ABSTRACT OF THE DISSERTATION

Effects of Age and Training on the Cytokine, Myokine and Endocrine Regulators of Glucose Metabolism in Standardbred Mares

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Age adversely affects the hypothalamic-pituitary-adrenal axis (HPAA) and glucose metabolism in horses. Changes may be attenuated with exercise training, but mechanisms behind these phenomena are unknown. Six old (22.0 ± 0.7 yrs; mean ± SE) and six young (7.3 ± 0.6 yrs) healthy, unfit Standardbred mares were utilized to test several hypotheses: 1) the HPAA response, plasma insulin and glucose concentrations differ in old and young mares during acute, exhaustive exercise vs. recovery, and differences are altered after exercise training; 2) aging and training alter cortisol, ACTH and glucose responses to endocrine stimulation tests; 3) old and young mares have different endocrine responses to a frequently sampled intravenous glucose tolerance test along with different cytokine profiles in blood, adipose and muscle tissues; and 4) exercise training would alter endocrine and cytokine profiles. A separate pilot study tested the hypothesis that equine blood, muscle and adipose tissue harbor different quantities of cytokine mRNA, and young horses (≤10 yrs) have different cytokine profiles in these tissues and blood compared to old (≥20 yrs) horses. Mares ran three
graded exercise tests, one pre-training, one after 8 weeks of training at 60% maximum heart rate, and the third at the conclusion of the study at 15 weeks. Training appears to condition the HPAA to a lesser response to acute, exhaustive exercise in both age groups. Old and young mares improved insulin sensitivity and pancreatic beta cell function after training, and there were significant differences in tissue cytokine profiles. In the pilot study, novel data demonstrated varied cytokine profiles in blood, adipose (abdominal and subcutaneous) and muscle tissues in horses of mixed breeds and backgrounds, but no age differences were noted. In summary, altered hormone and glucose concentrations in aged animals may adversely affect the ability to maintain and recover from strenuous exercise. Training was successful in partially reversing these changes. Exact mechanisms for the changes remain to be elucidated, but evidence presented here demonstrates that changes occur at multiple levels of the HPAA and involve factors including hormones, glucose and inflammatory cytokines.
Acknowledgements and Dedication

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I wish to dedicate this dissertation to the horses of the world, may you all live a safe, healthy and happy life, and I thank you for moving me to pursue my goals. To Something, Rummy, Cocoa, Emmy and ET, you are my inspiration!
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REVIEW OF LITERATURE
This study was funded by the New Jersey Agricultural Experiment Station, and the New Jersey State Equine Initiative. The aim was to further understand the effects of aging and exercise training on endocrine response, tissue cytokine profile and glucose homeostasis in Standardbred mares.

Introduction

Age has been shown to disrupt endocrine and cytokine functions involved in the control of the hypothalamic-pituitary-adrenal axis (HPAA) and whole body energy balance. Exercise training may attenuate some of these changes (Horohov et al., 1999; Malinowski et al., 2006). However, the underlying physiological causes for training-induced alterations remain unclear. A broader understanding of the mechanisms involving aging- and exercise training-related changes of the HPAA, glucose homeostasis and inflammatory processes in horses is necessary as data in this regard is limited.

Hormones of the HPAA include adrenocorticotropic hormone (ACTH) and cortisol, known to suppress pro-inflammatory cytokines (Chrousos, 1995). Concentrations of cortisol are known to decrease with age in horses (Malinowski et al., 2006). However, Horohov et al. (1999) concluded that older mares not only had lower plasma cortisol, but were consequently more resistant to exercise-induced immune suppression compared to young horses. Cortisol is also known to stimulate gluconeogenesis. A lack of production and/or response of cortisol with age may interfere with substrate mobilization as well as endocrine signals associated with the HPAA.
Combined, these physiological changes with age may have implications for exercise tolerance and recovery.

Exercise training in old mares has at least partially restored endocrine function lost with age (Malinowski et al., 2006). Exercise at appropriate intensities also affects the immune system (Horohov, 2004). Because endocrine and immune functions are tied together, it is important to understand how changes in one system may affect the other. Whether age-related changes occur in horses with respect to ACTH remains to be determined. Understanding hormonal changes during acute exercise, as well as before and after exercise training, may help pinpoint where the HPAA becomes altered, and if training can help reverse these deteriorations.

Interaction of HPAA hormones with those from other endocrine pathways (such as insulin and the catecholamines) affect cytokines, and the complex signaling pathways associated with a wide variety of physiological processes. Cytokines are small proteins that mediate inflammation. Some cytokines may promote or inhibit insulin-mediated signaling mechanisms and the secretion of certain hormones, and are also influenced by age and exercise. Cytokines secreted from adipose tissue cells are labeled as adipokines (Kahn and Flier, 2000, Arner, 2005), and may include cytokines (tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 beta (IL-1β) and interferon-gamma (IFN-γ)) and hormones (adiponectin, leptin). Thus, these proteins are important factors to consider when investigating the influence of the endocrine system on changes associated with age and exercise. In addition, data is limited with regard to the comparison of cytokine profiles in adipose and muscle tissues (largely responsible for insulin-mediated glucose clearance) in horses.
However, differences in the cytokine profiles of these tissues have been shown in humans (Arner, 2005; Fantuzzi, 2005). Increased risk of Type 2 diabetes is correlated with high amounts of abdominal fat in humans (Arner 1995), but it is unknown if the same etiology holds true in horses. Mechanisms of glucose homeostasis are well conserved among species (Kahn and Flier, 2000), therefore, knowledge gained in horses may be relevant to other species.

The interactions of the endocrine and immune systems are coordinated to maintain a biological balance that becomes impaired with age. Exercise training may result in adaptations that mediate such changes (Malinowski et al., 2002; Powell et al., 2002), however more data is needed to fully appreciate the mechanisms behind these alterations.

The Hypothalamic-Pituitary-Adrenal Axis (HPAA)

The HPAA is an effector limb of the stress system, the main function of which is to maintain homeostasis (Tsigos and Chrousos, 2002). This is accomplished in part by controlling cortisol secretion (Nieuwenhuizen and Rutters, 2007). When the stress system is activated, for example by exercise or threat to self, arousal, attention, motor reflexes and pain tolerance are increased, while appetite and immune-mediated inflammation are decreased (Chrousos, 1995).

The signaling cascade of the HPAA is multi-tiered, and a schematic of the normal axis is illustrated in Figure 1. In response to a challenge, or stressor, corticotropin releasing factor (CRF) is released from the hypothalamus, which in turn signals the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) (Nieuwenhuizen and


Rutters, 2007). ACTH signals the adrenal glands to release cortisol (Nieuwenhuizen and Rutters, 2007). Once cortisol binds to glucocorticoid receptors (GR), a cascade of negative feedback is initiated via other GRs on the pituitary gland and hypothalamus (Nieuwenhuizen and Rutters, 2007). The ability of cortisol to exert inhibitory effects on ACTH and CRF results in limited duration of tissue exposure to glucocorticoids, thus minimizing catabolic and immunosuppressive actions (Tsigos and Chrousos, 2002).

Cortisol is a glucocorticoid hormone (Andrews and Walker, 1999), so classified because of its role in glucose metabolism, production in the adrenal cortex and classification as a steroid hormone (de Graaf-Roelfsema et al., 2007). Generally accepted actions of cortisol include the modulation of immune function (Horohov et al., 1999), suppression of insulin and stimulation of gluconeogenesis and lipolysis (de Graaf-Roelfsema et al., 2007). Furthermore, cortisol exhibits diurnal variation in horses (Johnson and Malinowski, 1986). Peak cortisol concentration has been reported between 0700 and 0900 in nonpregnant mares, with the lowest concentration occurring between 1900 and 2300 (Johnson and Malinowski, 1986). Diurnal rhythms may be disrupted in response to a disruption of homeostasis (Tsigos and Chrousos, 2002).

Cortisol secretion has been shown to increase during strenuous exercise in man (Few, 1974). Humans of mixed training background also demonstrated increased plasma cortisol concentrations during an exhaustive exercise test on a bicycle ergometer (Schwarz and Kindermann, 1989). Similarly, cortisol and ACTH concentrations increased in response to strenuous exercise in young, human male athletes (Cinar et al., 2009), and in normal men during exercise tests on a bicycle ergometer (Coiro et al., 2007). Additionally, cortisol and ACTH increased in Thoroughbred horses during
exercise to fatigue (Nagata et al., 1999). In recovery from treadmill exercise in horses, plasma ACTH concentration peaked at the end of the test whereas cortisol peaked 20-30 minutes after cessation of a standard exercise test (Marc et al., 2000). Cortisol concentration in horses was similar to baseline measurements by 2 hours post-exercise (Church et al., 1987). It has been proposed that this delayed rise in cortisol in response to a challenge prevents the immune and inflammatory responses from over-reacting (Munck et al., 1984) without interfering with the appropriate activation of stress-defense mechanisms initiated by the sympathetic nervous system (DeVries et al., 2000). Additionally, this could be an adaptive response to training designed to increase protein synthesis and muscle glycogen stores as part of the recovery process (de Graaf-Roelfsema et al., 2007). It appears that an increase in HPAA hormone concentrations is a normal response to exercise, while a return to baseline measurements occurs within a few hours of cessation of exercise to prevent long-term exposure to hormone concentrations above normal.

Factors external to the HPAA may influence its activity. For example, argenine vasopression (AVP) acts synergistically with CRF to stimulate secretion of ACTH in response to a stressor, such as exercise (Chrousos, 1995). In horses, AVP increases in response to exercise in a similar manner that occurs in humans (McKeever and Hinchcliff, 1995). AVP perpetuates the concentration of CRF during exercise (and vice versa) at the level of the hypothalamus (Tsigos and Chrousos, 2002), with implications not only downstream on the HPAA, but for control of blood volume during exercise (McKeever and Hinchcliff, 1995). Interestingly, a positive correlation between ACTH and AVP was reported in the absence of CRF detection in pituitary venous blood of
Thoroughbred horses running on a treadmill (Alexander et al., 1991), suggesting that CRF is not the sole activator of the HPAA when exercise begins. Collectively, it could be speculated that multiple factors influence HPAA activation.

Additionally, the presence of cytokines associated with exercise may influence the HPAA. Interleukin-6 (IL-6) can enhance ACTH production, signaling the adrenal cortex to produce glucocorticoids (Moldoveanu et al., 2001). Circulatory concentrations of IL-6 rise quickly in response to various types, durations and intensities of exercise, (Moldoveanu et al., 2001), likely as a result of skeletal muscle contractions (Pedersen et al., 2003). IL-6 gene transcription occurs locally in contracting skeletal muscle, and IL-6 has been measured in high concentration in the blood from an exercising limb in humans (Pedersen et al., 2003b). In addition, IL-6 mRNA is downregulated when muscle glycogen availability is reduced (Pedersen et al., 2003b). Thus, IL-6 may influence ACTH secretion, possibly by acting as a sensor of carbohydrate availability (Wallenius et al., 2002). IL-6 can also act to potentiate the effects of glucocorticoids on the liver (Moldoveanu et al., 2001), therefore facilitating hepatic gluconeogenesis (Goldstein et al., 1993). In addition, Bethin et al. (2000) showed the presence of IL-6 receptors on corticotrophs in mice, as well as an ability of IL-6 to promote cortisol release at the level of the adrenal. Overall, upregulation of IL-6 during exercise could be part of the mechanism leading to a rise in cortisol with the aim of increasing substrate mobilization.

Markers of exercise performance, such as lactic acid and lactate, appear to influence ACTH secretion. Data collected from active college students revealed a positive correlation between plasma lactic acid and ACTH, and the authors suggested that lactic acid promoted ACTH secretion during exercise (Farrell et al., 1983; de Mierler et
Glucose and glycogen can be converted into lactic acid to produce ATP (Marlin and Nankervis, 2002), thus increased ACTH as result of a challenge to the stress system may be facilitating this process. In contrast, lactic acid infusion in sheep at rest did not appear to raise ACTH concentration, but HCl did (Wood and Isa, 1991). Lactic acid quickly dissociates into H+ and lactate, allowing the regeneration of NAD from NADH, and the continuation of glycolysis (Marlin and Nankervis, 2002). Interestingly, plasma ACTH and lactate concentrations have also shown positive correlations in human literature (Farrell et al., 1983; Heitkamp et al., 1998; Minetto et al., 2006) as well as in horses (Nagata et al., 1999). Lactate increases as a normal response exercise (Streltsova et al., 2006; Liburt et al., 2010), consequently increasing the presence of H+ ions (Marlin and Nankervis, 2002). It could therefore be speculated that as work is increased and/or prolonged, a signaling mechanism sensitive to H+ promotes ACTH secretion, and consequently cortisol in effort to promote gluconeogenesis and substrate mobilization.

In addition to physical challenges, age has been reported to affect HPAA activity. Old mares have shown a blunted response to cortisol after an exercise test before and after exercise training compared to young and middle aged mares, but the mechanisms behind such differences are unknown (Malinowski et al., 2006). Diminished cortisol concentration may have a negative impact on the ability to mobilize substrate and recover from exercise (Malinowski et al., 2006). Exercise training has attenuated this deficit to some degree (Malinowski et al., 2006; Liburt et al., unpublished data). Similarly, in humans, older, fit women demonstrated increased cortisol production compared to older, unfit women (Traustadottir et al., 2004). It was suggested that an increase in adrenal capacity as a consequence of fitness training was at least partially responsible for the
difference (Traustadottir et al., 2004). It has also been suggested that training results in an increase in adrenal sensitivity to ACTH, thus facilitating the change in cortisol concentration (Mastorakos et al, 2005). Mechanisms behind the age-related decline in hormone concentration remain to be fully understood.

Age has also been associated with pathology of the HPAA. Most notable is pituitary pars intermedia dysfunction (PPID), commonly known as equine Cushing’s syndrome. PPID is a spontaneous neurodegenerative disease that results from a loss of dopaminergic innervation of the pars intermedia for which there is no cure (McFarlane, 2007). PPID is phenotypically characterized by lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). Changes in morphology of the pituitary gland may affect its function (Miller et al., 2008). Size of the pars intermedia increased with age of the horse, with horses over the age of 20 years exhibiting a pars intermedia almost 3x the size of horses under 12 years (Miller et al., 2008). Hypertrophy of the pars intermedia is thought to result from a loss of dopamine inhibition (McFarlane et al., 2005), and may result in abnormally high concentrations of ACTH and cortisol (Miller et al., 2008). Thus, with advanced age comes increased likelihood of abnormal ACTH and cortisol secretion due to abnormal hypertrophy of the pars intermedia. The consequence of such a change may result in a loss of ability to maintain homeostasis via the HPAA.

PPID may also affect immune function. McFarlane and Holbrook (2008) reported evidence of a pro-inflammatory state in aged horses that exhibited increased interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Horses with PPID had increased expression of interleukin-8 (IL-8), but not IL-6 or TNF-α in the blood compared to age-matched controls and younger animals (McFarlane and Holbrook, 2008). It was
suggested that a possible reason for the lower expression of IL-6 and TNF-α in PPID horses compared to age-matched controls was chronic exposure to elevated levels of anti-inflammatory hormones, such as ACTH, α-MSH and β-endorphin, that occur with this pathology (McFarlane and Holbrook, 2008). Furthermore, authors concluded that horses with PPID have altered cytokine profiles compared to non-PPID animals, which may affect the ability of PPID horses to respond to pathogens (McFarlane and Holbrook, 2008).

Season is also a consideration when testing the responsiveness of the HPAA. Cortisol did not show seasonal variation in Standardbred mares tested in May, September and December, however sampling times were limited to daylight hours and may not have incorporated enough sample times to detect a difference (Liburt et al., unpublished). By contrast, other work led to the conclusion that only pregnant mares show seasonal variation in cortisol where barren mares did not (Flisinska-Bojaowska et al., 1991). Research indicated healthy horses and horses with equine metabolic syndrome did not show seasonal changes in cortisol, but both showed increases in plasma ACTH from August – October in the northeast United States (Place et al., 2010). More specifically, seasonal changes in plasma ACTH were reported to decrease as daylight decreases from the summer solstice to 12 hrs of daylight (Beech et al., 2009). Work by Donaldson et al. (2005) reported a significant difference in the reference ranges of plasma ACTH in pony mares and stallions in May (100% of subjects within reference range) and September (5% in 2002 and 8% in 2003 within reference range). Finally, Frank et al. (2010) reported that PPID did not affect the timing or duration of seasonal increases in ACTH in August, September and October, however values were higher in horses with this pathology.
compared to non-PPID animals. Taken together, alterations in plasma ACTH may affect data collected over multiple seasons, if not the HPAA itself, even if cortisol is not affected.

Data from the author’s lab has examined changes in the HPAA as associated with age and exercise training. It was concluded that with age, both the pituitary and adrenal glands became less sensitive to endocrine signals, and consequently, cortisol was inappropriately low in response to endocrine stimulation tests and acute exercise. After a period of exercise training, sensitivity and function of the pituitary and adrenal glands was improved, although not completely restored, in old mares. After training, patterns of ACTH, cortisol and glucose concentrations in old mares were more closely aligned with those exhibited by young mares. Thus, it was concluded that sensitivity of the aged pituitary and adrenal glands can be improved, although not completely restored, with exercise training.

**Glucose Homeostasis**

Insulin is one of the main hormones that controls blood glucose concentrations, and it is secreted by pancreatic β-cells. The insulin signaling cascade is well conserved among species from *C. elegans* to humans (Kahn and Flier, 2000), so knowledge of what occurs in humans and rodents may be applicable to the study of horses. A schematic illustrating normal maintenance of blood glucose is shown in Figure 2.

When mechanisms of glucose homeostasis fail, imbalances of insulin secretion and glucose clearance may result. A basic, working definition of insulin resistance is that in response to glucose intake, insulin is not effective in clearing glucose from the blood.
Insulin fails to stimulate glucose uptake into peripheral tissues such as fat and muscle. Insulin secretion by the pancreatic $\beta$-cells may be higher in an insulin resistant animal and may remain elevated for a longer time compared to an insulin sensitive animal. Hepatic gluconeogenesis may not be sufficiently suppressed in response to insulin, perpetuating elevated blood glucose concentration. Consequently, an insulin resistant animal’s blood glucose concentration may stay elevated longer than that of a normal animal. Thus, replenishment of glucose in skeletal muscles may be prolonged, or insufficient, resulting in a faster onset of fatigue during and delayed recovery from exercise.

Exercise is generally considered a valid way to improve insulin sensitivity in several species. Proposed mechanisms for the positive effects of exercise include reduction in liver and muscle glycogen, increased binding of insulin to the insulin receptor and increased glucose uptake by muscle cell glucose transporters in horses (Firshman and Valberg, 2007). Short-term exercise training has repeatedly been shown to improve insulin sensitivity in horses (Powell et al., 2002; Stuart-Hunt et al., 2006), but a single bout of exercise did not (Pratt et al., 2007). Previous work has shown that 12 weeks of exercise training improved insulin sensitivity in old (~27.0 yrs) Standardbred mares, and the improvement was greater in the old horses compared to young (~7 yrs) and middle aged (~15 yrs) mares (Malinowski et al., 2002). Longer-term training has also resulted in a decrease of glucose flux in Standardbred and Thoroughbred horses (Geor et al., 2002). Comparative studies reported that in sedentary humans, a single bout of exercise did not improve glucose or insulin response to a glucose challenge, but 7 days of exercise for 60 minutes at 68% VO$_2$max did (Reaven, 1995). Considering these data
together, it could be suggested that improvements in insulin sensitivity as a result of exercise may be similar in horses and humans.

The degree to which glucose tolerance decays with age in humans is modified by the amount of regular activity and body fat distribution, particularly abdominal obesity (Reaven, 1995). Insulin sensitivity declines with age in horses as well, with environment, diet and genetics all playing roles (Firshman and Valberg, 2007). Murine models have shown β-cell hyperplasia associated with age and decreasing insulin sensitivity (Reaven, 2005), but whether this phenomenon occurs in horses is unknown. Reaven’s review (1995) notes that in rats, the amount of insulin secreted by a pancreatic β-cell declines with age, regardless of strain, and the resulting pancreatic hyperplasia and increased mass of β-cells may be a compensatory mechanism. It was also noted that in both humans and rats, the maximal insulin response tended to remain the same while the glucose response tended to decline (Reaven, 1995). Therefore, the ability of insulin to stimulate glucose disposal appeared to decline with age (Reaven, 1995). Transgenic mice that over-expressed insulin in the liver also demonstrated an age-related decline in insulin receptor expression as well as glucose intolerance and hyperlipidemia (Kahn and Flier, 2000). These processes may also have implications for the decline in insulin sensitivity often seen with age in horses.

In order to understand the dynamics of glucose and insulin and to make observations about how well the body responds to a glucose/insulin challenge, the Minimal Model (MinMod) Analysis of Frequently Sampled Intravenous Glucose Tolerance Test (FSIGTT) may be used. The FSIGTT provides data regarding insulin sensitivity, glucose effectiveness and pancreatic β-cell function (Muniyappa, 2008). The
disposition index (DI), estimated using the MinMod, has been used to evaluate beta-cell function in humans (Bergman et al., 2002) and horses (Hoffman et al., 2003; Treiber et al., 2005). Evaluation of DI in horses may lend clues as to how insulin resistance develops in horses, and whether β-cell hyperplasia should be a factor to consider.

The MinMod requires several assumptions. The model assumes that glucose is instantaneously distributed, and that glucose clearance in response to glucose and/or insulin occurs at a similar rate (Muniyappa et al., 2008). Another assumption is that the glucose concentration at the end of the FSIGTT is the same as it was at the beginning (Muniyappa et al., 2008). Lastly, the MinMod assumes that endogenous plus exogenous insulin during the FSIGTT is above a certain threshold (Muniyappa et al., 2008).

The advantages of the MinMod include the fact that it can differentiate between insulin-dependent and insulin-independent glucose clearance (Treiber et al., 2006). This one test can provide data regarding insulin sensitivity, glucose effectiveness and pancreatic β-cell function (Muniyappa et al., 2008). The test is not without limitations. The MinMod puts the effects of insulin together for suppression of hepatic glucose production and peripheral glucose uptake, however the contribution of these may vary in individuals with compromised insulin sensitivity making such estimates unreliable (Muniyappa et al., 2008). Lastly, the MinMod protocol currently used in horses does not account for any urinary glucose spilling (Toth et al., 2010).

While it is known that insulin sensitivity declines with age in horses, and that exercise training improves insulin sensitivity, mechanisms behind these phenomena remain to be elucidated. Unpublished data from the author’s lab repeated findings that insulin sensitivity increased with exercise training in old and young Standardbred mares.
It was also observed that DI increases as well, and may be part of the mechanism responsible for improved glucose homeostasis in horses as a result of exercise.

**Inflammation**

The immune system is responsible for, among other things, the inflammatory response. The inflammatory response is the body’s way of protecting itself from infection and helping to heal injured tissue (Murphy et al., 2008b), and is also a natural and beneficial response to exercise that promotes protein synthesis (Pedersen et al., 1998). However, increased inflammation has also been associated with increased adipose tissue deposits and insulin resistance (Hotamisligil et al., 1995; Arner, 2005).

The immune system is regulated in part by cytokines, which are small proteins released from many cell types in the body (Murphy et al., 2008a). Cytokines may induce or inhibit intracellular signaling via specific binding to receptors (Murphy et al., 2008a). Cytokines can act in an autocrine, paracrine or endocrine fashion, affecting the cell that releases it, an adjacent cell or a distant cell, respectively (Murphy et al., 2008a). Individual cytokines usually serve multiple functions, and may serve different roles in different tissues (Petersen and Pedersen, 2005). In general, cytokines do not work alone, but rather in concert with each other, and with other cell types and hormones in the body (Roitt et al., 1998).

Cytokines are influenced by general disruptions in homeostasis. For example, age and stress are major factors known to depress immune function in horses (Horohov et al., 1999). Horohov et al. (1999) concluded that older horses a decreased proliferative response of peripheral blood mononuclear cells in response to mitogens, but not to
exercise. These researchers also saw decreased plasma cortisol concentrations in older mares compared to young, and suggested that old mares were resistant to exercise-induced immune suppression (Horohov et al., 1999). Combined, these physiological events may have contributed to the differences observed in immune response between age groups.

**Tumor Necrosis Factor – alpha (TNF-α) & Interleukin-6 (IL-6).** TNF-α has been associated with insulin resistance in humans, by mechanisms that include interference with insulin signaling, glucose transport protein 4 (GLUT-4) and decreased production of adiponectin (Arner, 2005). Studies of humans indicate that obese, premenopausal women had a 2.5-fold higher expression of TNF-α mRNA in adipose tissue compared to non-obese controls, as well as a decrease in TNF-α mRNA associated with weight loss (Hotamisligil et al., 1995). A review by Petersen and Pedersen (2005) observed that in humans, the pro-inflammatory cytokine TNF-α impaired insulin signaling, whereas IL-6 appears to act as an inflammatory mediator. Pedersen et al. (2003) observed that healthy elderly humans and elderly Type 2 diabetes patients had high quantities of TNF-α and IL-6 compared to young controls, and amounts of these cytokines were correlated with truncal fat mass. Additional data from human studies support a relationship between TNF-α, adipose tissue and insulin resistance (Ryden and Arner, 2007). Whether similar phenomena exist in horses is currently unknown.

Increased fat mass is associated with increased cytokine concentration. In addition, very strenuous exercise can be accompanied by an increase in circulating pro-inflammatory and inflammation responsive cytokines in a human model (Pedersen et al., 1998), in particular TNF-α and IL-6 (Vassilakopoulos et al., 2003). TNF-α is a pro-
inflammatory cytokine, and is an important stimulator of a second wave of cytokines, including IL-6 (Pedersen et al., 2003). TNF-α and IL-6 are tightly linked, with TNF-α stimulating IL-6 production (Pedersen et al., 2003b; Pedersen et al., 2003c). TNF-α may inhibit insulin-receptor signaling, whereas IL-6 suppresses TNF-α, increases insulin-stimulated glucose uptake, lipolysis and fat oxidation (Pedersen, 2007). It has also been suggested that IL-6 is an anti-inflammatory cytokine (Steensberg et al., 2003). Taken together, the presence of TNF-α during exercise is likely crucial for suppressing insulin and insulin-stimulated glucose uptake in order to keep circulating glucose readily available for work. IL-6 is necessary to moderate the TNF-α response (Pedersen, 2007), and possibly to facilitate glycogen replenishment post-exercise.

IL-6 enhances insulin-mediated glucose transport, glycogen synthesis and promotes glycogen storage and lipid oxidation in human models (Pedersen, 2007). In addition to its inhibitory effects on TNF-α, IL-6 stimulates the production of IL-1ra (IL-1 receptor antagonist) and IL-10, both of which counteract the production of pro-inflammatory cytokines (Pedersen, 2007).

IL-6 does not directly induce inflammation (Pedersen et al., 1998a), but rather serves to prevent tissue damage and activate repair processes (Moldoveanu et al., 2001). To accomplish this, IL-6 stimulates the release of ACTH and subsequent appearance of cortisol (a general anti-inflammatory mediator), resulting in suppression of the synthesis of the pro-inflammatory cytokines IL-1 and TNF (Moldoveanu et al., 2001). It appears that IL-6 is critical to augment adrenal function in response to immune system activation (Bethin et al., 2000). Glucocorticoids and catecholamines are key metabolic and stress hormones, and are able to moderate both pro-and anti-inflammatory cytokines (Elenekov
and Chrousos, 2002). Glucocorticoids potentiate the effects of IL-6 (and other cytokines) on hepatocytes (Moldoveanu et al., 2001). Negative feedback occurs as glucocorticoids inhibit macrophages, and thus IL-6 production is down-regulated (Moldoveanu et al., 2001). Similarly, more data exists to suggest that glucocorticoids and catecholamines (the major stress hormones) inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-12 and IFN-γ, while anti-inflammatory cytokines are stimulated, resulting in an important negative feedback system keeping the immune response in check (Elenkov and Chrousos, 2002).

It has been demonstrated that the release of IL-6 from working skeletal muscle is positively related to work intensity, glucose uptake and plasma epinephrine concentration, suggesting that IL-6 is linked to regulation of glucose during exercise, and/or that IL-6 works as a sensor of carbohydrate availability (Pedersen et al., 2003b). Furthermore, IL-6 knockout mice developed mature-onset obesity and insulin resistance, which was reversed by administration of IL-6 (Wallenius et al., 2002). Transcription of IL-6 mRNA is hindered when muscle glycogen availability is reduced (Pedersen et al., 2003b). It appears that when human subjects received a high dose of rhIL-6, whole body glucose disposal and oxidation rose significantly, but when subjects received physiological amounts of rhIL-6, no effect on glucose metabolism was noted (Pedersen et al., 2003b). Either way, IL-6 appears to play a considerable role in carbohydrate metabolism and possibly the detection of carbohydrate availability. IL-6 also acts on adipose tissue, inducing lipolysis and whole body lipid oxidation, thereby influencing the presence of free fatty acids the availability of substrate for energy (Pedersen et al., 2003b).
Horses over the age of 20 yrs demonstrate changes in their immune response that are comparable to what is seen in older humans (Horohov et al., 2002). General increases in systemic inflammation have been associated with age in horses, as in humans, with older animals showing increased cytokine production compared to young animals (Adams et al., 2009). Older, fat horses demonstrated a higher percentage of TNF-α positive lymphocytes and monocytes after stimulation compared to older, thin animals, and a decrease in body fat over time reduced these percentages (Adams et al., 2009). A reduction of body weight and fat in old horses resulted in decreased TNF-α and IFN-γ mRNA, and increased with weight gain and greater percent body fat (Adams et al., 2009). Insulin sensitivity was not measured in the study by Adams et al. (2009), but it was shown that obesity in aged horses certainly had an effect on the presence of inflammatory cytokines that influence mechanisms of insulin action. Additionally, in humans TNF-α and IL-6 from adipocytes may influence the function of pancreatic β-cells (Bonora et al., 2008). Therefore, visceral obesity within the liver, skeletal muscle and pancreatic islets likely influences insulin resistance, β-cell dysfunction and abnormal glucose regulation via secretion of TNF-α and IL-6 in humans as well (Bonora et al., 2008).

**Monocyte Chemotactic Protein – 1 (MCP-1).** In various species, MCP-1 is secreted by several cell types, and has been shown to increase expression in adipose tissue in a murine model (Tateya et al., 2010). MCP-1 is upregulated in adipose tissue in association with obesity, potentially in part by TNF-α (Arner, 2005). Increased MCP-1 resulted in the attraction of macrophages to adipose tissue and additional secretion of pro-inflammatory cytokines, events that are likely associated with the interference of insulin signaling and contribution to insulin resistance (Tateya et al., 2010).
MCP-1 is an inflammatory cytokine shown to contribute to insulin resistance in primates (Bose et al., 2009). Inhibition of the MCP-1 receptor improved insulin sensitivity in studies of mice fed a high fat diet, regardless of macrophage infiltration into adipose tissue (Tateya et al., 2010). Other work has shown that insulin suppresses MCP-1 (Dandona et al., 2004). Previous studies in rodents have demonstrated that MCP-1 increased expression in adipose tissue (Tateya et al., 2010), and had also been detected in human skeletal muscle (Confalonieri et al., 2000) and adipose tissue (Sell et al., 2006). MCP-1 was also found in equine skeletal muscle and adipose tissue (Liburt et al., unpublished). Considering comparative data, MCP-1 signaling may be an important factor with regard to maintenance of proper glucose concentrations (Tateya et al., 2010).

The presence of MCP-1 appears to be further influenced by exercise. White adipose tissue from healthy, young, exercise-trained rats has demonstrated differences in the inflammatory cytokines TNF-α and MCP-1 (Sakurai et al., 2009). Exercise training reduced MCP-1 in epididymal, retroperitoneal and subcutaneous adipose tissue in these rats, and reduced TNF-α in epididymal and retroperitoneal adipose tissue (Sakurai et al., 2009). Collectively, exercise training and reduction of body fat appear to be important factors for reducing the presence of MCP-1 and improving insulin sensitivity, but more research is necessary to better understand the role of this cytokine in glucose metabolism.

**Interleukin-1 beta (IL-1β).** The interleukin-1 family of cytokines has a strong influence on the innate immune system (Sims and Smith, 2010). Here, IL-1β is considered, as it is a main inducer of inflammatory processes (discussed below) in this family (Sims and Smith, 2010). IL-1β is principally produced by monocytes and macrophages and circulates systemically (Sims and Smith, 2010). IL-1β is a pro-
inflammatory cytokine, and its presence induces the upregulation of IL-1ra to counteract inflammation (Osborn et al., 2008), as well as the presence of other cytokines, particularly IL-6 (Dinarello, 1994).

In humans, IL-1β was associated with type-2 diabetes, and it has been shown that pancreatic beta cells are particularly susceptible to destruction by IL-1β (discussed in Maedler et al., 2009). Mice treated with IL-1β antibody showed improved glycemic control and pancreatic beta cell function, suggesting that IL-1β may be a therapeutic target for insulin resistance (Osborn et al., 2008). IL-6 appeared to upregulate IL-1ra, independently of TNF-α (Steensberg et al., 2003), suggesting another possible mechanism for manipulating cytokines that promote insulin sensitivity.

Expression of IL-1β had previously been found in human skeletal muscle up to 5 days post exertion (Cannon et al., 1989). In addition, expression of IL-1β was shown in adipose and muscle tissues of horses (Liburt et al., unpublished). However, exercise training reduced IL-1β in patients with chronic heart failure (Gielen et al., 2003). During exercise at 100% VO2max, human subjects experienced a suppression of IL-1β, possibly due to glucocorticoids released in response to the stress of exertion (DeRijk et al., 1997).

In addition to exercise, evidence suggests that IL-1β is influenced by endocrine factors, such as cortisol. As IL-1β appears to target the destruction of pancreatic beta cells, it is another critical cytokine to be considered in the assessment of insulin resistance and exercise training. Little information is available on the combination of age, exercise training and insulin sensitivity on IL-1β, especially in horses.
**Interferon-gamma (IFN-γ).** IFN-γ is another major pro-inflammatory cytokine (Mosmann and Sad, 1996). IFN-γ is a product of Th1 cells, but it is also produced by natural killer (NK) cells and activated B-cells (Ainsworth et al., 2003).

In vitro studies of human adipocytes treated with IFN-γ showed a decrease in insulin-stimulated glucose uptake along with a down-regulation of insulin receptor substrate -1 and GLUT-4 via JAK/STAT pathway activation (McGillicuddy et al., 2009). Additionally, IFN-γ potentially plays a role in the upregulation of chemoattractant cytokines (Bloomgarden, 2003), potentially increasing the population of other pro-inflammatory proteins. The combination of the aforementioned mechanisms suggest that IFN-γ affects glucose metabolism via intracellular signaling and by encouraging the release of other pro-inflammatory cytokines.

In horses, insulin resistance has been associated with chronic, low-grade inflammation (Vick et al., 2007; Vick et al., 2008). Research suggests that obesity leads to a general inflammatory state that influences the sensitivity of skeletal muscle, liver and fat to insulin in horses (Vick et al., 2007; Vick et al., 2008). Comparisons of cytokine mRNA in the adipose tissues of young and old horses before and after exercise training have not, to the best of our knowledge, been conducted. It also remains to be determined if variations exist between the metabolic activity of abdominal fat compared with subcutaneous fat from the neck crest in horses, and what possible influence such activity could exert on insulin sensitivity.

**Summary**
Endocrine signals and inflammatory processes appear to decline with age, but seem to improve with moderate exercise. Corticosteroid hormones affect cytokines, as well as gluconeogenesis and insulin action. Furthermore, cytokines influence the HPAA and glucose metabolism. Evaluation of the effects of age and training on the HPAA, inflammation in adipose and muscle tissue and glucose homeostasis will lead to a greater understanding of mechanisms of endocrine regulation and glucose metabolism.

The average lifespan of a horse in the 1980’s was 15-20 years. Today, through research and better care, that has improved to 25-30 years (Lewis, 1996). Consequently, the population of elderly equines is also growing (National Animal Health Monitoring & Surveillance Equine, 2005 Study). As with humans, the incidence of disorders affecting glucose regulation and energy balance appear to be increasing along with the number of older horses, and thus, there is a need and demand for research-based information to better support the health of these older athletic animals. There are many physiological similarities between horses and humans as they age and another benefit of the present research is that it gives us an opportunity to help the older horse, and to better understand factors leading to obesity and insulin resistance both in horses and humans.
References


**Figure Legends**

**Figure 1.** Schematic of the normal hypothalamic pituitary adrenal axis (HPAA). Courtesy of [http://www.montana.edu/wwwai/imsd/alcohol/Vanessa/vwhpa.htm](http://www.montana.edu/wwwai/imsd/alcohol/Vanessa/vwhpa.htm), accessed December 31, 2010.

**Figure 2.** Schematic of normal glucose homeostasis. Courtesy of [http://www.ekcsk12.org/faculty/jbuckley/leclass/lifeprocnts.html](http://www.ekcsk12.org/faculty/jbuckley/leclass/lifeprocnts.html), accessed December 31, 2010.
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CHAPTER 2:

RESPONSE OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS TO STIMULATION TESTS BEFORE AND AFTER EXERCISE TRAINING IN OLD AND YOUNG STANDARDBRED MARES
ABSTRACT: This study tested the hypothesis that exercise training alters the response of the hypothalamic-pituitary-adrenal axis (HPAA) to a series of endocrine stimulation tests in horses. Six old (O, 22.0 ± 0.7 yrs; mean ± SE) and six young (Y, 7.3 ± 0.6 yrs) unfit Standardbred mares ran 3 graded exercise tests (GXT): before (GXT1), after 8 wks of training (GXT2) and at study end at 15 wks (GXT3). Mares trained 3 d/wk at 60% maximum heart rate (HRmax). Each mare underwent 5 endocrine stimulation tests pre- and post-training: 1) control (CON), 2) adrenocorticotropin hormone (ACTHtest), 3) combined dexamethasone suppression/ACTH (DEX/ACTH), 4) dexamethasone suppression (DEX), and 5) combined DEX/corticotropin releasing factor (DEX/CRF). For CON, there was no difference in plasma cortisol between age groups pre-training ($P=0.19$), but young mares had a 102% higher (mean, $P=0.02$) plasma cortisol concentration than old mares post-training. The pre-training ACTHtest showed young mares had a 72% higher ($P=0.05$) overall plasma cortisol concentration compared to old. This age difference in cortisol disappeared in the post-training ACTHtest, but old mares still had lower cortisol concentrations at 30 min during the test, suggesting decreased adrenal response to ACTH stimulation. There was no effect of training cortisol concentrations for any test, and no effect of age in DEX, DEX/ACTH or DEX/CRF tests. In CON and DEX/CRF, there were no significant age differences in plasma ACTH in O vs. Y pre- or post-training. Pre-training, there was no age difference in glucose response to DEX, but post-training old mares had a 4% ($P=0.04$) lower overall plasma glucose concentration compared to young. Post-training, old mares had lower plasma glucose concentrations during DEX compared to pre-training ($P=0.02$), but young mares did not ($P=0.19$). Old and young mares both had lower plasma glucose concentrations post-
training during DEX/ACTH ($P<0.001$, $P=0.05$, respectively) and DEX/CRF ($P<0.001$, $P=0.003$, respectively) compared to pre-training. Both the pituitary and adrenal glands experience a decline in function with age, although the exact mechanisms remain unknown. Exercise training facilitates the counteraction of these deficits.
Introduction

Research has shown age-related changes in endocrine function in horses, where old mares exhibited reduced plasma cortisol concentrations compared to young mares (Horohov et al., 2002; Malinowski et al., 2006). Moderate exercise training attenuated, but did not reverse, the effect of age on the cortisol response to acute exertion (Malinowski et al., 2006). Similarly, Horohov et al. (1999) observed that older horses had decreased plasma cortisol concentrations in response to a strenuous exercise challenge compared to younger animals, consistent with other work (Malinowski et al., 2006). A change in the glucose and insulin responses to exercise in aged Standardbred mares has also been observed, where old mares had higher concentrations of plasma insulin and lower concentrations of plasma glucose compared to young and middle aged mares (Malinowski et al., 2002). Collectively, research supports the hypothesis that exercise influences the hypothalamic-pituitary-adrenal axis (HPAA) along with endocrine regulation of glucose homeostasis, but these mechanisms become impaired with age. The reasons behind these changes remain to be determined in horses.

The HPAA controls cortisol secretion (Nieuwenhuizen and Rutters, 2007). Corticotropin releasing factor (CRF) is released from the hypothalamus, and signals the anterior pituitary to secrete adrenocorticotropin hormone (ACTH) (Nieuwenhuizen and Rutters, 2007). ACTH signals the adrenal glands to release cortisol (Nieuwenhuizen and Rutters, 2007). Once free cortisol binds to glucocorticoid receptors (GR), a cascade of negative feedback is initiated via other GRs on the pituitary gland and hypothalamus (Nieuwenhuizen and Rutters, 2007) to keep the HPAA signals in check.
Cortisol is a glucocorticoid (steroid) hormone (Andrews and Walker, 1999) that normally rises in response to a physical, environmental or psychological challenge (de Graaf-Roelfsema et al., 2007). Generally accepted actions of cortisol include the modulation of immune function (Horohov et al., 1999), counteraction of insulin and stimulation of gluconeogenesis and lipolysis (de Graaf-Roelfsema et al., 2007). In addition, cortisol follows a diurnal rhythm, with the highest concentrations seen in the morning and lowest in the evening (Johnson and Malinowski, 1986).

In humans, abnormally low cortisol concentration is classified as Addison’s disease, with symptoms such as fatigue, muscle weakness and weight loss (Bethune, 1989), the latter of which has been anecdotally reported in many aged horses. Because a major function of cortisol is to stimulate gluconeogenesis and to modulate the immune system (de Graaf-Roelfsema et al., 2007), an aberrantly low cortisol response to exercise could result in decreased hepatic gluconeogenesis or an altered recovery response to an exercise challenge. Exercise training in horses appears to partially attenuate some of the endocrine deficits manifested with age, but does not completely reverse them (Malinowski et al., 2006). The point at which changes occur along the HPAA with age and the exact mechanisms behind altered endocrine and glucose concentrations observed in aged animals remain to be elucidated.

The average lifespan of a horse in the 1980’s was 15-20 years (Lewis, 1996). Today, through research and better care, that has improved to 25-30 years (Lewis, 1996). Consequently, the population of elderly equines is also growing (NAHMS Equine Study, 2005). The incidence of endocrine disorders affecting glucose regulation and energy homeostasis appear to be increasing along with the number of older horses, and thus,
there is a need and demand for research-based information to better support the health of these older athletic animals. Therefore, the present study used a series of endocrine stimulation tests to examine the hypothesis that the age-induced alteration in cortisol, ACTH and glucose concentrations are due to differences in the response of the HPAA in old horses compared to younger horses. Secondarily, it was hypothesized that exercise training would attenuate these differences.

**Materials and Methods**

*Animals & Diet*

Twelve unfit Standardbred mares were studied. Six mares were old (22.0 ± 0.7 yrs; mean ± SE) and 6 were young (7.3 ± 0.6 yrs) (Table 1). None of the mares showed outward physical signs of pituitary pars intermedia dysfunction (PPID), such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). The only apparent difference between the groups was age. Mares were housed on 2-acre dry lots with shelters and were given *ad libitum* access to grass hay and water. All were previously familiarized with the treadmill laboratory and associated procedures, and the Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment.

Horses were fed a commercially pelleted grain mix (~2 kg/d, divided into two feedings at 0700 and 1500). Hay samples were analyzed by a commercial laboratory (DairyOne Forage Laboratory, Ithaca, NY), and grain analysis was provided by the manufacturer (F.M. Brown’s Sons, Inc., Birdsboro, PA). On an as-fed basis, hay provided 0.95 Mcal/kg, and grain provided 1.4 Mcal/kg. Thus, mares’ approximate daily
intake was 28.3 Mcal/d, and 63.2% non-fiber carbohydrate. The calories provided were above the minimum requirements determined by the National Research Council (2007) for mature horses weighing 500 kg in moderate work, listed as 23.3 Mcal/d.

Endocrine Stimulation Tests

Before and after exercise training (Table 2), each mare received five endocrine stimulation tests in a randomized crossover design, with one week between each test: 1) Control (CON), 2) Adrenocorticotropic Hormone (ACTHtest), 3) Dexamethasone Suppression (DEX), 4) Combined Dexamethasone Suppression/ACTH (DEX/ACTH) and 5) Combined Dexamethasone Suppression/Corticotropin Releasing Factor (DEX/CRF). On all testing days except DEX, the morning grain meal was withheld; for DEX, only the evening grain meal was withheld. Grass hay was offered ad libitum during testing. Two baseline samples were drawn for each test at -30 min and 0.

For CON and ACTHtest, a dose of 10 mL normal saline or 0.05 mg ACTH (Cortrosyn™, Amphastar, Rancho Cucamonga, CA), respectively, was given at 0900, with sampling at 0.5, 1, 2, 4, 6, and 24 hrs. The dose of ACTH was chosen based on a previously reported dose response trial (Stewart et al., 2006).

For the DEX tests, mares received 0.04 mg/kg dexamethasone at 1700, with blood samples collected at 0.5, 1, 2, 4, 6, 15, 16, 17, 18 and 19 hrs. Dexamethasone doses were utilized based on previously published methods (Dybdal et al., 1994; Eiler et al., 1997).

For DEX/ACTH and DEX/CRF, dexamethasone was administered at 0900 (0.04 mg/kg) and samples drawn at 0.5, 1, 2 and 4 hrs. After the 4 hr blood sample, 0.05 mg ACTH or 500 µg CRF (human/rat, GenScript, Piscataway, NJ), respectively, was
administered, with additional samples drawn 4.5, 5, 6, 8, 24 and 28 hrs. Livesey et al. (1991) isolated CRF from equine hypothalami, and found that the purified peptide is equipotent with human CRF in an *in vitro* bioassay and in a human plasma binding protein assay. The CRF dose administered in the present study was based on human studies where the peptide was administered at 1 μg/kg (Schulte et al., 1984; Schurmeyer et al., 1987).

With respect to cortisol, a parallel control study was conducted to evaluate seasonal change in cortisol concentrations. Four mares participated in a pilot study that took place in December, 2008. The same 4 mares (1 old, 3 young) were part of the main study that ran through May and September, 2009. The CON test was run during all time periods, following the same protocol described above.

**GXT & Training Protocol**

Each mare underwent three graded exercise tests (GXTs) on a treadmill (Sato I, Equine Dynamics, Lexington, KY) set at a fixed 6% grade. The first GXT (GXT1) was conducted prior to training, the second (GXT2) after 8 wks training, and the third (GXT3) at the conclusion of the study after 16 wks (Table 2). The GXTs were conducted between 0800 and 1130. An indirect open-flow calorimeter (Oxymax-XL, Columbus Instruments, Inc., Columbus, OH) was used to measure gas exchange to determine each mare’s maximal oxygen uptake (VO₂max). Oxygen uptake was measured continuously during the test and recorded at 10 s intervals. VO₂max was defined as the point when there were no further increases in VO₂ despite increases in speed. Exercise capacity,
measured as run time to fatigue, was also calculated as described (McKeever and Malinowski, 1997).

The GXTs started at an initial speed of 4 m/s for 1 min. Speed was then increased to 6 m/s, followed by incremental increases of 1 m/s every 60 s until the horses reached fatigue. Fatigue was defined as the point where the horse could not keep up with the treadmill despite humane encouragement. At the point of fatigue, the treadmill was stopped, and 5 min of post-exercise calorimetric data was collected.

A heart rate monitor (Polar Equine, Lake Success, NY) was used to measure heart rate during GXTs. Heart rates were recorded at rest, and during the last 10 s of each treadmill step such that maximum heart rate and the HR versus speed relationship could be documented for each horse. Training intensity was calculated using this data so that each mare exercised at ~60% of HRmax. The range of recorded HRmax was 185-216 beats per minute (bpm). Thus during training, velocity was adjusted and HR kept between 111-130 bpm depending on the individual so as to maintain a submaximal relative work intensity of ~60% HRmax.

Training was conducted between 0600 and 1000 from June-October. Mares were exercised trained for an initial 8-wk period in a free-stall exercise machine (Equi-Ciser, Equi-Master International, Sudre, Alberta, Canada). The order of the groups was alternated every other session, so the same group was not always the first to train. Grain meals were withheld prior to training, but were provided at the conclusion of the training sessions. The training protocol was similar to that used previously (Malinowski et al., 2006), consisting of a 5 min warm-up walk, followed by 30 min trotting at a submaximal work intensity (~60% of HRmax), and ending with a 10 min cool down walk. One mare
was randomly assigned to wear a heart rate monitor during each training session to ensure HR was kept at an appropriate rate. Thus, mares trained aerobically, with heart rate below 160 beats per min, therefore staying below the anaerobic threshold (Marlin and Nankervis, 2002). In this way, mares would still achieve the physiological changes associated with aerobic exercise (Jimenez et al., 1998) without surpassing the anaerobic threshold.

After the initial 8 wk training period, mares completed GXT2 to measure changes in VO₂max and exercise capacity. The endocrine stimulation testing protocol described above was then repeated in subsequent weeks.

Mares continued to exercise train for an additional 7 wks at the same exercise intensity they reached during weeks 7-8 in the initial training period. Training at a steady intensity to avoid losing or gaining additional physiological changes due to exercise after GXT2. Mares completed their training sessions no less than 24 hr prior to their next endocrine stimulation test in order to avoid any influences of exercise on the tests (Golland et al., 1999). After completing the second round of endocrine stimulation tests, mares completed GXT3 to test for changes in VO₂max and exercise capacity between GXT2 and GXT3.

Sample Collection

On all testing days, catheters were inserted percutaneously into the jugular vein, using sterile technique and lidocaine anesthesia, approximately 30-45 min prior to the first blood sample. Blood samples (40 mL) were collected into pre-chilled tubes (40 mL, Vacutainer, Franklin Lakes, NJ), containing either EDTA or sodium heparin. Samples
were immediately centrifuged at 3000 x g at 4°C for 10 min. Plasma for hormone analyses was stored at -80°C until analysis. Plasma samples for glucose concentration were measured immediately (described below).

Assays and Sample Measurement

Plasma glucose was measured immediately in duplicate using an enzyme-electrode interface (ABL 800 Flex, Radiometer America, Westlake, OH). Plasma cortisol and ACTH were measured using commercially available radioimmunoassay (RIA) kits (ImmuChem™ Cortisol, MP Biomedical, Solon, OH and DiaSorin, Stillwater, MN, respectively). The cortisol RIA was previously validated for use in horses (Freestone et al., 1991), with sensitivity as low as 0.17 ug/dL according to the manufacturer. The justification for use of the ACTH RIA, designed for humans, was based on publications that describe equine ACTH as very similar to human ACTH, (Livesey, et al. 1991), as human and horse ACTH peptides have the same amino acid composition and biological activity (Ng et al., 1981). In addition, the same assay was being used by the Michigan State University Diagnostic Center for Population and Animal Health for measurement of equine ACTH (personal communication, Dr. Patricia Schenck, December 18, 2009), utilizing a reference range of 9-45 pg/mL. A serial dilution of the lowest standard in the ACTH RIA was made in our lab and it was found that the sensitivity and linearity of the assay did not extend below the lowest standard, 15 pg/ml, provided by the manufacturer.

For the measurement of cortisol, the within-assay CV’s were 8.8% for ACTHtest, 12.5% for CON, 7.0% for DEX, 15.0% for DEX/ACTH and 11.1% for DEX/CRF. For
measurement of plasma ACTH, the within-assay CV’s were 13.5% for CON and 5.6% for DEX/CRF.

**Statistical Analyses**

Data were analyzed for main effects using a two-way ANOVA for repeated measures (SigmaStat 3.1, SPSS, Chicago, IL). Where appropriate, *post-hoc* tests were employed using Student-Neuman-Keuls for pairwise multiple comparisons. Areas under the curve (AUC) were calculated for glucose and hormone concentrations (SigmaPlot 9.0, SPSS, Chicago, IL), and compared using appropriate t-tests. In the absence of normality, a Mann-Whitney Rank Sum test was used. The null hypothesis was rejected when $P \leq 0.05$.

Multiple linear regression analysis was used to determine which factors contributed to the predictive model between cortisol, ACTH (where appropriate) and glucose in old and young mares, pre- and post-training.

Caloric output estimates were calculated based on the relationship that 5 cal are burned for every 1 L of oxygen consumed (Swain, 2000) (Table 2). Average VO$_2$max and average wt in kg were used for calculating estimates in old and young mares.

**Results**

**VO$_2$max and Exercise Capacity.**

Old mares demonstrated a 28%, 20% and 22% lower ($P < 0.05$) VO$_2$max than younger mares during GXT1, GXT2 and GXT3, respectively. However, both old and young mares significantly increased their VO$_2$max after 8 wks of training by 18% and
10%, respectively. All mares reached VO₂max by the 9 m/s step of the GXT. Neither VO₂max nor exercise capacity changed significantly in either age group from GXT2 to GXT3 (Figure 1a-b). Estimated caloric output is outlined in Table 3a.

Old mares had a 26%, 20% and 17% lower (\(P=0.002\)) run time to fatigue compared to young mares during GXT1, GXT2 and GXT3, respectively (Figure 1b). In other words, old mares had a lower exercise capacity than young mares. However, both old and young mares increased their exercise capacity after 8 wks of training by 22% and 13%, respectively (Table 3b). Exercise capacity did not change significantly from GXT2 to GXT3. Mean treadmill speed reached during each GXT is outlined in Table 3c.

**Cortisol**

For CON, there was no difference between age groups pre-training (\(P=0.19\)) (Fig. 2a), although young mares had a mean cortisol concentration throughout the duration of the test that was 50% higher than old mares. Post-training, young mares had an overall average of 102% higher plasma cortisol concentration compared to old mares (\(P=0.02\)) (Figure 2b). Interestingly, there was no main effect of training on plasma cortisol concentration for old mares (\(P=0.78\)) or young mares (\(P=0.97\)). Additionally, there was no difference in AUC for cortisol in old or young mares pre- vs. post-training (Table 4).

For the pre-training ACTH test, young mares had a mean plasma cortisol concentration that was an average of 72% higher than old (\(P=0.05\)) over the course of the test. The age-related difference disappeared post-training (\(P=0.22\)). However, post-training, there was an age x time interaction at 30 min (\(P=0.02\)) where young mares had a 49% higher average cortisol concentration compared to old (Figure 3a-b). There was no
effect of training on plasma cortisol concentration for old mares ($P=0.86$) or young mares ($P=0.63$). There was also no significant difference in AUC for cortisol in old mares pre- vs. post-training or young mares pre- vs. post-training (Table 4). Pre-training, there was no difference in AUC of cortisol between old and young mares ($P=0.07$). However, post-training, young mares had an 81% (mean, $P=0.02$) higher AUC of cortisol compared to old.

During ACTHtest, peak plasma cortisol concentrations were at 30 min and 1 hr in old and young mares pre- and post-training. Pre-training, old mares had an average of a 202% and 245%, respectively, higher peak plasma cortisol concentration during ACTHtest compared to CON, where young mares were 219% and 181%, respectively, higher during ACTHtest compared to CON. Post-training, old mares showed an average of 295% and 294%, respectively, higher peak plasma cortisol during ACTHtest compared to CON, whereas young mares showed an average of 225% and 125%, respectively, overall higher peak plasma cortisol concentration during ACTHtest compared to CON.

There were no significant differences in cortisol response between age groups in the DEX, DEX/ACTH or DEX/CRF tests, pre- or post-training.

For DEX, there was no effect of training for old ($P=0.20$) or young ($P=0.15$) mares. The AUC of cortisol was not calculated for DEX due to very low concentrations of cortisol present during the tests (Figure 4a-b).

For DEX/ACTH, there was no effect of training on plasma cortisol concentration for old mares ($P=0.63$). Young mares experienced a 26% increase ($P=0.02$) in overall plasma cortisol concentration post-training in response to DEX/ACTH. Similarly, there was no difference in AUC of plasma cortisol between old and young mares pre-training
(\(P=0.20\)), but young mares had a 71% greater (mean, \(P=0.03\)) AUC for cortisol post-training compared to old (Table 4).

For DEX/CRF, there was no effect of training on plasma cortisol concentration for old mares (\(P=0.07\)), but there was for young mares (\(P=0.02\)). However, the AUC of plasma cortisol was 44% lower (mean, \(P=0.01\)) post-training for old mares compared to pre-training. Young mares had 52% lower AUC of plasma cortisol concentration (mean, \(P<0.001\)) post-training compared to pre-training (Table 4). There was an age x time interaction in DEX/CRF pre- and post-training (\(P<0.001\) and \(P=0.01\), respectively) for cortisol (Figure 5a-b). Pre-training, there were an age x time interactions at 0, 1 hr and 24 hr post CRF (1:00) (\(P=0.01, 0.01 \text{ and } 0.04\), respectively), and post-training at 24 hr post-DEX (9:00) and 24 hr post-CRF (1:00) (\(P=0.01\) and <0.05, respectively).

Because there was no effect of training on plasma cortisol concentration, data from the post-training CON study was included in the parallel control analysis. Regarding the parallel control study, there was no difference (\(P=0.57\)) in cortisol concentration during CON in December vs. May vs. September.

**ACTH**

Tables 5a-b illustrate mean plasma ACTH during the CON study as well as the range of plasma ACTH concentrations measured. During the CON study, there was no effect of training on plasma ACTH concentration for old (\(P=0.82\)) or young (\(P=0.19\)) mares. There was also no significant difference in plasma ACTH concentration between old and young mares pre-training (\(P=0.56\)), or post-training (\(P=0.07\)) (Figure 5a-b).
However, post-training, old mares had higher concentrations of plasma ACTH compared to young at 30 min (110% higher, mean, \(P=0.02\)) and 2 hr (137% higher, mean, \(P=0.01\)).

During DEX/CRF, there was no effect of training on plasma ACTH concentration for old \((P=0.70)\) or young \((P=0.58)\) mares. There was also no effect of age on plasma ACTH concentration pre- \((P=0.14)\) or post- \((P=0.29)\) training. However, post-training, old mares had a 122% higher (mean, \(P=0.01\)) average plasma ACTH concentration compared to young at time 0 (Figure 7a-b).

In CON pre- and post- and DEX/CRF pre- and post-, there were no differences by age or training in AUC for ACTH (Table 6).

**Glucose**

There was no effect of age or training on plasma glucose during CON or ACTHtest pre- or post-training.

For DEX, there was an effect of training for old \((P=0.02)\), but not young \((P=0.19)\) mares, where old mares had a lower mean plasma glucose post-training. Pre-training, there was no difference in plasma glucose concentration between old and young mares \((P=0.46)\), but post-training young mares had a 4% higher plasma glucose concentration compared to old \((P=0.04)\) (Figure 8a-b). Young mares had higher plasma glucose concentrations compared to old pre-training at 0800 and 1000 (10% higher (mean) \(P=0.01\); and 9% higher, \(P=0.02\), respectively) and post-training at 6 hr (7% higher (mean), \(P=0.01\), 0800 (9% lower, \(P=<0.05\)), 0900 (9% higher, \(P=<0.05\)), 1000 (7% higher, \(P=0.01\)), 1100 (5% higher, \(P=0.05\)) and 1200 (7% higher, \(P=0.02\)).
For DEX/ACTH, there was an effect of training on plasma glucose concentration for both old \((P<0.001)\) and young \((P=0.05)\) mares. Both age groups had lower glucose concentrations post-training. There was no effect of age on plasma glucose concentration during DEX/ACTH.

For DEX/CRF, there was an effect of training on plasma glucose concentration for both old \((P<0.001)\) and young \((P=0.003)\), where glucose decreased post-training in both age groups. There was an age x time interaction post-training in DEX/CRF at 24 hr-post DEX (9:00) and 24 hr-post CRF (1:00), where old mares had lower plasma glucose concentrations compared to young (Figure 9a-b). There was no effect of age on plasma glucose during DEX/CRF.

**Regression Analyses**

For old and young mares in CON, no significant relationship could be predicted between cortisol, ACTH and glucose pre-training. Post-training, no significant relationship between cortisol, ACTH and glucose could be predicted for young mares, but for old mares, glucose could be predicted from cortisol and ACTH \((R=0.65, P<0.001\) for cortisol, \(P=0.04\) for ACTH). No significant relationship between cortisol and ACTH was detected for old mares post-training in CON.

For ACTHtest, no significant relationship could be predicted between glucose and cortisol for old mares pre- or post-training, or for young mares pre- or post-training.

With respect to DEX, the relationship between cortisol and glucose could be predicted in both old \((R=0.49, P<0.001)\) and young \((R=0.62, P<0.001)\) mares pre-
training. Post-training during DEX, the relationship between cortisol and glucose could still be predicted for old mares (R=0.47, P<0.001) and young mares (R=0.79, P<0.001).

Regarding DEX/ACTH, no significant relationship between glucose and cortisol was observed for old or young mares pre-training. However, post-training the relationship between glucose and cortisol could be predicted for old mares (R=0.28, P=0.02), but not for young mares (R=0.13, P=0.27).

For DEX/CRF, the relationship between cortisol, ACTH and glucose was tested. For old mares pre-training, only the relationship between cortisol and glucose could be predicted (R=0.74, P<0.001). For young mares, glucose could be predicted from both cortisol and ACTH (R=0.66, P<0.001, P=0.04, respectively), however there was no significant relationship between ACTH and cortisol. Similarly, post-training, only the relationship between glucose and cortisol could be predicted for old mares (R=0.42, P<0.001), and young mares (R=0.68, P<0.001).

**Discussion**

This work was conducted to help determine where along the HPAA age alters the endocrine response to exercise in horses. A novel finding here suggests that with age, both the pituitary and adrenal glands become less sensitive to endocrine stimulation. Sensitivity of the aged pituitary and adrenal glands can be increased, although not completely restored in old mares, with exercise training.

Results of the GXTs support earlier published findings in horses (Malinowski et al., 2006), where VO$_2$max and exercise capacity were shown to decline with age. The increase in exercise capacity after training is likely due to increased cardiovascular
function, i.e. stroke volume, as a result of training (Betros et al., 2002). It was expected that VO\textsubscript{2}max and exercise capacity would remain the same between GXT\textsubscript{2} and GXT\textsubscript{3}, as training intensity and duration were not increased between these tests.

**Cortisol**

Results presented here indicate that with age, adrenal glands are moderately impaired in their response to ACTH in old mares. This overall effect appears to be attenuated with moderate exercise training. Additional work described a 40% lower GR content in the hippocampus in sedentary rats compared to exercised animals (Campbell et al., 2010), suggesting a possible mechanism for the increase in hormonal sensitivity observed in exercised animals. Similarly, Campbell et al. (2009) reported an initial 2.5-fold increase in adrenal sensitivity after 2 weeks of exercise training in rats, but this increase in sensitivity was gone by training wk-8. This information suggested an adaptation of the HPAA to exercise in rats (Campbell et al., 2009). Likewise, in trained human runners the HPAA was conditioned to a decreased response during exercise (Luger et al., 1987). Additionally, chronic hypercortisolism has been observed under basal conditions in trained human runners, compared to controls (Mastorakos et al., 2010). Here, young mares showed higher cortisol concentrations during CON post-training, where old mares did not. Considering the comparative literature, it could be speculated that adrenal insufficiency in aged animals is not completely reversed by exercise training.

In contrast to repeated findings in horses (Horohov et al., 2002, Malinowski et al., 2006), Traustadottir et al. (2005) showed that older unfit women had higher cortisol
responses to an exercise challenge compared to young unfit and older fit women. Data in horses (Horohov et al., 2002, Malinowski et al., 2006) has shown that old mares have lower cortisol responses to exercise, as well as lower cortisol concentrations during recovery. Considering this information together, it is possible that age-related changes in adrenal glands of humans and horses do not follow the same patterns of senescence.

ACTH

In the current study, reference ranges were utilized from the Michigan State University Diagnostic Center for Population and Animal Health (published online, September, 2010), where a normal range of equine ACTH concentration is considered 9-45 pg/mL. Only one old mare consistently demonstrated ACTH concentrations above the normal range, however this mare showed no hirsutism or abnormal fat distribution. Otherwise, mares fell in and out of the normal range throughout the course of the CON study, both pre- and post- training. Old mares had more time points above what is considered normal compared to young, however none of the mares showed physical signs associated with what could be diagnosed as PPID. In addition, all mares suppressed cortisol during the DEX test.

It has been suggested in humans that daily exercise leads to chronic ACTH secretion and adrenal hyperfunction, which, in laboratory animals, was accompanied by adrenal hypertrophy (Mastorakos et al., 2010). This may partially explain the increased AUC of ACTH concentration observed in old mares, as part of the mechanism at work to compensate for diminished cortisol secretion in this age group, as well as the increase in cortisol concentration observed in young mares.
Studies of people showed that older, unfit women had higher ACTH concentrations compared to young, unfit and older, fit women (Traustadottir et al., 2005). Mares demonstrated a similar pattern, although differences between old and young horses were not significant. It could be speculated that increased ACTH concentration is a consequence of primary adrenal insufficiency, which becomes altered to some degree in fit individuals (Dorin et al., 2003). Alternatively, as described above, regular exercise may promote ACTH hypersecretion (Mastorakos et al., 2010). A combination of primary adrenal insufficiency and, consequently, ACTH hypersecretion may be part of the mechanism behind elevated ACTH and diminished cortisol concentrations observed here in old mares. Although none of the old mares showed signs of PPID, old mares had a tendency to exhibit ACTH concentrations considered above the normal reference range. The possibility remains that physiological changes in the pituitary glands of old mares contributed to ACTH hypersecretion.

In the present study, old mares exhibited higher ACTH concentrations during CON post-training at 30 min and 2 hr. It has been reported elsewhere (Alexander et al. 1996) that ACTH secretion patterns were complex and irregular during circadian maximum (daytime). Such irregularity may contribute to the differences seen here in old horses compared to young, however, data is limited regarding circadian rhythm of ACTH in horses and changes with age.

Seasonal variation in glucocorticoid activity may result in altered target tissue sensitivity in men, depending on time of year (Walker et al., 1997). Research indicated healthy horses or horses with equine metabolic syndrome did not show seasonal changes in cortisol, but both showed increases in plasma ACTH from August – October in the
northeast United States (Place et al., 2010). Work by Donaldson et al. (2005) also found potential seasonal variations in plasma ACTH. Donaldson’s group noted a significant difference in the reference ranges of plasma ACTH in pony mares and stallions in May (100% of subjects within reference range) and September (5% in 2002 and 8% in 2003 within reference range). By contrast, other work led to the conclusion that only pregnant mares show seasonal variation in cortisol where barren mares did not (Flisinska-Bojaowska et al., 1991). The pre-training HPAA stimulation testing here was conducted in May, and post-training testing occurred in the last week of August through September. It is possible that season played a role in the differences, with old mares particularly affected. However, there was no difference in plasma ACTH concentration in old mares pre- (May) vs. post- (September) training in CON, (despite exercise training), so seasonal variation may not be a factor here. A parallel control study described here examined cortisol, but found no changes from December to May to September. However, samples for cortisol measurement were collected between 0830 and 1500, and not collected during the evening hours. As cortisol has a diurnal variation in horses (Johnson and Malinowski, 1986), the lack of an evening sampling time may have confounded the results. Although seasonal variations in ACTH cannot be ruled out, the fact that cortisol did not change in the time periods described could suggest that ACTH did not either, but this hypothesis remains to be investigated. Thus seasonal results are mixed, and even studies of seasonal variation of cortisol in humans have shown both within- and between-subject variation unexplained by menstrual cycle, behavioral, emotional or cognitive stress reactions (Hansen et al., 2001).
Glucose

Cortisol is known to promote gluconeogenesis, particularly in the liver (Bowen, 2006). Dexamethasone is a synthetic steroid hormone that is exponentially more potent than cortisol (Meikle and Tyler, 1977). Thus, it acts as a powerful anti-inflammatory agent and a diagnostic tool for pituitary and adrenal dysfunction (McEwen et al., 1997; Isidori et al., 2003). However, research in human Addison’s disease patients suggested that the peripheral actions of glucocorticoids, such as insulin suppression, play a larger role in regulating plasma glucose concentration (Malerbi et al., 1988). In light of the observed lower concentrations of cortisol and glucose in old mares here, it is possible that peripheral action, or lack thereof, of glucocorticoids is part of the mechanism behind age-related differences in glucose concentration.

On the other hand, Martin and Martin (1988) reported a decrease in maximal glucocorticoid responsiveness in the aged rat liver, suggesting a possible decrease in hepatic gluconeogenesis in response to glucocorticoid signals. Additionally, these researchers showed a higher amount of unoccupied dexamethasone binding sites in the cytosol of adrenalectomized old rats compared to young ones (Martin and Martin, 1988). This may be part of the mechanism behind the lower plasma glucose concentrations observed in old mares, most notably in tests involving dexamethasone. It does not appear that an assessment of glucocorticoid binding sites has been conducted in horses. However, if lower amounts of glucocorticoid binding sites are occupied, it is possible that a similar mechanism may be at work in the aged horse, contributing to lower blood glucose concentration.
In addition, regression analyses suggested that glucose and cortisol influence one another. Similarly, research in humans demonstrated that cortisol infusion directly resulted in increased glucose production via hepatic gluconeogenesis (Khani and Tayek, 2001). Earlier studies of rats also suggested that hepatic gluconeogenesis increases in response to cortisol (Haynes and Lu, 1969). Considering the results of DEX, plasma glucose rose markedly the morning after dexamethasone administration in young mares post-training. The same phenomenon did not occur to the same degree in old mares. It could be speculated that dexamethasone, acting here as a synthetic glucocorticoid, may be promoting hepatic gluconeogenesis (Martin and Martin, 1988), therefore increasing plasma glucose in young animals. This process appears impaired in the aged. When the glucose data of DEX/CRF are considered as well, the rise in ACTH may also be influencing the increase in plasma glucose noted in young mares, but to a lesser degree in old mares. DeVries et al. (2000) suggested that HPAA activation associated with exercise is due to fuel demands by working muscles, which is signaled by dropping glucose concentration. Thus, ACTH may help to increase plasma glucose concentration. Although the regression did not detect a relationship between glucose and ACTH during DEX/CRF, the possibility remains that there is still a physiological significance of the interaction of these two variables.

**DEX/CRF**

This appears to be the first report of a combined dexamethasone/corticotropin releasing factor endocrine stimulation test in horses. Equine CRF was not commercially available, so the present investigation took advantage of the fact that CRF is highly
conserved among species (Livesey et al., 1991), justifying the use of human/rat CRF. The combined dexamethasone-CRF test was described in humans by Yanovski et al. (1993) as a more accurate way to diagnose hypercortisolism compared to dexamethasone or CRF alone, hence the selection of the combined DEX/CRF test in the current investigation. It would also be appropriate to test horses with CRF alone, as the procedure in horses remains to be described.

The current data showed a lower AUC of plasma cortisol in response to DEX/ACTH in both old and young mares post-training, suggesting a physiological change due to chronic exercise. Similarly, research in trained human runners reported a decreased cortisol response to a CRH-stimulation test, while ACTH and cortisol responses were comparatively higher during treadmill tests similar to that described here (Luger et al., 1987). This suggests that training increases the capacity of the HPAA to handle a higher workload with less pituitary adrenal activation (Luger et al., 1987; Mastorakos et al. 2005).

**Summary and Conclusion**

A notable implication of this study is that older animals can ascertain benefits with respect to HPAA function by maintaining a moderate level of physical activity. Exercise sensitized the adrenal glands of young mares to ACTH to a greater degree than in old mares. Exercise also appeared to improved sensitivity of the pituitary gland to CRF in old and young mares, however in older mares, the pituitary may not be as sensitive to negative feedback from cortisol, thus perpetuating elevated concentrations of
plasma ACTH. Alternatively, exercise training may promote chronically elevated ACTH concentrations, thus increasing adrenal activity.

In conclusion, evidence presented here points to impaired function of both the pituitary and adrenal glands in horses with advancing age. The impairment is somewhat attenuated, but not completely reversed, by exercise training. Diminished cortisol concentration combined with low plasma glucose could have a negative impact on exercise tolerance and recovery, and cause alterations in substrate utilization in horses (Malinowski et al., 2006). Exercise training helps to attenuate the age-related deficiencies, but does not completely reverse them. This information should be taken into consideration with respect to diet, exercise and general management of the older horse.

Acknowledgements

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List of Tables

Table 1. Body weight in kg (expressed as mean ± SE) and range of body condition scores (Henneke et al., 1983) of mares throughout the study period.

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</tr>
<tr>
<td>BCS, range</td>
<td>4.5 – 6</td>
<td>4.5 – 6.5</td>
<td>4 – 5</td>
<td>4.5 – 7</td>
<td>4 – 5.5</td>
<td>5 – 6.5</td>
</tr>
</tbody>
</table>

Table 2. Timeline for study protocol.

<table>
<thead>
<tr>
<th>Endocrine Stimulation</th>
<th>GXT #1</th>
<th>8 wks training</th>
<th>GXT #2</th>
<th>Testing &amp; Training</th>
<th>GXT #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3a. Average maximal oxygen uptake (VO$_2$max, expressed as mean mL/kg/min ± SE) and caloric output for old and young mares during GXT1, GXT2 and GXT3. Superscripts denote significant differences in VO$_2$max.

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
<th>GXT3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Old, VO$_2$max (mL/kg/min)</strong></td>
<td>95 ± 6$^a$</td>
<td>112 ± 7$^b$</td>
<td>114 ± 6$^b$</td>
</tr>
<tr>
<td>100% VO$_2$max Calories/Min</td>
<td>231</td>
<td>278</td>
<td>281</td>
</tr>
<tr>
<td>60% VO$_2$max Calories/Min</td>
<td>139</td>
<td>167</td>
<td>168</td>
</tr>
<tr>
<td><strong>Young, VO$_2$max ± SE (mL/kg/min)</strong></td>
<td>112 ± 5$^c$</td>
<td>134 ± 8$^d$</td>
<td>139 ± 3$^d$</td>
</tr>
<tr>
<td>100% VO$_2$max Calories/Min</td>
<td>305</td>
<td>331</td>
<td>359</td>
</tr>
<tr>
<td>60% VO$_2$max Calories/Min</td>
<td>183</td>
<td>199</td>
<td>215</td>
</tr>
</tbody>
</table>

Table 3b. Average run time to fatigue (exercise capacity) during GXTs (expressed as mean run time in seconds ± SE). Superscripts denote significant differences in run time.

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
<th>GXT3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Old, Run Time to Fatigue, s</strong></td>
<td>260 ± 17$^a$</td>
<td>317 ± 19$^b$</td>
<td>314 ± 20$^b$</td>
</tr>
<tr>
<td><strong>Young, Run Time to Fatigue, s</strong></td>
<td>350 ± 10$^c$</td>
<td>395 ± 31$^d$</td>
<td>378 ± 16$^d$</td>
</tr>
</tbody>
</table>

Table 3c. Average maximal velocity reached during GXTs (expressed as mean m/s ± SE).

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
<th>GXT3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Old Maximal velocity, m/s</strong></td>
<td>8.8 ± 0.3</td>
<td>9.7 ± 0.3</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td><strong>Young, Maximal velocity, m/s</strong></td>
<td>10.3 ± 0.2</td>
<td>10.5 ± 0.2</td>
<td>10.5 ± 0.2</td>
</tr>
</tbody>
</table>
Table 4. Summary of AUC data for plasma cortisol concentration during endocrine stimulation tests, pre-and post-training in old and young mares. AUC was not calculated for DEX. Data expressed as mean ± SE. An asterisk (*) in a row indicates a significant difference between age groups. A symbol (‡) in a column indicates a significant difference within an age group pre- and post-training.

<table>
<thead>
<tr>
<th>Test</th>
<th>Training</th>
<th>Old</th>
<th>Young</th>
<th>P= O vs. Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>128 ± 35</td>
<td>183 ± 29</td>
<td>0.07</td>
</tr>
<tr>
<td>CON</td>
<td>Post</td>
<td>103 ± 8</td>
<td>162 ± 22*</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>P= within age</td>
<td>0.53</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>ACTHtest</td>
<td>Pre</td>
<td>184 ± 31</td>
<td>306 ± 52</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>151 ± 31</td>
<td>274 ± 34*</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>P= within age</td>
<td>0.40</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>DEX/ACTH</td>
<td>Pre</td>
<td>134 ± 25</td>
<td>184 ± 25</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>135 ± 25</td>
<td>231 ± 28*</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>P= within age</td>
<td>0.98</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>DEX/CRF</td>
<td>Pre</td>
<td>410 ± 29</td>
<td>388 ± 17</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>231 ± 32 ‡</td>
<td>187 ± 26 ‡</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>P= within age</td>
<td>0.01</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
**Table 5a.** Mean (expressed as mean pg/mL ± SE) plasma ACTH concentrations during CON test for old and young mares, pre- and post-training. Reference range utilized was 9-45 pg/mL.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Old, Pre</th>
<th>Young, Pre</th>
<th>Old, Post</th>
<th>Young, Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30 min</td>
<td>42 ± 4</td>
<td>38 ± 6</td>
<td>43 ± 8</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>0</td>
<td>45 ± 5</td>
<td>31 ± 9</td>
<td>42 ± 6</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>+30 min</td>
<td>43 ± 3</td>
<td>32 ± 5</td>
<td>48 ± 11</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>1 hr</td>
<td>52 ± 9</td>
<td>34 ± 3</td>
<td>39 ± 4</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>2 hr</td>
<td>40 ± 2</td>
<td>33 ± 5</td>
<td>57 ± 14</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>4 hr</td>
<td>43 ± 5</td>
<td>37 ± 8</td>
<td>42 ± 7</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>6 hr</td>
<td>50 ± 6</td>
<td>29 ± 6</td>
<td>43 ± 6</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>24 hr</td>
<td>48 ± 3</td>
<td>37 ± 5</td>
<td>41 ± 4</td>
<td>28 ± 6</td>
</tr>
</tbody>
</table>

**Table 5b.** Range of plasma ACTH concentrations in pg/mL during CON test for old and young mares, pre- and post-training. Reference range utilized was 9-45 pg/mL.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Old, Pre</th>
<th>Young, Pre</th>
<th>Old, Post</th>
<th>Young, Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30 min</td>
<td>34-61</td>
<td>27-54</td>
<td>29-76</td>
<td>12-45</td>
</tr>
<tr>
<td>0</td>
<td>31-61</td>
<td>10-47</td>
<td>26-64</td>
<td>15-43</td>
</tr>
<tr>
<td>+30 min</td>
<td>34-51</td>
<td>23-48</td>
<td>25-85</td>
<td>12-36</td>
</tr>
<tr>
<td>1 hr</td>
<td>29-88</td>
<td>28-43</td>
<td>28-56</td>
<td>16-56</td>
</tr>
<tr>
<td>2 hr</td>
<td>34-45</td>
<td>23-48</td>
<td>23-104</td>
<td>17-43</td>
</tr>
<tr>
<td>4 hr</td>
<td>30-61</td>
<td>27-62</td>
<td>26-71</td>
<td>17-58</td>
</tr>
<tr>
<td>6 hr</td>
<td>36-76</td>
<td>13-39</td>
<td>28-67</td>
<td>15-29</td>
</tr>
<tr>
<td>24 hr</td>
<td>42-64</td>
<td>24-48</td>
<td>27-57</td>
<td>18-55</td>
</tr>
</tbody>
</table>
**Table 6.** Summary of AUC data for plasma ACTH concentration during CON and DEX/CRF endocrine stimulation tests, pre- and post- training in old and young mares.

Data expressed as mean ± SE.

<table>
<thead>
<tr>
<th>Test</th>
<th>Training</th>
<th>Old</th>
<th>Young</th>
<th>P= O vs. Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>Pre</td>
<td>2685 ± 256</td>
<td>2019 ± 377</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>2623 ± 436</td>
<td>1539.3 ± 276</td>
<td>0.06</td>
</tr>
<tr>
<td>P= within age</td>
<td>0.99</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEX/CRF</td>
<td>Pre</td>
<td>2708 ± 186</td>
<td>1763 ± 446</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>3177 ± 750</td>
<td>2008 ± 318</td>
<td>0.11</td>
</tr>
<tr>
<td>P= within age</td>
<td>0.53</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. (a) Average maximal oxygen uptake (VO₂max) in old (■) vs. young (□) Standardbred mares, pre-training (GXT1), post-8 wks training (GXT2), and again once all post-training tests were completed (GXT3). (b) Average run time to fatigue. Expressed as mean ± SE. An asterisk (*) signifies a significant difference (P<0.05) between age groups, where δ signifies a difference from GXT1.

Figure 2. Control test measuring plasma cortisol (ug/mL). Expressed as means ± SE, (a) pre-training, and (b) post-training. Superscripts denote significant differences between age groups.

Figure 3. ACTH stimulation tests measuring plasma cortisol (ug/mL). Expressed as means ± SE, (a) pre-training, and (b) post-training. An asterisk (*) signifies a significant age x time interaction.

Figure 4. Dexamethasone suppression test measuring plasma cortisol (ug/mL). Expressed as means ± SE, (a) pre- and (b) post- training.

Figure 5. Combined dexamethasone suppression/CRF stimulation test measuring plasma cortisol (ug/mL). Expressed as means ± SE, (a) pre-training, and (b) post-training. An asterisk (*) signifies a significant age x time interaction.
**Figure 6.** Control test measuring plasma ACTH (pg/mL). Expressed as means ± SE. (a) Pre-training, and (b) post-training. An asterisk (*) signifies a significant age x time interaction at 30 min and 2 hr post ACTH dose post-training.

**Figure 7.** Combined dexamethasone suppression/CRF stimulation test measuring plasma ACTH (pg/mL). Expressed as means ± SE. (a) Pre-training, and (b) Post-training. An asterisk (*) signifies a significant age x time interaction.

**Figure 8.** Dexamethasone suppression test measuring plasma glucose (mmol/dL). Expressed as means ± SE. (a) Pre-training, there was no effect of age on plasma glucose, but there was an age x time interaction 0800 and 1000. (b) Post-training, there was an effect of age, where old mares had lower plasma glucose compared to young. There were also age x time interactions at 6 hr, 0800, 0900, 1000, 1100 and 1200. An asterisk (*) signifies a significant age x time interaction.

**Figure 9.** Combined dexamethasone suppression/CRF stimulation test measuring plasma glucose (mmol/dL). Expressed as means ± SE. (a) Pre-training, and (b) post-training. An asterisk (*) signifies a significant age x time interaction.
1A

[Bar chart showing VO2max across GXT1, GXT2, GXT3 for Old and Young groups.]

1B

[Bar chart showing Run Time to Fatigue across GXT1, GXT2, GXT3 for Old and Young groups.]
6A

Plasma ACTH (pg/mL)

Timepoint

6B

Plasma ACTH (pg/mL)

Timepoint
CHAPTER 3:
RESPONSE OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND
GLUCOSE HOMEOSTASIS DURING ACUTE EXERCISE TO FATIGUE,
BEFORE AND AFTER TRAINING IN STANDARDBRED MARES.
Abstract

This study tested the hypothesis that age and exercise training alter the response of the hypothalamic-pituitary-adrenal axis (HPAA), insulin and glucose during an acute exercise challenge to fatigue. Six old (22.0 ± 0.7 yrs) and six young (7.3 ± 0.6 yrs; mean ± SE) unfit Standardbred mares were tested. Mares ran a graded exercise test (GXT) before (GXT1) and after (GXT2) 8 wks of training. Blood samples were taken before (-30 and 0 min), during the last 10 s of each GXT step and at fatigue. Between GXTs, mares trained 3 d/wk at 60% maximum heart rate (HRmax). Plasma ACTH, cortisol and insulin concentrations were measured via RIA, and glucose and lactate concentrations were measured via enzyme-electrode interface. Data were analyzed using a two-way ANOVA for repeated measures and regression analysis. The null hypothesis was rejected when $P \leq 0.10$. During GXT1, there was an effect of time/intensity on plasma cortisol concentration in both age groups ($P < 0.001$), which disappeared during GXT2. Peak plasma ACTH concentration increased an average of 58% over baseline ($P < 0.001$) during GXT1, and 122% during GXT2 in both age groups. Old mares displayed an average 135% higher concentration ($P = 0.06$) of plasma insulin compared to young during GXT2. Plasma glucose increased with time/intensity during GXT1 and GXT2, but the overall plasma glucose response to exercise decreased by 7% and 8% in old and young, respectively, in GXT2. After training, ACTH was positively correlated with glucose in old and young mares ($R = 0.46$, $P = 0.002$ and $R = 0.28$, $P = 0.07$, respectively) and lactate ($R = 0.54$, $P < 0.001$ and $R = 0.50$, $P < 0.001$, respectively). Old mares may experience a reduction in the feedback mechanism between cortisol and ACTH that is not restored.
with training (R=0.15, \( P=0.39 \)). In conclusion, training appears to condition the HPAA to blunted response during acute exercise to fatigue. Training attenuates, but does not completely reverse, age-related changes in endocrine responses and glucose homeostasis.
**Introduction**

The cortisol response to an exercise challenge is blunted in old horses compared to young and middle aged animals (Malinowski et al., 2006). Exercise training partially reversed the low cortisol response in old mares, but did not restore it to the concentration of younger animals (Malinowski et al., 2006). Diminished cortisol concentration could have a negative impact on exercise tolerance and recovery, and cause alterations in substrate utilization in horses (Malinowski et al., 2006). However, data is limited with regard to the hypothalamic-pituitary-adrenal axis (HPAA) during an exercise challenge in horses. This information may be important because of the role of cortisol in glucose metabolism and substrate availability during work.

Cortisol, a glucocorticoid, influences carbohydrate metabolism and insulin secretion, and is known to suppress insulin and promote gluconeogenesis (Andrews and Walker, 1999). Cortisol secretion is controlled by the HPAA (Nieuwenhuizen and Rutters, 2007). In response to a physiological challenge, such as exercise, corticotropin releasing factor (CRF) is released from the hypothalamus, which stimulates the anterior pituitary to secrete adrenocorticotropin hormone (ACTH) (Nieuwenhuizen and Rutters, 2007). ACTH signals the adrenal glands to release cortisol (Nieuwenhuizen and Rutters, 2007). Once cortisol binds to a glucocorticoid receptor (GR), a cascade of negative feedback is initiated via other GRs on the pituitary gland and hypothalamus, keeping HPAA activity under control (Nieuwenhuizen and Rutters, 2007). Cortisol has been shown to follow a diurnal pattern in horses, with the highest levels seen in the morning and lowest in the evening (Johnson and Malinowski, 1986), a pattern similar to that
observed in humans (Van Cauter et al., 1996). Thus, it is important to consider time of
day and consistency of sampling times when considering cortisol.

Cortisol secretion has been shown to increase during strenuous exercise in man
(Few, 1974). Humans of mixed training background also demonstrated increased plasma
cortisol concentrations during an exhaustive exercise test on a bicycle ergometer
(Schwarz and Kindermann, 1989). Similarly, cortisol and ACTH concentrations
increased in response to strenuous exercise in young, human male athletes (Cinar et al.,
2009), and in normal men during exercise tests on a bicycle ergometer (Coiro et al.,
2007). Also in man, insulin concentration fell during exercise, likely as part of the
mechanism to stimulate hepatic glucose production (Borghouts and Keizer, 2000). In line
with data described in humans, Gordon et al. (2007) observed a 41% increase in plasma
cortisol and 35% decrease in insulin during an exercise test to fatigue in unfit female
horses. Additionally, cortisol and ACTH increased in Thoroughbred horses during
exercise to fatigue (Nagata et al., 1999). While it appears that an increase in HPAA
hormone concentration is a normal response to exercise, limited comparative data is
available with respect to age and training status on these and other parameters during an
exercise challenge.

Advanced age has been associated with lower basal plasma cortisol
concentrations in humans (Galbo, 2001). This association is supported by the work of
Traustadottir et al. (2006), who observed a decline in negative feedback sensitivity of the
HPAA in older women, which was attenuated by aerobic fitness. In aged Standardbred
mares, a blunted cortisol response to exercise was observed when compared to that of
younger animals (Malinowski et al., 2006). Twelve weeks of exercise training improved,
but did not completely reverse, the effect of age on the diminished cortisol response to acute exercise in horses (Malinowski et al., 2006). This work is consistent with that of Horohov et al. (1999), which demonstrated that older horses had decreased plasma cortisol concentrations compared to younger animals after a strenuous exercise challenge. Limited information is available regarding age-related endocrine changes during exercise in horses. While research suggests that exercise training attenuates inappropriately low concentrations of cortisol in aged animals, underlying mechanisms remain to be elucidated.

Factors external to the HPAA may influence its activity. For example, arginine vasopression (AVP) acts synergistically with CRF to stimulate secretion of ACTH in response to a stressor, such as exercise (Chrousos, 1995). AVP perpetuates the concentration of CRF during exercise (and vice versa) at the level of the hypothalamus (Tsigos and Chrousos, 2002), with implications not only downstream on the HPAA, but for control of blood volume during exercise (McKeever and Hinchcliff, 1995). In addition, the concentration of circulating interleukin-6 (IL-6) increases quickly in response to various types, durations and intensities of exercise (Moldoveanu et al., 2001). IL-6 can enhance ACTH production, signaling the adrenal cortex to produce glucocorticoids, and potentiate the effect of glucocorticoids on the liver (Moldoveanu et al., 2001). It could therefore be speculated that altered HPAA function observed in aged animals may have implications for other endocrine and immunological systems.

It is unknown exactly how the HPAA response to acute exercise is altered in aged horses, and how glucose homeostasis may be affected. Therefore, this study was designed to test the hypotheses that plasma cortisol, ACTH, insulin and glucose concentrations
differ between old and young horses during an acute bout of exercise to fatigue. Secondarily, it was hypothesized that differences would be altered after a period of moderate exercise training.

Materials and Methods

Animals & Diet

Twelve unfit Standardbred mares were studied. Six mares were old (22.0 ± 0.7 yrs; mean ± SE) and 6 were young (7.3 ± 0.6 yrs) (Table 1). None of the mares showed phenotypic signs of pituitary pars intermedia dysfunction (PPID), such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). The only apparent difference between the groups was age. Mares were housed on 2-acre dry lots with shelters and were given *ad libitum* access to grass hay and water. All were previously familiarized with the treadmill laboratory and associated procedures, and the Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment.

Graded Exercise Tests (GXTs) & Training Protocol

Each mare underwent two graded exercise tests (GXTs) on a treadmill (Sato I, Equine Dynamics, Lexington, KY) set at a fixed 6% grade, one prior to training (GXT1), and the second after 8 wks training (GXT2). All GXTs were conducted between 0800 and 1130. An indirect open-flow calorimeter (Oxymax-XL, Columbus Instruments, Inc., Columbus, OH) was used to measure gas exchange to determine each mare’s maximal oxygen uptake (VO₂max). Oxygen uptake was measured continuously during the test and
recorded at 10 s intervals. VO₂ max was defined as the point when there were no further increases in VO₂ despite increases in speed. Exercise capacity, measured as run time to fatigue, was also calculated as described (McKeever and Malinowski, 1997).

The GXTs started at an initial speed of 4 m/s for 1 min. Speed was then increased to 6 m/s, followed by incremental increases of 1 m/s every 60 s until horses reached fatigue. Fatigue was defined as the point where horses could not keep up with the treadmill despite humane encouragement. At the point of fatigue, the treadmill was stopped, and 5 min of post-exercise calorimetry data was collected.

A heart rate monitor (Polar Equine, Lake Success, NY) was used to measure heart rate during GXTs. Heart rates were recorded at rest, and during the last 10 s of each treadmill step such that maximum heart rate and the HR versus speed relationship could be documented for each horse. Training intensity was calculated using this data so that each mare exercised at ~60% of HR max. The range of recorded HR max was 185-216 beats per minute (bpm). Thus during training, velocity was adjusted and HR kept between 111-130 bpm depending on the individual so as to maintain a submaximal relative work intensity of ~60% HR max.

Training was conducted between 0600 and 1000 from June-August. Mares exercised trained for an 8 wk period in a free-stall exercise machine (Equi-Ciser, Equi-Master International, Sudre, Alberta, Canada). The order of the groups was alternated every other session, so the same group was not always the first to train. Grain meals were withheld prior to training, but were provided at the conclusion of the training sessions. The training protocol was similar to that used previously (Malinowski et al., 2006), consisting of a 5 min warm-up walk, followed by 30 min trotting at a submaximal
work intensity (~60% of HRmax), and ending with a 10 min cool down walk. One mare was randomly assigned to wear a heart rate monitor during each training session. This ensured that mares trained consistently and aerobically, with heart rate below 160 beats per min, therefore staying below the anaerobic threshold (Marlin and Nankervis, 2002). In this way, mares would still achieve the physiological changes associated with aerobic exercise (Jimenez et al., 1998) without surpassing the anaerobic threshold.

**Sample Collection and Measurement**

Catheters were inserted percutaneously into the jugular vein, using sterile techniques and lidocaine anesthesia. Catheterization occurred approximately 30-45 min prior to the first blood sample. Blood samples were collected at -30 min, 0, during the last 10 sec of each treadmill step and at fatigue. Blood samples (40 mL) were placed into pre-chilled tubes (Vacutainer, Franklin Lakes, NJ), containing either EDTA or sodium heparin and centrifuged at 3000 x g for 10 min at 4°C. Plasma samples for cortisol, ACTH and insulin measurement were stored at -80°C until analysis. Plasma for glucose and lactate concentration was measured immediately.

Plasma glucose and lactate were measured immediately via enzyme-electrode interface (ABL 800 Flex, Radiometer America, Westlake, OH). Plasma cortisol, ACTH and insulin were measured using commercially available radioimmunoassay (RIA) kits (ImmuChem™ Cortisol, MP Biomedicals, Solon, OH; DiaSorin, Stillwater, MN; Siemens, Los Angeles, CA respectively).

The RIAs for cortisol and insulin were previously validated in horses (Freestone et al., 1991), with sensitivities as low as 0.17 ug/dL and 1.2 uIU/mL, respectively,
according to the manufacturers. The justification for use of the ACTH RIA, designed for humans, was based on publications that describe equine ACTH as very similar to human ACTH, (Livesey, et al. 1991), as human and horse ACTH peptides have the same amino acid composition and biological activity (Ng et al., 1981). In addition, the same assay was being used by the Michigan State University Diagnostic Center for Population and Animal Health for measurement of equine ACTH (personal communication, Dr. Patricia Schenck, December 18, 2009) utilizing a reference range of 9-45 pg/mL. A serial dilution of the lowest standard in the ACTH RIA was made in our lab and it was found that the sensitivity and linearity of the assay did not extend below the lowest standard, 15 pg/ml, provided by the manufacturer.

For cortisol, the intra-assay CVs were 9.3% for GXT1 and 12.0% for GXT2, and the inter-assay CV was 11.0%. For insulin, the within-assay CV for both GXTs was 5.8%. For ACTH, the intra-assay CVs were 8.2% for GXT1 and 14.1% for GXT2, and the inter-assay CV was 19.7%.

_Statistical Analyses_

A two-way ANOVA for repeated measures was utilized to test for main effects of age and training on VO₂max, hormone and glucose concentrations. _Post hoc_ testing for pairwise multiple comparisons was via Student-Newman-Keuls where appropriate (SigmaStat 3.0, SPSS, Chicago, IL). The null hypothesis was rejected when _P_<0.10 in effort to protect against a Type II error (Little and Hills, 1978). Grubbs’ test was used to test for outliers.
Multiple linear regression analysis was used to determine which factors contributed to the predictive model between cortisol, ACTH (where appropriate), glucose and lactate in old and young mares, pre- and post-training. A t-test was used to compare slopes of the regression lines, and a test for coincidence, where appropriate, was used to test differences in slopes of regression lines and assess changes in set points of the endocrine response.

To investigate physiological events during exercise, statistical analyses and calculations were made for the RUN portion of the GXT (-30 min through 9 m/s). All mares reached VO₂max by the 9 m/s step of the GXT.

**Results**

**VO₂max and Exercise Capacity**

Old mares had a 28% lower (mean, \( P<0.05 \)) and 20% lower (\( P<0.05 \)) VO₂max compared to young mares in GXT1 (95 ± 6 vs. 122 ± 5, mean ± SE mL/kg/min, respectively) and GXT2 (112 ± 7 vs. 134 ± 8, respectively). After 8 wks of aerobic exercise training, old mares increased their VO₂max by 18% (mean, \( P<0.05 \)), and young mares increased their VO₂max by 10% (\( P<0.05 \)).

Old mares had a 26% (260 ± 17 vs. 350 ± 10 seconds, mean ± SE, respectively) and 20% (317 ± 19 vs. 395 ± 31, respectively) lower (\( P=0.002 \)) exercise capacity compared to young mares during GXT1 and GXT2, respectively. Along with VO₂max, both old and young mares increased their exercise capacity after 8 wks of training by 22% and 13%, respectively. Mean exercise capacity and maximal treadmill speed reached for each age group during each GXT are outlined in Table 2.
RUN phase.

Cortisol. There was no difference in cortisol with respect to age \((P=0.31)\) or age by time interaction \((P=0.80)\) during GXT1, however there was an effect of time/intensity \((P<0.001)\). Cortisol increased 26% at peak compared to baseline (Figure 1a). During GXT2, there was no effect of age \((P=0.78)\), time/intensity \((P=0.10)\) or age by time interaction \((P=0.53)\) (Figure 1b). There was no effect of training on cortisol concentrations during GXT1 vs. GXT2 for old mares \((P=0.74)\) or young mares \((P=0.99)\).

ACTH. During GXT1, there was no effect of age \((P=0.98)\) or age by time interaction \((P=0.97)\) on plasma ACTH concentration. However, ACTH showed an effect of time with a mean 58% increase in plasma ACTH concentration from baseline to peak at 9 m/s \((P<0.001)\) in both age groups (Figure 2a). Similarly, during GXT2, there was no effect of age \((P=0.42)\) or age by time interaction \((P=0.67)\). Again, an effect of time was observed with an overall mean 122% increase in plasma ACTH concentration from baseline to peak at 9 m/s \((P<0.001)\) in both age groups (Figure 2b). There was no effect of training on ACTH concentrations during GXT1 vs. GXT2 for old mares \((P=0.17)\) or young mares \((P=0.42)\).

Glucose. During GXT1, there was no effect of age \((P=0.61)\) or age by time interaction \((P=0.99)\), but there was an overall effect of time \((P<0.001)\), with a 7% increase in plasma glucose concentration from baseline to peak (Figure 3a) in both age groups. Similarly, during GXT2, there was no effect of age \((P=0.63)\) or age by time interaction \((P=0.28)\), but there was an effect of time \((P<0.001)\) with an 8% increase in plasma glucose concentration from baseline to peak in both groups (Figure 3b). There
was no effect of training on glucose concentrations during GXT1 vs. GXT2 for old mares
\((P=0.18)\) or young mares \((P=0.23)\).

**Insulin.** During GXT1, there were no effects of age on plasma insulin
concentration \((P=0.89)\) (Figure 4a). During GXT2, old mares had an overall mean 135%
higher \((P=0.06)\) concentration of plasma insulin compared to young mares (Figure 4b).
Old mares showed no difference \((P=0.61)\) in plasma insulin concentration in GXT1 vs.
GXT2. However, young mares had a 60% lower (mean, \(P=0.09\)) concentration of plasma
insulin during GXT2 compared to GXT1.

**Lactate.** There were no age differences in plasma lactate concentration during
GXT1 \((P=0.74)\) or GXT2 \((P=0.79)\). Overall, old mares had lower mean plasma lactate
during GXT2 compared to GXT1 \((P=0.04)\), but young mares experienced no change
\((P=0.21)\) (data not shown).

**Regression.** Since no age differences were observed for plasma concentrations of
cortisol, ACTH or glucose, data were pooled for multiple linear regression analysis.
Multiple linear regression analysis suggested that in the RUN phase of GXT1, there was
a strong association between plasma glucose and cortisol concentrations \((R=-0.83,
P=0.02)\), but not from ACTH concentration \((P=0.35)\). However, for GXT2, plasma
glucose also had strong associations with both cortisol \((R=-0.83, P=0.03)\) and ACTH
\((R=0.05, P<0.001)\).

Slopes of the lines of ACTH vs. cortisol for GXT1 vs. GXT2 were significantly
different \((P<0.05)\) for old mares, but there was no difference \((P>0.10)\) for young mares
(Figure 5 a-b). The test for coincidence also revealed no difference for young mares
\((P>0.10)\).
Slopes of the regression lines for ACTH vs. glucose were different for old and young mares during RUN ($P<0.05$ for both age groups) (Figures 6a-b).

For cortisol vs. glucose, slopes of the regression lines for GXT1 and GXT2 were different for old and young mares during RUN ($P<0.05$ for both age groups) (Figures 7a-b).

For ACTH vs. lactate, slopes of the regression lines for GXT1 and GXT2 were different for young mares but not for old. However, the y-intercepts were significantly different for old mares (Figures 8a-b).

**Discussion**

Results of this investigation demonstrate that exercise training affects the HPAA during acute exercise to fatigue. A schematic of the normal workings of the HPAA is illustrated in Figure 9. It appears that chronic, sub-maximal exercise training results in decreased adrenal sensitivity to ACTH, perhaps as an adaptive mechanism to tolerate a higher workload with lower adrenal activation (Luger et al., 1987).

Limited data regarding adaptations of the HPAA to aerobic exercise training is available in horses. Such adaptations have been reported in humans (Luger et al., 1987) and rats (Campbell et al., 2010). In addition, increases over baseline measures of blood lactate, epinephrine, norepinephrine and cortisol were recorded to begin in horses working 48% VO$_{2}$max, but did not reach the anaerobic threshold until horses exercised at approximately 80% VO$_{2}$max (Jimenez et al., 1998). This suggests that training above the anaerobic threshold is not necessary to achieve an endocrine response to training. A training intensity of 60% HRmax for a duration of 12 wks has been previously reported...
to increase not only VO$_2$max, but to affect cortisol in old and young horses (Malinowski et al., 2006). Continued exercise at a heart rate below approximately 160 bpm maintains work below the anaerobic threshold (Marlin and Nankervis, 2002). The training regimen described here did not tax the cardiovascular system nearly this high. Nevertheless, it was clearly adequate to produce increases in VO$_2$max and exercise capacity. Thus, the exercise training protocol here was sufficient to develop an endocrine and cardiovascular adaptation to aerobic exercise, without conditioning the system at or above the anaerobic threshold.

Data described here is consistent with the work of Church et al. (1987) who reported no significant effect of training on plasma cortisol, ACTH or insulin response to a standardized exercise test. Here, only old mares showed an effect of training on plasma insulin concentrations, where Church et al. (1987) did not make comparisons between age groups.

In the present investigation, ACTH increased during exercise without a concurrent rise in cortisol post-training. Training abolished the increase in plasma concentration of cortisol in response to acute exercise. There is no other work in horses in this regard, however comparative research may provide insight as to the mechanisms of action at work. Highly trained human runners have shown a diminished HPAA response during acute exercise to fatigue compared to sedentary and moderately trained groups, suggesting that the HPAA of trained athletes becomes conditioned to a blunted response (Luger et al., 1987). Additionally, research with rats observed an initial 2.5-fold increase in adrenal sensitivity after 2 wks of exercise training, but the increase in sensitivity was gone by training week-8 (Campbell et al., 2009). The latter observation suggests an
attenuated response of the HPAA after exercise training in rats (Campbell et al., 2009). Similar physiological events appear to occur in the horse, where exercise training conditions the HPAA to a blunted response during an acute, physical challenge.

The highest concentration of plasma ACTH reported here was at 9 m/s both before and after training. The difference in plasma ACTH concentration at 9 m/s from GXT1 to GXT2, although not statistically significant, is likely physiologically significant. Here, plasma ACTH was positively correlated with increased plasma glucose and lactate. As lactate has a role in hepatic gluconeogenesis (Marlin and Nankervis, 2002), increased ACTH may be an adaptation to training that influence mechanisms responsible for promoting the adequate supply of substrate for aerobic metabolism.

Furthermore, trained human runners had a higher ACTH concentration during high-intensity treadmill work compared to a oCRH stimulation test (Luger et al., 1987). It was concluded that as an adaptive response to training, a higher workload was tolerated with less pituitary-adrenal activation (Luger et al., 1987), and that other factors influenced the increase in plasma ACTH concentration during intense exercise (Luger et al., 1987; Mastorakos et al., 2005). Suggested factors included arginine vasopressin (AVP) (Mastorakos et al., 2005), lactic acid (Farrell et al., 1983) and interleukin-6 (Moldoveanu et al., 2001). While AVP and IL-6 were not assessed here, their potential influence on the HPAA cannot be ruled out. It would appear that in horses, as described in humans, training did, in fact, condition the HPAA to tolerate an exercise challenge to fatigue with less adrenal activation.

The sympathetic nervous system may also influence the HPAA and ACTH secretion (Sircar, 2008). During exercise and recovery, the parasympathetic and
sympathetic nervous systems act oppositely, but synergistically, allowing for rapid
trends in cardiac parameters (Fitzgerald et al., 2009). Intense exercise activates the
sympathetic nervous system, and hormones such as epinephrine and norepinephrine
(McKeever, 1993; de Graaf-Roelfsema et al., 2007). The concentrations of these
hormones are correlated with exercise intensity and the withdrawal of parasympathetic
tone (Jimenez et al., 1998). Binding of adrenergic receptors promotes ACTH secretion in
response to a challenging event (Sircar, 2008). Additionally, epinephrine has been
shown to increase lactate concentration in skeletal muscle of humans exercising at 70-
86% VO2max (Kjaer et al., 2000). Increased concentration of plasma lactate contributes
to the continued process of gluconeogenesis via the Cori cycle, the end result of which
provides energy to the working animal (Marlin and Nankervis, 2002). Data from the
present study concur with that found in studies of humans (Farrell et al., 1983) and horses
(Nagata et al., 1999) that reported positive correlations between ACTH and plasma
lactate concentrations. The combination of increased ACTH and lactate during intense
exercise may therefore function to provide a continuous source of energy for aerobic
work. However, evidence of an age-related decline in sympathetic nervous system
function has been reported in man (Piccirillo et al., 2001). Examination of the roles of
the sympathetic and parasympathetic responses to exercise and recovery was beyond the
scope of the present investigation. The possibility remains that age-related changes in the
sympathetic and parasympathetic responses influence ACTH, cortisol, glucose, insulin
and lactate, but these questions remain to be answered.

In the current study, both old and young mares experienced an increase in plasma
glucose during both GXTs. Increased plasma glucose concentration was also observed in
horses running at 55% VO\textsubscript{2}max for 60 min (Geor et al., 2002). Furthermore, epinephrine and norepinephrine are known to increase during exercise (Jimenez et al., 1998). These catecholamines promote increased blood glucose concentration and inhibition of insulin, which are essential events for energy balance in the fight-or-flight response (Sircar, 2008). Epinephrine not only inhibits insulin-stimulated glucose clearance in horses (Geor et al., 2000), but has been shown to increase the rate of hepatic gluconeogenesis in humans (Dufour et al., 2009). This may, in part, explain why hepatic glucose release exceeds peripheral glucose uptake during intense exercise in humans, causing an increase in plasma glucose concentration (Kjaer, 1998). The liver clearly plays an important role in the supply of glucose to working muscle in human and rat models (Hoene and Weigert, 2010). Also in man, hepatic glucose output increases with continuous muscular work, contributing to the availability of substrate for energy use (Bergstrom and Hullman, 1967). Therefore, it could be speculated that the liver is likely to be at least partially responsible for the increase in plasma glucose observed during exercise in horses as well. Additional research in this area would help answer this question.

As expected, there was a negative correlation between ACTH and cortisol in young mares. However, there appeared to be a disruption in this relationship in old mares. This is in line with data in aged human women (Traustadottir et al., 2006). Although slopes of the regression lines differed for ACTH vs. cortisol in old mares during GXT1 and GXT2, the correlations between these parameters were not significant. This suggests a reduction of the feedback mechanism between cortisol and ACTH with age that was not restored with training.
Interestingly, positive correlations existed between ACTH and glucose, as well as ACTH and lactate concentrations. These data support the speculation that ACTH contributes to increased glucose concentrations, and that by promoting lactate, gluconeogenesis may continue during exercise. Moreover, these relationships could be part of an adaptive response to exercise that ensures sufficient substrate is available for aerobic work. Additional research is warranted to further investigate these theories.

Training eliminated the effects of exercise intensity on cortisol during GXT2, but glucose continued to increase in both age groups. During exercise, cortisol may not be the main signal to increase plasma glucose concentration. Insulin concentrations did not change during GXT1 or GXT2, suggesting other influences on glucose concentration. Considering comparative research discussed above, it could be suggested that epinephrine, ACTH or lactate may serve as potent contributors to increased hepatic gluconeogenesis and plasma glucose concentration. Such determinations were beyond the scope of the current study, but warrant further investigation.

**Summary and Conclusion**

This study provides valuable insight into the workings of the HPAA and glucose metabolism during intense exercise as affected by age and training status. Exercise training appears to condition the HPAA to a decreased response during acute exercise to fatigue. It is likely that factors outside the HPAA, particularly epinephrine, influence its action. In addition, the relationship between cortisol and ACTH appears to become more synergistic with training, but may be reduced with age. ACTH may be part of an endocrine signal involved with upregulation of plasma glucose concentration. The
relationship between glucose and cortisol is less clear, but may be different in old and young mares or have influences from outside the HPAA. Overall, moderate exercise training appears to have a positive effect on the HPAA and glucose homeostasis in horses, and to help restore age-related deficits to some degree. Therefore, moderate exercise should remain part of a horse’s routine as tolerated with advancing age.

Acknowledgements

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List of Tables

**Table 1.** Body weight in kg (expressed as mean ± SE) and range of body condition scores (BCS) (Henneke et al., 1983) of mares throughout the study period.

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Old (kg)</td>
<td>Young (kg)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>486 ± 14</td>
<td>500 ± 16</td>
</tr>
<tr>
<td>BCS, range</td>
<td>4.5 – 6</td>
<td>4.5 – 6.5</td>
</tr>
<tr>
<td></td>
<td>Old (kg)</td>
<td>Young (kg)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>496 ± 13</td>
<td>494 ± 20</td>
</tr>
<tr>
<td>BCS, range</td>
<td>4 – 5</td>
<td>4.5 – 7</td>
</tr>
</tbody>
</table>

**Table 2.** Average maximal velocity reached during GXTs (expressed as mean m/s ± SE).

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old Maximal velocity, m/s</td>
<td>8.8 ± 0.3</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>Young Maximal velocity, m/s</td>
<td>10.3 ± 0.2</td>
<td>10.5 ± 0.2</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** RUN only data for Cortisol during (a) GXT1, where letters denote changes over time, and (b) GXT2. Expressed as mean ± SE. An asterisk (*) denotes a significant difference from baseline for both age groups.

**Figure 2.** RUN only data for ACTH during (a) GXT1 and (b) GXT2. Expressed as mean ± SE. An asterisk (*) denotes a significant difference from baseline for both age groups.

**Figure 3.** RUN only data for plasma glucose during (a) GXT1 and (b) GXT2. Expressed as mean ± SE. An asterisk (*) denotes a significant difference from baseline for both age groups.

**Figure 4.** RUN only plasma insulin data for old and young mares from (a) GXT1 and (b) GXT2. Expressed as mean ± SE.

**Figure 5.** Regression graphs comparing ACTH vs. cortisol during GXT1 (——) and GXT2 (- - -) in (a) RUN only, old mares and (b) RUN only, young mares.

**Figure 6.** Regression graphs comparing ACTH vs. glucose during GXT1 (——) and GXT2 (- - -) in (a) RUN only, old mares and (b) RUN only, young mares.

**Figure 7.** Regression graphs comparing glucose vs. cortisol during GXT1 (——) and GXT2 (- - -) in (a) RUN only, old mares and (b) RUN only, young mares.
Figure 8. Regression graphs comparing ACTH vs. lactate during GXT1 (——) and GXT2 (---) in (a) RUN only, old mares and (b) RUN only, young mares.

Figure 9. Illustration of the normal HPAA, courtesy Virginia Maryland Regional College of Veterinary Medicine website.
Old Mares

ACTH = (1.390 * Cortisol) - 78.329
P = 0.39
R = 0.13

ACTH = - (3.598 * Cortisol) - 78.958
P = 0.64
R = 0.08

Cortisol, ug/dL

Young Mares

ACTH = - (5.911 * Cortisol) + 112.249
P = 0.07
R = 0.28

ACTH = - (4.639 * Cortisol) + 91.434
P = 0.20
R = 0.21

Cortisol, ug/mL
6A

Old Mares

ACTH = (2.377 * Glucose) - 137.129
P = 0.002
R = 0.46

ACTH = (-0.192 * Glucose) + 82.785
P = 0.88
R = 0.02

Glucose, mmol/dL

6B

Young Mares

ACTH = (2.406 * Glucose) - 160.015
P = 0.07
R = 0.28

ACTH = (1.597 * Glucose) - 97.151
P = 0.13
R = 0.24

Glucose, mmol/dL
7A

**Old Mares**

Cortisol = - (0.0784 * Glucose) - 12.373  
P = 0.08  
R = 0.91

Cortisol = - (0.0231 * Glucose) + 6.901  
P = 0.63  
R = 0.079

---

7B

**Young Mares**

Cortisol = - (0.128 * Glucose) - 19.106  
P = 0.01  
R = 0.43

Cortisol = - (0.0385 * Glucose) - 9.790  
P = 0.40  
R = 0.13
**Old Mares**

- ACTH = (7.93 \times \text{Lactate}) + 32.72
- P < 0.001
- R = 0.68

**Young Mares**

- ACTH = (6.11 \times \text{Lactate}) + 38.23
- P = 0.005
- R = 0.44

- ACTH = (11.75 \times \text{Lactate}) + 28.54
- P < 0.001
- R = 0.50
CHAPTER 4:
RESPONSE OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND
GLUCOSE HOMEOSTASIS IN RECOVERY FROM ACUTE EXERCISE,
BEFORE AND AFTER TRAINING IN STANDARDBRED MARES.
Abstract

This study tested the hypothesis that age and exercise training alter the response of the hypothalamic-pituitary-adrenal axis (HPAA), insulin and glucose during recovery from an acute exercise challenge to fatigue. Six old (22.0 ± 0.7 yrs) and six young (7.3 ± 0.6 yrs; mean ± SE) unfit Standardbred mares were tested. Mares ran a graded exercise test (GXT) before (GXT1) and after (GXT2) 8 wks of training. Blood samples were taken before (-30 and 0 min) and after GXTs (2, 5, 30 min, 1, 2, 4 and 24 h). Plasma ACTH, cortisol and insulin concentrations were measured via RIA, and glucose and lactate concentrations were measured via enzyme-electrode interface. Data were analyzed using a two-way ANOVA for repeated measures and regression analysis. Areas under the curve were calculated and compared with appropriate t-tests. The null hypothesis was rejected when $P \leq 0.10$. Both age groups increased VO$_2$max and exercise capacity from GXT1 to GXT2 ($P < 0.05$), but old mares were consistently lower (28% and 20% lower VO$_2$max, and 26% and 20% lower exercise capacity, respectively). After GXT1, young mares had an overall 49% greater mean plasma cortisol concentration compared to old ($P = 0.03$), but this difference disappeared after GXT2. There was no effect of age on plasma ACTH after GXT1 ($P = 0.72$) or GXT2 ($P = 0.32$). Plasma ACTH increased an average of 188% over baseline at 2 min after GXT1, and 148% after GXT2. There was no age difference in plasma glucose after GXT1 ($P = 0.21$) or GXT2 ($P = 0.33$). There was no age difference in plasma insulin after GXT1 ($P = 0.24$), but there was after GXT2 ($P = 0.02$). For plasma lactate, young mares had higher concentrations at 2- and 5 min after GXT1 ($P = 0.08$) compared to old. This age effect was not apparent after GXT2. Regression analysis suggests that ACTH and glucose increase together in both age groups after training.
(R=0.42, \( P<0.001 \), pooled). A relationship between ACTH and lactate existed in old and young mares before (R=0.63, \( P<0.001 \) and R=0.58, \( P<0.001 \), respectively) and after (R=0.56, \( P<0.001 \) and R=0.67, \( P<0.001 \), respectively) training. In conclusion, training attenuates overall age-related declines in HPAA response to an exercise challenge.
Introduction

The cortisol response after exercise to fatigue is blunted in aged horses (Malinowski et al., 2006), but the mechanisms responsible for this phenomenon are poorly understood. Diminished cortisol concentration could cause altered substrate mobilization, resulting in reduced ability to recover from exercise (Malinowski et al., 2006). Interestingly, exercise training partially reversed the low cortisol response after an acute exercise challenge in old horses, which is likely important for maintenance of endocrine and glucose homeostasis (Malinowski et al., 2006), but the underlying reasons behind this change remain to be elucidated. Additional data is limited with regard to the effects of aging and training on the hypothalamic-pituitary-adrenal axis (HPAA) during recovery from an exercise challenge in horses.

Cortisol, a glucocorticoid, influences carbohydrate metabolism and insulin sensitivity, and is known to suppress insulin and promote gluconeogenesis (Andrews and Walker, 1999). Cortisol secretion is controlled by the HPAA (Nieuwenhuizen and Rutters, 2007). Corticotropin releasing factor (CRF) is released from the hypothalamus, and stimulates the anterior pituitary to secrete ACTH (Nieuwenhuizen and Rutters, 2007). ACTH signals the adrenal glands to release cortisol (Nieuwenhuizen and Rutters, 2007). Once cortisol binds to a glucocorticoid receptor (GR), a cascade of negative feedback is initiated via other GRs on the pituitary gland and hypothalamus keeping the HPAA activity under control (Nieuwenhuizen and Rutters, 2007). Cortisol has been shown to follow a diurnal pattern in horses, with the highest levels seen in the morning and lowest in the evening (Johnson and Malinowski, 1986), a pattern similar to that
observed in humans (Van Cauter et al., 1996). Thus, it is important to consider time of day and consistency of sampling times when considering cortisol.

Studies of exercise recovery have shown that plasma cortisol increased in humans (Traustadottir et al., 2004; Mastorakos et al., 2005), rats (Kim et al., 2008) and horses (Church et al., 1987; Horohov et al., 1999; Marc et al., 2000; Malinowski et al., 2006; Gordon et al., 2007). In recovery from treadmill exercise in horses, plasma ACTH concentration peaked at the end of the test whereas cortisol peaked 20-30 min after cessation of a standard exercise test (Marc et al., 2000). Similarly, college-aged men and women exhibited increased plasma ACTH, cortisol and glucose after submaximal and exhaustive exercise challenges (Farrell et al., 1983), as did male students (Kindermann et al., 1982). Taken together, part of a normal endocrine response to exercise recovery involves an increase in HPAA hormones and plasma glucose concentration, as well as a delayed increase in insulin. However, the mechanisms behind physiological events that occur after exercise are less studied in horses with respect to training status and age.

Age has been associated with lower basal plasma cortisol concentrations in humans (Galbo, 2001) and horses (Horohov et al., 1999; Malinowski et al., 2006). This association is supported by the work of Traustadottir et al. (2006), who observed a decline in negative feedback sensitivity of the HPAA in older women that was attenuated by improvements in aerobic fitness. Comparative data discussed here suggests that age-related deficits in HPAA function may be affected by training, but the exact part(s) of the HPAA that become impaired with advancing age have not been described in horses.

In addition to the endocrine response of the HPAA, glucose homeostasis is challenged by age and exercise. Exercise training improved overall insulin sensitivity in
horses ranging in age from young to old (Malinowski et al., 2002; Powell et al., 2002). In aged people, insulin resistance occurred predominantly in skeletal muscle, with hepatic sensitivity largely unaffected (Scheen, 2005). However, Martin and Martin (1988) reported a decrease in maximal glucocorticoid responsiveness in the aged rat liver, suggesting a possible decrease in hepatic gluconeogenesis in response to glucocorticoid signals. Taken together, diminished glucocorticoid responsiveness of the liver combined with low cortisol levels in aged animals could result in deficiencies in substrate mobilization and utilization, impairing the ability to sustain and recover from exercise. Additional investigation of age-related deficits in glucose homeostasis and the effects of exercise training are warranted to further understand the effects of age and exercise on glucose homeostasis.

The point(s) along the HPAA (hypothalamus, pituitary and/or adrenal glands) at which age-related changes occur remain to be elucidated. The effects of continued exercise training on the HPAA during recovery also remain to be clarified (Mastorakos et al., 2005), particularly in horses. Thus, the purpose of this investigation was to better understand the effects of age and moderate exercise training on the HPAA and glucose homeostasis in recovery from an acute bout of exercise to fatigue in horses. The hypotheses tested were that old and young mares would differ in their endocrine recovery responses to acute exercise to fatigue, but that responses would be modified by exercise training. Additionally, it was hypothesized that exercise would attenuate detrimental effects of aging on the HPAA and glucose homeostasis.

**Materials and Methods**
Animals & Diet

Twelve unfit Standardbred mares were studied. Six mares were old (22.0 ± 0.7 yrs; mean ± SE) and 6 were young (7.3 ± 0.6 yrs) (Table 1). None of the mares showed phenotypic signs of pituitary pars intermedia dysfunction (PPID), such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). The only apparent difference between the groups was age. Mares were housed on 2-acre dry lots with shelters and were given *ad libitum* access to grass hay and water. All were previously familiarized with the treadmill laboratory and associated procedures, and the Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment.

Graded Exercise Tests (GXTs) & Training Protocol

Each mare performed two graded exercise tests (GXTs) on a treadmill (Sato I, Equine Dynamics, Lexington, KY) set at a fixed 6% grade, one prior to training (GXT1), and the second after 8 wks training (GXT2). The GXTs were conducted between 0800 and 1130. An indirect open-flow calorimeter (Oxymax-XL, Columbus Instruments, Inc., Columbus, OH) was used to measure gas exchange to determine each mare’s maximal oxygen uptake (VO2max). Oxygen uptake was measured continuously during the test and recorded at 10 s intervals. VO2max was defined as the point when there were no further increases in VO2 despite increases in speed. Exercise capacity, measured as run time to fatigue, was also calculated as described (McKeever and Malinowski, 1997).

The GXTs started at an initial speed of 4 m/s for 1 min. Speed was then increased to 6 m/s, followed by incremental increases of 1 m/s every 60 s until horses reached
fatigue. Fatigue was defined as the point where horses could not keep up with the treadmill despite humane encouragement. At the point of fatigue, the treadmill was stopped, and 5 min of post-exercise calorimetry data was collected.

A heart rate monitor (Polar Equine, Lake Success, NY) was used to measure heart rate during GXTs. Heart rates were recorded at rest, and during the last 10 s of each treadmill step such that maximum heart rate and the HR versus speed relationship could be documented for each horse. Training intensity was calculated using this data so that each mare exercised at ~60% of HRmax. The range of recorded HRmax was 185-216 beats per minute (bpm). Thus during training, velocity was adjusted and HR kept between 111-130 bpm depending on the individual so as to maintain a submaximal relative work intensity of ~60% HRmax.

Training was conducted between 0600 and 1000 from June-August. Mares exercised trained for an 8 wk period in a free-stall exercise machine (Equi-Ciser, Equi-Master International, Sudre, Alberta, Canada). The order of the groups was alternated every other session, so the same group was not always the first to train. Grain meals were withheld prior to training, but were provided at the conclusion of the training sessions. The training protocol was similar to that used previously (Malinowski et al., 2006), consisting of a 5 min warm-up walk, followed by 30 min trotting at a submaximal work intensity (~60% of HRmax), and ending with a 10 min cool down walk. One mare was randomly assigned to wear a heart rate monitor during each training session. This ensured that mares trained consistently and aerobically, with heart rate below 160 beats per min, therefore staying below the anaerobic threshold (Marlin and Nankervis, 2002).
In this way, mares would still achieve the physiological changes associated with aerobic exercise (Jimenez et al., 1998) without surpassing the anaerobic threshold.

Sample Collection & Measurement

Catheters were inserted percutaneously into the jugular vein, using sterile techniques and lidocaine anesthesia. Catheterization occurred approximately 30-45 min prior to the first blood sample. Samples were collected at -30 min, 0, 30 min, 2-, 5-, 30 min, 1-, 2-, 4- and 24 hrs. Blood samples (40 mL) were placed into pre-chilled tubes (Vacutainer, Franklin Lakes, NJ), containing either EDTA or sodium heparin and centrifuged at 3000 x g for 10 min at 4°C. Plasma samples for cortisol, ACTH and insulin measurement were stored at -80°C until analysis. Plasma cortisol, ACTH and insulin were measured using commercially available radioimmunoassay (RIA) kits (ImmuChem™ Cortisol, MP Biomedicals, Solon, OH; DiaSorin, Stillwater, MN; Siemens, Los Angeles, CA respectively). Plasma glucose and lactate were measured immediately via enzyme-electrode interface (ABL 800 Flex, Radiometer America, Westlake, OH).

The RIAs for cortisol and insulin were previously validated in horses (Freestone et al., 1991), with sensitivities as low as 0.17 ug/dL and 1.2 uIU/mL, respectively, according to the manufacturers. The justification for use of the ACTH RIA, designed for humans, was based on publications that describe equine ACTH as very similar to human ACTH, (Livesey, et al. 1991), as human and horse ACTH peptides have the same amino acid composition and biological activity (Ng et al., 1981). In addition, the same assay was being used by the Michigan State University Diagnostic Center for Population and
Animal Health for measurement of equine ACTH (personal communication, Dr. Patricia Schenck, December 18, 2009) utilizing a reference range of 9-45 pg/mL. A serial dilution of the lowest standard in the ACTH RIA was made in our lab and it was found that the sensitivity and linearity of the assay did not extend below the lowest standard, 15 pg/ml, provided by the manufacturer.

For cortisol, the intra-assay CVs were 9.3% for GXT1 and 12.0% for GXT2, and the inter-assay CV was 11.0%. For insulin, the within-assay CV for both GXTs was 5.8%. For ACTH, the intra-assay CVs were 8.2% for GXT1 and 14.1% for GXT2, and the inter-assay CV was 19.7%.

Statistical Analyses & Calculations

A two-way ANOVA for repeated measures was utilized to test for main effects of age and training. Post hoc testing for pairwise multiple comparisons was via Student-Newman-Keuls where appropriate (SigmaStat 3.0, SPSS, Chicago, IL). The null hypothesis was rejected when \( P \leq 0.10 \) in effort to protect against a Type II error (Little and Hills, 1978). Grubbs’ test was used to test for outliers.

Areas under the curve (AUC) were calculated for glucose and hormone levels (SigmaPlot 9.0, SPSS, Chicago, IL). Appropriate t-tests were used to compare means between two groups, old vs. young or pre- vs. post-training. In the absence of normality, a Mann-Whitney Rank Sum test was used.

Multiple linear regression analysis was used to determine which factors contributed to the predictive model between cortisol, ACTH (where appropriate), glucose and lactate in old and young mares, pre- and post-training. A t-test was used to compare
slopes of the regression lines, and a test for coincidence, where appropriate, was used to compare overall differences in regression lines and assess changes in the gain on the endocrine response.

**Results**

Old mares demonstrated a 28% lower (mean, $P<0.05$) and 20% lower ($P<0.05$) VO$_{2\text{max}}$ compared to young mares in GXT1 (95 ± 6 vs. 122 ± 5 mL/kg/min, mean ± SE, respectively) and GXT2 (112 ± 7 vs. 134 ± 8 mL/kg/min, respectively). After 8 wks of aerobic exercise training, old mares increased their VO$_{2\text{max}}$ by 18% (mean, $P<0.05$), and young mares increased their VO$_{2\text{max}}$ by 10% ($P<0.05$).

Old mares had a 26% lower (260 ± 17 vs. 350 ± 10 seconds, mean ± SE, respectively) and 20% (317 ± 19 vs. 395 ± 31, respectively) lower ($P=0.002$) exercise capacity compared to young mares during GXT1 and GXT2, respectively. Along with VO$_{2\text{max}}$, both old and young mares increased their exercise capacity after 8 wks of training by 22% and 13%, respectively. Mean maximal treadmill speeds reached for old and young mares during each GXT are outlined in Table 2.

**RECOVERY phase.**

*Cortisol.* After GXT1, young mares had an overall mean 49% greater plasma cortisol concentration compared to old ($P=0.03$). There were age by time interactions ($P=0.001$) immediately after exercise, as well as a change in cortisol over time (Figure 1a). Plasma cortisol peaked at 30 min, with a 14% increase over baseline in old mares, and 51% increase over baseline in young mares. After GXT2, the effect of age
disappeared ($P=0.26$), as did the age by time interaction ($P=0.19$), however there remained an overall effect of time ($P<0.001$) (Figure 1b) with a mean 41% increase in plasma cortisol concentration at peak at 30 min compared to baseline. There was no effect of training on cortisol concentrations during GXT1 vs. GXT2 for old mares ($P=0.44$) or young mares ($P=0.89$).

There was no difference in AUC of cortisol concentration between old and young mares after GXT1 ($P=0.15$) or GXT2 ($P=0.24$) (Table 3a). No differences were seen in the AUC of cortisol in old mares ($P=0.42$) or young mares ($P=0.73$) from GXT1 to GXT2.

**ACTH.** After GXT1, again, there was no effect of age ($P=0.72$) or age by time interaction ($P=0.15$), but there was an overall effect of time ($P<0.001$), with a mean 188% increase over baseline in plasma ACTH concentration at 2 min (Figure 2a). Similar results were observed after GXT2, with no difference in age ($P=0.32$) or age by time interaction ($P=0.60$), but there was a change over time ($P<0.001$). Because there was no age difference after GXT2, data were pooled for old and young mares, where peak ACTH concentration at 2 min was a mean 148% over baseline (Figure 2b), and a mean 31% increase ($P=0.006$) in peak plasma ACTH concentration at 2 min from GXT1 to GXT2 was apparent. Plasma ACTH concentration was higher for old mares after GXT2 compared to GXT1 ($P=0.02$), but there was no difference for young mares ($P=0.47$).

There was no age difference in AUC for ACTH after GXT1 ($P=0.17$), but old mares had a mean overall 96% higher AUC for ACTH compared to young after GXT2 ($P=0.06$) (Table 3b). Old mares had a mean overall 58.5% greater AUC of ACTH after
GXT2 compared to GXT1 during RECOVERY ($P=0.05$). Young mares did not demonstrate differences in AUC of ACTH in GXT1 vs. GXT2 during RECOVERY ($P=0.20$).

**Glucose.** After GXT1, there was no effect of age ($P=0.21$) or age by time interaction ($P=0.56$) on plasma glucose concentration, but there was an effect of time with a mean peak increase of 40% at 5 min compared to baseline ($P<0.001$) (Figure 3a). Likewise, after GXT2, there was no effect of age ($P=0.33$) or age by time interaction ($P=0.90$). However, there was effect of time, where the mean peak concentration of plasma glucose at 5 min increased 45% over baseline measurements ($P<0.001$) (Figure 3b). There were no effects of training on plasma glucose during recovery from GXT1 vs. GXT2 for old mares ($P=0.98$) or young mares ($P=0.89$).

There was no difference in AUC of plasma glucose concentration between old and young mares after GXT1 ($P=0.68$) or GXT2 ($P=0.92$). Both old (6.8% lower, $P=0.10$) and young (8.2% lower, $P=0.08$) mares had a lower AUC of glucose following GXT2 compared to GXT1 (Table 3b).

**Insulin.** There were no age related differences in plasma insulin after GXT1 ($P=0.24$) (Figure 4a). After GXT2 old mares had an overall mean plasma insulin concentration that was 61% higher ($P=0.02$) compared to young (Figure 4b). Neither young nor old mares experienced a change in plasma insulin during RECOVERY due to training ($P=0.53$, $P=0.44$ respectively).

**Lactate.** There was no age difference in plasma lactate concentration between old and young mares after GXT1 ($P=0.21$) or GXT2 ($P=0.54$) (Figure 5 a-b). However, there was a significant age by time interaction during GXT1 ($P=0.08$), where young
mares had a higher concentration of plasma lactate at 2- and 5 min. This difference disappeared after GXT2. Additionally, old mares had increased plasma lactate concentration after GXT2 compared to GXT1 ($P=0.01$), but young mares did not ($P=0.94$).

**Regression.** Multiple linear regression analyses were separated by age for the RECOVERY phase of GXT1, due to the age-related difference in plasma cortisol concentration. For old mares, no prediction could be made, as no relationship between plasma cortisol, ACTH and glucose appeared to be found ($R=0.14$, $P=0.65$). However, for young mares, a weak, but significant, prediction could be made between plasma glucose and ACTH concentrations ($R=0.16$, $P<0.001$). After GXT2, there were no age differences between plasma cortisol, ACTH and glucose, so data were pooled. Again, plasma glucose could be predicted from plasma ACTH concentrations ($R=0.15$, $P<0.001$, pooled).

ACTH and cortisol were compared for GXT1 and GXT2. A correlation between ACTH and cortisol could not be predicted for old mares in GXT1 ($R=0.01$, $P=0.98$) or GXT2 ($R=0.07$, $P=0.65$). Similarly for young mares, no correlation was detected in GXT1 ($R=0.13$, $P=0.36$) or GXT2 ($R=0.22$, $P=0.11$). There were no significant differences in the slopes for old mares ($P>0.10$) or young mares ($P>0.10$) during RECOVERY (data not shown).

Slopes of the regression lines for ACTH vs. glucose were different for old and young mares during RECOVERY ($P<0.05$ for both) (Figures 6a-b).

For cortisol vs. glucose, slopes of the regression lines for GXT1 and GXT2 indicated no relationship between these parameters for old or young mares ($P=0.51$, pooled).
R=0.09 and \( P=0.12 \), R=0.22, respectively). However, slopes of the lines for cortisol vs. glucose from GXT1 and GXT2 were different for old and young mares during RECOVERY (\( P<0.05 \) for all) (data not shown).

For plasma lactate vs. ACTH, there was a significant difference in the slopes of the regression lines for old mares (Figure 7a). There were no differences in the slopes of the regression lines, y-intercept or test for coincidence for young mares (Figure 7b).

**Discussion**

In horses, moderate exercise training attenuates the age-related decline in VO\(_2\)\text{max} and exercise capacity, as well as the HPAA response and lactate production in recovery from acute exercise to fatigue. Most notably, the age difference in plasma cortisol concentration was absent after GXT2. In addition, plasma glucose concentrations remained elevated over a longer period of time after GXT2, suggesting an adaptive response to training and increased potential for replenishment of glycogen stores. Increased ACTH concentration after training may contribute to increased plasma glucose and lactate, as these variables are strongly correlated.

Limited data regarding adaptations of the HPAA to aerobic exercise training is available in horses. Such adaptations have been reported in humans (Luger et al., 1987) and rats (Campbell et al., 2010). However, in horses, increases over baseline measures of blood lactate, epinephrine, norepinephrine and cortisol were observed to begin at 48% VO\(_2\)\text{max}, but did not reach the anaerobic threshold until horses exercised at approximately 80% VO\(_2\)\text{max} (Jimenez et al., 1998). This suggests that training above the anaerobic threshold is not necessary to achieve an endocrine response to training. A
training intensity of 60% HRmax for a duration of 12 wks has been previously reported to increase not only VO$_2$max, but to affect cortisol in old and young horses (Malinowski et al., 2006). In addition, continued exercise at a heart rate below approximately 160 bpm maintains work below the anaerobic threshold (Marlin and Nankervis, 2002). The training regimen described here did not tax the cardiovascular system nearly this high. Nevertheless, it was clearly adequate to produce increases in VO$_2$max and exercise capacity in both age groups. Thus, the exercise training protocol here was sufficient to develop an endocrine and cardiovascular response to aerobic exercise, without conditioning the system at or above the anaerobic threshold. It should be noted that one limitation of the study was the individual variation in exercise capacity. Not all mares reached fatigue at the same time, as some were able to run longer than others. Consequently, mares may have begun the recovery process with some variation in physiological states.

Data described here is consistent with the work of Church et al. (1987) who reported no significant effect of training on plasma cortisol or insulin response to a standardized exercise test. Here, only old mares showed an effect of training on plasma ACTH concentrations, where Church et al. (1987) did not make comparisons between age groups. In addition, cortisol concentration in horses was similar to baseline measurements by 2 hrs post-exercise, but hyperinsulinemia also occurred at this time point (Church et al., 1987). Very similar data was reported in the present study after GXT2. It could be suggested that the timing of these events is coordinated to promote replenishment of glycogen stores during exercise recovery.
Presently, training eliminated the effect of age on plasma cortisol concentration during recovery. On the contrary, while work by Malinowski et al. (2006) showed an increase in plasma cortisol concentration after acute exercise in old mares post-training, the difference was not enough to eliminate the disparity compared to young and middle-aged mares. However, research in humans may provide insight into the reason for the difference observed in the current study. Similar to data reported here, older, fit women demonstrated increased cortisol production compared to older, unfit women (Traustadottir et al., 2004). It was suggested that an increase in adrenal capacity as a consequence of fitness training was at least partially responsible for the difference (Traustadottir et al., 2004). It has also been suggested that training results in an increase in adrenal sensitivity to ACTH, thus facilitating the change in cortisol levels (Mastorakos et al. 2005). When considering the comparative literature, it could be hypothesized that old mares also developed increased adrenal sensitivity to ACTH and/or increased adrenal capacity as a result of exercise training.

Cortisol stimulates substrate mobilization and increases free fatty acid pools, thus providing the building blocks for protein synthesis (de Graaf-Roelfsema et al., 2007). Cortisol did not increase during acute exercise after a period of training, yet it spiked 30 min into recovery. It has been proposed that this delayed rise in cortisol in response to a challenge prevents the immune and inflammatory responses from over-reacting (Munck et al., 1984) without interfering with the appropriate activation of stress-defense mechanisms initiated by the sympathetic nervous system (DeVries et al., 2000). Additionally, this could be an adaptive response to training designed to increase protein synthesis and muscle glycogen stores as part of the recovery process (de Graaf-
Roelfsema et al., 2007). An increased ability of the aged animal to manage these processes will likely improve exercise tolerance and facilitate recovery.

Peak plasma ACTH concentrations were observed during recovery at 2 min. Current observations are in line with that reported in humans (DeVries, et al., 2000; Traustadottir et al., 2004), where peak ACTH concentrations were observed within 5 min of cessation of exhaustive exercise. Additionally, plasma ACTH and lactate concentrations were positively correlated here, a phenomenon widely reported in human literature (Farrell et al., 1983; Heitkamp et al., 1998; Minetto et al., 2006) as well as in horses (Nagata et al., 1999). Lactate serves as substrate for the process of gluconeogenesis, the end result of which provides energy to the working animal (Marlin and Nankervis, 2002). In addition, epinephrine has been shown to increase lactate concentration in skeletal muscle of humans exercising at 70-86% VO₂max (Kjaer et al., 2000). Epinephrine has also been reported to promote ACTH secretion (Sircar, 2008) and hepatic gluconeogenesis in humans (Dufour et al., 2009), and inhibit glucose clearance in horses (Geor et al., 2000). Although epinephrine was not measured in the present investigation, comparative studies suggest it is a common link influencing the rise in ACTH, lactate and glucose seen here after exercise. Taken together, it could be speculated that increases in epinephrine are at least part of the mechanism responsible for increased plasma glucose and ACTH concentrations immediately after intense exercise.

In the current study, both old and young mares experienced an increase in plasma glucose that peaked 5 min after exercise, and just after the peak in ACTH. This observation is similar to that in horses by Geor et al. (2002). Interestingly, plasma glucose concentrations increased in the absence of an increase in cortisol or decline in
insulin, suggesting other elements at work. Data from other species may lend clues as to the mechanisms behind this phenomenon. During intense exercise in humans, hepatic glucose release exceeds peripheral glucose uptake, causing an increase in plasma glucose concentration (Kjaer, 1998). In this way, the liver functions to supply glucose to working muscle in human and rat models (Hoene and Weigert, 2010). Catecholamines have been reported to remain elevated in young men 2 hr following exhaustive exercise (Bahr et al., 2008), potentially perpetuating hepatic gluconeogenesis and the prolonged elevation of plasma glucose concentration observed after training. The prolonged elevation in plasma glucose after GXT2 may be a consequence of prolonged hepatic glucose output in response to fatigue-inducing exercise, and therefore likely part of the adaptive response to training that is responsible for making glucose available for replenishment of glycogen stores in skeletal muscles.

After exercise training, mares in both age groups experienced an overall decrease in plasma glucose concentration in recovery from acute exercise. Other work reported in horses concluded that moderate-intensity training similar to that described here resulted in a decreased rate of glucose production and whole-body glucose uptake during exercise, a decreased rate of muscle glycogen breakdown and increased the contribution of fat for energy utilization (Geor et al., 2002). Later work reported that trained horses had improved glucose clearance in response to acute exercise (Treiber et al., 2006). Other research with rats described enhanced glucose transport and glycogen synthesis in skeletal muscle in response to exercise, partially due to an increase in insulin sensitivity (Richter et al., 1982). One could therefore speculate that a combination of these events
occurred as an adaptive response to training, possibly as a way to spare glycogen and maintain energy stores.

Training resulted in an increase in overall plasma insulin concentration in old mares, but not young. Other research has shown that exercise improves insulin sensitivity in horses (Malinowski et al., 2002; Powell et al., 2002; Stewart-Hunt et al., 2006; Firshman and Valberg, 2007), humans (Coggan, 1991; Reaven, 1995; Bloem and Chang, 2008) and rodents (Richter et al., 1981), but didn’t necessarily report changes in insulin concentrations before and after a training period. Considering that overall concentrations of plasma glucose decreased after GXT2, it could be suggested that training improved insulin sensitivity in both age groups, but old mares still required more insulin to achieve glucose clearance compared to young. Thus, insulin sensitivity may have improved in old mares, but was not completely restored after exercise training.

During recovery, regression analysis suggested that ACTH and cortisol did not influence each other over time after GXT1 or GXT2, in old or young mares. Similarly, another study of incremental exercise in horses showed no relationship between plasma cortisol and ACTH during exercise (Nagata et al., 1999). These data suggest that factors outside the HPAA, discussed above, may be influencing changes in ACTH and cortisol during recovery from acute exercise.

The positive correlation between ACTH and glucose existed after both GXT1 and GXT2 for young mares, but only after GXT2 in old mares. DeVries et al. (2000) suggested that HPAA activation associated with exercise is due to fuel demands by working muscles, which is signaled by decreasing glucose levels. Training appeared to reverse the age-related decline in the relationship between ACTH and glucose. In
recovery, this relationship may be part of the mechanism involved for making substrate available for replenishment of glycogen stores. In addition, positive correlations between ACTH and lactate were observed in both age groups before and after training. Lactate is known to be a substrate for hepatic gluconeogenesis (Marlin and Nankervis, 2002). These data support the speculation that ACTH contributes to increased glucose concentrations, and that by promoting lactate, gluconeogenesis may continue after exercise. Moreover, these relationships could be part of an adaptive response to exercise that ensures sufficient substrate is available for recovery from aerobic work. The implication of this would likely contribute to expedited replenishment of glucose stores in muscle, therefore speeding recovery in aged animals.

The difference observed in the slopes of regression lines for cortisol and glucose from GXT1 to GXT2 in both age groups suggests a physiological significance, if not a statistical one. Training appears to affect the dynamic between cortisol and glucose during recovery, but additional research is needed to clarify the mechanism at work.

**Summary and Conclusion**

In summary, training eliminated all age-related differences, except for insulin, suggesting a restoration of HPAA signaling in the aged animal. The peak in plasma glucose in the absence of an increase in cortisol suggests that other factors are responsible for substrate mobilization. Epinephrine is a likely candidate, as it may remain elevated into recovery. Speculatively, epinephrine may have promoted hepatic gluconeogenesis and inhibited glucose clearance well into the recovery phase. In addition, insulin concentration spiked up at the 2 hr mark after GXT2 in both age groups and glucose
levels began to subsequently decrease. This likely occurred in effort to promote glucose uptake and replenish glycogen stores. However, it appears that old mares require more insulin to clear a relatively similar amount of glucose as young mares. Therefore, old mares may be experiencing lower insulin sensitivity compared to young mares even after a period of exercise training. As ACTH and glucose and ACTH and lactate are also positively correlated, one could be acting as a marker of availability of the other, resulting in altered concentrations of these parameters. Figure 8 represents the normal HPAA. Collectively, it could be speculated that these events are choreographed to promote protein synthesis, substrate availability and glycogen replenishment to maximize recovery from exercise to fatigue.

Exercise training has been associated with a reduction in pituitary-adrenal activation in response to exercise (Mastorakos and Pavlatou, 2005). Here, it has again been shown that training attenuates age-related declines in adrenal function and insulin sensitivity. Other factors, such as epinephrine, may be part of the mechanism that activates the HPAA in effort to maintain sufficient blood glucose levels and facilitate lactate production, but further investigation is warranted to investigate this hypothesis. Although training may not completely reverse the effects of aging, it is a valid method for improving the ability of aged animals to recover from exercise.

**Acknowledgements**

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Anthony Sachetti, as well as the graduate and undergraduate research students for assistance with data collection and processing and horse handling.
References


List of Tables

**Table 1.** Body weight in kg (expressed as mean ± SE) and range of body condition scores (BCS) (Henneke et al., 1983) of mares throughout the study period.

<table>
<thead>
<tr>
<th></th>
<th>GXT1</th>
<th>GXT2</th>
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<tbody>
<tr>
<td></td>
<td>Old</td>
<td>Young</td>
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<tr>
<td><strong>Weight, kg</strong></td>
<td>486 ± 14</td>
<td>500 ± 16</td>
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<td><strong>BCS, range</strong></td>
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**Table 2.** Average maximal velocity reached during GXTs (expressed as mean m/s ± SE).

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<tr>
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<tr>
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<td>Old</td>
<td>Young</td>
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<td><strong>Maximal velocity, m/s</strong></td>
<td>8.8 ± 0.3</td>
<td>9.7 ± 0.3</td>
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Table 3. Summary of area under the curve calculations for old and young mares during the RECOVERY phase of GXT1 and GXT2 for a) between age groups, and b) within age groups. Expressed as mean ± SE. An asterisk (*) indicates a significant difference between measurements.

3a.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td><strong>Cortisol</strong></td>
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<tr>
<td>GXT1</td>
<td>121 ± 14</td>
<td>158 ± 19</td>
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<td>GXT2</td>
<td>145 ± 31</td>
<td>167 ± 31</td>
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<td><strong>ACTH</strong></td>
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<td>GXT2</td>
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3b.

<table>
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<th>GXT2</th>
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<tbody>
<tr>
<td><strong>ACTH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old</td>
<td>1512 ± 168</td>
<td>2397 ± 463</td>
<td>0.06*</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Old</td>
<td>2856 ± 42</td>
<td>2661 ± 88</td>
<td>0.10*</td>
</tr>
<tr>
<td>Young</td>
<td>2909 ± 105</td>
<td>2671 ± 36</td>
<td>0.08*</td>
</tr>
</tbody>
</table>
List of Figures

**Figure 1.** RECOVERY only data for cortisol during (a) GXT1, and (b) GXT2. Expressed as means ± SE. A symbol (#) denotes a significant age x time interaction, where an asterisk (*) denotes a significant change from baseline.

**Figure 2.** RECOVERY only data for ACTH during (a) GXT1 and (b) GXT2. Expressed as means ± SE. An asterisk (*) denotes a significant change from baseline for both age groups.

**Figure 3.** RECOVERY only data for plasma glucose during (a) GXT1 and (b) GXT2. Expressed as means ± SE. An asterisk (*) denotes a significant change from baseline for both age groups.

**Figure 4.** RECOVERY only plasma insulin data for old and young mares at (a) GXT1 and (b) GXT2. Expressed as means ± SE. Old mares had a significantly higher overall plasma insulin concentration after GXT2 compared to young mares. An asterisk (*) indicates a significant age by time interaction at 2- and 24 hrs after GXT2.

**Figure 5.** RECOVERY only plasma lactate data for old and young mares at (a) GXT1 and (b) GXT2. Expressed as means ± SE. A symbol (#) denotes a significant age x time interaction.
Figure 6. Regression graphs comparing ACTH vs. glucose during RECOVERY from GXT1 (——) and GXT2 (- - -) in (a) old mares, and (b) young mares.

Figure 7. Regression graphs comparing ACTH vs. lactate during RECOVERY from GXT1 (——) and GXT2 (- - -) in (a) old mares, and (b) young mares.

Figure 8. Outline of the normal HPAA, courtesy Virginia Maryland Regional College of Veterinary Medicine website.
6A

**Old Mares**

ACTH = (1.670*Glucose) - 86.276  
P<0.001  
R=0.48

ACTH = (0.475*Glucose) + 9.422  
P=0.38  
R=0.13

Glucose, mmol/dL

6B

**Young Mares**

ACTH = (1.538*Glucose) - 118.043  
P<0.001  
R=0.52

ACTH = (1.095*Glucose) - 52.245  
P<0.001  
R=0.52

Glucose, mmol/dL
7A

**Old Mares**

- GXT #1
- GXT #2

ACTH = (4.82 * Lactate) + 42.80

P < 0.001

R = 0.63

7B

**Young Mares**

- GXT #1
- GXT #2

ACTH = (6.52 * Lactate) + 37.80

P < 0.001

R = 0.67

ACTH = (5.84 * Lactate) + 35.56

P < 0.001

R = 0.58
CHAPTER 5:
THE EFFECT OF EXERCISE TRAINING ON INSULIN SENSITIVITY, FAT AND MUSCLE TISSUE CYTOKINE PROFILES AND BODY COMPOSITION OF OLD AND YOUNG STANDARDBRED MARES
ABSTRACT: This study tested the hypothesis that old and young mares exhibit different endocrine responses to a frequently sampled intravenous glucose tolerance test (FSIGT) along with different cytokine profiles in blood, adipose and muscle tissues. It was also hypothesized that exercise training would alter endocrine and cytokine profiles. Six old (22.0 ± 0.7 yrs) and six young (7.3 ± 0.6 yrs; mean ± SE) unfit Standardbred mares were tested pre- and post-training. Training consisted of exercise 3d/wk for 15 wks at ~60% maximum heart rate (HRmax). Plasma insulin and glucose concentrations were measured via RIA and enzyme-electrode interface, respectively. Blood samples (B) and biopsies of adipose tissue from the neck crest (NF) and middle gluteal muscle (RM) were collected pre- and post-training for measurement of tissue differences in TNF-α, IL-6, IL-1β, MCP-1 and IFN-γ mRNA. Body composition was measured prior to pre- and post-training FSIGTs. Minimal Model analysis of FSIGT and repeated measures ANOVA were used to examine data. The null hypothesis was rejected when $P\leq0.10$.

After exercise old and young mares improved insulin sensitivity (Si) ($P=0.08$, $P=0.01$, respectively), glucose effectiveness (Sg) ($P=0.004$, $P=0.01$, respectively), and disposition index (DI) ($P=0.04$, $P<0.001$, respectively), but acute insulin response to glucose (AIRg) increased in young mares only ($P=0.02$). All mares increased body weight post-training by an average of 2.3%. Old mares had 48% less rump fat post-training ($P=0.004$) compared to young. Old mares had lower area under the curve (AUC) for plasma glucose in response to FSIGT pre- ($P<0.001$) and post- ($P<0.001$) training compared to young. Old mares had a lower ($P=0.06$) average RQ of TNF-α in NF compared to RM. Old mares had a higher RQ of IL-6 in NF compared to young ($P=0.08$). Overall, old mares showed a higher RQ of IFN-γ mRNA ($P=0.003$) post vs. pre training. In
conclusion, exercise training improves pancreatic beta cell function and insulin sensitivity in old and young horses, and also results in increased fat-free mass. In addition, differences in cytokine profiles exist in muscle, adipose tissue and blood.
Introduction

Insulin resistance (IR) is a general decrease in sensitivity or responsiveness to metabolic actions of insulin, such as insulin-mediated glucose disposal and inhibition of hepatic glucose production (Kahn, 1978). Insulin sensitivity declines with age (Malinowski et al., 2002). The association of IR and equine health problems, such as laminitis, is of growing concern (Geor, 2008). Inflammatory cytokines also influence insulin sensitivity in humans (Pedersen et al., 2003; Dyck et al., 2006) and horses (Vick et al., 2007). Adipose tissue is known to secrete cytokines, and abdominal fat is more strongly linked to IR than subcutaneous fat in people (Kissebah and Krakower, 1994; Pedersen et al., 2003). It is unknown if a similar relationship exists in horses, or if equine blood, subcutaneous fat and skeletal muscle have the same or different cytokine profiles.

Moderate training modulates age-related changes to the endocrine and immune systems that can influence IR (Horohov et al., 1999; Malinowski et al., 2002). Regular exercise protects against tumor necrosis factor-alpha (TNF-α)-induced insulin resistance in humans, in part by increased production of interleukin-6 (IL-6) in contracting muscle (Petersen and Pedersen, 2005). Similarly in horses, insulin sensitivity improved after 12 wks of exercise training in young (~7 yrs), middle aged (~15 yrs) and old (~27 yrs) mares (Malinowski et al., 2002). It could be speculated that cytokines may be part of a similar mechanism in the horse.

Comparisons of TNF-α, IL-6, interleukin-1beta (IL-1β), monocyte chemotactic protein-1 (MCP-1) and interferon-gamma (IFN-γ) mRNA in the blood, adipose and muscle tissues of young and old horses before and after exercise training have not been conducted. To better understand the etiology of IR in horses, this study was designed to
test several hypotheses. First, it was hypothesized that exercise training affects insulin sensitivity in old and young horses. Second, age-related differences in the relative quantity (RQ) of cytokine mRNA exist. Finally, cytokine profiles of circulating blood, adipose tissue and middle gluteal muscle differ, and cytokine profiles will be affected by exercise training.

Materials and Methods

*Animals & Diet.*

Twelve unfit Standardbred mares were studied. Six mares were old (22.0 ± 0.7 yrs; mean ± SE) and 6 were young (7.3 ± 0.6 yrs). None of the mares showed phenotypic signs of pituitary pars intermedia dysfunction (PPID), such as lack of coat shedding, hirsutism or abnormal fat distribution (Miller et al., 2008). The only known difference between the groups was age. All horses were previously familiarized with the treadmill laboratory and associated procedures, and the Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment.

Mares were housed on 2-acre dry lots at Rutgers University and were offered ~2 kg (divided into two feedings at 0700 and 1500) of a commercially available grain ration. Grass hay, salt and water were provided *ad libitum.* Hay samples were analyzed by a commercial laboratory (DairyOne Forage Laboratory, Ithaca, NY), and grain analysis was provided by the manufacturer (F.M. Brown’s Sons, Inc., Birdsboro, PA). On an as-fed basis, hay provided 0.95 Mcal/kg, and grain provided 1.4 Mcal/kg. Thus, mares’ approximate daily intake was 28.3 Mcal/d, and 63.2% non-fiber carbohydrate. The calories provided were above the minimum requirements determined by the National
Research Council (2007) for mature horses weighing 500 kg in moderate work, listed as 23.3 Mcal/d. Weights, rump fat measurements and body condition scores throughout the study are described in Table 1.

**Modified Frequently Sampled Intravenous Glucose Tolerance Test (FSIGT)**

Two mares were randomly selected for testing each day for 6 d until all 12 were complete. Mares were brought into stalls the morning of the test at 0630 and weighed on an electronic scale. The grain meal was withheld, but grass hay and water were available *ad libitum*. Between 0700 and 0800, a jugular catheter was inserted percutaneously using sterile techniques and lidocaine anesthesia. The protocol for the FSIGT followed previously published methods (Hoffman et al., 2003), as described below.

Baseline blood samples (30 mL) were collected at 0830 and 0859. At approximately 0900, the test began. A glucose bolus of 0.3 g/kg BW (dextrose solution 50%, Phoenix Pharmaceutical, Inc, St. Joseph, MO) was infused via the catheter over a period of 2 min. Twenty min after the glucose dose, an insulin bolus (Humulin R, Eli Lilly & Co, Indianapolis, IN) of 30 mU/kg of BW was administered through the catheter. The goal was to mimic the physiological response of insulin to a glucose surge and reduce the risk of hypoglycemia (Hoffman et al., 2003). No mares showed signs of hypoglycemia during or after the test.

The test lasted for ~3 hr, during which time samples were collected at -30, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min after glucose administration. Samples were placed in pre-chilled tubes containing sodium heparin (Vacutainer, Franklin Lakes, NJ) and centrifuged at
3000 x g at 4°C for 10 min. Plasma for insulin analysis was frozen at -80°C until analysis. For insulin analysis, samples were measured in duplicate via radioimmunoassay (RIA) (Coat-A-Count, Siemens, Los Angeles, CA), previously validated for use in horses (Freestone et al., 1991). The CV for the insulin assay pre-training was 18.6%, and post-training was 11.4%. The inter-assay CV was 13.7%. Plasma glucose was measured immediately in duplicate via enzyme-electrode interface (ABL 800 Flex, Radiometer America, Westlake, OH).

**Graded Exercise Tests (GXTs) & Training Protocol**

Each mare performed 3 graded exercise tests (GXTs), pre-training (GXT1), post-8 weeks training (GXT2), and at the end of the study totaling 15 weeks training (GXT3). An equine treadmill (Sato I, Equine Dynamics, Lexington, KY) set at a fixed 6% grade was used for GXTs. All GXTs were conducted between 0800 and 1130, and 3 mares ran each day until all were complete.

An indirect open-flow calorimeter (Oxymax-XL, Columbus Instruments, Inc., Columbus, OH) was used to measure gas exchange to determine each mare’s maximal oxygen uptake (VO$_2$max). Oxygen uptake was measured continuously during the test and recorded at 10 s intervals. VO$_2$max was defined as the point when there were no further increases in VO$_2$ despite increases in speed. Exercise capacity, measured as run time to fatigue, was also calculated as described (McKeever and Malinowski, 1997).

The GXTs started at an initial speed of 4 m/s for 1 min. Speed was then increased to 6 m/s, followed by incremental increases of 1 m/s every 60 s until horses reached fatigue. Fatigue was defined as the point where horses could not keep up with
the treadmill despite humane encouragement. At the point of fatigue, the treadmill was stopped, and 5 min of post-exercise calorimetry data was collected.

A heart rate monitor (Polar Equine, Lake Success, NY) was used to measure heart rate during GXTs. Heart rates were recorded at rest, and during the last 10 s of each treadmill step such that maximum heart rate and the HR versus speed relationship could be documented for each horse. Training intensity was calculated using this data so that each mare exercised at ~60% of HRmax. The range of recorded HRmax was 185-216 beats per minute (bpm). Thus during training, velocity was adjusted and HR kept between 111-130 bpm depending on the individual so as to maintain a submaximal relative work intensity of ~60% HRmax.

Mares exercised trained for an initial 8 wk period in a free-stall exercise machine (Equi-Ciser, Equi-Master International, Sudre, Alberta, Canada). The order of the groups was alternated every other session, so the same group was not always the first to train. Grain meals were withheld prior to training, but were provided at the conclusion of the training sessions. The training protocol was similar to that used previously (Malinowski et al., 2006), consisting of a 5 min warm-up walk, followed by 30 min trotting at a submaximal work intensity (~60% of HRmax), and ending with a 10 min cool down walk. One mare was randomly assigned to wear a heart rate monitor during each training session. This ensured that mares trained consistently and aerobically, with heart rate below 160 beats per min, therefore staying below the anaerobic threshold (Marlin and Nankervis, 2002). In this way, mares would still achieve the physiological changes associated with aerobic exercise (Jimenez et al., 1998) without surpassing the anaerobic threshold.
After 8 wks of training, mares ran GXT2 to test for changes in VO\textsubscript{2}\text{max} and exercise capacity from GXT1. GXT2 replaced one training day on the Equi-Ciser, but mares trained the two other days as usual. The FSIGT protocol described above was then repeated in subsequent weeks. Mares completed their training sessions no less than 48 hr prior to their post-training FSIGT in order to avoid any hormonal influences of exercise on the test (Golland et al., 1999) and to allow for glycogen repletion in the muscles (Waller et al., 2009).

Mares continued to exercise train at the same level they reached during weeks 7-8 in the initial training period for 6 additional wks to in effort to maintain VO\textsubscript{2}\text{max} and exercise capacity without fluctuation. Mares trained for a total of 15 wks between the pre- and post-training FSIGTs. After completing the post-training FSIGT, mares performed GXT3 to test for any further changes in VO\textsubscript{2}\text{max} and exercise capacity.

**Tissue Biopsies**

Adipose and muscle tissue biopsies were collected twice during the study – first, prior to any exercise training or exercise test, and second, after the 15-wk training period had been completed. Approximately 30 min prior to biopsies and administration of sedation, blood samples (4 mL) were collected into PAXgene\textsuperscript{TM} Blood RNA Tubes (PAXGene\textsuperscript{TM}, Qiagen, Inc., Santa Clarita, CA) for subsequent measurement of TNF-\textalpha, IL-6, IL-1\beta, MCP-1 and IFN-\gamma mRNA. A Bergstrom biopsy needle was used to collect fat samples from the neck crest, dorsal to the nuchal ligament (midline, approximately 2 cm ventral from mane hair line) using sterile techniques. Muscle biopsies were also collected from the middle gluteal muscle. In all cases, mares were sedated with xylazine.
(0.5 mg/kg) and given lidocaine anesthesia (8-10 cc) at both the neck crest and gluteal muscle biopsy sites. Mares received 1 g phenylbutazone orally immediately following biopsies, and 24 hrs later, and monitored for any signs of discomfort or infection. Samples were cut into 0.25 cm pieces if necessary and placed in RNALater (Qiagen, Valencia, CA). Samples were stored at room temperature for 24 hrs, then at -20°C until analysis. Blood and tissue samples were analyzed for cytokine mRNA of TNF-α, IL-6, IL-1β, MCP-1 and IFN-γ, using RT-PCR.

**Cytokine Quantification**

Quantification of cytokines was completed using real time-polymerase chain reaction (RT-PCR). Total RNA was subsequently isolated from the PAXgene™ Blood RNA tubes using spin columns according to the manufacturer’s instructions. For adipose and muscle samples, tissues were physically disrupted using sterile zirconium oxide beads in a mixing mill (Retsch MM301 Mixing Mill, Clifton, NJ) to homogenize the tissues in microcentrifuge tubes containing RNA-Stat60 Reagent (Tel-Test Inc., Friendswood, TX). Total RNA was isolated from the samples using chloroform extraction, isopropanol precipitation and ethanol wash, according to the manufacturer’s instructions.

The RNA was quantified using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). In all cases, OD_{260/280} ratios were greater than 1.9 and RNA yields were greater than 50 μg/mL. One microgram of RNA was reverse transcribed into cDNA in an 80μl reaction containing a master mix of 16 μL of AMV Buffer (5x), 16 μL of MgCl₂, 4 μL of dNTP, 1 μL of RNasin, μL oligo dT and 0.5 μL of AMV reverse
transcriptase (Promega, Madison, WI). Cytokine-specific cDNA was then amplified and quantified by “real-time” PCR (ABI Systems 7900 Fast Real-Time PCR System, Foster City, CA) using the Taq thermostable DNA polymerase and primers based on our sequences for equine cytokines and beta-glucoronidase (β-gus) (Breathnach et al., 2006). Specific primers and FAM-labeled probes for each cytokine and β-gus, provided as Assay-by-Design kits (ABI, Foster City, CA), were added to 10 μL reactions in 384-well plates containing the 5 μL Taq polymerase (TaqMan Gene Expression Master Mix, ABI), 0.5 μL of primer-probe, and 4.5 μL of cDNA. The following PCR conditions were employed: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected using β-glucoronidase as an internal control for each sample (Breathnach et al., 2006). Relative differences in cytokine mRNA expression resulting from exercise were determined by relative quantification. Relative quantification (RQ) provides accurate comparison between the initial levels of target cDNA in a sample without requiring that the exact copy number be determined (Livak and Schmittgen, 2001). The pre-exercise samples for each group of horses (young and old) were averaged and the group average used as the calibrator for subsequent calculation of RQ and the change in cytokine gene expression post-expression relative to the calibrator for its respective group.

**Body composition**

Mares were weighed on an electronic scale each week during the study, prior to the training session and grain meal. Body condition scores (Henneke et al., 1983) and
rump fat measurements were evaluated by the same person prior to each of the three GXTs.

Rump fat thickness was measured via ultrasound (Aloka SSD-500, Wallingford, CT). Distance was measured between the point of hip and point of rump bilaterally. The ultrasound was placed at the midpoint of the sacral bone and approximately 5-10 cm below midline to measure rump fat thickness in cm (Westervelt et al., 1976). Percent body fat was calculated using the Westervelt equation (Westervelt et al., 1976), where:

\[ \% \text{fat} = 8.64 + 4.70(\text{rump fat in cm}). \]

This method has been used previously (Westervelt et al., 1976; Gordon et al., 2007; Adams et al., 2009) for quantification of body fat percentage in horses.

Fat-free mass was determined as described (Kearns et al., 2006). Fat mass was determined by multiplying % fat and total body mass. Fat-free mass was calculated by subtracting fat mass from total body mass (Kearns et al., 2006).

*Statistical Analyses*

A two-way repeated measures ANOVA was used to evaluate differences between old and young mares with respect to tissue cytokine composition, glucose and insulin pre- and post- training during the FSIGT and body weights measured at each GXT (SigmaStat 3.1, SPSS, Chicago, IL). Areas under the curve were calculated for plasma glucose (AUCg) and insulin (AUCi) (SigmaPlot 9.0, SPSS, Chicago, IL). Appropriate t-tests were used to compare means between two groups, old vs. young or pre- vs. post-training, similar to previously published techniques (Toth et al., 2009).
Glucose effectiveness (Sg), insulin sensitivity (Si), acute insulin response to glucose (AIRg) and disposition index (DI) were calculated using minimal model software (MinMod Millenium, Version 6.02, Boston et al., 2003) as described (Hoffman et al., 2003; Toth et al., 2009). Again, appropriate t-tests were used to compare means between two groups, old vs. young or pre- vs. post-training to detect differences between Sg, Si, AIRg and DI and in rump fat thickness.

Pearson Product Moment was used to determine correlations between Si and each cytokine investigated (data not shown).

The null hypothesis was rejected when $P \leq 0.10$ to protect against a Type II error (Little and Hills, 1978). Post hoc testing for separation of means (Student-Neuman-Keuls) was used where appropriate.

**Results**

**GXTs & Training**

Old mares demonstrated a 28%, 20% and 22% lower ($P < 0.05$) VO$_2$max than younger mares during GXT1, GXT2 and GXT3, respectively. However, both old and young mares significantly increased their VO$_2$max after 8 wks of training by 18% and 10%, respectively (Table 2a).

Old mares had a 26%, 20% and 17% lower ($P = 0.002$) run time to fatigue (exercise capacity) compared to young mares during GXT1, GXT2 and GXT3, respectively. However, both old and young mares increased their exercise capacity after 8 wks of training by 22% and 13%, respectively (Table 2b).
There was no significant difference in plasma glucose or insulin concentration between old and young mares during the FSIGT pre- or post-training (Figures 1 and 2). However, there was a significant age by time interaction for plasma glucose post-training ($P<0.001$). *Post hoc* analysis revealed age differences at 1, 2, 4, 5, 6, 7, 8, 10 and 16 min, where old mares had lower plasma glucose compared to young. There were no age by time interactions pre- or post-training for plasma insulin.

There was no difference in AUCi in old vs. young mares pre-training ($P=0.69$, data not shown). Post-training, old mares had a mean 31% greater ($P=0.10$) AUCi compared to young. Old and young mares had decreased AUCi post-training compared to pre-training, although the difference was not significant within either age group.

There was an effect of training, but not age, on AUCg. Old mares had a mean 25% lower AUCg post-training compared to pre- ($P<0.001$), where young mares were a mean 18% lower post-training ($P<0.001$) (data not shown). There was no difference in AUCg between old and young mares pre- ($P=0.45$) or post-training ($P=0.26$) (data not shown).

*Minimal Model Parameters.* Upon data analysis, it was revealed that 3 old mares were insulin resistant pre-training ($Si<1.0 \text{ L x mU}^{-1} \text{ x min}^{-1}$), and 2 of these 3 had impaired glucose effectiveness ($Sg<1\% \text{ min}^{-1}$). One young mare was insulin resistant, but did not have an impaired $Sg$. Post-training, none of the mares were insulin resistant, and none had impaired responses to glucose. MinMod data are summarized in Table 3.

There was no difference in $Si$ with old vs. young mares pre-training ($P=0.68$), but young mares were more insulin sensitive compared to old mares post-training ($P=0.03$).
Old and young mares improved Si post- compared to pre- training ($P=0.08$, $P=0.01$, respectively).

There was no difference in Sg with old vs. young mares pre-training or post-training ($P=0.90$ and $P=0.91$ respectively). Old and young mares increased Sg post-compared to pre-training ($P=0.004$, $P=0.01$ respectively).

There was a difference in AIRg in old vs. young mares pre-training ($P=0.004$), and post-training ($P=0.03$). In both cases, old mares had an increased insulin response to glucose compared to young mares. Old mares did not experience a change in AIRg pre vs. post training ($P=0.57$), but young mares had an improved insulin response to glucose post-training ($P=0.02$).

Old mares had a higher DI pre-training compared to young ($P=0.07$), but there was no difference post-training ($P=0.51$). Old and young mares differed pre vs. post training ($P=0.04$, $P<0.001$, respectively), where DI increased post-training in both age groups (Table 3).

**Tissue Biopsies & Cytokine Analysis.**

**TNF-α.** There were no effects of age or training on TNF-α in NF, B or RM. Old mares had a lower ($P=0.06$) average RQ of TNF-α in NF compared to RM (Figure 3). There were no significant correlations between Si and TNF-alpha in any of the tissues, pre- or post-training.

**IL-6.** There was no effect of training for IL-6 in NF, B or RM. Analysis within age groups revealed the following. Young mares had a higher RQ of IL-6 ($P=0.08$) in B compared to NF, but old mares did not. Old mares had a higher RQ of IL-6 in NF
compared to young ($P=0.08$) (Figure 4). There was a negative correlation between Si and IL-6 in NF post-training ($P=0.07$, $R=-0.54$) only.

**MCP-1.** There was no effect of age or training for MCP-1 in NF, B or RM. Analysis within age groups revealed the following. Old mares showed a tissue variation in MCP-1 ($P=0.07$) with a higher RQ in NF compared to B, but young mares did not. There were no significant changes for old or young mares in the overall RQ of MCP-1 mRNA due to training (Figure 5). There was a positive correlation between Si and MCP-1 in NF post-training ($P=0.08$, $R=0.53$), but no correlations were evident pre-training.

**IFN-γ.** Within NF, there was a significant effect of age ($P=0.001$), training ($P=0.02$) and an age x training interaction ($P=0.02$) on IFN-γ. Pre-training, there was no age difference in the RQ of IFN-γ in NF, but post-training, old mares had a higher RQ of IFN-γ ($P<0.001$) compared to young. Overall, old mares showed a higher RQ of IFN-γ mRNA ($P=0.003$) post vs. pre training. Young mares did not show a difference in IFN-γ pre- vs. post-training (Figure 6). There was no correlation between Si and IFN-γ in any of the tissues pre- or post-training.

**Other comparisons.** In RM samples, there were no significant effects of age or training on IL-6, IL-1β, MCP-1 or IFN-γ. For NF, there were no significant effects of age or training on TNF-α, IL-1β and MCP-1. For B, there were no significant effects of age or training on TNF-α, IL-6, IL-1β, MCP-1, or IFN-γ. There was no correlation of Si with IL-1β in any tissue, pre- or post-training. An illustration of IL-1β data is shown in Figure 7.

**Body Composition.** A t-test was used to compare body weight and rump fat thickness in old vs. young mares pre-training, and again post-training. There was no
effect of age \((P=0.38)\) on body weight (Table 2a), as measured just prior to each of the 3 GXTs, but there was an effect of training \((P=0.003)\), where all mares increased body weight from GXT1 to GXT3 an average of 2.3\%. At GXT1, there were no age differences measured in rump fat thickness \((P=0.12)\). At GXT2, after 8 weeks of training, old mares had an average of 48\% less rump fat compared to young \((P=0.04)\). At GXT3, the age difference in rump fat thickness remained \((P=0.01)\), where old mares had an average of 57\% less than young mares (Table 2b). The change in rump fat thickness from GXT2 to GXT3 was not significant in old \((P=0.26)\) or young \((P=0.55)\) mares (Table 2c). Data describing fat-free mass is listed in table 2d. There was no main effects of age on fat free mass \((P=0.39)\), but there were effects of training \((P=0.01)\). Old mares increased fat free mass from GXT1 to GXT3 \((P=0.01)\), but young mares did not \((P=0.11)\).

The ranges of body condition scores recorded prior to each GXT are outlined in Table 2e.

**Discussion**

This is the first study to report the effects of both age and exercise training on Si, Sg, AIRg and DI, combined with tissue cytokine profiles in horses. Data suggests that while training improves Si in old and young mares, the improvement is more profound in younger animals. Aged animals still appear to require higher concentrations of insulin to clear glucose. In addition, IL-6 and MCP-1 in adipose tissue may influence Si in horses.

In response to exercise in horses, increases over baseline measures of blood lactate, epinephrine, norepinepherine and cortisol were observed to begin at 48\%
VO₂max, but did not reach the anaerobic threshold until horses exercised at approximately 80% VO₂max (Jimenez et al., 1998). This suggests that exercising above the anaerobic threshold is not necessary to achieve an endocrine response to training. In addition, exercise at a heart rate below approximately 160 bpm maintains work below the anaerobic threshold (Marlin and Nankervis, 2002). The training regimen described here did not tax the cardiovascular system nearly this high. Nevertheless, it was clearly adequate to produce increases in VO₂max and exercise capacity in both age groups. Thus, the exercise training protocol here was sufficient to develop an endocrine and cardiovascular response to aerobic exercise, without conditioning the system at or above the anaerobic threshold. Therefore, improved β-cell function as a result of aerobic exercise could account for the increased insulin concentration observed post-training in old and young mares.

Earlier work demonstrated that old horses needed the most insulin to properly manage an oral glucose tolerance test compared to young and middle-aged horses, but all age groups improved their insulin response after a training regime (Malinowski et al., 2002). Here again, old mares had a higher AUCi compared to young post-training. While exercise training appears beneficial for management of glucose homeostasis, it does not appear to completely reverse the age-related decline in insulin sensitivity.

The Minimal Model Analysis of Frequently Sampled Intravenous Glucose Tolerance Test (MinMod) is a valid model to evaluate mechanisms of impaired glucose tolerance, and it provides estimates of metabolic indices regarding insulin sensitivity, glucose effectiveness and pancreatic β-cell function (Bergman et al., 1981; Boston et al., 2003; Muniyappa, 2008). The DI, estimated as part of the MinMod, has been used to
evaluate β-cell function in humans (Bergman et al., 1981; Bergman et al., 2002) and horses (Hoffman et al., 2003; Treiber et al., 2005). Moderate exercise training has been shown to improve DI in humans (Slentz et al., 2009), and has now been shown to improve DI in both old and young horses.

It should be noted that urinary glucose spilling (UGS) was not accounted for in the present investigation. Toth et al. (2009) recently reported that UGS occurs during the FSIGT as described here, where AUCg, AUCi and AIRg had a linear relationship with dextrose that reached a plateau at 200 mg/kg. Here, 300 mg/kg dextrose was administered, following previously published methods (Hoffman et al., 2003). Urine was not collected or analyzed for glucose concentration in the present investigation. It is possible that UGS affected the outcomes described here, and contributed to lower plasma glucose observed in old mares during the FSIVGT prior to the insulin bolus.

In the present investigation, plasma insulin was measured. Connecting peptide (C-peptide) and insulin are secreted from the pancreas in a 1:1 ratio (Rubenstein et al., 1969; Toth et al., 2010). It has been suggested that C-peptide may be a more accurate assessment of pancreatic beta cell secretion of insulin, as the liver clears up to 50% of insulin from the plasma (Polonsky et al., 1986). C-peptide is not readily cleared by the liver (Stoll et al., 1970; Toth et al., 2010). Thus, the consideration of C-peptide may affect interpretation of data collected and analyzed from the FSIGT. In addition, hepatic insulin clearance has been reported to decline with age in nondiabetic humans (Iozzo et al., 1999). If the same is true in horses, this may partially explain the higher insulin concentrations observed in older mares or partially skew data interpretation. Additional study is needed to investigate this further.
In addition to the endocrine effects, exercise influences immune functions associated with inflammation and insulin sensitivity (Horohov et al., 1999; Malinowski et al, 2002). Here, no age or training differences were observed in RQs of TNF-α in rump muscle. Such observations vary from findings that a bout of exercise attenuated the overexpression of the TNF-α gene in the muscle of TNF receptor 1 and 2 knockout mice (Keller et al., 2004). The present study involved exercise training, whereas work by Keller et al. (2004) used a 1 hr bout of exercise, so comparisons should be made with caution. There may be differences in the effects of a single bout of exercise on TNF-α versus exercise training, as well as the timing of biopsy samples.

In a murine model, IL-6 knockout mice developed mature-onset obesity and insulin resistance, which was reversed by the infusion of IL-6, suggesting that IL-6 helped improve insulin sensitivity (Wallenius et al., 2002). It has also been demonstrated that the release of IL-6 from working skeletal muscle is positively related to work intensity, glucose uptake and plasma epinephrine concentration, suggesting that IL-6 may be linked to regulation of glucose homeostasis during exercise, and/or that IL-6 may work as a sensor of carbohydrate availability (Pedersen et al., 2003b). On the contrary, in obese and overweight humans, IL-6 in the blood and incidence of insulin resistance were increased compared to subjects with a normal body mass index (Gnacinska et al., 2010). In the present study, a negative correlation between Si and IL-6 in subcutaneous adipose tissue from the neck was observed post-training. Whether this indicates a possible divergent role for IL-6 in equine blood, skeletal muscle and adipose tissue warrants additional investigation.
While MCP-1 expression was positively correlated with Si in adipose tissue of the neck in the present investigation, studies of mice fed a high fat diet showed that inhibition of the MCP-1 receptor improved insulin sensitivity regardless of macrophage infiltration into adipose tissue (Tateya et al., 2010). Other work has shown that insulin suppresses MCP-1 (Dandona et al., 2004). In mice, chronically elevated MCP-1 induced insulin resistance and macrophage infiltration into adipose tissue (Tateya et al., 2010). Additionally, circulating MCP-1 was not associated with insulin sensitivity in healthy men (Chacon et al., 2007). It appears that MCP-1 in the horse may function differently in circulation and adipose tissue, but likely plays some role in tissue insulin sensitivity.

*In vitro* studies of human adipocytes treated with IFN-γ showed a decrease in insulin-stimulated glucose uptake along with a down-regulation of insulin receptor substrate -1 and GLUT-4 via JAK/STAT pathway activation (McGillicuddy et al., 2009). IFN-γ is a known stimulator of MCP-1 (Struyf et al., 1997), and here, old mares not only had generally higher levels of IFN-γ, but elevated levels of MCP-1 mRNA in NF compared to young. This etiology could be a partial reason for the age-related decline in insulin sensitivity.

Along with cytokine profiles, body composition is an important factor to consider in studies of glucose metabolism (Reaven, 1995; Pedersen et al., 2003). Young mares experienced no change in rump fat thickness after training, but these results are consistent with that seen in humans (Nishida et al., 2004). Old mares had less rump fat thickness after 8- and 15-weeks of training compared to young mares. Rump fat thickness is the directly measured parameter that reflects fat mass. An increase in body mass without a concurrent increase in rump fat thickness indicates an increase in fat free mass, the latter
reflecting primarily muscle mass. In response to exercise, free fatty acids are mobilized from adipose tissue (Marlin and Nankervis, 2002). Use of fat for fuel ultimately results in glycogen sparing, possibly delaying the onset of fatigue (Marlin and Nankervis, 2002). Consequently, it could be speculated that old mares in particular rely more heavily on fat oxidation in the type of exercise described here in effort to spare glucose. This may at least partially explain the decrease in fat mass observed in old mares by training wks 8 and 15.

Interestingly, aerobic exercise promoted fat oxidation in older, obese adult humans (Solomon et al., 2008). Additionally, sedentary older men demonstrated an impaired ability to oxidize carbohydrate after a single bout of exercise (Krishman et al. 2002). Studies of humans exercising at ~63% VO$_2$max for 60 min over 9 d resulted in a 24% increase in lipid oxidation (Tunstall et al., 2002). Mild to moderate exercise training in humans is known to promote lipid oxidation, whereas increases in exercise intensity promote carbohydrate utilization (Brooks and Mercier, 1994). Additionally, other work reported in horses concluded that moderate-intensity training similar to that described here resulted in a decreased rate of glucose production and whole-body glucose uptake during exercise, a decreased rate of muscle glycogen breakdown and increased the contribution of fat for energy utilization (Geor et al., 2002). The training protocol in the current study was considered moderate intensity at 60% HRmax. Considering this data together, one could speculate that decreased rump fat in old mares combined with lower plasma glucose present during exercise is suggestive of a shift in substrate utilization for exercise, with an increased reliance on fat as an energy source.
Furthermore, loss of skeletal muscle, or sarcopenia, has been described in aging humans (Evans, 2010; Koopman, 2010). Consequences of sarcopenia may include increased risk of insulin resistance, decreased muscle mass, strength and function and an overall decrease in basal metabolic rate (Evans, 2010). Interestingly, no difference in fat free mass was observed between unfit old and young mares in the present study. However, here, old mares experienced an increase in fat free mass after completion of training. Therefore, it could be speculated that the increase in fat free mass at least partially accounted for improved insulin sensitivity, particularly in old mares.

**Summary and Conclusion**

In their review, Kahn and Flier (2000) note that the insulin signaling cascade is well conserved between species, from *C. elegans* to humans, so findings in an equine model may be relevant to understanding mechanisms of IR in other animals. Equine adipose tissue was shown to express TNF-α, along with MCP-1 and IL-6 *in vivo*. Novel findings showed improvements in the DI of old and young mares after exercise training. A decrease in IL-6 mRNA in adipose tissue from the neck was associated with an increase in insulin sensitivity. Tissue cytokine profiles differed, for all cytokines studied except IL-1β. To the best of our knowledge, this is the first time these cytokines have been compared in old and young horses both pre- and post-exercise training. Finally, body mass increased in all mares from GXT1 to GXT3, but only old mares experienced a decrease in body fat.

In conclusion, it has again demonstrated here that exercise training improves insulin sensitivity in both old and young horses, although it does not appear to be
completely restored in old mares. Proposed mechanisms for the positive effects of exercise include reduction in liver and muscle glycogen, increased binding of insulin to the insulin receptor and increased glucose uptake by muscle cell glucose transporters (Firshman and Valberg, 2007). Differences in body composition in old and young mares could have far-reaching effects on hormones associated with glucose homeostasis and regulation of energy intake and substrate utilization during exercise. Future studies examining endocrine mediators and substrate utilization in old and young horses before and after exercise training are warranted. Continued work in examining the inflammatory influence on glucose metabolism is essential for a greater understanding of the mechanisms contributing to the etiology of glucose homeostasis, age, cytokines and body composition in horses.

Acknowledgements

Funding provided by the New Jersey Agricultural Experiment Station and State Equine Initiative. Special thanks to Rutgers University Animal Care Supervisor Joanne Powell and Senior Animal Caretaker Anthony Sachetti, and to Ashley McClune, Veterinary Assistant of Centenary College for her assistance with collection of biopsies. Additional thanks to Jessica Suagee and Dr. Jill McCutcheon at Virginia Polytechnic and State University for help with biopsy techniques, and Dr. Amanda Adams at the Gluck Equine Research Center of the University of Kentucky for assistance with cytokine analysis.
References


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Table 1. (a) Weight in kg; (b) rump fat thickness in cm for old and young mares, presented as mean ± SE; (c) mean rump fat changes; (d) fat free mass in kg presented as mean ± SE; and (e) range of body condition scores recorded in old and young mares for pre- (GXT1) and post- (GXT2 and GXT3) training. Different superscripts represent significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
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<tr>
<td></td>
<td>Old, kg</td>
<td>Young, kg</td>
<td></td>
</tr>
<tr>
<td>Pre, GXT1</td>
<td>486 ± 14</td>
<td>500 ± 16</td>
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<tr>
<td>Post, GXT2</td>
<td>496 ± 13</td>
<td>494 ± 20</td>
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<tr>
<td>Post, GXT3</td>
<td>492 ± 11</td>
<td>516 ± 16</td>
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<tr>
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<tr>
<td></td>
<td>Old, cm</td>
<td>Young, cm</td>
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<tr>
<td>Pre, GXT1</td>
<td>0.61 ± 0.1 a</td>
<td>0.89 ± 0.1 a</td>
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<tr>
<td>Post, GXT2</td>
<td>0.43 ± 0.1 a</td>
<td>0.78 ± 0.1 b</td>
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</tr>
<tr>
<td>Post, GXT3</td>
<td>0.38 ± 0.1 a</td>
<td>0.87 ± 0.1 b</td>
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<tbody>
<tr>
<td></td>
<td>P=</td>
<td></td>
<td>P=</td>
</tr>
<tr>
<td>GXT1 vs. GXT2</td>
<td>0.27 a</td>
<td>0.47 a</td>
<td></td>
</tr>
<tr>
<td>GXT1 vs. GXT3</td>
<td>0.18 a</td>
<td>0.76 a</td>
<td></td>
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<tr>
<td>GXT2 vs. GXT3</td>
<td>0.26 a</td>
<td>0.55 a</td>
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<table>
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<tr>
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<th>Fat Free Mass, kg</th>
<th>Old</th>
<th>Young</th>
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<tr>
<td>Pre, GXT1</td>
<td>422 ± 11 a</td>
<td>438 ± 12 a</td>
<td></td>
</tr>
<tr>
<td>Post, GXT2</td>
<td>430 ± 10 a</td>
<td>447 ± 11 a</td>
<td></td>
</tr>
<tr>
<td>Post, GXT3</td>
<td>441 ± 10 b</td>
<td>450 ± 13 ab</td>
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<th>Body Condition Score, range</th>
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<th>Young</th>
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<tr>
<td>Pre, GXT1</td>
<td>4.5 – 6</td>
<td>4.5 – 6.5</td>
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</tr>
<tr>
<td>Post, GXT2</td>
<td>4 – 5</td>
<td>4.5 – 7</td>
<td></td>
</tr>
<tr>
<td>Post, GXT3</td>
<td>4 – 5.5</td>
<td>5 – 6.5</td>
<td></td>
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Table 2. Mean ± SE values for (a) maximal oxygen uptake (VO2max) and (b) exercise capacity, measured as run time to fatigue in seconds, for young and old horses for all GXTs. An asterisk (*) within a sample time denotes a significant difference due to age. Within an age group the symbol denotes that the mean was significantly different from pre-training and the same symbol indicates that there was no difference between 8 and 15 weeks.

a. Maximal Oxygen Uptake (mL/kg/min)

<table>
<thead>
<tr>
<th></th>
<th>GXT 1 Pre-Training</th>
<th>GXT 2 8 weeks</th>
<th>GXT 3 15 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>122 ± 5 a*</td>
<td>134 ± 8 b*</td>
<td>139 ± 3 b*</td>
</tr>
<tr>
<td>Old</td>
<td>95 ± 6 a</td>
<td>112 ± 7 b</td>
<td>114 ± 6 b</td>
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</table>

b. Exercise Capacity (run time to fatigue in seconds)

<table>
<thead>
<tr>
<th></th>
<th>GXT 1 Pre-Training</th>
<th>GXT 2 8 weeks</th>
<th>GXT 3 15 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>350 ± 10 a*</td>
<td>395 ± 31 b*</td>
<td>378 ± 16 b*</td>
</tr>
<tr>
<td>Old</td>
<td>260 ± 17 a</td>
<td>317 ± 19 b</td>
<td>314 ± 20 b</td>
</tr>
</tbody>
</table>
Table 3. Minimal model data for old and young mares, pre- and post-training. Data is expresses as mean ± SE. Symbols denote significant differences within Si, Sg, AIRg or DI.

<table>
<thead>
<tr>
<th></th>
<th>Old</th>
<th>Young</th>
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<tbody>
<tr>
<td></td>
<td>Si ( (L \times \text{mU}^{-1} \times \text{min}^{-1}) )</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1.3±0.4 (^a)</td>
<td>1.5±0.2 (^a)</td>
</tr>
<tr>
<td>Post</td>
<td>2.3±0.2 (^b)</td>
<td>3.4±0.4 (^c)</td>
</tr>
<tr>
<td></td>
<td>Sg ( (\text{min}^{-1}) )</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>0.015±0.009 (^d)</td>
<td>0.014±0.003 (^d)</td>
</tr>
<tr>
<td>Post</td>
<td>0.030±0.002 (^e)</td>
<td>0.029±0.002 (^e)</td>
</tr>
<tr>
<td></td>
<td>AIRg ( (\text{mU} \times \text{min} \times \text{L}^{-1}) )</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>492.2±117 (^f)</td>
<td>113±49 (^g)</td>
</tr>
<tr>
<td>Post</td>
<td>423.3±61.5 (^f)</td>
<td>59.9±24.5 (^h)</td>
</tr>
<tr>
<td></td>
<td>DI ( (\text{AIRg} \times \text{SI}) )</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>372.4±81.5 (^i)</td>
<td>185.5±42.5 (^j)</td>
</tr>
<tr>
<td>Post</td>
<td>1020±177.3 (^k)</td>
<td>868.1±133.8 (^k)</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Mean plasma glucose and insulin pre-training in (a) old and (b) young mares. Data is presented as mean ± SE.

Figure 2. Mean plasma glucose and insulin post-training in (a) old and (b) young mares. Data is presented as mean ± SE.

Figure 3. Graph comparing the RQ of TNF-alpha mRNA, expressed as mean ± SE. Different superscripts denote significant differences between tissues.

Figure 4. Graph comparing the RQ of IL-6 mRNA, expressed as mean ± SE. Different superscripts denote significant differences within a tissue, and an asterisk (*) denotes a significant age difference within a tissue.

Figure 5. Graph comparing the RQ of MCP-1 mRNA, expressed as mean ± SE. Different superscripts denote significant differences between tissues.

Figure 6. Graph comparing the RQ of IFN-gamma mRNA, expressed as mean ± SE. Different superscripts denote significant differences between tissues, and an asterisk (*) denotes an age difference within a tissue.

Figure 7. Graph comparing the RQ of IL-1 beta mRNA, expressed as mean ± SE.
MCP-1 mRNA

IFN-gamma mRNA

Tissue

Rump Muscle  Neck Fat  Blood

Old, Pre  Young, Pre  Old, Post  Young, Post

a  b

a  b
CHAPTER 6:

INFLAMMATORY CYTOKINE PROFILES OF EQUINE BLOOD, ADIPOSE
AND MUSCLE TISSUE – A PRELIMINARY REPORT
Abstract

This study tested the hypotheses that blood, muscle and adipose tissue differ in inflammatory cytokine profiles in horses. Secondarily, it was hypothesized that young horses (≤10 yrs) would have different cytokine profiles in these tissues and blood compared to old (≥20 yrs) horses. Fifteen horses of mixed breed and background were admitted to the study based on medical history and veterinary evaluation. A blood sample (B) was collected prior to euthanasia. Within 30 min of euthanasia, neck adipose (NF), omental adipose (OF) and skeletal (middle gluteal) muscle (RM) samples were collected. Samples were analyzed via real time-polymerase chain reaction (RT-PCR) for relative quantities (RQ) of cytokine mRNA. Expression of mRNA for the cytokines tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), monocyte chemoattractant protein-1 (MCP-1) and interferon-gamma (IFN-γ) were measured using the 7900HT Fast Real-Time PCR System. Data were analyzed using the Friedman repeated measures analysis of variance on ranks for non-normal data sets, and a t-test for normal data sets. The null hypothesis was rejected when \( P \leq 0.05 \). There were no differences between old and young horses (\( P > 0.05 \)). The RQ of TNF-α mRNA was a mean 155% higher in B compared to RM (\( P < 0.05 \)); IL-6 was a mean 165% higher in NF and 363% higher in OF compared to B (\( P < 0.05 \) for both); IL-1β was higher in B compared to RM, NF and OF (1140%, 1025% and 920.8%, means, respectively, \( P < 0.05 \) for all three comparisons); MCP-1 was a mean 1009% higher in RM and 1646% higher NF compared to B (\( P < 0.05 \) for both); and IFN-γ was 177% higher in B compared to NF (\( P < 0.05 \)). This study presented novel data demonstrating varied cytokine profiles in equine blood, adipose and muscle tissues.
Introduction

Cytokines are inflammatory proteins active throughout the body, including adipose and muscle tissues, and are influenced by age and exercise in horses (Horohov et al., 1999). Recent research has determined an association between the increase in inflammatory proteins and the decrease in metabolic activity and subsequent fat deposition (Adams et al., 2009). Cytokines are secreted from many cell types, and have been linked to insulin resistance and glucose homeostasis in human (Arner, 2005; Pedersen, 2007), murine (Wallenius et al., 2002) and equine (Vick et al., 2007; Vick et al., 2008) models. Metabolically active adipocytes secrete biologically active proteins called adipokines (Arner 2005, Kahn and Flier 2000), which may include the cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and interferon gamma (IFN-γ) and hormones (adiponectin, leptin). However, simultaneous measurements of these cytokines in equine blood, skeletal muscle and adipose tissue (subcutaneous and abdominal) have not been made.

Tumor necrosis factor-α is a pro-inflammatory cytokine that stimulates, among other things, the release of IL-6 (Pedersen et al., 2003; Pedersen 2007); thus, TNF-α and IL-6 are tightly linked (Pedersen et al., 2003b; Pedersen et al., 2003c). TNF-α has been directly associated with insulin resistance in humans by mechanisms that include binding insulin receptors and inhibiting signaling, reducing glucose transport protein 4 (GLUT-4) translocation to the cell surface and decreasing production of adiponectin (Arner 2005; Bezaire and Langin, 2009). Truncal fat mass in humans has been associated with
increased presence of TNF-α and insulin resistance in humans (Reaven, 1995), but it is unknown if the same relationship exists in horses.

To moderate the action of TNF-α, IL-6 induces a negative feedback cascade on TNF-α, and also stimulates the production of IL-1 receptor antagonist (IL-1ra) and IL-10, both of which counteract the production of pro-inflammatory cytokines (Pedersen, 2007), and may help attenuate excessive inflammation associated with physiological stressors such as exercise. Consequently, IL-6 appears to have the ability to inhibit TNF-α-induced insulin resistance (Pedersen et al., 2003b).

The interleukin-1 family of cytokines has a strong influence on the innate immune system (Sims and Smith, 2010), and here, the primary variant considered is IL-1β. IL-1β is principally produced by monocytes and macrophages, and it circulates systemically (Sims and Smith, 2010). IL-1β is a pro-inflammatory cytokine, and its presence in tissues or circulation induces the upregulation of IL-1ra to counteract inflammation (Osborn et al., 2008), as well as the presence of other cytokines, particularly IL-6 (Dinarello, 1994). IL-1β has been associated with type 2 diabetes in humans, and it has been shown that pancreatic beta cells are particularly susceptible to destruction by IL-1β (Maedler et al., 2009), possibly by inducing oxygen free-radicals, lipid peroxidation or aldehyde production in the islets (Rabinovitch et al., 1996). Thus, IL-1β is an important factor when considering the effects of inflammation on glucose metabolism.

Cytokines are attracted to tissues via chemotactractant proteins. For example, MCP-1 is secreted by several cell types, such as monocytes, epithelial and smooth muscle cells (Tateya et al., 2010). Expression of MCP-1 is upregulated in adipose tissue and is associated with obesity in a murine model (Tateya et al., 2010). Theoretically, MCP-1-
induced inflammation causes the recruitment of macrophages to adipose tissue, a process that can accentuate the increased secretion of pro-inflammatory cytokines (Tateya et al., 2010). The long-term exposure to pro-inflammatory cytokines likely contributes to insulin resistance (Tateya et al., 2010). This speculated action is supported by the observation that MCP-1 has been shown to contribute to insulin resistance in primates (Bose et al., 2009).

Another major pro-inflammatory cytokine is IFN-$\gamma$ (Mosmann and Sad, 1996). IFN-$\gamma$ is a product of Th1 cells, but it is also produced by natural killer (NK) cells and activated B-cells (Ainsworth et al., 2003). In vitro studies of human adipocytes treated with IFN-$\gamma$ showed a decrease in insulin-stimulated glucose uptake along with a down-regulation of insulin receptor substrate -1 and GLUT-4 via JAK/STAT pathway activation (McGillicuddy et al., 2009). Such changes prevent proper insulin signaling, and a decrease in activation of GLUT-4, resulting in prolonged and elevated blood glucose levels.

Exercise and disease in humans often produce a shift in the Th1/Th2 balance favoring the pro-inflammatory Th1 production, although the mechanism causing this shift is not known (Ibfelt et al., 2002). Research has shown changes in the endocrine (McKeever and Malinowski, 1999), adipokine (Kearns et al., 2006) and immune (Horohov et al., 1999) response to age and training in an equine exercise model. Horses over the age of 20 yrs showed diminished immune responses that are comparable to what is seen in older people (Horohov et al., 2002). General increases in systemic inflammation have been associated with age in horses, as in humans, with older animals showing increased cytokine production compared to young animals (Adams et al., 2009).
A reduction in body weight and fat in old horses resulted in a decrease in TNF-α and IFN-γ mRNA, and increased when weight and percent body fat were gained (Adams et al., 2009). Taken together, research suggests that a reduction in body fat along with exercise may help to reduce the presence of cytokines associated with insulin resistance.

In addition to the action of cytokines, differences between the metabolic activity of abdominal (visceral) and subcutaneous fat and muscle tissues have been demonstrated in humans (Kahn and Flier, 2000; Fantuzzi, 2005). Abdominal adipose tissue is known to secrete pro-inflammatory cytokines in both humans (Pedersen, 2003) and horses (Vick et al., 2009). Excessive deposits of abdominal adipose tissue have been associated with increased risk of Type 2 diabetes in humans (Pedersen, 2003). Whether or not the distribution of body fat in horses affects the risk for developing IR remains to be determined.

Simultaneous comparison of cytokine profiles in adipose tissue from the neck crest (dorsal to the nuchal ligament) and omentum (abdomen), as well as middle-gluteal muscle tissue and blood circulation of horses of varied background, and of young vs. old animals is lacking. The cytokine profiles of tissues collected from different areas of the body may lend clues to the metabolic activity and inflammatory mechanisms at work in equine adipose and muscle tissue. Therefore, this study was designed to test the hypothesis that cytokine profiles differ in omental fat, subcutaneous fat, middle-gluteal muscle and blood in horses. Secondarily, it was hypothesized that cytokine profiles differ in old (20 yrs and older) vs. young (10 years and younger) animals.

Materials and Methods
Animals

Horses were enrolled in the study based on diagnostic evaluation and a specific set of exclusion and inclusion criteria (discussed below). The Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment. One animal in the study was from the Gluck Equine Research Center, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Evaluations of horses were made by attending veterinarians and owners. There were no restrictions on age, and horses in the study (n=15) ranged in age from 8-25 yrs. Horses were of mixed breed, sex and background (Table 1). Stallions were excluded due to handling concerns. Other exclusion criteria included acute illness, catastrophic injury, systemic infection, animals receiving antibiotics, treatment for Cushing’s syndrome, the presence of metastatic cancer, unexplained weight loss or those not eating or drinking water normally. Additional exclusion criteria were use of non-steroidal anti-inflammatory medications within 10 d preceding euthanasia, or any other anti-inflammatory pharmaceutical or recent surgery (within 6 months). A board-certified large animal surgeon conducted all euthanasia procedures, necropsies and supervised and assisted with sample collections.

Horses accepted into the study were transported to Rutgers University 12-24 h prior to euthanasia. However, because of logistics, one animal was euthanized at the University of Kentucky under similar protocol, and samples were collected in accordance with procedures described here. Prior to euthanasia, animals were housed on 1-acre dry lots, with ad libitum access to grass hay and water. From arrival until euthanasia, horses
were kept in paddocks alone as per University quarantine protocols, but could easily see other horses. Background diet was not controlled due to the nature of the study.

For purposes of comparing tissues in young vs. old horses, animals 10 years and under were considered young (n=2), and animals 20 years and over were considered old (n=7).

**Sample collection**

Prior to euthanasia, blood samples (B) (4 mL) were collected via venapuncture into PAXGene™ tubes (Qiagen, Santa Clarita, CA) for measurement of the relative quantity (RQ) of TNF-α, IL-6, IL-1β, MCP-1 and IFN-γ mRNA via RT-PCR. Horses were then catheterized percutaneously (Angiocath, 14 gauge, Becton Dickinson, Inc., Parsippany, NJ) in the jugular vein. Once catheterized, horses were sedated with Xylazine (0.3-0.5 mg/kg IV), then euthanized with phetobarbital sodium (78 mg/kg IV). Immediately after euthanasia, biopsy sites for obtaining neck crest adipose tissue (NF) and muscle (M) were shaved and aseptically prepared. Fat samples from the neck crest were collected at the mid-point of the neck, approximately 2 cm ventral from the mane hairline, dorsal to the nuchal ligament. A 5 cm x 5 cm “U-shaped” sterile incision was made through the skin and subcutaneous tissues and reflected back. A sample of adipose tissue (approximately 1.5 cm x 1.5 cm) was resected using a #10 scalpel blade. Muscle biopsies were collected from the middle-gluteal muscle using a Bergstrom biopsy needle as previously described (Lehnhard et al., 2004). Omental fat (OF) was collected using sterile techniques via a paralumbar fossa surgical approach. Tissue samples were cut into ~0.25 cm pieces and placed into 4 mL low-temperature freezer vials containing no less
than 1.5 mL RNALater™ (Qiagen, Santa Clarita, CA). Blood and tissue samples remained at room temperature for 24 hrs, then stored in a freezer at -20°C until analysis.

**Real Time-Polymerase Chain Reaction (RT-PCR)**

The blood samples for measurement of TNF-α, IL-6, IL-1, MCP-1 and IFN-γ mRNA were collected into PAXgene™ Blood RNA Tubes (PAXGene™, Qiagen, Inc., Santa Clarita, CA). Total RNA was subsequently isolated from the tubes using spin columns according to the manufacturer’s instructions. For adipose and muscle samples, tissues were physically disrupted using the bead-beating method. Sterile zirconium oxide beads were used in collaboration with a mixing mill (Retsch MM301 Mixing Mill, Clifton, NJ) to homogenize the tissues in microcentrifuge tubes containing RNA-Stat60 Reagent (Tel-Test Inc, Friendswood, TX). Total RNA was isolated from the samples using chloroform extraction, isopropanol precipitation and ethanol wash, according to the manufacturer’s instructions.

The RNA was quantified using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). In all cases, OD$_{260/280}$ ratios were greater than 1.9 and RNA yields were greater than 50 μg/mL. One microgram of RNA was reverse transcribed into cDNA in an 80μl reaction containing a master mix of 16 μL of AMV Buffer (5x), 16 μL of MgCl$_2$, 4 μL of dNTP, 1 μL of RNasin, μL oligo dT and 0.5 μL of AMV reverse transcriptase (Promega, Madison, WI). Cytokine-specific cDNA was then amplified and quantitated by “real-time” PCR (ABI Systems 7900 Fast Real-Time PCR System, Foster City, CA) using the Taq thermostable DNA polymerase and primers based on our sequences for equine cytokines and beta-glucoroidase (β-gus) (Swiderski et al., 1999;
Breathnach et al., 2006). Specific primers and FAM-labeled probes for each cytokine and β-gus, provided as Assay-by-Design kits (ABI, Foster City, CA), were added to 10 μL reactions in 384-well plates containing the 5 μL Taq polymerase (TaqMan Gene Expression Master Mix, ABI), 0.5 μL of primer-probe, and 4.5 μL of cDNA. The following PCR conditions were employed; 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, as recommended by the manufacturer.

Differences in RNA isolation and cDNA construction between samples were corrected using β-actin as an internal control for each sample (Breathnach et al., 2006). Relative differences in cytokine mRNA expression were determined by relative quantification. Relative quantification provides accurate comparison between the initial levels of target cDNA in a sample without requiring that the exact copy number be determined (Livak and Schmittgen, 2001). The average ΔCT for the entire group was selected as the calibrator and the change in cytokine gene expression post-expression relative to the calibrator was then determined for each sample.

Statistical analyses

Data were reduced and then analyzed using the Friedman repeated measures analysis of variance on ranks for non-normal data sets, and a t-test for normal data sets to compare RQs of cytokine mRNA of different tissues (SigmaStat version 3.1 and SigmaPlot version 9.0, SPSS, Chicago, IL). Post hoc analysis, where appropriate, used the Tukey Test for pairwise multiple comparisons. For comparison of age groups, the Kruskal-Wallis Rank Sum test was utilized for non-normal data, and a t-test was used to
compare data sets with normal distribution. The null hypothesis was rejected when
$P \leq 0.05$.

Results

Effect of Age

For TNF-α, IL-6, IL-1β, MCP-1 and IFN-γ, no difference ($P > 0.05$) in the RQ of
cytokine mRNA was observed in young compared to old horses in NF, OF, RM or B
(data not shown). Therefore, the data were pooled for all horses and analyzed for
differences between blood and tissues.

Tissues and blood

The RQ of TNF-α mRNA was a mean 155% higher in blood compared to muscle
($P < 0.05$) (Figure 1). For IL-6, neck adipose tissue exhibited a mean 165% greater RQ of
mRNA compared to muscle ($P < 0.05$), and a mean 363% greater RQ of mRNA in
omental adipose tissue compared to muscle ($P < 0.05$) (Figure 2). There was a
significantly higher RQ of IL-1β mRNA in blood compared to neck adipose tissue,
omental adipose tissue and muscle (1025%, 921% and 1140%, means, respectively,
$P < 0.05$ in all 3 cases) (Figure 3). Regarding MCP-1, RQ of mRNA was 1009% higher in
muscle vs. blood ($P < 0.05$) and 1646% higher in neck adipose tissue vs. blood ($P < 0.05$)
(Figure 4). The RQ of IFN-γ mRNA in blood was 177% higher compared to neck
adipose tissue (Figure 5).

Discussion
The major finding in this investigation was that middle-gluteal muscle, adipose tissue from the neck and omentum, and blood have different cytokine profiles in horses. This novel information is crucial for considering the role of cytokines in metabolic processes.

Response of the equine immune system and hormones, that modulate inflammation, such as cortisol, have been shown to decline with age in horses (Horohov et al., 1999; Malinowski et al., 2006), so it was unexpected to find no age-related difference in tissue cytokine profile here. In response to an exercise challenge to fatigue, research has shown no difference between young and middle aged mares (~7 yrs and 15 yrs, respectively) in VO2max and cortisol responses (Malinowski et al., 2006), or to plasma insulin (Malinowski et al., 2002). However, old (~27 yrs) mares differed from the younger age groups (Malinowski et al., 2002; Malinowski et al., 2006). Thus, in effort to separate out differences between tissues in young and old animals, the age-related comparison was limited to young (10 years and under) and old (20 years and over) horses. Since the analysis was limited to 2 young animals and 7 old, sample numbers were limited and, consequently, statistical power was low. Other factors could have contributed to the lack of age difference observed, including reason for euthanasia, treatment and management history. Therefore, comparative data presented here with respect to age should be interpreted with caution.

Data were pooled for the collective analysis of tissue cytokine profiles (n=15). Little expression of TNF-α mRNA was detected in adipose tissue from the neck and omentum. However, the higher RQ of TNF-α mRNA in blood indicates the cytokine was present to some degree in the circulation. It is known that TNF-α mRNA expression
increases in circulation in response to exercise in horses (Streltsova et al., 2006; Liburt et al., 2009), but animals in the present investigation were at rest prior to euthanasia. Studies of humans indicate that obese, premenopausal women had a 2.5-fold higher expression of TNF-α mRNA in adipose tissue compared to non-obese controls, as well as a decrease in TNF-α mRNA associated with weight loss (Hotamisligil et al., 1995). In the present study, neither body condition nor body composition were measured, and obese and lean horses were not compared. Here, investigators took a snapshot of cytokine mRNA in horses immediately following euthanasia. As TNF-α was observed in the circulation, the origination of circulating TNF-α was likely not from cells in adipose or muscle tissue sites sampled, and may have been related to the biological reason for euthanasia. Future studies in horses are warranted for tissue comparisons of obese and non-obese animals, TNF-α and insulin sensitivity and evaluation of changes in these variables in response to diet and exercise.

Work by Vick et al. (2008) did not detect TNF-α, IL-6 or IL-1β in unstimulated, mature adipocytes sampled from the rumps of 2 aged mares at necropsy. Presently, TNF-α and IL-1β were quantified in adipose tissue from the neck and omentum, but expression was low for both. The adipose tissue samples in the present investigation were sampled from the neck crest and omentum, compared to the rump as in Vick et al. (2008). Additionally, IL-6 was expressed in adipose tissue from the neck and omentum. This data collectively supports the notion that adipose tissue from different parts of the body exhibit varied cytokine profiles, and that adipose tissue in horses secretes TNF-α, IL-6 and IL-1β. It could therefore be speculated that cytokines may have varied metabolic effects in different tissues throughout the body.
Interestingly, IL-6 mRNA was present in higher RQ in adipose tissue from the neck and omentum compared to blood. These results are similar to that reported for humans, where IL-6 mRNA was present in higher concentrations in subcutaneous adipose tissue compared to plasma (Nielsen et al., 2009). Additionally, IL-6 enhances insulin-mediated glucose transport into muscle tissue, glycogen synthesis and glycogen storage and lipid oxidation in a human model (Pedersen, 2007). IL-6 plays a role in lipolysis and whole body lipid oxidation (Pedersen et al., 2003b), therefore increased expression of IL-6 observed in adipose tissue may be indicative of lipid oxidation.

With regard to IL-1β, there was a greater expression in blood compared to adipose tissue from the neck and omentum. Vascular endothelial cells are known to synthesize IL-1β, more so than adipose or muscle cells (Dinarello, 1988), which may partially account for this observation. Research showed that mice treated with IL-1β antibody demonstrated improved glycemic control and pancreatic beta cell function (Osborn et al., 2008). Consequently, the presence of IL-1β is important to consider as it may be a therapeutic target for insulin resistance (Osborn et al., 2008). However, the exact role of IL-1β in equine tissue as it relates to glucose homeostasis remains to be elucidated.

Other studies in rodents have demonstrated that MCP-1 has increased expression in adipose tissue (Tateya et al., 2010). MCP-1 has been detected in human skeletal muscle (Confalonieri et al., 2000) and adipose tissue (Sell et al., 2006) as well, and has now been shown to have a presence in equine skeletal muscle and adipose tissue. Studies of mice fed a high fat diet showed that inhibition of the MCP-1 receptor improved insulin sensitivity regardless of macrophage infiltration into adipose tissue (Tateya et al., 2010), and other work has shown that insulin suppresses MCP-1 (Dandona et al., 2004).
together, MCP-1 signaling may be an important factor with regard to glucose homeostasis (Tateya et al., 2010). Here, greater expression of MCP-1 mRNA was present in equine adipose tissue from the neck crest and middle gluteal muscle compared to the blood circulation. These results are similar to that found in humans (Nielsen et al., 2009), suggesting similar patterns at work in horses and humans.

IFN-γ potentially plays a role in the upregulation of chemoattractant cytokines (Bloomgarden, 2003). However, the presence of IFN-γ in the circulation was relatively high compared to MCP-1, and MCP-1 was elevated in adipose tissues where IFN-γ was not. Adipose tissue does not appear to be a primary site for synthesis of IL-1β (Dinarello, 1988), which may partially account for the difference. IFN-γ also has anti-viral properties, and it up-regulates reactive oxygen species (ROS) and nitrogen production in order to prime microphages for anti-tumor and antimicrobial activity (Ainsworth et al., 2003). Upregulation of ROS may also promote IL-1β secretion, which may at least partially explain the increased levels of IL-1β in circulation seen here.

It is generally accepted that cytokines such as those described here can influence insulin signaling, and consequently the activity of GLUT-4 (Arner 2005; Bezaire and Langin, 2009). Earlier work (Manso Filho et al., 2007) discovered a greater expression of GLUT-4 in adipose tissue compared to muscle. The fact that greater RQs of IL-6 and MCP-1 mRNA were found in adipose tissue compared to muscle or blood in the present study could be associated with the higher quantities of GLUT-4 in adipose tissue observed by Manso Filho et al. (2007). As stated earlier, IL-6 is known to facilitate insulin signaling, in part by counteracting the prohibitive action of TNF-α. Since insulin
stimulates tissue uptake of glucose by GLUT-4, the presence of IL-6 in adipose tissue is likely important for this process.

Data from the current study represent a preliminary comparison of equine adipose and muscle tissues and blood samples with regard to cytokine profiles. The evidence presented confirms that the quantities of cytokine mRNA present in the blood, adipose and muscle tissues of horses vary. Additional work is needed to evaluate glucose tolerance and insulin sensitivity along with body composition in effort to draw conclusions about the status of glucose metabolism and the presence of cytokines. Due to the nature of the investigation, such testing was not completed, and single blood samples were not sufficient for evaluation of insulin or cortisol status.

A limitation of the investigation is that mRNA, not actual protein, was measured. However, mRNA levels provide a window to observe potential protein production. Due to the nature of the investigation, background of the subjects could not be completely controlled for. Finally, reason for euthanasia or the euthanasia process itself may have influenced the presence of cytokines in blood and tissues after death, but data in this context is limited.

**Summary and Conclusion**

The aim of the investigation was to gain preliminary insight into the differences of inflammatory cytokines in different tissues and blood sampled in the horse. Proposed roles for the cytokines measured here are described in Table 2. Evidence that differences in cytokine mRNA do exist in blood circulation and in tissues sampled from different areas of the equine body was presented. Additional investigations with larger sample
numbers of young and old horses may help draw out differences in tissue cytokines that were not detected here, as well as to confirm parallelisms, or lack thereof, between inflammatory processes in horses and humans. This knowledge may help in the diagnosis and treatment of inflammatory-related diseases, such as equine metabolic syndrome and laminitis.

Acknowledgements

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References


Table 1. Characteristics of horses included in study.

<table>
<thead>
<tr>
<th>#</th>
<th>Age*</th>
<th>Sex**</th>
<th>Breed</th>
<th>Reason for Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8, Y</td>
<td>M</td>
<td>Paint</td>
<td>Chronic Lameness</td>
</tr>
<tr>
<td>2</td>
<td>8, Y</td>
<td>M</td>
<td>Suffolk Punch</td>
<td>Squamous cell carcinoma of the eye</td>
</tr>
<tr>
<td>3</td>
<td>13, MA</td>
<td>G</td>
<td>Peruvian Paso Fino</td>
<td>DSLS***</td>
</tr>
<tr>
<td>4</td>
<td>15, MA</td>
<td>M</td>
<td>Thoroughbred</td>
<td>Chronic laminitis</td>
</tr>
<tr>
<td>5</td>
<td>16, MA</td>
<td>M</td>
<td>Standardbred</td>
<td>Unmanageable behavior</td>
</tr>
<tr>
<td>6</td>
<td>19, MA</td>
<td>G</td>
<td>Thoroughbred</td>
<td>Chronic lameness</td>
</tr>
<tr>
<td>7</td>
<td>19, MA</td>
<td>M</td>
<td>Standardbred</td>
<td>Chronic lameness</td>
</tr>
<tr>
<td>8</td>
<td>19, MA</td>
<td>M</td>
<td>Standardbred</td>
<td>Chronic laminitis</td>
</tr>
<tr>
<td>9</td>
<td>20, O</td>
<td>M</td>
<td>Standardbred</td>
<td>Chronic lameness</td>
</tr>
<tr>
<td>10</td>
<td>20, O</td>
<td>G</td>
<td>Appendix</td>
<td>Chronic lameness</td>
</tr>
<tr>
<td>11</td>
<td>20, O</td>
<td>M</td>
<td>Thoroughbred</td>
<td>Age</td>
</tr>
<tr>
<td>12</td>
<td>21, O</td>
<td>M</td>
<td>Standardbred</td>
<td>Arthritis, low mobility, weight loss</td>
</tr>
<tr>
<td>13</td>
<td>23, O</td>
<td>G</td>
<td>Paso Fino</td>
<td>Chronic laminitis</td>
</tr>
<tr>
<td>14</td>
<td>24, O</td>
<td>M</td>
<td>Standardbred</td>
<td>DSLS***, Equine Cushings syndrome</td>
</tr>
<tr>
<td>15</td>
<td>25, O</td>
<td>M</td>
<td>Quarter Horse cross</td>
<td>Chronic lameness</td>
</tr>
</tbody>
</table>

*Age in years, classified as Young (Y), Middle Aged (MA) or Old (O)
**M=mare, G=Gelding
***DSLS=Degenerative Suspensory Ligament Desmitis
Table 2. Proposed roles of cytokines studied, considering present data and comparative literature.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Proposed role</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Pro inflammatory. Present in adipose tissue to inhibit insulin signaling and GLUT-4.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Inflammatory mediator. Present in adipose tissue to promote lipolysis, promote glucose uptake and inhibit TNF-α. Lower in muscle due to lack of strenuous exercise.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Pro inflammatory. Elevated in blood due to production by vascular endothelial cells; general inflammatory mediator. Possibly high due to reason for euthanasia.</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Chemokine. Present in adipose tissue to attract macrophages that produce TNF-α and IL-6.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Pro inflammatory. Present in adipose and muscle tissue to downregulate GLUT-4.</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Average RQ of TNF-α in adipose tissue from the neck crest (neck fat), omentum (omental fat), middle gluteal muscle (rump muscle) and blood. Different superscripts represent significant differences. Expressed as mean ± pooled SE.

Figure 2. Average RQ of IL-6 mRNA for adipose tissue from the neck crest (neck fat), omentum (omental fat), middle gluteal muscle (rump muscle) and blood. Different superscripts represent significant differences. Expressed as mean ± pooled SE.

Figure 3. Average RQ of IL-1β mRNA for adipose tissue from the neck crest (neck fat), omentum (omental fat), middle gluteal muscle (rump muscle) and blood. Different superscripts represent significant differences. Expressed as mean ± pooled SE.

Figure 4. Average RQ of MCP-1 mRNA for adipose tissue from the neck crest (neck fat), omentum (omental fat), middle gluteal muscle (rump muscle) and blood. Different superscripts represent significant differences. Expressed as Mean ± Pooled SE.

Figure 5. Average RQ of IFN-γ mRNA for adipose tissue from the neck crest (neck fat), omentum (omental fat), middle gluteal muscle (rump muscle) and blood. Different superscripts represent significant differences. Expressed as Mean ± Pooled SE.
SUMMARY

The present research confirmed that as a horse ages, plasma concentrations of cortisol, ACTH, glucose and insulin are altered. With respect to the hypothalamic-pituitary-adrenal axis (HPAA), it appears that both the pituitary and adrenal glands become less sensitive to stimulation by the appropriate hormones with advancing age. In addition, the endocrine regulation of glucose homeostasis changes, and pancreatic beta cells become less efficient over time. Exercise training reverses these changes to some degree, but does not completely reverse them.

During exercise, plasma concentrations of cortisol, adrenocorticotropin hormone (ACTH) and glucose concentration increase with exercise intensity in both old and young mares. However, the increase in cortisol did not occur after a period of moderate exercise training. Exercise training appears to condition the HPAA to a decreased response during acute, exhaustive exercise, with factors outside this axis affecting the increase in ACTH post-training. Speculatively, sympathetic nervous activity may be contributing to the increase in ACTH concentration, as well as the production of lactate and glucose for energy use.

During intense exercise in trained horses, young mares experienced a decrease in plasma insulin concentration, but old mares did not. In recovery from intense exercise pre-training, young mares had a higher concentration of plasma cortisol compared to old, but the age difference disappeared post-training. During exercise recovery post-training, old mares had a higher plasma ACTH concentration compared to young. Increased plasma ACTH without a concurrent rise in cortisol post-training suggests that the adrenal response is attenuated after training, or that factors outside the HPAA (adrenaline, IL-6 or
lactate, for example) are promoting ACTH secretion. As old mares tended to have lower plasma cortisol concentrations than young mares, perhaps the higher concentrations of ACTH served as a compensatory mechanism to increase cortisol, promote gluconeogenesis and suppress insulin in response to acute, exhaustive exercise.

Moderate exercise training attenuates age-related changes in endocrine mediators of the HPAA and glucose homeostasis to some degree. Post-training, old mares’ response to an ACTH stimulation test resulted in higher plasma cortisol concentrations compared to pre-training, toward that which young mares experienced. The response may help improve exercise tolerance and speed recovery. However, old mares had a lower plasma cortisol concentration during the post-training Control test, and a tendency for higher ACTH concentrations after DEX/CRF test. These observations suggest that exercise training helps restore sensitivity of the pituitary and adrenal glands to endocrine signals to some degree, but does not completely reverse age-related changes. This data provides further evidence that exercise training resulted in an attenuated adrenal response to a challenge while promoting ACTH concentrations. In addition, exercise training lowered the insulin response during exercise in young mares, but not in old. Post training, no mares tested insulin resistant in a separate study. Taken together, old mares could be said to have an impaired sensitivity to insulin, even after exercise training, without full-blown insulin resistance.

The results of these studies yielded important information. The hypothalamic-pituitary-adrenal axis reacts differently in response to exercise, recovery from exercise and standing stimulation. Collective evidence suggests an improvement in the sensitivity of both the pituitary and adrenal glands in response to exercise, particularly in old mares.
However, age-related deficits do not appear to be completely reversed with cardiovascular exercise training alone.

In addition, it has again been shown that moderate exercise training improves insulin sensitivity and glucose tolerance. Novel information presented here describes an improvement in pancreatic beta cell function, especially in aged mares, after training. However, old mares still required higher amounts of insulin to clear a relatively similar amount of glucose as young mares. With the growing number of horses experiencing insulin resistance, this is critical information for the management of such conditions. The influence of inflammatory cytokines cannot be ruled out, as it was shown that there are differences in blood, muscle and subcutaneous adipose tissue of old and young horses. IL-6 was negatively correlated with insulin sensitivity in adipose tissue, but has been reported to promote glucose clearance skeletal muscle. A possible divergent role for IL-6 may exist in different tissue types. MCP-1 was positively correlated with insulin sensitivity in adipose tissue, but has been associated with insulin resistance in other studies. These cytokines may have different action in different tissues, but definitive answers remain to be elucidated.

Furthermore, new evidence presented here demonstrated that the presence of inflammatory cytokines differs in skeletal muscle, adipose tissue of the neck, adipose tissue of the blood and in adipose tissue of the omentum. While it remains to be determined exactly how the differences in cytokine presence influence endocrine regulators of the HPAA and glucose homeostasis, it is important to understand that such differences exist in various tissues, much like in other species such as humans and rats.
Whether hormone replacement therapy is an option for aged horses with decreased cortisol concentrations remains to be investigated. Furthermore, it is quite likely that cytokines have a wide influence on endocrine etiologies, and such pathways remain to be investigated. Future research should address changes in the sympathetic response to exercise in old and young mares, and further investigate the role of cytokines in glucose homeostasis in these age groups. As the population of aging horses grows, it is critical that exercise training remains part of the regular routine. The benefits that training offers include a more balanced HPAA response, greater insulin sensitivity, and improved glucose homeostasis. The quantity of exercise needed to achieve endocrine and physiological benefits is not excessive, and many older equines should be able to tolerate continued activity at an appropriate intensity with the appropriate management.
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