestly [10]. Alpha-MSH and β-END potentiate the effects of corticotropin to cause adrenal dysfunction. However, only 10-15% of horses with PPID develop hypertrophy and hyperplasia of the adrenal cortex, which results in an increase in corticosteroid production and loss of the normal circadian pattern of corticosteroid secretion [71].

Haritou et al. [72] reported seasonal changes in circadian peripheral plasma concentrations of melatonin, serotonin, dopamine, and cortisol in aged horses with PPID. Six horses and ponies with PPID were matched with six controls to test the hypothesis that aged horse respond differently to changes in season because of deficiency in melatonin production. They also examined the link between the presence or absence of the clinical signs of PPID and peripheral plasma concentration of serotonin, dopamine, and cortisol. Results showed that the 24-h pattern of plasma melatonin concentrations during the four seasons of the year were similar in both groups, indicating that impaired melatonin output is unlikely to play a role in PPID. However, serotonin profiles were affected by season, with lower serotonin detected in PPID horses in the summer and winter. The total amount of dopamine released was dependent

on season and markedly lower in PPID horses versus controls. These results implicate both serotonin and dopamine in the pathogenesis of the disease [72].

1.5.1 Effects of insulin resistance, PPID, diet, and season on resting glucose and insulin concentrations

Resting blood glucose concentrations are tightly controlled by insulin. In a healthy animal, this relationship is functional and communication is free flowing. In an unhealthy animal, however, this relationship can be hard to maintain. Insulin resistance (IR) is an endocrine disorder associated with altered glucose and insulin concentrations. Impaired insulin signaling alters normal cell functions in the liver, pancreas, and skeletal muscle [73-74].

Tissues are less responsive to insulin when IR develops [34]. Hyperinsulinemia develops and IR results in abnormal glycemic and insulinemic responses to oral or IV glucose and/or insulin challenges [73-74]. Insulin resistance is a major concern in horses because of its link to laminitis [75]. It has also been shown to affect ponies in the UK where insulin sensitivity varied with season [76]. In one study, ponies that were prone to laminitis had significantly higher serum insulin concentrations in the summer months, when compared to the non-laminitis prone group [76]. However, no differences were detected between the same groups during the winter. Carter et al. [77] also found that blood insulin and leptin concentrations predicted the occurrence of laminitis in ponies.

Pituitary pars intermedia dysfunction has been associated with altered blood glucose and insulin concentrations in horses. Horses with PPID are frequently, but not always, insulin resistant and have elevated serum glucose and insulin levels [78]. Interestingly, treatment for PPID does not always return these values back to normal [79-80]. Insulin concentrations are also affected by diet. In a group of ponies that were predisposed to laminitis, a significantly

higher median serum insulin concentration was detected on a pasture diet, and decreased when only hay was fed [81].

Horses are hindgut fermenters that are evolutionarily adapted to graze continuously on plants [82]. Plants contain carbohydrates in three forms: simple sugars (e.g., glucose, fructose, sucrose), storage molecules (e.g., starch, fructans), and structural polysaccharides (e.g., hemicelluloses, cellulose) [83]. The simple sugars and storage molecules make up the nonstructural carbohydrate (NSC) portion of the plant [83]. In the horse, carbohydrates are digested either by hydrolysis to simple sugars in the small intestine (SI), or fermented by bacteria to volatile fatty acids mostly in the hindgut, and slightly in the stomach [82]. Hydrolysable carbohydrates enter the small intestines and are hydrolyzed by enzymes, primarily pancreatic α-amylase [82]. These carbohydrates include disaccharides, some oligosaccharides, and starch. The end products of hydrolysis by enzymes are disaccharides and oligosaccharides. These sugars are broken down further to simple sugars (glucose, galactose, and fructose) by enzymes along the brush border of the SI tract and absorbed.

Fermentation of carbohydrates such as starches resistant to enzymatic hydrolysis, hemicelluloses, cellulose, fibers, and some oligosaccharides, namely fructans and galactans, occurs principally in the equine hind gut. Carbohydrates are fermented by bacteria to produce volatile fatty acids (e.g., acetate propionate, butyrate, lactate, and valerate) [82]. Volatile fatty acids are absorbed along a pH gradient by passive diffusion, usually in the form of free acids. Propionate supplies the liver and muscle for glycogen storage, while acetate and butyrate provide carbon for adipose synthesis [82].

The differential insulin response to pasture grazing or grains are of particular importance. Over 80% of horses in the United States have some access to pasture, and more than 90% of farms feed grain in addition to hay or pasture [84]. Insulin concentrations were

higher when horses were grazing on spring pasture grass, compared to the diet provided in stalls [85]. Only one study has been performed to assess the effects of season on glucose and insulin concentrations in horses [2]. Glucose concentrations did not change significantly throughout the year and insulin concentrations changed significantly over time, but there was no discernable pattern. Pasture samples were not collected, but carbohydrates may have been responsible for these alterations over time.

1.5.2 Diagnosis of PPID

Diagnosis of PPID is problematic. Single-sample tests have been developed for screening purposes and include resting ACTH, cortisol, insulin, and glucose concentrations [86]. Previously in horses, ACTH concentrations had been the most reliable test for diagnosing PPID. Testing for ACTH is simple and can be obtained from a single blood sample. The reliability of this test, however, is under review due to diurnal and seasonal changes of ACTH within each horse [71, 87].

Resting ACTH concentrations are high in horses with PPID because POMC synthesis increases with hyperplasia/neoplasia of the pars intermedia [88]. However, resting hormone and metabolite concentrations are potentially affected by season, diet, and stress, and this affects the accuracy of results. One important confounding factor is season and this was revealed when high resting ACTH concentrations (consistent with PPID) were detected in plasma samples collected in September from otherwise healthy horses [1]. In this study, 15 pony mares (all pregnant during the study), 14 pony stallions, and 10 non-pregnant horse mares were evaluated at four time points over 12 months (September 2002, January, May, and September 2003). Only one high (> 35 pg/mL) ACTH concentration was detected in January and May, whereas the majority of results were high at the September sampling times; only 5%

and 8% of results were < 35 pg/mL in these healthy ponies and horses in September 2002 and September 2003, respectively. This was the first observation that healthy horses and ponies can exhibit quantitatively “abnormal” ACTH concentrations during different seasons, indicating that false-positive diagnosis of PPID may be made based on blood sampling in the late summer and autumn seasons.

As an alternative, “response tests” are often used to diagnose PPID, including the dexamethasone suppression test (DST) and the thyrotropin-releasing hormone (TRH) stimulation test [3, 89]. Donaldson et al. [1] also discovered seasonal variation in DST results within normal horses. Healthy horses and ponies did not suppress as well in September, compared with January. Beech et al. [3] also found an association between number of daylight hours and TRH response test results for diagnosing PPID in a population of 48 horses There was evidence of higher resting ACTH concentrations and abnormal results in healthy horses during the late summer months [3]. Season therefore affects resting ACTH concentrations and other diagnostic tests for PPID, so more research is needed to examine seasonal hormonal changes in horses.

Alpha-MSH has only recently been implicated as a possible diagnostic hormone for diagnosing PPID [90]. No difference was seen in the hormone at the two times (1200 and 1600 hours) during the day showing diurnal stability, but a distinct seasonal effect was found in horses and ponies, with the ponies having a more profound increase in α-MSH in the fall when compared to the horses. As a diagnostic test for PPID, α-MSH showed no more promise than ACTH [88].

Cortisol concentrations should not be measured to diagnose PPID because cortisol in horses has a circadian rhythm, fluctuations can be caused by stress, and the reference range for resting cortisol concentrations is wide [8, 91]. In some species however, namely the feline,

cortisol has no circadian rhythm [92], making it useful to perform diagnostic test at any time of the day. The circadian rhythm confounds the use of cortisol measurement as an indicator of PPID in horses, even though horses affected with PPID have a diminished diurnal response to cortisol [89]. Many studies report normal cortisol concentrations in horses affected with PPID [10, 89].

1.6 Statement of the problem

Diagnostic testing for PPID relies upon resting hormone and metabolite concentrations, yet potential confounding factors have not been evaluated across different months of the year. The purpose of this project was therefore to further evaluate the effects of season and diet on blood ACTH, insulin, glucose, and leptin concentrations in healthy horses and those affected by PPID.

CHAPTER 2

Association of season and pasture grazing with blood hormone and metabolite concentrations in horses with presumed pituitary pars intermedia dysfunction

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2.1 Abstract

Background: Pituitary pars intermedia dysfunction (PPID) is a risk factor for pasture-associated laminitis, which follows a seasonal pattern.

Hypothesis: Hormonal responses to season differ between PPID and unaffected horses.

Animals: Seventeen horses aged 8 to 30 years (14 horses ≥ 20 years of age).

Methods: Longitudinal observational study. Blood was collected monthly from August 2007 until July 2008, after pasture grazing and again after overnight stall confinement. Blood hormone and metabolite concentrations were measured and pasture grass samples were analyzed to determine carbohydrate content. Analysis of variance analyses for repeated measures were performed.

Results: Mean ACTH concentrations varied significantly over time ($P < 0.001$) with higher concentrations detected in August, September, and October, compared with November to April. Pasture × time effects were detected for glucose and insulin concentrations, with peaks observed in September. Horses were retrospectively allocated to PPID (n = 8) and control (n = 9) groups on the basis of plasma ACTH concentrations. Changes in insulin concentrations over time differed in the PPID group, when compared to the control group. Insulin concentrations were positively correlated with grass carbohydrate composition.

Conclusions and clinical importance: Pituitary pars intermedia dysfunction did not affect the timing or duration of the seasonal increase in ACTH concentrations, but higher values were detected in affected horses. Insulin concentrations differed between groups, but hyperinsulinemia was rarely detected. Glucose and insulin concentrations peaked in September when horses were grazing on pasture, which could be relevant to the seasonal pattern of laminitis.

2.2 Introduction

Pituitary pars intermedia dysfunction (PPID), which is also known as Equine Cushing's Disease, has been associated with laminitis in horses [79, 93-95], but the mechanisms responsible for this association have not been fully elucidated. One potential explanation for this association is that horses with PPID are insulin resistant [96]. Insulin resistance (IR) is an important predisposing factor for pasture-associated laminitis in ponies, cortisol antagonizes the action of insulin within tissues [97-98], and some PPID-affected horses have reduced insulin sensitivity [5, 78]. Hyperinsulinemia is detected in some, but not all, horses and ponies with PPID and hyperglycemia occurs in a smaller number of animals [99-101].

The incidence of pasture-associated laminitis follows a seasonal pattern that might be relevant to the association between PPID and laminitis. Laminitis develops between March and May in ponies in Virginia [4, 97], and between September and May in a group of 40 horses that included 28 animals with suspected PPID [93]. This increase in laminitis incidence in September is of interest because it coincides with seasonal up-regulation of the hypothalamic-pituitary-adrenal axis in equids [1, 3, 102]. Plasma concentrations of ACTH and alpha melanocyte-stimulating hormone are higher in September, and false positive dexamethasone suppression test results occur more frequently at this time of the year [1, 102].

Seasonal alterations in hormone concentrations warrant further examination because they appear to coincide with a higher incidence of laminitis in the autumn [93]. Furthermore, it must be determined whether these seasonal alterations are more profound in horses with PPID, which could explain the association between this disorder and laminitis. We therefore hypothesized that hormonal responses to season would differ between PPID and unaffected horses. It was also hypothesized that changes in pasture grass composition would induce seasonal alterations in glucose and insulin concentrations and PPID would affect these responses. Blood hormone and metabolite concentrations and responses to pasture grazing were therefore examined across a 12-month period.

2.3 Materials and Methods

Animals—Seventeen adult light breed horses (9 mares; 8 geldings) ranging in age from 8 to 30 years (14 horses aged > 20 years) were included in the study. None of the horses included in this study were receiving medical treatment for PPID.

Experimental design—A longitudinal study was performed across a 12-month period extending from August 2007 until July 2008. Horses from a facility located in Kingston, Tennessee within the South-eastern region of the United States were included in the study. Evaluations were performed during the first week of every month and consisted of visits to the farm on two consecutive days. Blood samples were collected via jugular venipuncture between 0800 and 1000 on both days. On day 1, blood samples were collected from horses after they were brought in from pasture and housed in stalls. Physical measurements and grass samples were also obtained on day 1 after all blood samples had been collected. Horses were subsequently returned to pasture until 1800 to 1900 when they were brought back to their stalls for the night. Two flakes of hay were given to each horse, but no grain or additional hay was provided until blood samples had been collected the following morning (day 2). The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Feeding and management practices – Horses were routinely housed on pasture, except for a 30-minute to 2-hour period between 0700 and 0900 when they were brought into stalls for feeding. A 12% protein sweet feed or a complete pelleted feed for senior horses was fed in the morning, with amounts varying according to the individual horse and time of year. Hay was fed during the winter months. Feed amounts were recorded.

Physical measurements – Body weight was measured by weight tape and the body condition score was assessed using the 1 to 9 scale described by Henneke et al [103]. Neck measurements were obtained as previously described [104]. Any abnormalities of the haircoat, including dullness, longer hair length, and curling of the hair, were recorded at this time. These observations were subjective and made by different investigators throughout the year.

Blood variables – Blood was collected into tubes containing potassium EDTA, sodium heparin, or no anticoagulant. Tubes were chilled on ice (plasma) and then placed in racks within coolers containing ice packs or left at ambient temperature to clot for 1 hour (serum) before being transferred to a cooler for transportation. Plasma and serum were harvested by low-speed (1,000 × *g*) centrifugation within 2 hours of collection and then stored at –80 ºC for further analysis.

Serum insulin concentrations were measured using a radioimmunoassay kit[1] previously validated for equine sera [105] and revalidated by our laboratory within 6 months of samples being analyzed.

Plasma glucose, triglyceride, and cholesterol concentrations were measured using colorimetric assays[2,3] and an automated discrete analyzer.[4] Nonesterified fatty acid (NEFA) concentrations were measured by using an enzymatic colorimetric test kit[3] and microtiter plate reader.[5] For all assays performed on site, measurements were performed in duplicate with all samples analyzed on the same day, and intra-assay coefficients of variation of <5% were required for acceptance of results, with the exception of insulin, which had a cut-off value of 10%.

Frozen plasma samples were packaged with ice packs and sent via overnight mail to the Animal Health Diagnostic Center at Cornell University[6] for measurement of plasma ACTH

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[1] Coat-A-Count insulin radioimmunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA
[2]Glucose, Roche Diagnostic Systems Inc, Somerville, NJ
[3] Wako Chemicals USA, Richmond, VA
[4] Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ
[5] ELx800 Microplate Reader, Bio-Tek, Winooski, VT
[6] Animal Health Diagnostic Center, Cornell University, Ithaca, NY

concentrations. A chemiluminescent ACTH immunoassay[7] previously validated [95] for use with equine plasma was used, with samples analyzed in duplicate. A reference range of 9 to 35 pg/mL was provided by the laboratory.

Pasture grass analysis – Wire exclusion cages were maintained on pastures. One grass sample was collected from each pasture between 0900 and 1000 on day 1 using electric shears, with the stems cut approximately 1 cm above the ground. Samples were placed in plastic bags and then immediately transferred to a cooler that contained ice packs, which remained closed at all other times. Samples were transported to the laboratory within 2 hours of collection and stored at – 20 ºC. Carbohydrate analysis was performed by the Dairy One Forage Laboratory.[8] Carbohydrate composition was determined by wet chemistry analysis and amounts of ethanol-soluble carbohydrates, water-soluble carbohydrates, and starches were measured. Depending upon the pastures being used at different times, data from 2 to 7 samples were pooled for each month.

Statistical analysis – Normality was assessed by examining plotted results and performing Shapiro-Wilk tests. Adrenocorticotropin hormone and insulin data required logarithmic transformation to fit a normal distribution before statistical tests were performed. Geometric mean values with 95% confidence intervals are displayed for these variables. Mean SD values are reported for glucose and NEFA concentrations. Mixed-model ANOVA for repeated measures was performed by use of statistical software[9] to determine the effects of time (month), and subsequently group (PPID versus control), on measured variables. Effects of pasture

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[7]Immulite adrenocorticotropin hormone chemiluminescent assay, Siemens Medical Solutions Diagnostics, Los Angeles, CA
[8] Dairy One Forage Laboratory, Ithaca, NY
[9] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC

grazing were also included in the same model for all variables, with the exception of ACTH because this variable was only measured on day 1. When a significant effect was detected, the Bonferroni test for multiple comparisons was used to identify significant differences among least squares means. Effects of sex, initial body weight, and the amount of feed provided were also examined, but were subsequently removed from the model because they did not affect results. Pearson correlation coefficients were calculated for mean blood variable concentrations and mean pasture grass carbohydrate percentages. Significance was defined at a value of $P < 0.05$.

2.4 Results

All horses remained healthy throughout the study, with the exception of one horse that required tissue debridement and application of a foot cast because of recurrent sole abscesses. Glucose, insulin, and lipid data from this horse were excluded from the analysis because marked hyperinsulinemia was detected, with a peak insulin concentration of 955 μU/mL observed in April. Another horse suffered from chronic degenerative joint disease of both carpi and received phenylbutazone intermittently during the study.

Time effects were significant for body weight ($P < 0.001$), neck circumference ($P < 0.001$), and BCS ($P < 0.001$; Table 2.1). Mean body weight (via weight tape) was highest in December and mean mid-neck circumference was lowest in June. There was no discernable pattern for BCS.

Plasma ACTH concentrations were significantly ($P < 0.001$) affected by time, with higher mean values detected in August, September, and October compared with the November to April period (Figure 2.1). Effects of pasture grazing on plasma ACTH concentrations were not assessed because this hormone was measured once each month.

Time ($P < 0.001$), pasture ($P < 0.001$), and pasture × time ($P < 0.001$) effects were significant for glucose concentrations, with a peak in September when horses were grazing on pasture (Figure 2.2). Insulin concentrations also peaked in September when samples were collected after grazing and time ($P < 0.001$), pasture ($P < 0.001$), and pasture × time ($P < 0.001$) effects were significant for this variable (Figure 2.3). A positive correlation (r = 0.22; $P = 0.002$) existed between mean ethanol-soluble carbohydrate content of the grass reported on a dry matter basis and mean insulin concentrations measured in grazing horses. Monthly mean ethanol-soluble carbohydrate, water-soluble carbohydrate, and starch content (dry matter basis) within pasture grass ranged from 2.0 to 9.1%, 1.6 to 12.7%, and 1.0 to 2.0% across the 12-month sampling period. Pasture, time, and pasture × time effects were also significant for triglyceride and NEFA concentrations, with higher NEFA concentrations detected after stall confinement. Total cholesterol concentrations were affected by pasture and time, but did not follow a recognizable seasonal pattern.

Horses were subsequently allocated to PPID (n = 8) and control (n = 9) groups on the basis of plasma ACTH results. A presumptive diagnosis of PPID was made when plasma ACTH concentrations exceeded 35 pg/mL on ≥ 3 occasions between December and June. Five of 8 horses in the PPID group had persistently elevated plasma ACTH concentrations throughout this 7-month time period. Horses in the PPID group ranged in age from 18 (estimated) to 30 years (median; 28.5 years) compared with 8 to 26 years (median; 21 years) for the control group. Both groups contained 7 horses that were > 20 years of age. Five mares and 3 geldings were included in the PPID group and the control group contained 4 mares and 5 geldings. Breeds represented in the PPID group included Arabian (n = 1), Arabian/Quarter Horse (1), Quarter Horse (2), Saddlebred (1), mixed breed (1), and Thoroughbred (1), whereas the control group contained Appaloosa (1), Quarter Horse (3), Thoroughbred (4), and

Tennessee Walking Horse (1) horses. Initial mean ± SD body weight was 449 ± 88 kg for the PPID group and 493 ± 51 kg for the control group. The amount of feed provided to each horse varied by individual animal and over time, but mean values did not differ significantly between groups (2.4 ± 0.6 versus 2.4 ± 0.5 lb/day for PPID and control groups, respectively; $P = 0.963$). Abnormalities of the haircoat were noted in 6 of 8 horses from the PPID group and 4 of 9 horses in the control group. Three horses aged 27, 29, and 30 years in the PPID group exhibited a long curly haircoat consistent with hirsutism and fat redistribution. One of these horses was the animal that developed sole abscesses. Haircoat abnormalities recorded for other horses included subjective observations of longer hair length and dullness. There were no reports of laminitis, polyuria, or polydipsia and the owner did not raise concerns about accelerated weight loss in any of the horses.

Physical examination variables did not differ significantly between groups. Plasma ACTH concentrations were higher in the PPID group ($P < 0.001$), but the group × time effect was not significant ($P = 0.847$; Figure 2.4). Plasma ACTH concentrations >35 pg/mL were detected at 1 (n = 1), 2 (n = 3) or 3 (n = 4) of the August, September, and October time points in 8 of 9 horses from the control group, but did not exceed this cut-off value in the remaining horse. In contrast, plasma ACTH concentrations were persistently elevated between August and October in the PPID group. Maximum ACTH values were 1,250 pg/mL and 105 pg/mL for PPID and control groups, respectively.

Overall mean insulin concentrations did not differ significantly between PPID and control groups ($P = 0.185$), but a group × time effect ($P = 0.037$) was detected (Figure 2.5). The group × pasture × time interaction ($P = 0.962$) was not significant. A significant group × pasture effect ($P = 0.004$) was also detected for NEFA concentrations (Figure 2.6).

Table 2.1 Physical examination measurements for 17 horses across a 12-month sampling period.

Month	**Mean ± SD (n = 17)**		
	Body Weight (kg)	**BCS**	**Mid Neck Circumference [50]**
Aug	472 ± 72[cd]	5.5 ± 1.0[bc]	90.9 ± 8.7[ab]
Sept	481 ± 75[abcd]	6.0 ± 1.5[ab]	90.7 ± 7.6[ab]
Oct	488 ± 67[ab]	6.5 ± 1.5[a]	92.9 ± 7.6[a]
Nov	488 ± 60[a]	5.0 ± 1.5[c]	90.7 ± 6.7[ab]
Dec	493 ± 68[a]	6.5 ± 1.5[ab]	92.0 ± 8.0[ab]
Jan	484 ± 72[abc]	6.0 ± 1.5[abc]	92.6 ± 8.4[ab]
Feb	490 ± 68[a]	6.5 ± 1.5[ab]	89.4 ± 6.8[ab]
Mar	480 ± 68[abcd]	6.0 ± 1.5[abc]	89.3 ± 6.7[b]
Apr	480 ± 67[abcd]	6.0 ± 1.0[abc]	91.0 ± 6.9[ab]
May	479 ± 68[abc]	6.0 ± 1.5[abc]	90.9 ± 6.6[ab]
Jun	466 ± 64[d]	6.0 ± 1.5[abc]	85.6 ± 7.8[c]
Jul	472 ± 66[bcd]	6.0 ± 2.0[abc]	90.2 ± 7.9[ab]
Time effect	$P < 0.001$	$P < 0.001$	$P < 0.001$

[abcd]Within a column, values with different superscripts differ significantly ($P < 0.05$)

BCS = Body condition score

Figure 2.1

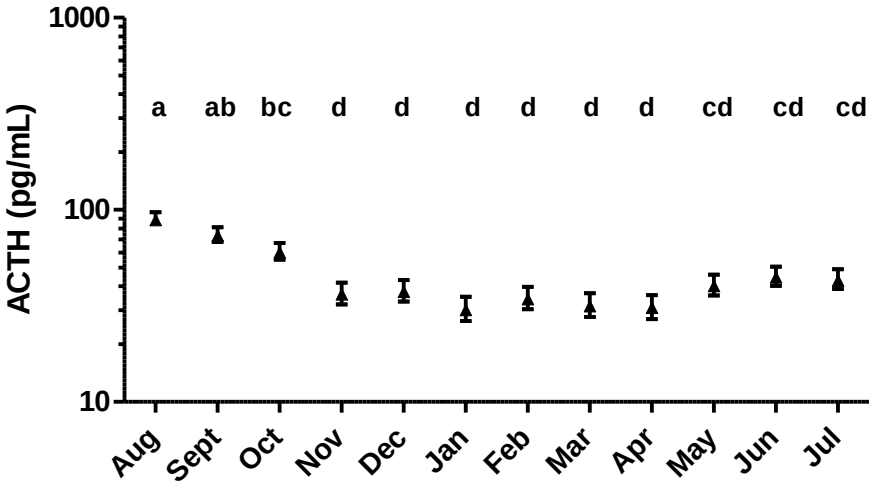


Fig 2.1. Geometric mean (95% confidence interval) plasma adrenocorticotropin hormone (ACTH) concentrations collected after pasture grazing (day 1) for 17 horses across a 12-month sampling period. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale. A significant effect of time ($P < 0.001$) was detected. Letters indicate significant differences among time points.

Figure 2.2

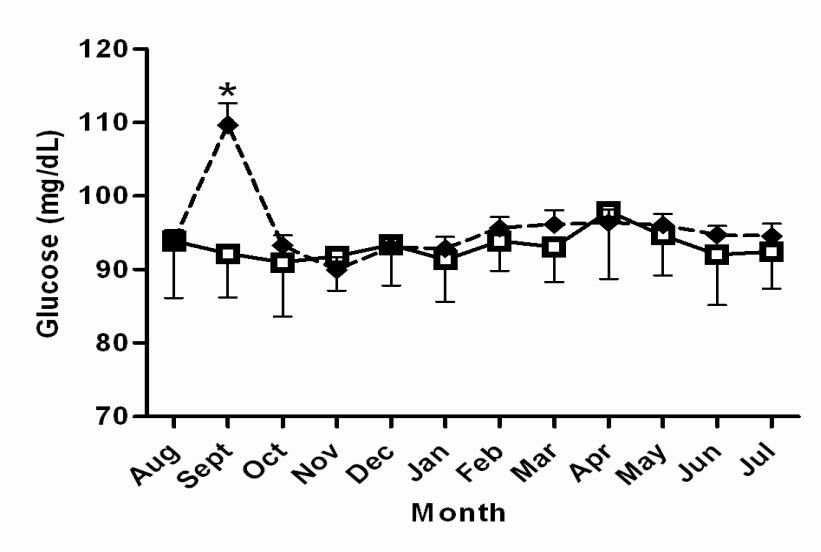


Fig 2.2. Mean ± SD plasma glucose concentrations for 16 horses across a 12-month sampling period. Blood samples were collected immediately after horses were brought in from pasture (dashed line; black diamonds) and then again the next morning after confinement in stalls overnight (solid line; white squares). Pasture ($P < 0.001$), time ($P < 0.001$), and pasture × time ($P < 0.001$) effects were detected, but the group × pasture × time interaction ($P = 0.874$) was not significant. Asterisk indicates that the mean glucose concentration in September on pasture was significantly higher than mean values for all other time points.

Figure 2.3

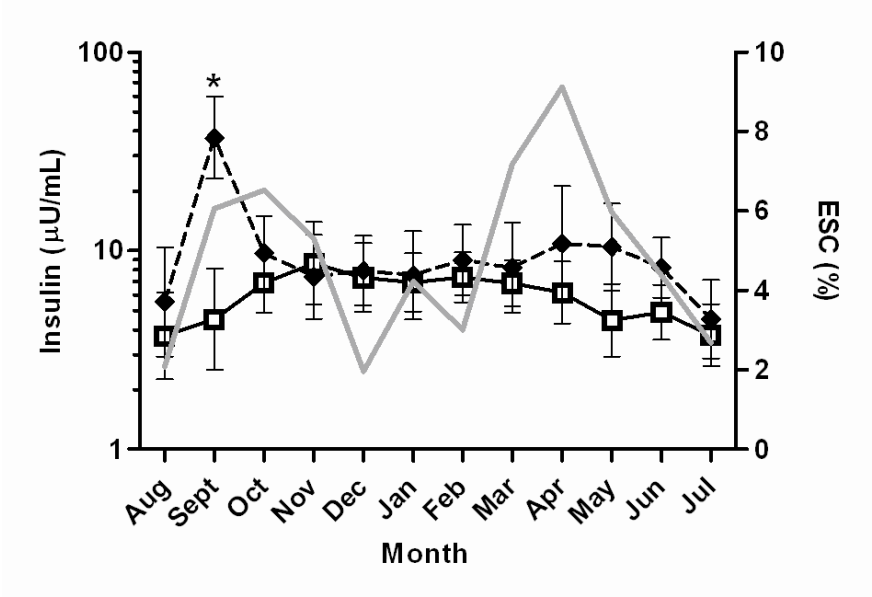


Fig 2.3. Geometric mean (95% confidence interval) serum insulin concentrations for 16 horses across a 12-month sampling period. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale. Blood samples were collected immediately after horses were brought in from pasture (dashed line; black diamonds) and then again the following morning after confinement in stalls overnight (solid line; white squares). Insulin data were log-transformed prior to statistical analysis. Pasture ($P < 0.001$), time ($P < 0.001$), and pasture × time ($P < 0.001$) effects were detected, but the group × pasture × time interaction ($P = 0.962$) was not significant. Ethanol-soluble carbohydrate (ESC) content of the pasture grass is also displayed as a grey line (y axis on the right); values represent percent dry matter content values for pooled grass samples collected every month. A positive correlation ($r = 0.22$; $P = 0.002$) was detected between log insulin concentrations and ESC. Asterisk indicates that the mean insulin concentration in September on pasture was significantly higher than mean values for all other time points.

Figure 2.4

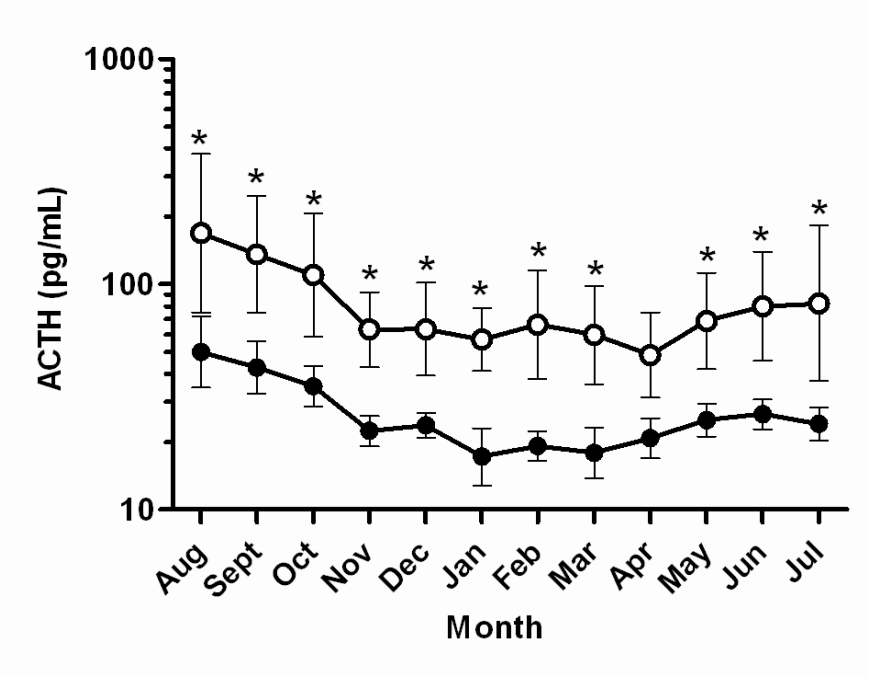
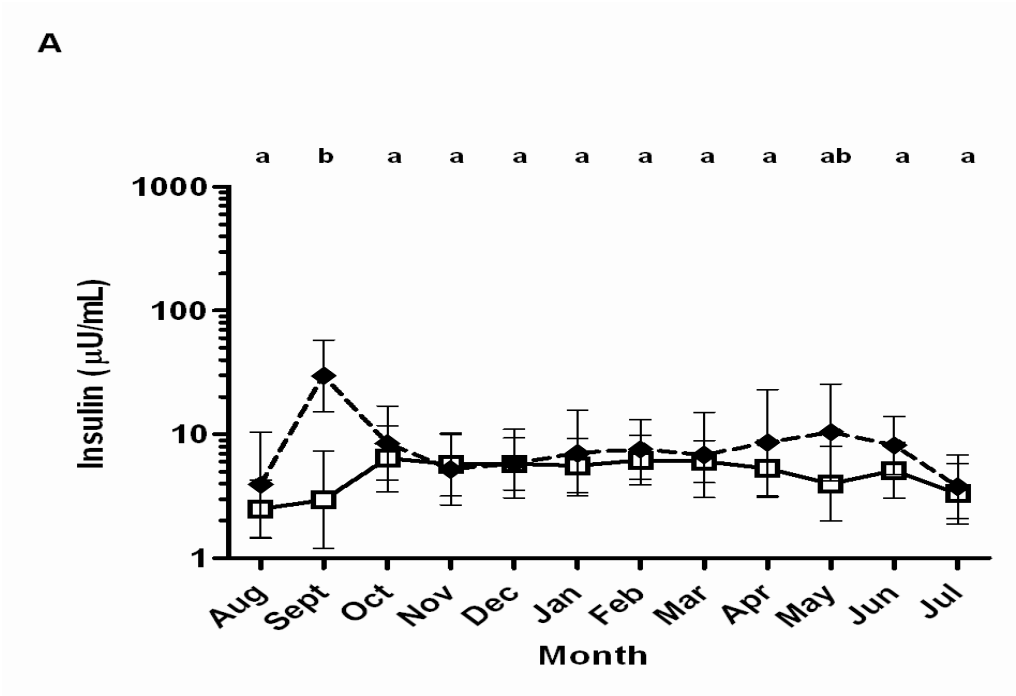


Fig 2.4. Geometric mean (95% confidence interval) plasma adrenocorticotropin hormone (ACTH) concentrations for 8 horses with presumptive pituitary pars intermedia dysfunction (PPID group; white circles) and 9 unaffected horses (control group; black triangles) across a 12-month sampling period. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale. Group ($P < 0.001$) and time ($P < 0.001$) effects were detected, but the group × time interaction ($P = 0.847$) was not significant. Asterisk indicates a significant difference between groups at that time point.

Figure 2.5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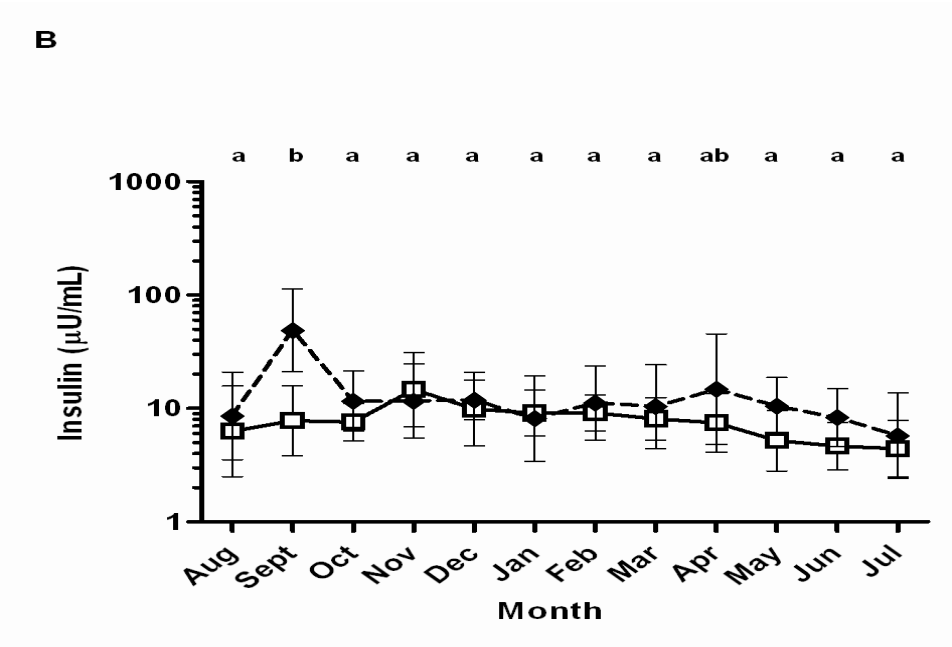
10
1
Insulin (µU/mL)
Aug Sept Oct Nov Dec Jan Feb Mar Apr May Jun Jul
Month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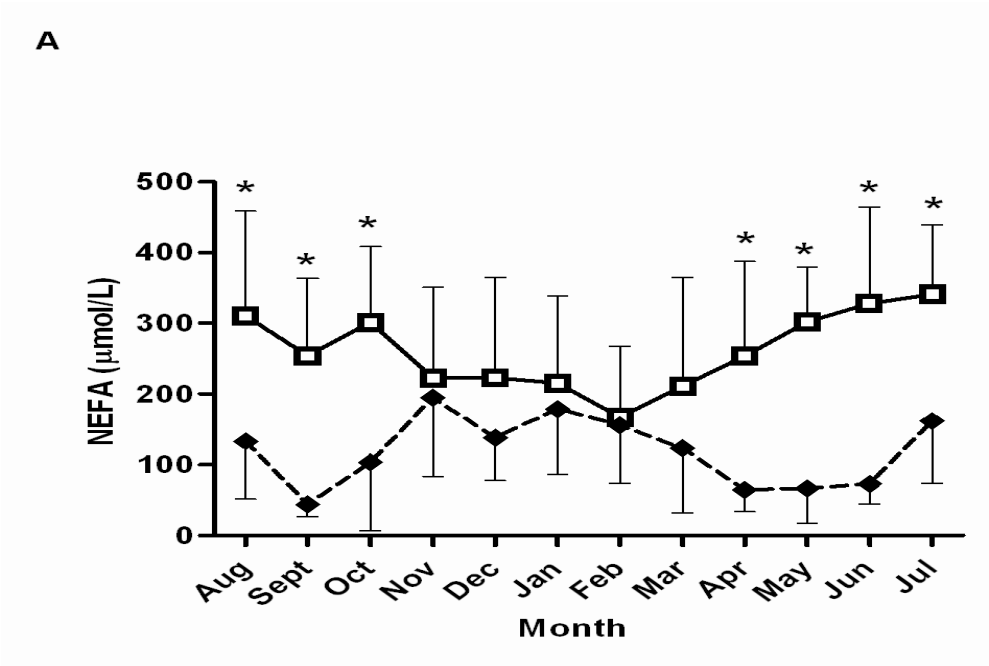
Insulin (µU/mL)
Aug Sept Oct Nov Dec Jan Feb Mar Apr May Jun Jul
Month

Fig 2.5. Geometric mean (95% confidence interval) serum insulin concentrations for 9 unaffected horses (control group; Panel A) and 7 horses with presumptive pituitary pars intermedia dysfunction (PPID group; Panel B) after grazing on pasture (dotted line; black diamonds) or following overnight stall confinement (solid line; white squares). Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale. Group × time ($P = 0.037$) and pasture × time ($P < 0.001$) effects were detected, but the group × pasture × time interaction ($P = 0.784$) was not significant. Letters indicate significant differences among monthly mean values for samples collected after pasture grazing. Mean values after stall confinement did not differ significantly over time.

Figure 2.6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NEFA (µmol/L)
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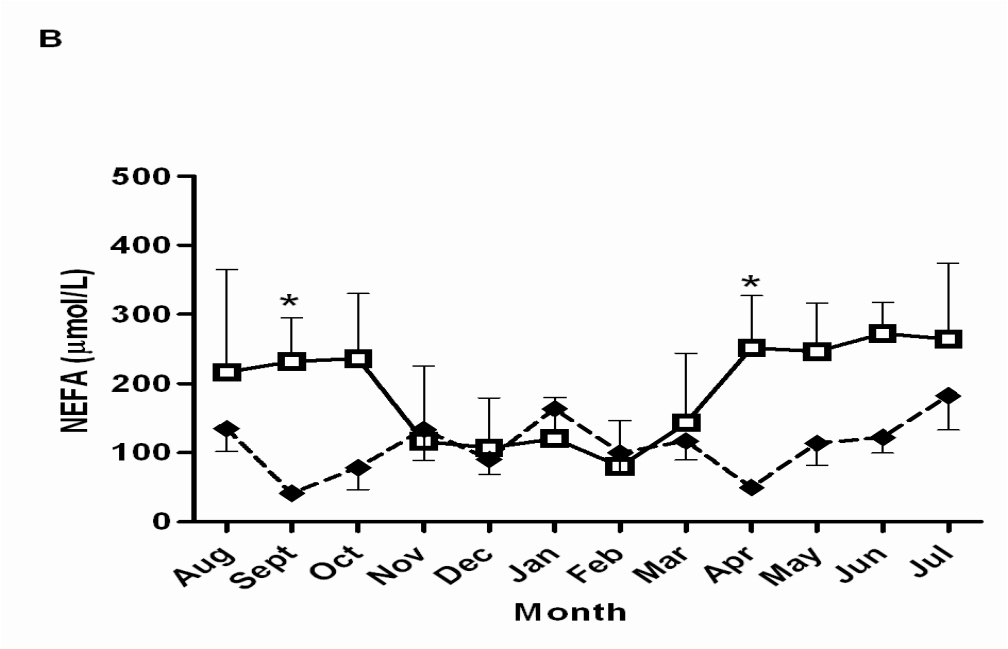
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NEFA (µmol/L)
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Fig 2.6. Mean ± SD plasma non-esterified fatty acid (NEFA) concentrations for 9 unaffected horses (control group; Panel A) and 7 horses with presumptive pituitary pars intermedia dysfunction (PPID group; Panel B) after grazing on pasture (dotted line; black diamonds) or following overnight stall confinement (solid line; white squares). Pasture ($P < 0.001$), time ($P < 0.001$), pasture × time ($P < 0.001$), and group × pasture ($P = 0.004$) effects were detected, but the group × pasture × time interaction ($P = 0.945$) was not significant. Asterisk indicates a significant ($P < 0.05$) difference between means values for stall confinement and pasture conditions.

2.5 Discussion

Horses in this study exhibited up-regulation of the hypothalamic-pituitary-adrenal axis in the late summer and autumn, as evidenced by significantly higher plasma ACTH concentrations. Horses grazing on pasture had higher glucose and insulin concentrations in September and insulin concentrations were positively correlated with carbohydrate composition when horses were grazing on pasture. Pituitary pars intermedia dysfunction did not alter the timing or duration of this seasonal change in ACTH concentrations, although higher concentrations were detected in affected animals. Variation in insulin concentrations over time differed between groups, but hyperinsulinemia was rarely detected when horses were sampled after stall confinement.

A presumptive diagnosis of PPID was made in this study on the basis of plasma ACTH concentrations exceeding 35 pg/mL on three or more occasions outside of the late summer and autumn period. This method was selected because the owner would not permit other diagnostic

tests to be performed because of concerns about inducing laminitis. However, three horses in the PPID group had clinical signs consistent with the disorder, including overt hirsutism and fat redistribution. Other results from the haircoat examinations must be evaluated within the context of the methods used because observations were subjective and some signs, such dullness of the haircoat are nonspecific. Objective criteria for diagnosing haircoat abnormalities attributable to PPID should be employed in future studies to address this deficiency. Hirsutism has been used as a gold standard for PPID in one previous study [95], which supports the allocation of three horses to the PPID group. However, all other horses in the PPID group were allocated on the basis of ACTH concentrations alone, so results should be interpreted accordingly.

Horses with presumed PPID did not differ with respect to the timing or duration of the seasonal alterations in plasma ACTH concentrations, but higher concentrations were detected in this group, and this was most apparent in August, September, and October. Diagnostic testing has been avoided during this time period since Donaldson et al [1] published their finding of elevated ACTH concentrations in September, but warrants re-evaluation in light of our findings. In the future, it might be possible to test horses in the late summer/autumn season if season-specific reference ranges for ACTH are established. Three of 8 horses in the PPID group also had ACTH concentrations that were within reference range on one or more occasion between December and July, which suggests that test accuracy could be improved by collecting more than one blood sample throughout the year.

Adrenocorticotropin hormone was measured using a chemiluminescent immunoassay that has previously been validated for the measurement of ACTH in equine plasma by Perkins et al [95]. A cut-off value of 35 pg/mL has been adopted in previous studies [1, 3, 93], but upper limits of 45 pg/mL [106-107], 50 pg/mL [79], or 70 pg/mL [101] have also been used for diagnosis of

PPID. A seasonal rise in plasma ACTH concentrations was detected in this study when values from August, September, and October were compared with those from December to April, consistent with previous reports [1, 3]. Resting ACTH concentrations were significantly higher in horses during the autumn, compared with the winter and spring. Results of these previous studies and the one reported here indicate that horses undergo up-regulation of the hypothalamic-pituitary-adrenal axis during the late summer and autumn.

Seasonal alterations in ACTH concentrations are likely to be linked to changes in photoperiod, with the reduction in daylight hours triggering alterations in the hypothalamic-pituitary-adrenal axis. Melatonin is thought to play an important role in this process because it has a circadian rhythm of low concentrations during the day followed by higher concentrations at night, and this pattern changes with season as days get shorter [50, 72]. Horses also gain body fat in response to decreasing photoperiod length, presumably in preparation for winter [50]. Seasonal weight gain was observed in this study, although weight tape and neck circumference measurements may have been confounded by the growth of winter haircoats.

Insulin concentrations differed between groups, but hyperinsulinemia (> 20 µU/mL; reference range for laboratory) was rarely observed, except in response to pasture grazing. There was little evidence of reduced insulin sensitivity in this study, but insulin concentrations are affected by alterations in pancreatic output as well as tissue insulin sensitivity, so this situation requires further investigation. Insulin and glucose data from one mare were excluded from statistical analyses because of severe lameness and marked hyperinsulinemia. Interestingly, this mare had a history of obesity, regional adiposity, and laminitis before losing weight and developing hirsutism in recent years. This suggests that she suffered from equine metabolic syndrome [108] prior to the development of PPID, and therefore makes it difficult to determine the cause of hyperinsulinemia in this animal. Results of this study differ from those of

previous reports because PPID has previously been associated with hyperinsulinemia [78, 99, 101, 107]. It should be noted, however, that only horses were examined in the study reported here, whereas PPID groups contained ponies in the previous studies. Blood samples were also collected under fed conditions in the aforementioned studies. These points could be relevant because ponies have lower insulin sensitivity when compared with horses [67, 109], and results of this study indicate that insulin concentrations are affected by feeding conditions.

Higher glucose and insulin concentrations were detected in horses after pasture grazing and were affected by changes in season, with peaks detected in September. Insulin concentrations also increased again in April, but this peak was not statistically significant. A weak correlation existed between mean insulin concentrations and mean ethanol-soluble content of the pasture grass when post-grazing results were examined. Ethanol-soluble carbohydrates include simple sugars [110], so higher glucose and insulin concentrations appeared to correspond with increases in sugar intake on pasture. The ethanol-soluble carbohydrate content of the pasture grass may also reflect its growth, so these may have been times of the year when the grass was more abundant because of increased rainfall and sunlight [111]. Unfortunately, grass intake could not be measured in this study.

There were several statistically significant alterations in blood lipid variables, but only NEFA concentrations followed a recognizable pattern. Higher NEFA concentrations were detected when blood samples were collected on day 2 after horses experienced a period of fasting. This is a normal physiological response to reduced feed intake because negative energy balance stimulates hormone-sensitive lipase and stored triglycerides are hydrolyzed to yield glycerol and fatty acids, which are used for energy [112-113]. A significant group × pasture effect was also detected for this variable, with stall confinement having a greater effect on NEFA concentrations in control horses. The lower response detected in the PPID group was

unexpected because hyperadrenocorticism and IR would be expected to increase hormone-sensitive lipase activity [114]. One explanation for this finding may be that PPID-affected horses remain calmer when deprived of feed, although no differences in demeanor were recognized in this study.

Retrospective studies provide evidence of an association between PPID and laminitis in horses and ponies [79, 93-95], with one report demonstrating that laminitis occurred more commonly in September and May [93]. Laminitis was not detected in the study reported here, but glucose and insulin concentrations peaked in September when plasma ACTH concentrations were elevated. Although not statistically significant, a second insulin peak was also observed in the spring when a higher incidence of pasture-associated laminitis has been reported in ponies [4, 97]. Interestingly, the insulin peak detected in September was higher than the one observed in April, despite similar increases in pasture grass ESC content. This suggests that up-regulation of the hypothalamic-pituitary-adrenal axis accentuated the insulinemic response, although further research is required to examine this relationship. Differences in total grass intake might also explain this discrepancy.

In conclusion, our hypothesis that horses with PPID would respond differently to changes in season was partially supported because changes in insulin concentrations over time were more pronounced in affected horses. However, seasonal changes in ACTH concentrations did not differ significantly in timing or duration between groups. Further studies are required to establish season-specific reference ranges for ACTH and determine whether the magnitude of the seasonal increase in hormone concentrations can serve as a diagnostic test for PPID in horses. Our results also demonstrate that pasture grazing raises glucose and insulin concentrations at specific times of the year in horses. Glucose and insulin

concentrations peaked in September at the same time that ACTH concentrations were elevated, and this convergence of risk factors may be relevant to the development of laminitis.

CHAPTER 3

Effects of season on adrenocorticotropin hormone (ACTH) concentrations in horses with pituitary pars intermedia dysfunction (PPID) from 15 different farms in East Tennessee.

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3.1 Abstract

Background: We have previously reported a seasonal increase in adrenocorticotropin hormone (ACTH) concentrations in horses from a single farm in Tennessee, with significantly higher concentrations detected in September and October. Since all of the horses included in our previous study were from the same farm, this study was undertaken to determine whether seasonal alterations in ACTH concentrations occur in horses from different farms in the area.

Hypothesis: Horses from different locations, with varying diets and husbandry practices, will show the same seasonal changes in hormone concentrations.

Animals: Eighteen horses median aged 19.8 (8-45 years) from 15 different farms

Methods: Longitudinal prospective study. Blood was collected monthly from August 2007 until July 2008. Blood hormone and metabolite concentrations were measured. Analysis of variance analysis for repeated measures was performed.

Results: Horses ranging in age from 8 to 45 years (median; 19.8 years) were retrospectively allocated to PPID (n=12) and healthy (n=6) groups based upon ACTH concentrations measured in the spring. Plasma ACTH concentrations were significantly affected by season (time effect; $P < 0.001$), with a significantly higher mean ± SD concentration (155.5 ± 292.6 pg/mL) detected in August, compared with all other months. There was no effect of season on glucose or insulin concentrations.

Conclusions and clinical importance: Plasma ACTH concentrations were significantly higher in August in healthy aged horses and those with PPID. Disease status did not affect the timing or magnitude of the seasonal peak in ACTH, suggesting that this function is not affected by PPID. Differences among farms with respect to husbandry did not affect seasonal ACTH responses.

3.2 Introduction

Pituitary pars intermedia dysfunction (PPID) is a common disorder in older horses and ponies [68]. This endocrine disorder is thought to develop when there is a loss of dopaminergic inhibition, which leads to an increased rate of melanotrope proliferation [115]. Without dopamine inhibition, more pro-opiomelanocortin (POMC)-derived peptides are synthesized and released and this leads to the development of clinical signs. These peptides include α-melanocyte stimulating hormone (α-MSH), β-endorphin, corticotrophin-like intermediate lobe peptide (CLIP), and adrenocorticotropic hormone (ACTH) [14]. Clinical signs of PPID include hirsutism, muscle wasting, abnormal fat distribution, glucose intolerance, lethargy, infertility, polydipsia and polyuria [116]. Horses with PPID can also suffer from insulin resistance (IR) [96].

Laminitis is a systemic disease that manifest in the foot [117]. According to USDA surveys, laminitis is the second most common cause for a horse or pony to be presented for veterinary care, second only to colic [118]. Horses and ponies with PPID show a greater susceptibility to laminitis and may be at greater risk for developing laminitis if they currently suffer from insulin resistance (IR) [75, 93]. Treiber et al. [97] found that insulin-resistant ponies developed clinical laminitis in the spring after grazing on pasture, and IR predicted the occurrence of laminitis when the same herd was reexamined by Carter et al. [4] two years later. Donaldson et al. [93] reported that laminitis was more common in September and May which suggests that season influences disease susceptibility.

The study of seasonal alterations in blood hormone and metabolite concentrations is important because this knowledge will improve our understanding of equine physiology. It is also important because testing for PPID is currently confusing with normal horses having elevated ACTH concentrations in the autumn months [1].

This study is a continuation of an earlier study in which we discovered that PPID did not affect the timing or duration of the seasonal increase in ACTH concentrations, but higher values were detected in affected horses. These horses were all located on one farm in Kingston, Tennessee.

The purpose of this study is to test the hypothesis that horses from different locations, with varying diets and husbandry practices, will show the same seasonal changes in hormone concentrations.

3.3 Materials and Methods

Animals—Eighteen adult horses (7 mares; 11 geldings) from fifteen different farms located in East Tennessee were included in the study. Ages ranged from 8 to 45 years.

Experimental design—A prospective longitudinal study was performed across a 12-month period beginning in August 2007 to July 2008. Horses were located on fifteen different farms located in East Tennessee. Horses with previous histories of PPID were included, along with control animals from the same farm, where available. Evaluations were performed the first week of each month. Blood samples were collected via jugular venipuncture between 0800 and 1100 for all horses. Horses were brought in from pasture on the morning of each blood sampling, with the feeding of concentrates delayed until after the sampling. Physical measurements were also obtained after blood samples had been collected. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Feeding and management practices—Horses were managed differently among the fifteen farms. Most horses had variations of pasture turnout during cooler times and stall confinement during warmer times of the day or night. All horses received some amount of concentrate during the day. Amounts of exercise also varied among farms. Therapies varied among farms. Briefly, 10 of 18 horses were on levothyroxine treatment, 4 were on Pergolide®, and 2 were on Smartpak™ therapy. Two of the 18 horses wore grazing muzzles when out on pasture, and 1 horse was kept in a dry lot with very limited access to pasture.

Physical measurements—Body weight was measured by weight tape and body condition score assessed using the 1 to 9 scale described by Henneke et al [103]. Neck measurements were taken with the horse being restrained so that the head and neck were in a normal upright position. The distance from the poll to the cranial aspect of the withers was measured. Neck

circumference was then measured perpendicular to this line at 0.25, 0.50, and 0.75 of the distance from the poll to the withers with a measuring tape (Figure 3.1) [104].

Blood variables—Blood was collected into tubes containing potassium EDTA, sodium heparin, or no anticoagulant. Tubes for plasma collection were placed immediately on ice to chill, and tubes for serum collection were left at ambient temperature to clot. After transportation to the laboratory, plasma and serum were harvested by low-speed (1,000 × *g*) centrifugation within 2 hours of collection and then stored at –80 ºC until further analysis. Serum insulin concentrations were measured using a radioimmunoassay kit[1] previously validated for equine sera [105]; an intra-assay coefficient of variation of <10% was required for acceptance of result. Plasma glucose concentrations were measured using a colorimetric assay[2] and an automated discrete analyzer[3], an intra-assay coefficient of variation of <5% was required for acceptance of results. Frozen plasma samples were shipped overnight with ice packs to the Animal Health Diagnostic Center at Cornell University[4] for measurement of ACTH concentrations by chemiluminescent assay.

Statistical Analysis—Data was analyzed by mixed model ANOVA for repeated measures using statistical software[5] to determine the effects of group (PPID versus control) and time (month). Shapiro-Wilks test were examined for normality. Husbandry practices were not included as a main effect because of the small number of horses at each location. Adrenocorticotropic hormone and insulin data required $\log_{10}$-transformation to fit normal data,

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[1]Coat-A-Count insulin radioimmunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA
[2] Glucose, Roche Diagnostic Systems Inc, Somerville, NJ
[3] Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ
[4] Animal Health Diagnostic Center, Cornell University, Ithaca, NY
[5] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC

and mean ± SD values for untransformed data are reported. The Bonferroni test for multiple comparisons was used to compare least squares means if the model was significant. Significance was defined at a value of $P < 0.05$.

3.4 Results

Horses were retrospectively allocated to PPID (n=12) and control (n=6) groups on the basis of ACTH concentrations measured in the spring. Of the 12 horses included in the PPID group, 4 did not have normal ACTH concentrations (<35 pg/mL) throughout the study, 1 had normal ACTH concentrations on one occasion, 2 had normal ACTH concentrations on 3 occasions, 2 had normal ACTH concentrations on 5 occasions, 2 had normal ACTH concentrations on 6 occasions, and one horse had normal ACTH concentrations on 7 occasions. Horses in the PPID group ranged in age from 9 to 45 years (median=21 years), and horses in the control group ranged in age from 8 to 27 (median=17.5 years). The PPID group contained 4 mares and 8 geldings and the control group contained 3 mares and 3 geldings. Breeds represented in the PPID group included Arabian (n=6), Morgan (2), Quarter Horse (1), Tennessee Walking Horse (1), Arabian/Appaloosa (1), and mixed breed (1). Breeds represented in the control group included Thoroughbred (n=2), Tennessee Walking Horse/Paso Fino (1), Pony (1), Tennessee Walking Horse (1), and Percheron/Quarter Horse (1). Average initial body weights for each group (mean ± SD) were PPID (442 ± 75 kg) and control (483 ± 41 kg). There was no effect of group on body weight ($P = 0.240$). A time effect ($P = 0.013$) was detected for body weight with horses weighing the most in November (weight ± SEM , 467 ± 17 kg) and the least in June (444 ± 14 kg). None of the other physical measurements changed significantly over time.

Most horses had access to pasture throughout the study. One horse in the PPID group developed laminitis during the month of March and remained on stall confinement for the duration of the study. There were no other health problems reported across the 12-month period.

Effects of group ($P = 0.020$) and time ($P < 0.001$) were significant for plasma ACTH concentrations, but PPID did not alter the timing or magnitude of these changes (group × time; $P = 0.497$). Horses in the control group had mean plasma ACTH concentrations that exceeded 35 pg/mL in July, August, and September (Figure 3.2). Each control horse had a plasma ACTH concentration >35 pg/mL detected on at least one occasion in July, August, September, or October. Ten of eleven PPID horses had plasma ACTH concentrations >35 pg/mL detected August, September, and November. Ten of twelve PPID horses had plasma ACTH concentrations >35 pg/mL in December. A 12th horse was added to the study in December. In January, 11 of 12 PPID horses still remained over the limit for normal ACTH values. Maximum ACTH values reached were 1,250 pg/mL (upper limit of detection) and 89.6 pg/mL for PPID and control groups, respectively. Effects of group ($P = 0.907$) and time ($P = 0.874$) were not significant for serum insulin concentrations.

Figure 3.1

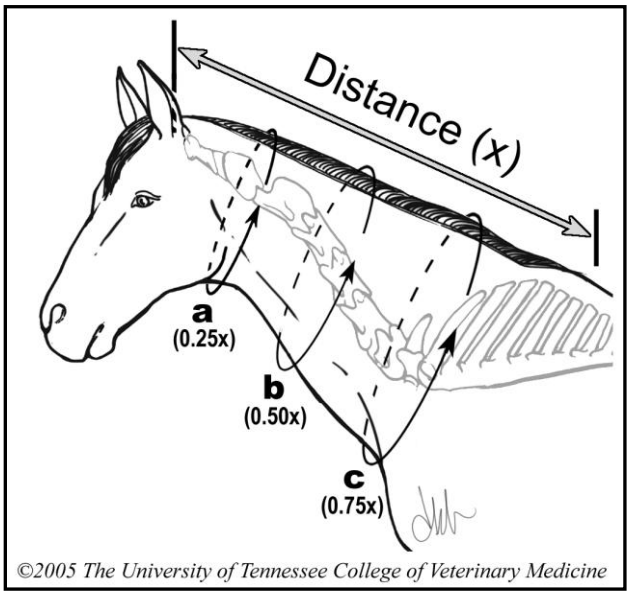

Fig 3.1. Illustration of a procedure used to measure neck circumference in horses, a = 0.25 of the distance from poll to withers, b = 0.50 of the distance from poll to withers, c = 0.75 of the distance from poll to withers. Reprinted with permission.

Figure 3.2

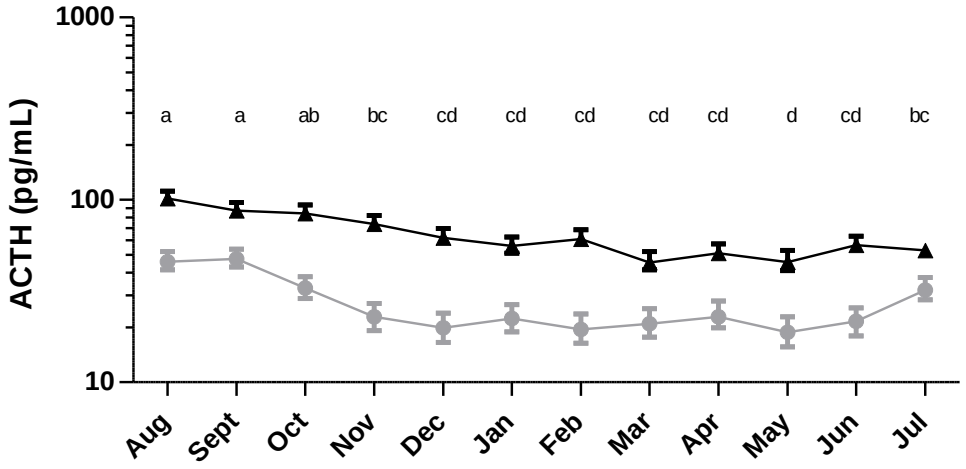

Fig 3.2. Geometric mean (95% confidence intervals) plasma adrenocorticotropin hormone (ACTH) concentrations in 12 horses with pituitary pars intermedia dysfunction (PPID group; black triangles) and 6 healthy horses (control group; gray circles) across a 12-month sampling period. Data were log-transformed prior to analysis, and displayed on a logarithmic scale. Group ($P = 0.02$) and time ($P < 0.001$) effects were detected, but the group × time interaction ($P = 0.497$) was not significant. For PPID and control groups combined, letters with different superscripts indicate significant differences among time points.

3.5 Discussion

Results of this study support our previous findings that ACTH concentrations increase in the late summer in both healthy and PPID affected horses. We also demonstrated that this response occurs independently of location or husbandry practices.

Results of this study indicate that false positives results will be obtained if plasma ACTH concentrations are measured in August to diagnose PPID. Beech et al. have investigated the possibility of using α-MSH concentrations as an alternative test for PPID [88], but this hormone has the same seasonal fluctuations as ACTH. It has been proposed that the seasonal increase in ACTH and α-MSH results from the loss of hypothalamic dopaminergic inhibitory control [11]. Dopamine production is lower in horses with PPID, and this is the reason for increased ACTH concentrations [119]. However, seasonal alterations in ACTH concentrations were still observed in horses with PPID, suggesting dopamine is not the principal hormone regulating this response [88].

Leptin is an adipokine that plays a major role in the negative control of feeding [38]. It acts on both the pars distalis and the pars intermedia to regulate corticotrophin-releasing hormone and ACTH secretion [38]. Adrenocorticotropic hormone, α-MSH, and β-MSH inhibit food intake when administered to the brain in rodents [14]. In humans, a defect in N-terminal acetylation of desacetyl-α-MSH could contribute to the development of obesity. This acetylation reaction is regulated by leptin and dopamine, two hormones involved in energy homeostasis [14]. Seasonal changes in leptin have been reported in many species [38, 43-44], with leptin resistance occurring in the autumn. Leptin resistance results from a reduction in the density or activity of leptin receptors within the hypothalamus [45] and may explain why horses gain weight in the late summer and autumn. Higher ACTH concentrations in August might therefore be the result of leptin resistance.

Insulin concentrations were not affected by season in this study. This finding is consistent with other seasonal studies [2, 88], but differs from results of our previous study, in which we detected higher fed insulin concentrations in the spring and fall. The most likely possibility for this discrepancy is differences in owner care of each horse. Ten of the horses in this study were on levothyroxine supplements. Treatment with levothyroxine has been shown to increase insulin sensitivity [120], possibly explaining why we detected no effect of season on insulin concentrations in this study. Some of the horses in this study wore grazing muzzles or were kept on a dry lot; limited access to pasture grass would affect changes in insulin concentrations. In our previous study, none of the horses were on therapies so insulin concentrations were not being controlled.

In conclusion, this study confirms that healthy and PPID affected horses' exhibit seasonal changes in ACTH concentrations regardless of location, diet, or husbandry practices. Further studies are required to determine whether leptin resistance plays a role in this process. When testing for PPID, many considerations need to be taken, blood samples should be collected outside of the July to October period, or season-specific reference ranges should be established. Results of this study suggest that the reference range for ACTH should be widened to 100pg/mL in the late summer and autumn.

CHAPTER 4

Association of season with plasma leptin concentrations in horses

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4.1 Abstract

Background: We have previously reported a seasonal increase in adrenocorticotropin hormone (ACTH) concentrations in horses in Tennessee, with significantly higher concentrations detected in September and October. Metabolic hormones such as leptin signal seasonal reproductive activity in mares, so changes in the blood concentrations of this adipokine may trigger the seasonal increase in ACTH.

Hypothesis: Plasma leptin concentrations will increase in the late summer prior to the seasonal increase in ACTH. It was further hypothesized that leptin concentrations differ significantly between horses with PPID and unaffected animals.

Animals: Seventeen horses from one location in Kingston, Tennessee

Methods: Longitudinal prospective study. Blood was collected monthly from August 2007 until July 2008. Blood hormone and metabolite concentrations were measured. Mixed model analysis of variance was performed.

Results: Mean leptin concentrations varied significantly over time ($P < 0.001$) with lower concentrations detected in July compared with November. Leptin and ACTH concentrations

followed an opposite seasonal pattern. Leptin and insulin concentrations were positively correlated. Horses were retrospectively allocated to PPID (n = 8) and control (n = 9) groups on the basis of plasma ACTH concentrations, and group x time effects were not significant. Conclusions and clinical importance: Leptin concentrations peaked after the seasonal increase in plasma ACTH concentrations, so our hypothesis was not supported. There was also no effect of PPID on leptin concentrations. Results suggest that ACTH suppresses leptin concentrations in horses, but further studies are required to determine cause-and-effect relationships.

4.2 Introduction

Leptin is an adipokine released by adipocytes [74] that acts with dopamine to regulate energy homeostasis via the acetylation of desacetyl-alpha melanocyte stimulating hormone to active alpha melanocyte-stimulating hormone (α-MSH) within the pituitary [14]. Endogenous α-MSH is critical for maintaining body weight in mice [14]. Leptin and α-MSH are anorexigenic hormones that act at the hypothalamic level to reduce appetite [121]. In many animals, including horses, body fat mass is positively correlated with circulating leptin concentrations [33, 46, 50, 122].

Leptin also signals reproductive cues in many species that follow seasonal breeding cycle [47, 50]. It has also been noted that higher concentrations of leptin are associated with continued reproductive activity throughout the winter in aged mares [50]. Leptin receptors are found within the hypothalamus, pituitary and pineal gland in cattle [123] and this suggests that leptin and melatonin coordinate appetite regulation and reproduction across different seasons.

Studies of seasonal breeding mammals have shown that leptin concentrations increase with long photoperiods and decrease with short photoperiods [47, 50, 124]. It has also been

suggested that leptin resistance develops in the autumn as a result of receptor resistance, post-receptor transduction failure, or decreased transport of leptin across the blood-brain barrier [47].

The purpose of this study was to investigate relationships between plasma adrenocorticotropin hormone (ACTH), leptin, and insulin concentrations in horses. We have previously reported a seasonal increase in ACTH concentrations in horses in Tennessee, with significantly higher concentrations detected in September and October. Since leptin signals seasonal reproductive activity in mares, we hypothesized that plasma leptin concentrations will increase in the late summer prior to the seasonal increase in ACTH. It was further hypothesized that leptin concentrations differ significantly between horses with PPID and unaffected animals.

4.3 Materials and Methods

Animals—Seventeen adult horses (9 mares; 8 geldings) ranging in age from 8 to 30 years (mean age 22.8 years) were included in the study.

Experimental design—A prospective longitudinal study was performed across a 12-month period beginning in August 2007 to July 2008. Horses were located on a single location in southeastern region of the United States, Kingston, Tennessee. Evaluations were performed the first week of each month. Blood samples were collected on two consecutive days via jugular venipuncture between 0800 and 1100 for all horses. Physical measurements were also obtained after blood samples had been collected. Horses were turned out to pasture, then returned to their stalls between 1800 and 1900 each night, two flakes of hay were given each horse until the next morning. Blood samples were collected the following morning, horses were fed their concentrates and returned to pasture. Only blood samples collected on the second day

after overnight stall confinement were used in this study. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Physical measurements—Body weight was measured by weight tape and the body condition score was assessed using the 1 to 9 scale described by Henneke et al [103]. Neck measurements were obtained as previously described in Chapter 3.

Plasma leptin concentrations—Leptin was measured in plasma collected on Day 2 (fasted conditions) using a radioimmunoassay kit[1] previously validated for equine serum or plasma. An intra-assay coefficient of variation of <10% was required for acceptance of results. Please refer to Chapter 2 for descriptions of other metabolite analyses.

Statistical Analysis—Data were analyzed by mixed model ANOVA for repeated measures using statistical software[2] to determine effects of group (PPID versus control) and time (month) on measured variables. Shapiro-Wilks tests were performed to determine normality. Adrenocorticotropic hormone, insulin, and leptin data required $\log_{10}$-transformation to fit normal distributions. Geometric mean values with 95% confidence intervals are displayed. The Bonferroni test for multiple comparisons was used to compare least squares means if main effects were significant. Spearman correlation coefficients were calculated for blood concentrations. Significance was defined at a value of $P < 0.05$.

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[1] Multi-Species Leptin RIA kit, Millipore Corporation, Billerica, MA
[2] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC

4.4 Results

Horses were retrospectively allocated to PPID (n = 8) and control (n = 9) groups on the basis of plasma ACTH results. Plasma ACTH concentrations exceeding 35 pg/mL on ≥ 3 occasions between December and June determined the PPID diagnosis. Initial mean ± SD body weight was 449 ± 88 kg for the PPID group and 493 ± 51 kg for the control group. Time effects were significant for body weight ($P < .001$), mid neck circumference ($P < .001$), and BCS ($P < .001$). Body weight was the highest from November to February and lowest in June. The highest mid-neck circumference was detected in October and the lowest value was recorded in June. Body condition did not follow a discernable pattern.

Adrenocorticotropic hormone, insulin, and glucose values were reported in Chapter 2. Mean Day 2 plasma leptin concentrations were significantly ($P < 0.001$) affected by time, with the highest mean value detected in November and the lowest mean value in July (Figure 4.1). There was no significant correlation between ACTH and leptin concentrations ($r_s = 0.017$; $P < 0.806$; Figure 4.2), but concentrations appeared to mirror one another. There was no significant group × time effect ($P = 0.770$) detected for any of the variables examined (Figure 4.3). Insulin and leptin concentrations were positively correlated ($r_s = 0.52$; $P < 0.001$; Figure 4.4). Leptin concentrations were also positively correlated with BCS ($r_s = 0.34$; $P < 0.001$) and blood glucose ($r_s = 0.27$; $P < 0.001$) concentrations.

Figure 4.1

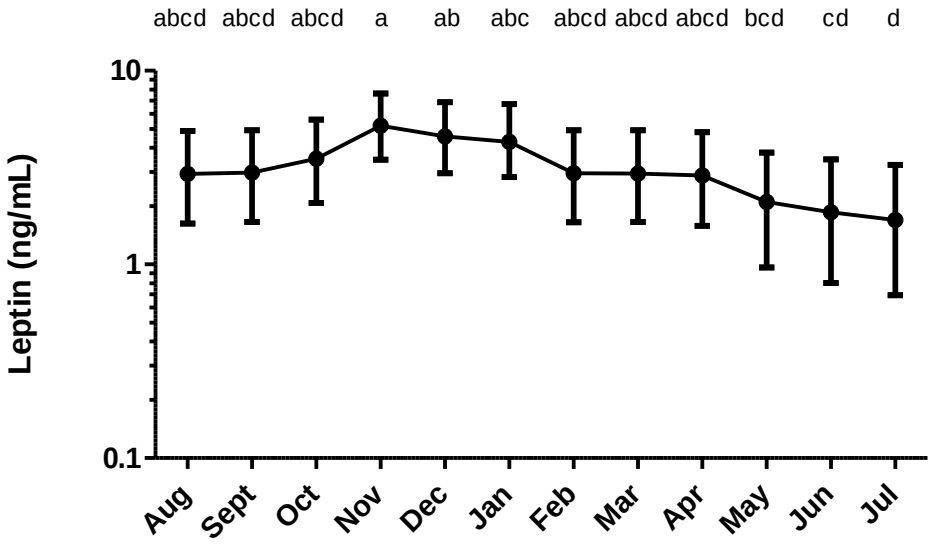

Fig. 4.1 Geometric mean (95% confidence intervals) plasma leptin concentrations for 17 horses across a 12-month period. Blood was collected between 0900 and 1000 after horses were confined in stalls overnight. Grass hay was fed the night before, but no other feed was provided until after samples were collected. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale. A significant time effect ($P < .001$) was detected. Letters indicate significant differences among time points.

Figure 4.2

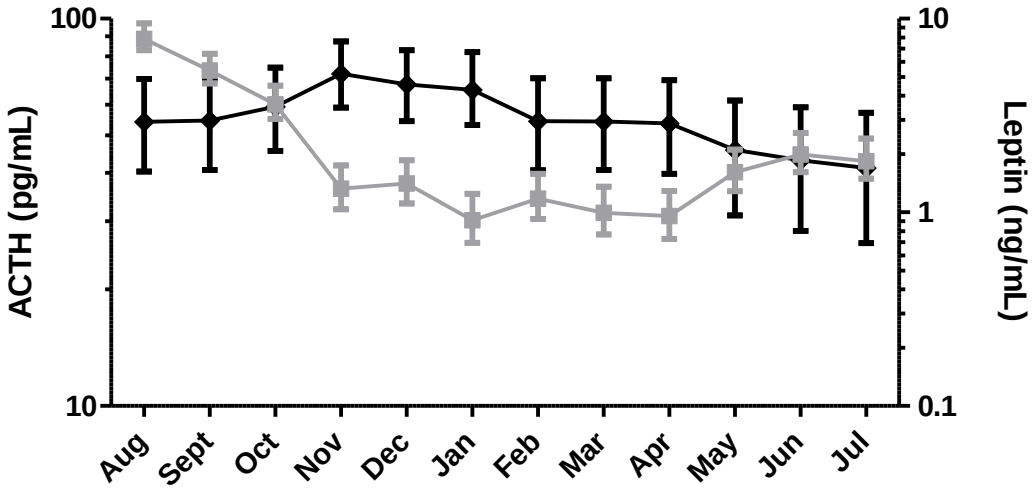

Fig 4.2 Geometric mean (95% confidence intervals) plasma adrenocorticotropin hormone (ACTH; gray squares) and leptin (black diamonds) concentrations across a 12-month sampling period in 17 horses. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale.

Figure 4.3

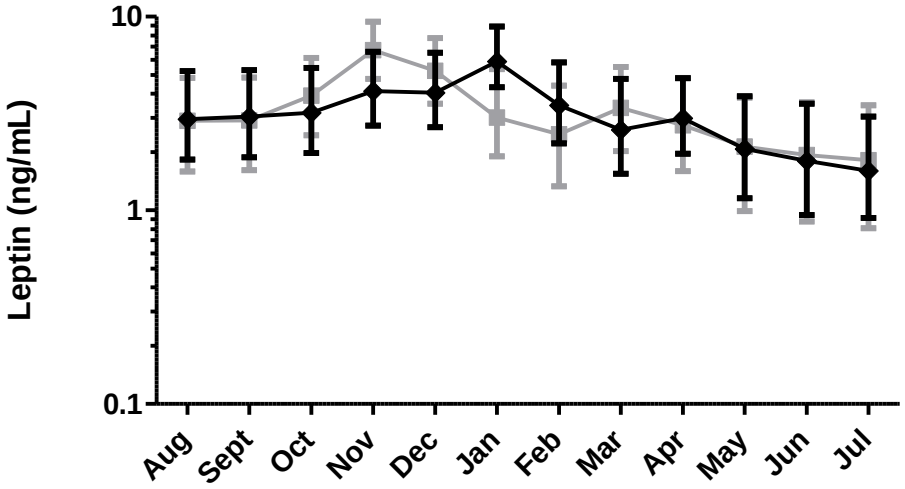

Fig 4.3 Geometric mean (95% confidence interval) plasma leptin concentrations for 9 unaffected horses (control group; black diamonds) and 8 horses with presumptive pituitary pars intermedia dysfunction (PPID group; grey squares) across a 12-month sampling period. Time ($P < 0.001$) effect was detected, but group ($P = 0.948$) and group × time ($P = 0.770$) effects were not significant. Data were log-transformed prior to statistical analysis and are displayed on a logarithmic scale.

Figure 4.4

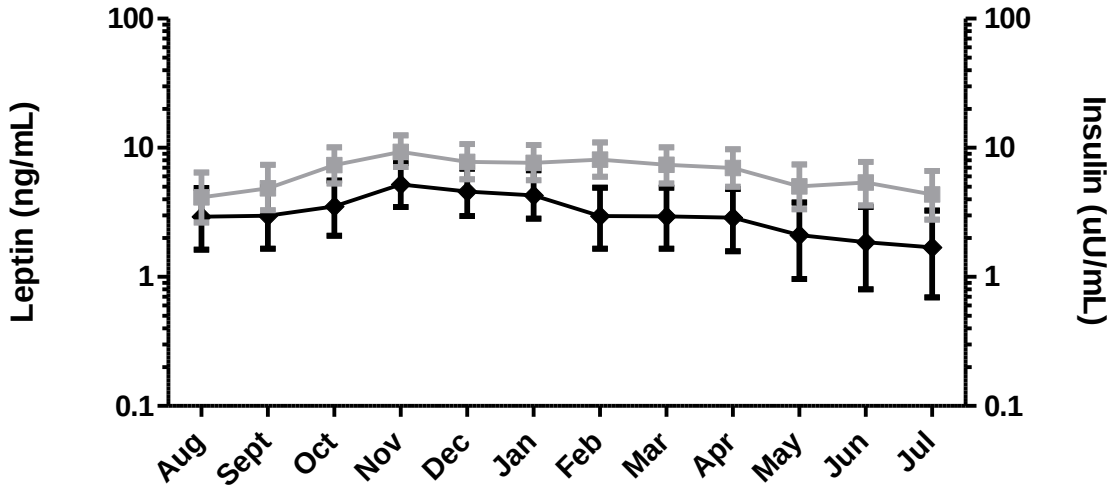

Fig 4.4 Geometric mean (95% confidence interval) serum insulin concentrations (grey squares) and plasma leptin concentrations (black diamonds) in 17 horses across a 12-month sampling period. A positive correlation ($r_s = 0.52$; $P < 0.001$) was detected between leptin and insulin concentrations.

4.5 Discussion

Plasma ACTH and leptin concentrations appeared to mirror one another in this study. Horses had lower leptin concentrations during long photoperiods and then higher concentrations as photoperiod shortened, with the highest mean concentration in November. Leptin was positively correlated with insulin, body condition score, and glucose concentrations. Mean neck circumference was highest in October and lowest in June, and these time points both fall one before the highest and lowest leptin concentrations were recorded, respectively.

Leptin concentrations increase in response to insulin in human adipocytes *in vitro* [125] and *in vivo* [126]. High leptin concentrations have also been detected in horses with elevated insulin concentrations in previous studies [51, 104]. A positive correlation between insulin and leptin concentrations was detected in this study, which included horses with PPID. There was no effect of PPID on resting leptin concentrations or responses over time. Leptin concentrations were positively correlated with body condition score in this study, and this is consistent with findings in other species [46, 127-128].

In humans, increased visceral fat deposition has been associated with higher plasma leptin concentrations [42]. One interesting finding in the current study is that the highest and lowest mid-neck circumference values were recorded one month prior to maximum and minimum mean plasma leptin concentrations, respectively. However, one potential confounder factor is the increased hair growth that occurs in the autumn, which would result in higher circumference values. It has recently been shown that adipose tissue from the neck of horses is more metabolically active than other fat depots [4], so additional studies are required to assess this situation.

Leptin resistance has been observed in sheep, Siberian hamsters, and humans [12, 44-45]. This process is thought to occur in the late summer as the hypothalamus-pituitary-adrenal axis up-regulates in preparation for winter. Suppressed responses to leptin allow increased feed intake and weight gain before the winter when food becomes scarce, and resistance is accompanied by higher plasma leptin concentrations. There was no support for our hypothesis that leptin resistance would develop before or during the seasonal ACTH increase in horses. Leptin concentrations peaked in November, compared to September and October for ACTH. Our results in older horses suggest ACTH is a key factor increasing appetite in preparation for winter.

Cortisol and ACTH are inversely related to leptin in humans [129] and this was observed in the study reported here. Lower leptin concentrations were detected in horses during the summer, which differs from results of a previous study [47]. Zieba et al. [47] concluded that leptin resistance occurs during longer days because leptin is less stimulatory during the summer. The age of horses included in this study might also have affected results. Fitzgerald and McManus [50] reported that seasonal anestrus is delayed in older mares (> 10 years of age) and this response is mediated by leptin. Horses in the study reported here had a mean age of 22.8 years, with 14 of 17 horses being ≥ 20 years old. If the signal for leptin to induce seasonal anestrus is diminished in aged mares, leptin secretion may be more responsive to inhibition by ACTH.

This is the first study to examine the effects of PPID on leptin concentrations in horses. Considering the inverse relationship between ACTH and leptin in humans, lower plasma leptin concentrations were expected in horses with PPID. In contrast, leptin activates α-MSH synthesis and secretion to decrease appetite [14]. Beech et al. [88] found PPID affected horses and ponies had significantly higher α-MSH when compared to the control group of horses and ponies, during all seasons. If PPID horses have higher circulating α-MSH, than it is conceivable that leptin concentrations would decrease to compensate. This study did not support either theory because leptin concentrations did not differ significantly between groups.

In conclusion, plasma leptin concentrations were affected by season in older horses, with the highest concentrations detected in November and a nadir observed in July. Leptin concentrations followed an opposite seasonal pattern to ACTH. Pituitary pars intermedia dysfunction did not affect resting leptin concentrations or responses to changes in season. More research is required to understand associations between ACTH and leptin in horses.

CHAPTER 5

General Summary and Future Directions

Studies reported here focus upon associations among season, diet, and pituitary pars intermedia dysfunction (PPID) and blood concentrations of adrenocorticotropic hormone (ACTH), insulin, glucose, and leptin in horses. Testing horses for PPID is problematic due to the seasonal variation in blood hormone concentrations and stimulation test responses. Understanding associations between the animal and its environment might improve diagnostic testing and increase our understanding of the disorder.

Our results provide evidence that horses affected with PPID elicit the same seasonal upregulation of the hypothalamus-pituitary-adrenal axis as healthy horses, regardless of diet, and husbandry habits. A total of 35 horses were included in this project, with 20 horses affected by PPID. Results show that PPID had no effect on timing or duration of the ACTH peak concentrations seen in the late summer and early fall. Horses with PPID did however, have higher resting ACTH concentrations when compared to the healthy horses. This finding led to the conclusion that horses could be tested during autumn months with the proper reference range (> 100 pg/mL) used for diagnosis.

Pasture grazing had significant effects on serum glucose and insulin concentrations across different seasons. This is the first study to investigate the specific effects of pasture grazing and season on horses affect with PPID. In our first study, horses were housed on pasture continuously, with no grazing restrictions or supplements administered. A positive correlation was detected between serum insulin concentrations and the ethanol-soluble

carbohydrate content of pasture grass. Horses also displayed a seasonal response to diet, with a peak in glucose and insulin concentrations observed in September. There was a second lower peak in serum insulin concentrations observed in the spring when the ESC content of the grass increased. This peak may have been lower than the one detected in September because less grass was consumed. Unfortunately, we were unable to measure grass intake in our study.

In our second study, horses were located on many different farms and had varying amounts of pasture turnout and exercise. Also, 10 of 18 horses were receiving levothyroxine, a known therapy to improve insulin sensitivity [120]. Our horses from this study did not have a significant seasonal increase in insulin concentrations when grazing on pasture, as seen in the previous study. These findings indicate that an insulin response to grazing is less likely to occur with turnout restrictions and levothyroxine treatment.

One important finding was that hyperinsulinemia was rarely detected in horses with PPID, and only after pasture grazing. It has been previously reported that horses with PPID have high resting insulin concentrations [78-79]. It can be hypothesized that diet-induced hyperinsulinemia, exacerbated by pasture grass carbohydrates, combined with higher ACTH concentrations seen in horses with PPID, increases the risk of laminitis. Our present study did not support this hypothesis, because we did not see a significant difference in insulin responses to pasture grazing and season between horses with PPID and unaffected animals. One explanation for the difference is that horses with PPID in previous studies may have suffered from equine metabolic syndrome prior to the development of PPID. A prospective study is needed to support this theory.

Horses affected with PPID in our research studies had higher NEFA concentrations compared to healthy horses. Melanocortin-2 receptors on adipose tissue allow circulating ACTH to bind, thus triggering lipolysis [19]and the release of free fatty acids. Our findings of

higher NEFA concentrations in horses affected with PPID suggest that higher plasma ACTH concentrations in these horses could be triggering lipolysis. This may be an area for further investigation in the future.

In our third study, we hypothesized that horses would develop leptin resistance in the late summer, triggering increased appetite due to the loss of negative feedback from leptin. Higher plasma leptin concentrations were expected in the late summer. Our results showed that leptin concentrations depressed in late summer, followed by a steady increase with a peak in November. This peak could be attributed to increased body mass, and therefore increased leptin production. Adrenocorticotropic hormone increased prior to the increase in leptin, and may be the trigger to increase appetite in horses or alter body fat composition. Future studies are needed to investigate the role of leptin and ACTH in the seasonal upregulation of appetite and body fat mass in horses.

We also hypothesized that leptin concentrations would differ between healthy horses and those affected by PPID. Adrenocorticotropic hormone inhibits leptin secretion from the adipocytes, and horses with PPID have higher circulating ACTH concentrations. One would therefore expect horses with PPID to have lower concentrations of leptin. Leptin concentrations were statistically the same in the two groups, so this hypothesis was not supported. Both groups also displayed the same seasonal pattern in leptin secretion.

One potential reason for us failing to detect differences between groups was the cut-off value used to diagnosis PPID. Many studies have reported cut-off values of 35 pg/mL [1, 3, 93], 45 pg/mL [106-107], 50 pg/mL [79], and 70 pg/mL [101] . It is possible that allocating horses to different groups on the basis of higher cut-off values may have revealed more differences.

In summary, preparation for winter appears cued by season in horses and involves increased ACTH concentrations. This upregulation is retained in horses with PPID, a disorder

associated with loss of dopaminergic inhibition to the pars intermedia of the pituitary. The seasonal rise in plasma ACTH concentrations is followed by an increase in leptin concentrations, which suggests an increase in adiposity or the development of leptin resistance.

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