THYROID STATUS IN EXERCISING HORSES AND LAMINITIC PONIES

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Key Words: Horse, Thyroid, Exercise, Laminitis, Thyrotropin releasing hormone

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(ABSTRACT)

The objective of these studies was to contribute to the understanding and assessment of thyroid function in horses. The first study evaluated methods of assessing thyroid function in horses, including validation of an enzyme immunoassay (EIA) for measuring equine thyroid hormones and development and assessment of a thyrotropin releasing hormone (TRH) response test. Our data indicated that EIA is an acceptable method for the measurement of total (T) and free (F) thyroxine (T_4) and triiodothyronine (T_3) in equine plasma. Its measurements are not equivalent to values obtained by radioimmunoassay (RIA), but they can be calibrated to predict corresponding RIA values. A protocol was developed for TRH response tests involving administration of 1 mg TRH intravenously, with blood sample collection immediately before, 2.5, 5.0, and 24 h after administration. Analysis of plasma TT_4, FT_4, TT_3, and FT_3 revealed that the magnitude of hormone response was best approximated by the area under the curve of hormone plotted against time and by the absolute change in thyroid hormone concentration. Baseline concentrations, peak concentrations, and percent of baseline values were not as well able to predict the magnitude of hormone response. The second study assessed the effects of exercise and feed composition on thyroid status. Thirteen mature Arabian geldings, adapted to either a high sugar and starch (SS) or high fat and fiber (FF) feed, underwent 15 wk of exercise training followed by a treadmill exercise test. The TRH response tests performed before training, after training, and the morning after the exercise test revealed that the exercise test decreased the TT_4 and FT_4 response, whereas feeding of high levels of sugars and starches increased the response of TT_3 and FT_3. During the first four weeks of training, increased TT_4 and FT_4 concentrations occurred simultaneously with increased nonesterified fatty acid concentrations, decreased triglyceride concentrations, and increased insulin sensitivity. The increase in TT_4 and FT_4 may have provided the cellular signaling necessary for increased lipolysis and insulin sensitivity. These metabolic changes facilitate the increases in lipid and carbohydrate metabolism that are needed to fulfill the additional energy requirements of regular

exercise. The third study assessed thyroid status in ponies with different laminitic histories. Total T_4, FT_4, TT_3, and FT_3 were measured during March and May 2004 in 126 ponies that were categorized as either previously laminitic (PL; n = 54) or never laminitic (NL; n = 72) and evaluated for current laminitis in May (CL; n = 13). Decreased concentrations of TT_4 and FT_4 were found in PL ponies when compared to NL ponies in March (P = 0.018, 0.020) and May (P = 0.018, 0.001). However, TT_4 and FT_4 concentrations in CL ponies were not different than concentrations in NL ponies in May (P = 0.82, 0.72), and when retrospectively separated out in March, were not different than NL ponies (P = 0.90, 0.84). Therefore, basal thyroid hormone concentrations are not useful as a predictor or hormonal characteristic of pasture-associated laminitis. The decreased TT_4 and FT_4 in PL ponies may be an indication of a response or compensation to laminitis and may facilitate the metabolic changes necessary to cope with the disease.

Key Words: Horse, Thyroid, Exercise, Laminitis, Thyrotropin releasing hormone

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CHAPTER I
Introduction

The action of thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), are vitally integrated into many body functions, including growth, reproduction, lactation, exercise, carbohydrate and lipid metabolism, energetics and thermogenesis. A proper understanding of thyroid function and assessment is important in helping horses reach their full potentials athletically, reproductively, developmentally, and metabolically.

Hypothyroidism is more frequently reported than hyperthyroidism as the form of thyroid dysfunction in horses. Clinical signs that had been previously associated with hypothyroidism include obesity, cresty neck, laminitis, and infertility (Johnson, 2002; Riddle and Gutierrez, 2003). However, experimental studies have shown that thyroidectomized horses do not develop these clinical signs (Lowe et al., 1974; Vischer et al., 1999; Frank et al., 1999, 2003a, 2003b, 2004). Symptoms that do result from thyroidectomy include rough hair coat, lower rectal temperature, lower heart rate, decreased weight gains, decreased feed consumption, and exercise intolerance.

Nevertheless, current treatment for obesity, laminitis, and infertility often includes oral levothyroxine supplementation. Estimates of the cost of exogenous thyroid hormone supplementation exceed $1 million per year (Riddle and Gutierrez, 2003). A lack of a unified, satisfactory method of evaluating thyroid status has resulted in many horses being placed on thyroid hormone supplementation without diagnosis or without proper diagnosis of thyroid gland dysfunction.

In humans, thyrotropin (TSH) and free T_4 (FT_4) measurements are often taken to evaluate thyroid function; in dogs, TRH response tests are often used; and in horses, basal concentrations of T_4 are used. Diagnosis of thyroid dysfunction does not depend exclusively on thyroid hormone concentrations in other species because many environmental and physiological factors influence the basal thyroid hormone concentrations. Still, equine veterinary practice depends almost exclusively on these measurements. This dependence on basal thyroid hormone concentrations may be partly due to the lack of commercial means of measuring TSH and administering TRH, and may also be due to a lack of understanding of thyroid function and assessment.

The integration of thyroid hormone actions into essentially all aspects of metabolism make a proper understanding of thyroid function and assessment critical in preserving and promoting equine health and performance. The objectives of my studies were to:

1. Evaluate and develop methods of assessing thyroid status in horses;
2. Assess the effects of exercise and feed composition on thyroid status in mature geldings; and
3. Assess thyroid status in healthy ponies compared to previously laminitic ponies and in ponies during the first day or two of clinical laminitis.

CHAPTER II

Literature Review

Thyroid Physiology

Thyroid hormones are formed by the iodination of tyrosine residues on thyroglobulin within the thyroid gland. These iodinated thyronines are initially in the form of monoiodotyrosine (MIT) or diiodotyrosine (DIT), which are then coupled to form triiodothyronine (T_3) or thyroxine (T_4) (Figure 1).

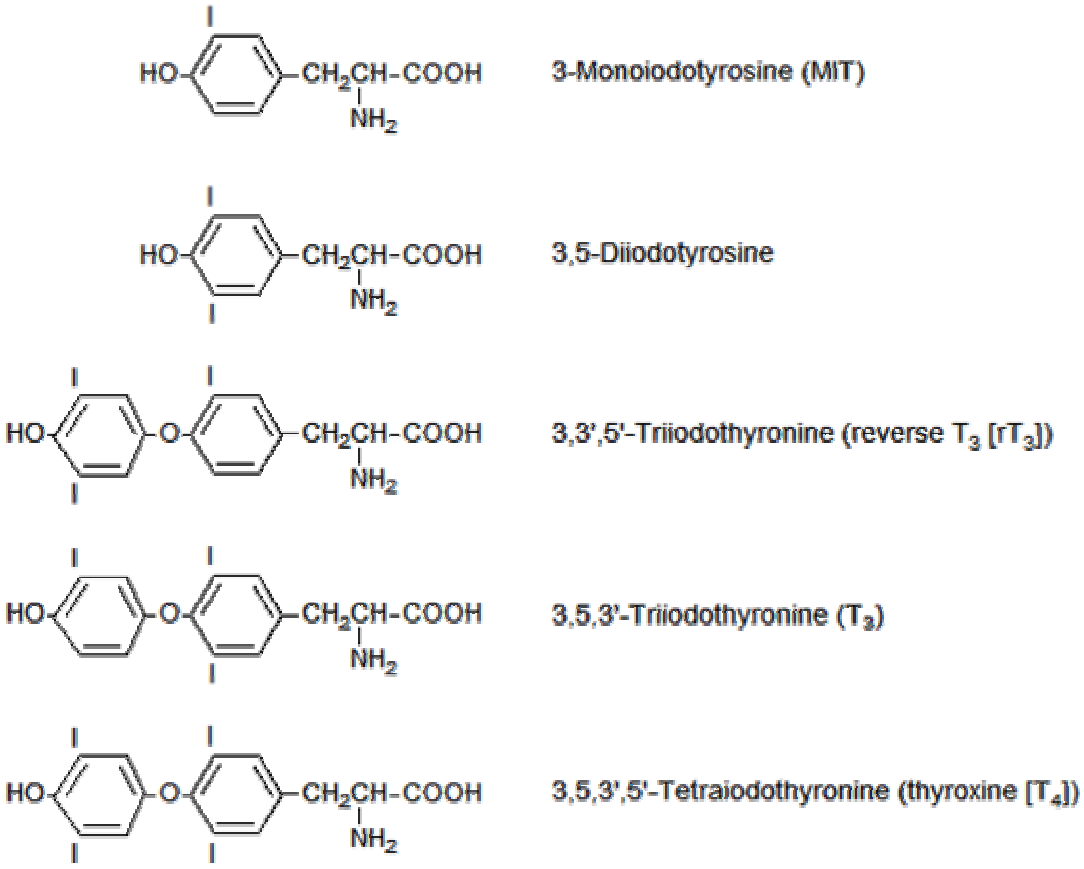

Figure 1. Structure of the major iodothyronines produced within the thyroid gland.

The thyroid gland is a bilobed organ that straddles the trachea just below the larynx. The isthmus is the area connecting the two lobes and is only a narrow remnant of tissue in the horse (Kaneko, 1997). Although the gland is small in size at approximately

0.20% of body weight, it is highly vascularized and has a large blood flow relative to its size. The thyroid gland consists of follicles which are composed of an outer layer of follicular cells creating an inner lumen. Inside the lumen is the clear, viscous colloid, containing thyroglobulin for the synthesis and storage of thyroid hormones.

Iodine is mainly absorbed in its water soluble form of iodide (I^-) through the intestinal mucosa or any moist body surface, including other mucosa and broken epithelia. Iodide is transported into thyroid follicular cells from circulation by a trapping enzyme that uses ATP for active transport of iodide across a steep concentration gradient. This enzyme is stimulated by thyrotropin (TSH). Once in the follicular cells, peroxidase oxidizes iodide to a free radical, I*. The I* binds to tyrosine residues on the thyroglobulin molecule at the 3 and/or 5 positions of the phenyl groups to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). At the follicular cell membrane bordering the lumen, a DIT and MIT or DIT are coupled by oxidative condensation to form T_4 or T_3. The iodinated thyroglobulin, or colloid, is then released into the lumen of the follicle for storage.

Thyrotropin stimulates the release of thyroid hormones by endocytosis of colloid into follicular cells. These vesicles then merge with lysosomes, where proteases hydrolyze the colloid to release MIT, DIT, T_3, and T_4. MIT and DIT are degraded and recycled within the thyroid gland by microsomal tyrosine deiodinases, while T_3 and T_4 are released into circulation by simple diffusion (Figure 2). In humans, approximately 90% of the hormone released from the thyroid gland is T_4 and 10% is T_3 (Kaneko, 1997).

The synthesis and secretion of thyroid hormones from the thyroid gland is regulated through the hypothalamus-pituitary-thyroid axis. Thyrotropin releasing hormone (TRH) is released from the hypothalamus to stimulate thyrotropin (TSH) secretion from the anterior pituitary. Thyrotropin circulates to the thyroid gland to stimulate the release of T_4 and T_3 into the bloodstream. Thyroxine and T_3 are either carried by binding proteins in the bloodstream, or circulate as free hormones to peripheral body tissues. While all T_4 is produced in the thyroid gland, most T_3 is produced by deiodination of T_4 in peripheral tissue. Triiodothyronine, the more active thyroid hormone, is then available to induce its physiological effects by inducing gene transcription in target cells and by

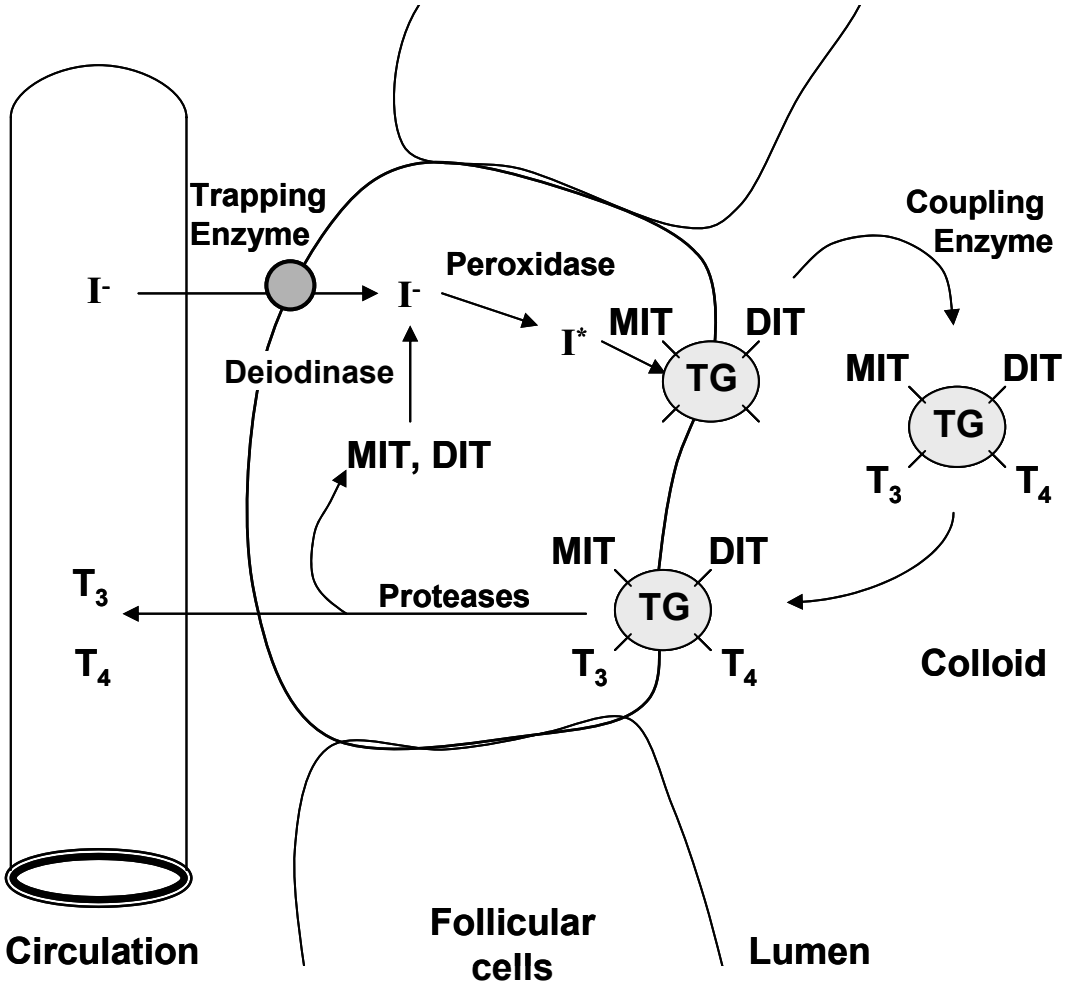

Figure 2. Mechanism of thyroid hormone synthesis and secretion. Adapted from Kaneko, 1997.

feedback regulation to the hypothalamus and pituitary, regulating the synthesis and release of TRH and TSH (Larsen et al., 1979).

Thyroid hormones are transported in blood by thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA), and albumin, with interspecies differences in the proportion of hormone bound to each protein. In horses, the percentage of T_4 bound to TBG, TBPA, and albumin are 61%, 22%, and 17%, respectively (Larsson et al., 1985). TBG is the major binding protein, with a binding constant for T_4 of approximately 10^{10}

L/mol, and for T_3 of approximately 10^9 L/mol. Consequently, only about 0.05% of T_4 is unbound in circulation, whereas 0.5% of T_3 is free in circulation in the horse (Anderson et al., 1988). Thyroid hormone binding to carrier proteins in circulation serves several functions. Binding of the lipid-soluble thyroid hormones to water-soluble proteins allows them to be transported readily through plasma. It also protects the hormones from excretion because the bound form does not pass through the renal glomerular membrane. Protection of T_4 from excretion and catabolism extends its longevity in circulation, with a half-life in horses of approximately 50 h (Katovich et al., 1974). Since a vast majority of the hormones are protein bound, there is a large, readily accessible reservoir for when active hormone is needed (Kaneko, 1997).

The free hormone fraction can be influenced by circulating metabolites or drugs that may inhibit hormone attachment to binding proteins, the concentration of binding proteins, and the affinity of the hormone for binding proteins. Free fatty acids (FFA) may displace thyroid hormones from binding proteins, especially when concentrations of FFA are increased during exercise or nonthyroidal illness (Liewendahl et al., 1992). Nonsteroidal anti-inflammatory drugs (NSAIDs) can alter thyroid hormone binding to serum proteins through competition of binding sites. Phenylbutazone administration in horses causes decreased serum thyroid hormone concentrations, and may be a factor to consider when evaluating thyroid function (Ramirez et al., 1997).

Elevated concentrations of thyroid hormones are seen in newborn foals because of the high concentration of strong affinity binding proteins, which allows a greater amount of thyroid hormones to bind to these carrier proteins in circulation (Irvine & Evans, 1975). Higher levels of thyroid hormones are needed in young animals to maintain thermogenesis and rapid growth of the musculo-skeletal and nervous systems. Concentrations of TBG are also increased during pregnancy as a result of decreased degradation. The rate of TBG degradation is dependent on its sialic acid content (Ain et al., 1987). During physiological states of high estrogen concentrations, including pregnancy, there are increased proportions of TBG molecules with higher sialic acid content, consequently decreasing overall degradation rates and elevating serum levels of TBG. Increases in circulating TBG induce greater hormone binding, which decreases hormone transfer and degradation and increases serum total T_4 and T_3 concentrations. A

new equilibrium develops between free and bound hormones, resulting in normal free T_4 and T_3 concentrations. Therefore, measurement of free hormone fractions is important in determining the bioavailability of thyroid hormones.

Transfer of thyroid hormones between plasma and interstitial fluid is rapid for liver and kidney, and slow for muscle, skin, fat, and brain (Kaptein et al., 1987). Slowly equilibrating tissue has small capillary pores, restricting the transfer of carrier proteins and allowing mostly free thyroid hormones into interstitial fluid. Conversely, rapidly equilibrating tissue has large vascular pores for the transfer of protein-bound thyroid hormones into interstitial fluid (Mayerson et al., 1960).

Once thyroid hormones are transferred out of circulation, a new equilibrium is formed between the bound and unbound state within the interstitial fluid. Only free hormones are transported across cellular membranes by transporter proteins.

Transport of thyroid hormones into cells is carrier mediated and dependent on free hormone fraction, cellular ATP concentration, the number of transporters per cell, and in some cases on the Na^+ gradient over the plasma membrane (Christensen et al., 1954; Hennemann et al., 2001). Transporters that have been identified include Na^+-dependent organic anion transporters (NTCP), Na^+-independent organic anion transporters (OATP), and L type heterodimeric amino acid transporters (Friesema et al., 1999; Friesema, 2001). Extracellular factors that affect carrier mediated transport include free hormone concentration; competition by circulating amino acids for transporters, especially tryptophan, phenylalanine, tyrosine, and leucine; and competition for transporters by metabolites with structural similarities to thyroid hormones which are increased during nonthyroidal illness (NTI) and starvation, including bilirubin and FFA (Lim et al., 1993).

The ATP-dependent transport of T_4 into cells is rate limiting for subsequent thyroid hormone metabolism, including intracellular T_4 deiodination to T_3 (Hennemann et al., 1986). During starvation, diminished thyroid hormone concentrations may be due to reduced intracellular ATP concentrations, which decreases the transport of T_4 into the cell and limits its conversion to T_3 (De Jong et al., 1992). The effects of decreased cellular uptake of T_4 may contribute to the increase in serum T_4 and decrease in serum T_3 concentrations seen in fasted chickens (Reyns et al., 2002). The bioavailability of thyroid

hormones is dependent on their transport into cells; therefore circulating concentrations of thyroid hormones cannot accurately indicate their intracellular bioactivity.

Although all circulating T_4 is produced and secreted from the thyroid gland, the majority of T_3 is produced peripherally by intracellular iodothyronine selenodeiodinases. There are three identified iodothyronine deiodinases: type 1, 2, and 3 deiodinases (D1, D2, and D3; respectively). Liver, kidney, and skeletal muscle tissue contain D1; brain, pituitary, and brown adipose tissue contain D2, and central nervous system tissues contain D3. There are two types of deiodinase activity that these enzymes can possess, 5'-deiodination and 5-deiodination, producing either T_3 (the active form) or rT_3 (reverse T_3, the inactive form) from T_4, respectively. Free rotation of the outer phenolic ring of the iodothyronine limits the enzymes from differentiating between the 3 and 5 positions of the phenolic rings, so that only these two types of deiodination are needed for complete deiodination (Bianco et al., 2002).

Deiodinase activity is influenced by many factors, including insulin levels, dietary composition, selenium intake, cold exposure, and lactation. Incubation of cultured rat hepatocytes with insulin or glucose increased T_3 neogenesis, indicating a stimulatory effect on hepatic T_4 5'-deiodinase activity (Gavin et al., 1987). The influence of insulin on deiodinase activity may be the reason why T_3 neogenesis is increased by the carbohydrate rather than the protein portion of the diet (Gavin et al., 1988). Cold exposure increases deiodinase activity in swine, probably to support increased thermogenesis (Reed et al., 1994). During lactation in cows, liver deiodinase activity decreases and mammary deiodinase activity increases to compensate for the increased metabolism in the lactating udder, causing an inverse relationship between serum thyroid hormone concentrations and milk production (Pezzi et al., 2003).

After T_4 deiodination, T3, the active form of thyroid hormone, moves through the cytoplasm and binds to specific thyroid hormone nuclear receptors. These thyroid hormone receptors (TR) then bind to thyroid hormone response element (TRE) sites on DNA, located upstream to the promoters where transcription of thyroid hormone-responsive genes is initiated (Figure 3). Binding of T_3 to the receptors results in either stimulation or inhibition of gene transcription into mRNA, which is then translated into the proteins that create the thyroid hormone responses (Greenspan, 2004).

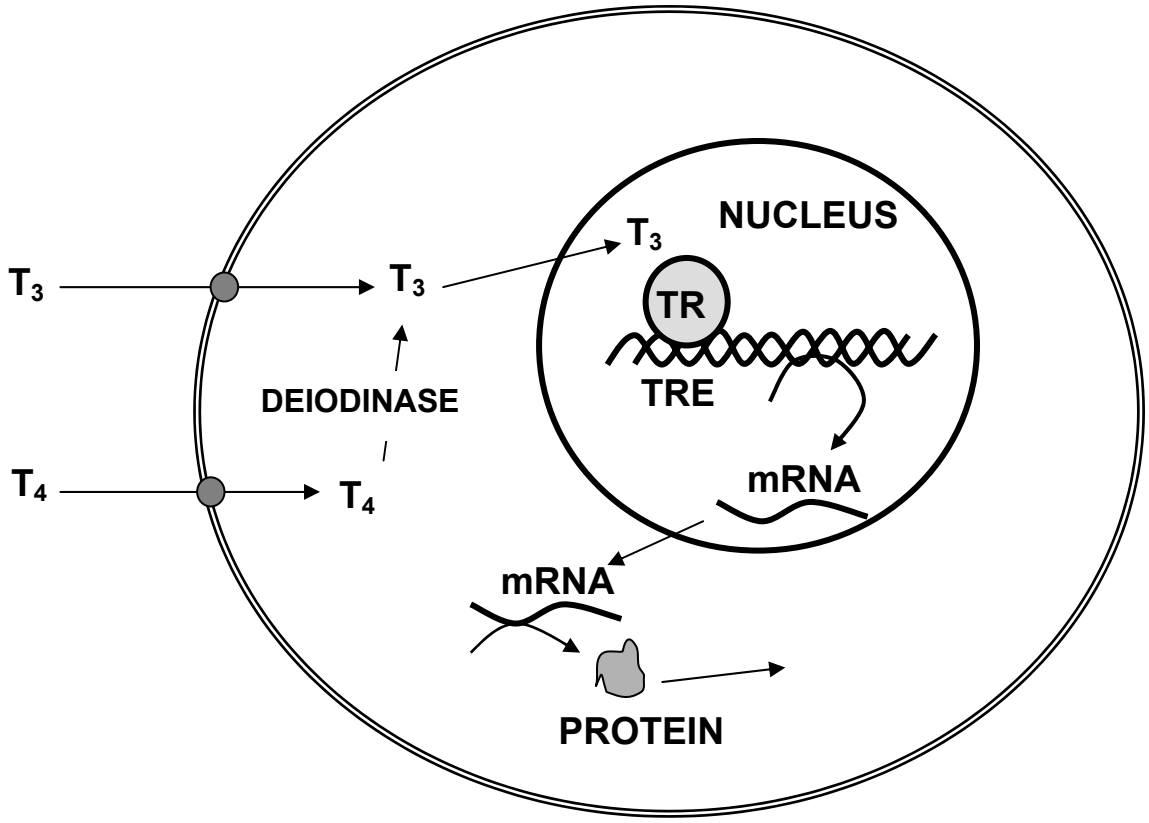

Figure 3. Cellular mechanism of thyroid hormone action. Adapted from Greenspan, 2004.

Degradation of iodothyronines involves deiodination, deamination, decarboxylation, and conjugation. Most iodine produced by deiodination circulates back to the thyroid gland. Conjugation of thyroid hormones with sulfates or glucuronides occurs within the liver and kidney. In animals, a mechanism of thyroid hormone metabolism is its conjugation with glucuronide by UDP-glucuronosyltransferase (UDP-GT). This occurs within hepatocyte microsomes, with subsequent excretion of T_4- and T_3-glucuronide conjugates into bile. Microsomal enzyme inducers have been shown to increase T_4 UDP-GT activity, including phenobarbital (PB), a drug that has been used in horses and dogs to prevent epileptic seizures and in cattle for detoxification of organochlorine insecticide poisoning (Hood et al., 2003). An increase in UDP-GT activity and subsequent increase in T_4 and T_3 excretion into bile eventually depletes

thyroid hormone reserves to a point where the decrease in circulating thyroid hormone concentrations cannot be compensated for by increased thyroid gland secretion.

Hypothyroidism is often described as a deficiency in circulating thyroid hormones. Primary hypothyroidism results from dysfunction of the thyroid gland, secondary hypothyroidism from dysfunction of the pituitary, and tertiary hypothyroidism from dysfunction of the hypothalamus. However, dysfunction of the hypothalamus-pituitary-thyroid axis is not the only reason for decreases in circulating thyroid hormone concentrations. Serum thyroid hormone concentrations are not only reliant on thyroid hormone synthesis and release, but also on peripheral regulatory mechanisms, including transfer between circulation and tissue sites, production of T_3 via peripheral deiodination, hormone binding to serum carrier proteins, and excretion rates. The occurrence of low circulating thyroid hormone concentrations in the presence of normal thyroid gland function is often referred to as the euthyroid sick syndrome (ESS) or nonthyroidal illness (NTI). Although these terms are usually used in human medicine, the same occurrences are evident in animals (Aceves et al., 1985; Wolf et al., 1995). This is often seen as a result of starvation, illness, or other stressors, and may be viewed as a defense mechanism to reduce metabolism and conserve energy (De Groot, 1999).

Nutritional factors influencing thyroid function

Selenocysteine (Sec), a rare amino acid with selenium in its chemical composition, is part of the amino acid sequence in the catalytic site of type 1-deiodinase, D1 (Berry et al., 1991). Since selenium is necessary for D1 synthesis and activity, selenium deficiency inhibits hepatic deiodination of T_4, causing increased T_4 and decreased T_3 concentrations (Beckett et al., 1989). The selenium requirement of the horse is 0.1 mg/kg of diet, with a maximal tolerable level of 2 mg/kg of diet (NRC, 1989).

Iodine intake is necessary for thyroid hormone synthesis because of its integration into the chemical structure of thyroid hormones. Daily requirements are relatively small because of the efficient iodine trapping mechanism of the thyroid gland. When intake drops below the necessary amount, gland hypertrophy (goiter) and eventually hypothyroidism result. Excessive iodine intake can result in either increased or decreased

thyroid hormone production. Decreased thyroid hormone concentrations may occur from the Wolff-Chaikoff effect, where organic binding of iodine within the thyroid gland is blocked when the level of plasma inorganic iodine is raised above a certain critical level (Wolff & Chaikoff, 1949). However, if the excessive iodine intake is large enough, it may override the Wolff-Chaikoff effect, causing iodide-induced thyrotoxicosis (Fradkin & Wolff, 1983). Iodine requirements of horses are 0.1 to 0.6 mg/kg of diet, with a maximal tolerable dietary concentration of 5 mg/kg DM (NRC, 1989).

Thyroid status may be influenced by dietary energy intake. Considerable differences were found between studies involving the effects of decreased caloric intake on thyroid status in horses. In horses, short term feed restriction (4 d), or fasting, results in decreases of circulating total and free T_4 and T_3 (Messer et al., 1995b). Long term feed restriction, or undernutrition, resulted in increases in total T_4 while free T_4 remained constant, and total and free T_3 decreased (Suwannachot et al., 2000). Conversely, another study reported that long term dietary energy and/or protein restriction did not alter circulating thyroid hormone concentrations (Stricker et al., 1995).

Overfeeding, or overnutrition, in humans results in increased T_3 levels, while T_4 concentrations remain constant (Danforth et al., 1979; Utiger, 1982). These results are seen during overnutrition regardless if the component of the diet overfed was carbohydrate, fat, or protein (Danforth et al., 1979). However, dietary composition may affect circulating thyroid hormone concentrations. Incubation of cultured rat hepatocytes with insulin or glucose increased T_3 neogenesis, indicating a stimulatory effect on hepatic T_4 5'-deiodinase activity (Gavin et al., 1987). The influence of insulin on deiodinase activity may be the reason why T_3 neogenesis is increased by the carbohydrate rather than the protein portion of the diet (Gavin et al., 1988).

Assessment of thyroid status

Basal thyroid hormone concentrations. Basal thyroid hormone concentrations are often measured to determine the amount of hormone available to tissues. Because the uptake of thyroid hormones into cells is dependent on the free hormone fraction, measuring free T_4 and T_3 will help determine the bioavailability of thyroid hormone (Kaptein et al., 1994). However, measurement of total or free thyroid hormone

concentrations may not accurately represent thyroid gland activity. Peripheral regulatory factors such as deiodination, serum binding proteins, vascular and cellular transfer, and catabolism will influence circulating thyroid hormone concentrations in response to thyroid gland activity.

Basal thyroid hormone concentrations may change depending on a number of environmental and physiological variables, so taking a basal thyroid hormone concentration at a single time period is not a reliable indication of thyroid status. Environmental factors, such as acclimatization to cold, daily rhythmicity, feed intake, and season, alter circulating thyroid hormone concentrations in horses (Danforth et al., 1979; Flisinska-Bojanowska et al., 1991; McBride et al., 1985; Messer et al., 1995b; Suwannachot et al., 2000). Physiological state, including age, gender, pregnancy, lactation, and illness, may also affect thyroid status (Anderson et al., 1988; Flisinska-Bojanowska et al., 1991; Irvine & Evans, 1975). Because of the high variability in basal values it is difficult to determine a reference range and classify a measurement as "normal". There is considerable variability between measurements taken during different research studies (Table 1).

Table 1. Thyroid hormone concentrations in adult horses. Data were compiled and converted to the same concentration units and summarized as means ± SD.

Investigators	Number of observations	Total T_4, µg/dL	Total T_3, ng/mL	Free T_4, ng/dL	Free T_3, pg/mL
Anderson et al., 1988	69	1.56 ± 0.67	0.68 ± 0.85	0.59 ± 0.32	3.22 ± 1.50
Bayly et al., 1996	4	0.30 ± 0.08	0.85 ± 0.07	0.73 ± 0.05	1.95 ± 0.21
Breuhaus, 2002	12	1.00 ± 0.44	0.64 ± 0.33	0.95 ± 0.27	1.35 ± 0.74
Messer et al., 1995b	6	1.54 ± 0.33	0.66 ± 0.25	0.90 ± 0.13	1.33 ± 0.53
Sojka et al., 1993	12*	1.59 ± 0.68	0.37 ± 0.12	0.16 ± 0.08	NA

NA = Not available
*8 FT_4 observations

Function tests. In order to evaluate the capabilities of the thyroid gland, its sensitivity to a stimulus may be measured. Both exogenously administered TSH and TRH have been used in horses to induce an increase in production and secretion of thyroid hormones from the thyroid gland (Beech & Garcia, 1985; Breuhaus, 2002; Chen & Li, 1986; Harris et al., 1992; Lothrop & Nolan, 1986; Messer et al., 1995a; Morris & Garcia, 1983). Circulating thyroid hormone concentrations are taken before and after

TSH or TRH administration to determine the magnitude of thyroid hormone response. In a TRH response test, thyroid gland response is dependent on production and release of endogenous TSH from the anterior pituitary, whereas in TSH stimulation tests exogenous hormone directly stimulates the thyroid gland. Therefore, administration of TRH evaluates both pituitary and thyroid gland function, whereas TSH administration evaluates only thyroid gland function.

Thyroid gland sensitivity may also be evaluated by the measurement of circulating TSH concentrations along with T_4 and T_3 concentrations, which is a method of diagnosis often used in human medicine (Greenspan, 2004). Decreased thyroid gland sensitivity would result in increased TSH and decreased or normal thyroid hormone concentrations. However, in horses a commercially available method of measuring TSH is not available; therefore response tests have been used to evaluate thyroid gland sensitivity. There are inter-species differences in the amino acid structure of TSH, making the human TSH assay ineffective in measuring equine TSH concentrations (Figure 4). Inter-species differences in TSH structure could potentially cause antibody production with repeated use in TSH stimulation tests and result in decreased effectiveness in causing a response. Conversely, TRH structure is similar between species and is easily accessible, making it a more practical choice than TSH for response tests (Harris et al., 1992) (Figure 5).

Studies have found peaks in T_4 between 4 and 10 h and in T_3 between 2 and 4 h after various doses of TRH have been administered intravenously, corresponding to peak concentrations of approximately 250 % of baseline and 300% of baseline in T_4 and T_3, respectively (Table 2). The peak increase in T_3 occurs before the peak increase in T_4, perhaps because of preferential release of T_3 after a large stimulation of the thyroid gland by TSH. The T_3 response gives more immediate results than T_4, which may provide a more practical means to clinicians of measuring thyroid response (Oliver & Held, 1985).

Thyroid function may be altered temporarily by factors such as NTI, oral levothyroxine supplementation, season, glucocorticoids, or phenylbutazone, resulting in changes in response test results. In cats and dogs, NTI may influence the results of stimulation tests, preventing differentiation between hypothyroidism and nonthyroidal illness. It has been suggested that the best way to evaluate thyroid function during severe

TSH-α

Identities = 85/119 (71%), Positives = 98/119 (82%), Gaps = 4/119 (3%)

```
Horse:  1 MDYYRKHAAVILATLSVFLHILHSFPDGEFTTQDCPECKLRENKYFFKLGVPIYQCKGCC  60
          MDYYRK+AA+ L TLSVFLH+LHS PD     QDCPEC L+EN +F + G PI QC GCC
Human:  1 MDYYRKYAAIFLVTLSVFLHVLHSAPD----VQDCPECTLQENPFFSQPGAPILQCMGCC  56

Horse: 61 FSRAYPTPARSRKTMLVPKNITSESTCCVAKAFIRVTVMGNIKLENHTQCYCSTCYHHK  119
          FSRAYPTP RS+KTMLV KN+TSESTCCVAK++ RVTVMG  K+ENHT C+CSTCY+HK
Human: 57 FSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHK  115
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TSH-β

Identities = 123/138 (89%), Positives = 132/138 (95%), Gaps = 0/138 (0%)

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Horse:   1  MTAIFLMSMVFGLACGQTMSFCIPTEYMMHVERKECAYCLTINTTICAGYCMTRDINGKL  60
            MTA+FLMSM+FGLACGQ MSFCIPTEY MH+ER+ECAYCLTINTTICAGYCMTRDINGKL
Human:   1  MTALFLMSMLFGLACGQAMSFCIPTEYTMHIERRECAYCLTINTTICAGYCMTRDINGKL  60

Horse:  61  FLPKYALSQDVCTYRDFMYKTVEIPGCPDHVTPYFSYPVAVSCKCGKCNTDYSDCIHEAI  120
            FLPKYALSQDVCTYRDF+Y+TVEIPGCP HV PYFSYPVA+SCKCGKCNTDYSDCIHEAI
Human:  61  FLPKYALSQDVCTYRDFIYRTVEIPGCPLHVAPYFSYPVALSCKCGKCNTDYSDCIHEAI  120

Horse: 121  KANYCTKPQKSYVVEFSI  138
            K NYCTKPQKSY+V FS+
Human: 121  KTNYCTKPQKSYLVGFSV  138
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Figure 4. Amino acid sequence of alpha and beta subunits of equine thyrotropin (TSH) compared to human TSH. Analysis performed by BLASTP 2.2.12 (http://www.ncbi.nlm.nih.gov/BLAST/).

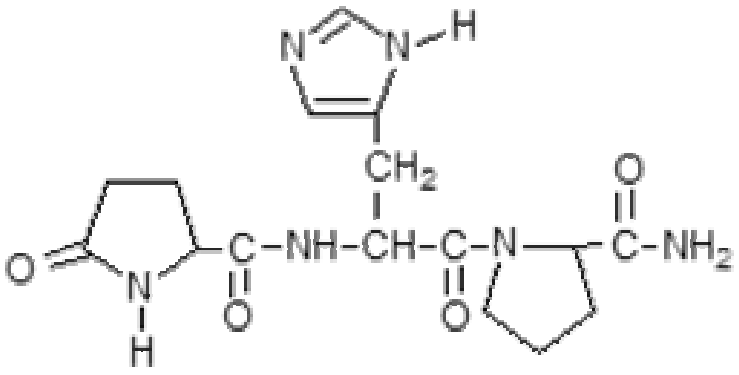

Figure 5. Structure of thyrotropin-releasing hormone (TRH), (pyro)Glu-His-Pro-(NH_2).

Table 2. The TRH response test in adult horses. Data were calculated from published data and compiled and summarized as means.

Investigators	TRH dose, mg	T_4 peak, h[a]	T_4 POB[b], %	T_3 peak, h[a]	T_3 POB[b], %	n
Beech & Garcia, 1985	1.0	4	166	3	250	12
Chen & Li, 1986	0.5	6	225	2	287	6
	1.0	4	242	4	321	6
	3.0	10	242	2	310	6
	5.0	6	367	4	310	6
Harris et al., 1992	0.2	4	225	NA	NA	15
	0.5	4	277	NA	NA	15
	1.0	4	355	NA	NA	15
Lothrop & Nolan, 1986	1.0	4	198	2	298	11
Sommardahl et al., 2005[c]	1.2	4	160	2	220	4

[a]Hour after TRH administration that the sample was taken
[b]Percent of baseline (POB) was calculated as
(peak concentration / baseline concentration) * 100%
[c]Results are an average of untreated horses during all weeks
NA = Not available

NTI is to first resolve the illness and then determine whether there is thyroid gland dysfunction (Panciera, 2001). Oral levothyroxine supplementation decreases the concentrations of total T_4, total T_3, free T_3, and TSH, but not FT_4 in response to TRH injection (Sommardahl et al., 2005). Higher concentrations of free thyroid hormones in circulation may negatively feedback to downregulate TSH synthesis and secretion pathways. Since the TSH response to TRH injection is blunted, pituitary gland function is inhibited in horses supplemented with levothyroxine. Responses to thyroid stimulation tests may demonstrate seasonal variability; however this variability may be species specific and has not been studied in horses. In lactating cows, greater responses are seen in spring and summer than in winter (Perera et al., 1985). Similarly, in reindeer the smallest response was during winter; however the greatest response was in autumn (Timisjarvi et al., 1994). Conversely, the greatest responses were seen during winter in humans (Harrop et al., 1985). Inter-species differences in seasonal variation in thyroid gland sensitivity may have evolved in order to increase thyroid hormone availability at times necessary for lactation, thermogenesis, or increased metabolic rate. Increases in endogenous or exogenous glucocorticoids may depress responses to stimulation tests (Banos et al., 1979; Messer et al., 1995a). Even though phenylbutazone treatment

decreases baseline concentrations of thyroid hormones, there is an increase in thyroid hormone response to TSH in phenylbutazone treated horses (Morris & Garcia, 1983). Phenylbutazone displaces thyroid hormones from plasma protein-binding sites, rather than decreasing production of T_4.

Although TRH response tests are often used to evaluate thyroid gland function, they may also be used to evaluate pituitary gland function and diagnose pituitary adenomas. Baseline cortisol concentrations are lower in horses suspected of having a pituitary adenoma and increase after TRH administration, returning to baseline within 24 h. This response is different than what is seen in normal horses, where cortisol concentrations decrease or increase then decrease after TRH administration, decreasing below baseline concentrations between 5 and 24 h (Beech & Garcia, 1985; Eiler et al., 1997). More distinct analysis may be performed when used in conjunction with a dexamethasone suppression test, where increases in cortisol are only seen in horses with pituitary adenomas (Eiler et al., 1997).

Measurement of thyroid hormone concentrations. Concentrations of thyroid hormones in equine plasma are often measured by radioimmunoassay (RIA). Thyroid hormone RIA use an analog procedure, where an ^{125}I-labeled T_4 or T_3 analog competes with plasma thyroid hormone for binding sites of a T_4 or T_3 specific antiserum immobilized to a polypropylene tube.

An alternative analysis to RIA used in human hormone diagnostics includes enzyme immunoassays (EIA), which eliminates the handling of radioactive materials. The EIA procedure is similar to RIA, given that plasma thyroid hormone competes with an enzyme-conjugated thyroid hormone analog for binding sites on an antibody coated well.

For free thyroid hormone concentrations, equilibrium dialysis assays are often used because they minimize disturbances of the bound to unbound equilibrium (Christofides et al., 1999). These disturbances may become most apparent during states of altered serum T_4 binding capacity, such as pregnancy or illness, at which time an assay involving equilibrium dialysis may be a more reliable method.

There are no equine specific RIA or EIA assays, however since thyroid hormones have the same chemical composition across species, unlike peptide hormones, an assay for one species should theoretically work for other species. Differences in thyroid hormone affinities to antibodies, buffer characteristics, or interfering biomolecules make assay validation necessary to confidently use measured values. Measurement of equine total T_4, free T_4, and total T_3 by RIA has been validated previously (Sojka et al., 1993).

Assays are often validated for accuracy, precision, and specificity. Accuracy is the extent to which the measurement of the hormone in a sample agrees with the amount that is present (Reimers et al., 1981). Accuracy may be evaluated by measuring the recovery after the addition of known amount of hormone. Accuracy may also be evaluated by comparison of measurements of the assay to be validated with the measurements of an assay that is known to be accurate, or the "gold standard" (Solter & Farner, 2000). Precision is the variability in repeated measurements of the same sample. Intra-assay variability measures the precision within one run of an assay, whereas inter-assay variability measures the precision of an assay over a time period. Specificity is the extent of freedom from interference by substances other than the one intended to be measured (Reimers et al., 1981). Specificity may be evaluated by determining the percent recoveries after serial dilutions of a sample and by measuring cross-reactivity of each antibody with substances chemically similar to the hormone trying to be measured, and biologic specificity can be evaluated by measuring hormone concentrations after a biologically activated stimulation of hormone levels.

Thyroid function and exercise

Exercise imparts additional stressors on the body above those of daily maintenance; therefore proper thyroid hormone action is especially critical during these times of increased metabolic rate. Among the many roles during exercise, thyroid hormones initiate the transcription of genes necessary for cardiac function, glucose transport, neuromuscular excitability, and nutrient metabolism (Clausen, 1998; Clement et al., 2002; Danzi & Klein, 2004; Romero et al., 2000).

Studies measuring thyroid hormone concentrations in horses after athletic training or exercise often find increased concentrations as compared to the basal state. Increases

in T_4 and T_3 concentrations were observed in horses 1 h after 15 min swimming (Garcia et al., 1986). It has been suggested that T_4 could be used to monitor training reserves in racehorses, after finding that T_4 concentrations increased during the latter stages of training (Takagi et al., 1974). Conversely, mild exercise training in Shetland ponies did not cause any changes in basal thyroid hormone concentrations (Suwannachot et al., 2000).

Studies have also found effects of training on thyroid hormone production and metabolism. Horses in training had a significantly greater increase in T_4 four hours after TRH administration when compared to horses out of work, representing an increased sensitivity of the pituitary-thyroid axis (Harris et al., 1992). Athletic conditioning in horses increases T_4 secretion rate and decreases protein-bound iodine (representative of thyroid hormones), indicating an increase in metabolism and excretion of thyroid hormones with training (Irvine, 1967).

Increases in thyroid hormone concentrations, production, and metabolism may facilitate the physiological changes seen with exercise. Thyroid hormones have a pronounced affect on the cardiovascular system (Danzi & Klein, 2004). T_3 mediates peripheral vasodilation, causing a decrease in arterial resistance and diastolic blood pressure. The effects of thyroid hormones on the heart include increasing cardiac contractility and cardiac output. Thyroidectomy in horses lowers resting heart rates, respiratory rates, and cardiac output (Frank et al., 2002). Exercise intolerance has been reported in hypothyroid horses, along with reduced distances to fatigue and lower maximal oxygen, maximal heart rate, and maximal velocity during a treadmill exercise test. The cardiovascular system of the thyroidectomized horse is less affected by β-adrenergic stimulation, indicating a decrease in number or function of their receptors.

Another mechanism that thyroid hormones influence the cardiovascular system is through regulation the expression of mRNA encoding myosin heavy chain (MHC) (Izumo et al., 1986). Mysosin heavy chain, the principal unit in myosin, is a structural protein largely responsible for exerting the contractile forces of heart and skeletal muscle. Whether thyroid hormones exert positive or negative regulation is tissue specific. This regulation may play a role in changes in muscle structure or contractility seen with athletic conditioning.

Thyroid hormones may contribute to the mechanism by which insulin sensitivity increases in exercising horses. In rats, thyroid hormones increase insulin stimulated glucose transport in skeletal muscle and adipose tissue by increasing the expression of GLUT1 and GLUT4 proteins (Romero et al., 2000; Shimizu et al., 2002; Weinstein et al., 1994). Similarly, athletic conditioning in rats results in an increase in cellular uptake of glucose due to an increased expression of GLUT mRNA (Ploug et al., 1990). In horses, increases in insulin sensitivity are also observed in response to oral levothyroxine supplementation and exercise, however glucose transporter concentrations were not measured (Frank et al., 2005; Treiber et al., 2005).

Thyroid hormones are important in maintaining ion gradients in cardiac and skeletal muscle through transcription of genes that encode ion transporters. Calcium concentration gradients across cellular membranes play an important signaling role in contraction coupling of muscle cells. Intracellular calcium concentrations are tightly regulated by proteins, including Na^+/Ca^{2+} exchangers (NCX), inositol 1,4,5-trisphosphate (IP_3) receptors, and ryanodine receptors (RyRs). An upregulation of mRNA levels of NCX, RyRs, and IP_3 receptors occurred after T_3 treatment in rats (Hudecova et al., 2004).

Skeletal muscle excitability is dependent on the action of membrane bound Na^+, K^+-ATPase for the regulation of Na^+ and K^+ concentration gradients across the sarcolemma. Regulation can occur by changes in either activity or concentration of Na^+, K^+-ATPases. Thyroid hormones stimulate the synthesis of Na^+, K^+-ATPases to increase their concentrations and provide long-term regulation of ion gradients (Clausen, 1998). However, changes in T_3 concentrations brought about by feed restriction in ponies only slightly influenced skeletal muscle Na^+, K^+-ATPase concentration; and no differences were seen in thyroid hormone or Na^+, K^+-ATPase concentrations as a result of mild exercise training (Suwannachot et al., 2000).

Gene expression may not only be regulated by thyroid hormone concentrations, but also by thyroid hormone receptor (TR) expression. Athletic conditioning of aged rats increased TR expression in cardiac muscle, and consequently increased MHC and sarcoplasmic reticulum Ca^{2+}-ATPase mRNA levels (Iemitsu et al., 2004). These changes in gene expression brought about by training improved cardiac function and contractility of aged hearts.

Thyroid hormones are involved in basically all aspects of energy metabolism by inducing the transcription of genes that encode enzymes necessary for protein, carbohydrate, and lipid metabolism (Clement et al., 2002). Some of the metabolic pathways influenced by thyroid hormones include glycolysis, gluconeogenesis, glycogen metabolism, the citric acid cycle, cholesterol metabolism, β-oxidation, and coupled and uncoupled respiration. Thyroid hormones also influence basal metabolic rate through activation of transcription of genes for uncoupling protein (UCP) (Solanes et al., 2005). UCP plays a major role in energy expenditure and thermogenesis by uncoupling respiration from ATP production to increase energy metabolism. Acute exercise upregulates UCP, whereas endurance training downregulates UCP (Schrauwen et al., 2003). This is in agreement with exercise-induced increases in metabolic rate and training-induced increases in mechanical energy efficiency.

Thyroid function and laminitis

Laminitis is a painful and disabling disease in which there is separation of the secondary epidermal and dermal lamellae of the hoof (Pollitt, 1996). Horses and ponies affected by laminitis often experience a decreased quality of life and usefulness, which may eventually lead to a loss of life itself (Breuhaus, 2004). The cause of laminitis is incompletely understood, and probably has multiple causal mechanisms with multiple component causes within each mechanism.

The role of thyroid function in the pathogenesis of laminitis is not understood, with the results of experimental studies conflicting with clinical observations and practice. However, the importance of thyroid hormone action in energy metabolism makes it a key area of interest when studying metabolic disorders, such as laminitis.

Thyroid hormones take part in carbohydrate metabolism by inducing the transcription of genes that encode enzymes necessary for gluconeogenesis, glycogen metabolism, the citric acid cycle, and coupled and uncoupled respiration (Clement et al., 2002). They also facilitate the regulation of plasma glucose concentrations by stimulating GLUT1 and GLUT4 expression in skeletal muscle and adipose tissue, increasing insulin stimulated glucose transport (Romero et al., 2000; Shimizu et al., 2002; Weinstein et al., 1994). These activities of thyroid hormones are part of the mechanism

in which oral levothyroxine supplementation increases insulin sensitivity and accelerates insulin disposal in resting horses (Frank et al., 2005).

Thyroid hormones may facilitate the changes in carbohydrate metabolism seen in laminitis, a state associated with insulin resistance (Coffman & Colles, 1983). Facilitation of these physiological changes may be especially critical in the hoof, since chronic laminitis is characterized by loss of GLUT1 and GLUT4 in laminar keratinocytes (Mobasheri et al., 2004). Also, *in vitro* studies of horse hoof explants indicate that the integrity of the explants was dependent on consumption of glucose (Pass et al., 1998).

Thyroid hormones play a critical role in all aspects of lipid metabolism, including synthesis, mobilization, and especially degradation (Pucci et al., 2000). They stimulate lipolysis, increasing TG utilization and mobilization from adipose tissue and appearance rates of plasma NEFA. Not only do thyroid hormones increase β-oxidation by increasing available NEFA, but they also induce lipogenesis in the liver. They decrease lipoprotein concentrations by decreasing TG availability for VLDL synthesis and by accelerating clearance of lipoproteins from circulation through an increased hepatic LDL receptor number and activity. Thyroidectomy in horses causes increases in VLDL, LDL, TG, and total cholesterol, whereas NEFA decreased (Frank et al., 1999). It has been suggested that although thyroidectomy may cause changes in blood lipid parameters, hypothyroidism does not interfere with their ability to adapt to dietary changes and it does not appear to be a factor in the pathogenesis of hyperlipemia in horses (Frank et al., 2003a, 2003b, 2004, 2005).

The actions of thyroid hormones in carbohydrate and lipid metabolism are important to consider when exploring the mechanisms in which thyroid function may be related to laminitis, especially since laminitis may be associated with obesity, insulin resistance, and in some cases dyslipidemia. Hypothyroidism has been proposed as a predisposing factor for laminitis, and suggestions of low thyroid hormone levels in horses affected with laminitis have been made (Colles and Jeffcott, 1977; Johnson, 2002). However, the results of experimental studies do not always support these propositions (Graves et al., 2002; Hood et al., 1987).

One study found that although serum levels of total T_4 and T_3 were depressed in horses with carbohydrate-induced acute laminitis, horses with chronic laminitis had

elevated T_3 concentrations and similar T_4 concentrations with respect to control values. Thyroid function, as determined by TSH stimulation tests, was similar for control horses and those affected with chronic laminitis (Hood et al., 1987). In another study, horses with obesity-associated laminitis and hyperinsulinemia had similar total T_4 and free T_4 responses and greater total T_3 and free T_3 responses to TRH response tests as horses with chronic lameness due to musculoskeletal disorders (Graves et al., 2002). Baseline thyroid hormone concentrations were similar between the two groups. The results of these studies indicate that thyroid gland dysfunction does not play a role in the development of laminitis and circulating thyroid hormone concentrations may not be depressed in laminitic horses.

The assumption that hypothyroidism is related to obesity-associated laminitis is often made (Johnson, 2002), perhaps because hypothyroidism has been suggested as a predisposing factor for obesity through a decrease in basal metabolic rate. However, thyroid hormone concentrations and their metabolism are normal in obese humans (Roti et al., 2000) and experimental thyroidectomy in adult horses does not induce laminitis or obesity (Lowe et al., 1974; Vischer et al., 1999; Frank et al., 1999, 2003a, 2003b, 2004).

The incidence of clinical observations of depressed circulating thyroid hormone concentrations may be explained by changes in peripheral hormone metabolism brought about by the disease state itself. Often times, basal thyroid hormone concentrations do not accurately represent thyroid gland function. Environmental and physiological factors influence the circulating levels of thyroid hormones. In humans, illness may cause abnormalities in thyroid hormone metabolism, resulting in the development of NTI. These subjects have normal thyroid gland function, but abnormal concentrations of thyroid hormones. In critically ill patients, T_4 deiodination to T_3 (the active form) is decreased, whereas T_4 deiodination to rT_3 (the inactive form) is increased and rT_3 degradation is decreased (Peeters et al., 2003). These changes in peripheral deiodinase activity result in decreased plasma T_3 and increased rT_3 concentrations. T_4 may be increased in mild illness and normal or decreased during critical illness. Another factor influencing thyroid hormone concentrations and deiodinase activity during critical illness may be the inhibition of T_4 transport into hepatocytes by elevated concentrations of

bilirubin and NEFA (Lim et al., 1993). Laminitis may have similar physiological effects as critical illness, resulting in changes in thyroid hormone metabolism.

Another nonthyroidal factor influencing thyroid hormone concentrations could include the drugs administered to laminitic horses. Phenylbutazone competes with thyroid hormones for binding sites on serum carrier proteins, consequently depressing circulating concentrations of thyroid hormones (Ramirez et al., 1997). There is no decrease in thyroid gland function with this decrease in serum hormone levels; conversely there is an increase in thyroid hormone response to TSH in phenylbutazone treated horses (Morris & Garcia, 1983).

Oral thyroid hormone supplementation is commonly used in the management of laminitis; however controlled studies have not been performed to determine whether thyroid hormone supplementation is beneficial during acute episodes of laminitis. Beneficial effects of thyroid hormone supplementation may result from weight loss caused by creating a state of iatrogenic hyperthyroidism. Levothyroxine supplementation has been shown to cause weight reduction in horses, perhaps through decreasing energy efficiency and enhancing lipolysis (Frank et al., 2005). While the use of thyroid hormone supplementation has been suggested as an acceptable treatment for obesity (Krotkiewski, 2000), human medicine has largely shifted away from this approach because of side effects of inappropriate supplementation and availability of alternative treatments. Tachycardia, increased heart rate, palpitations, atrial arrhythmia, widened pulse pressure, increased systolic and decreased diastolic pressures, dyspnea, exercise intolerance, and in severe cases congestive heart failure may occur from high circulating thyroid hormone concentrations (Danzi & Klein, 2004). Another possible mechanism for beneficial effects of supplementation during laminitis may be that thyroid hormones stimulate vasodilation by increasing β-adrenergic receptor number and sensitivity, which may increase circulation to the laminitic hoof (Breuhaus, 2004).

CHAPTER III

Methods of assessing thyroid status in horses

ABSTRACT: Methods of measuring thyroid status were validated and evaluated, including the validation of an enzyme immunoassay (EIA) for the measurement of total (T) and free (F) thyroxine (T_4) and triiodothyronine (T_3) in equine plasma, and the development and evaluation of a thyrotropin releasing hormone (TRH) response test protocol. Enzyme immunoassays for TT_4, FT_4, TT_3, and FT_3 were validated for specificity, accuracy, and precision. The TRH response tests were performed by administration of 1.0 mg TRH intravenously, with blood samples collected immediately before, 2.5, 5.0, and 24 h after administration. Eleven horses received TRH injections and two control horses received saline injections for each of three TRH response tests. The magnitude of the hormone response was approximated by the area under the curve (AUC) of the increments above the baseline concentrations plotted against time. The change in thyroid hormone concentration (ΔTH), baseline concentrations, percent of baseline (POB) values, and peak concentrations were compared to AUC using linear regression. During EIA validation, concentrations measurably increased after TRH administration, peaking at 171% to 225% of baseline, confirming biological specificity. Measurements of RIA and EIA concentrations were systematically different ($P < 0.001$), but correlated for TT_4 ($r^2 = 0.85$), FT_4 ($r^2 = 0.90$), TT_3 ($r^2 = 0.98$), and FT_3 ($r^2 = 0.94$), allowing calibration to establish accuracy. Inter- and intra-assay coefficients of variation varied between hormones, ranging from 0.77% to 17% and 3.5% to 33%, respectively. Data from the TRH response tests revealed that was the best predictor of the magnitude of hormone response was ΔTH, with AUC and ΔTH correlated for TT_4 ($r^2 = 0.92$), FT_4 ($r^2 = 0.96$), TT_3 ($r^2 = 0.80$), and FT_3 ($r^2 = 0.58$). The magnitude of hormone response, measured as either AUC or ΔTH, was not correlated to baseline measurements, indicating that baseline measurements do not accurately assess pituitary-thyroid axis responsiveness. Measuring thyroid hormone concentrations in equine plasma using EIA is a viable method; however results cannot be compared to RIA measurements. The use of TRH response tests to assess thyroid function should include the calculation of AUC

or ΔTH, which measure absolute changes in hormone concentrations, for a more critical and accurate evaluation than relative changes in hormone concentrations.

Key Words: Horse, Thyroid hormone, Thyrotropin releasing hormone, Enzyme immunoassay, Validation

Introduction

Basal thyroid hormone concentrations, thyroxine (T_4) and triiodothyronine (T_3), are often measured to determine the amount of hormone available in circulation. Since the uptake of thyroid hormone into cells is dependent on the free hormone fraction, measuring free T_4 (FT_4) and free T_3 (FT_3) may help determine the bioavailability of thyroid hormones (Kaptein et al., 1994). However, measurement of total or free thyroid hormone concentrations may not accurately represent thyroid gland activity. Peripheral regulatory factors such as deiodination, serum binding proteins, vascular and cellular transfer, and catabolism will influence circulating thyroid hormone concentrations in response to thyroid gland activity.

In order to evaluate the capabilities of the thyroid gland, its sensitivity to a stimulus may be measured. Thyrotropin releasing hormone (TRH) has been used to induce an increase in production and secretion of thyroid hormones from the thyroid gland. Circulating thyroid hormone concentrations are taken before and after TRH administration to determine the magnitude of thyroid hormone response. In a TRH response test, thyroid gland response is dependent on production and release of endogenous TSH from the anterior pituitary, therefore administration of TRH evaluates both pituitary and thyroid gland function.

Concentrations of thyroid hormones in equine plasma are often measured by radioimmunoassay (RIA). Thyroid hormone RIA use an analog procedure, where an ^{125}I-labeled T_4 or T_3 analog competes with plasma thyroid hormone for binding sites of a T_4 or T_3 specific antiserum immobilized to a polypropylene tube. An alternative analysis to RIA used in human hormone diagnostics includes enzyme immunoassays (EIA), which eliminates the handling of radioactive materials. The EIA procedure is similar to RIA, since plasma thyroid hormone competes with an enzyme-conjugated thyroid hormone analog for binding sites on an antibody coated well.

There are no equine specific RIA or EIA, however since thyroid hormones have the same chemical composition across species, unlike peptide hormones, an assay for one species should theoretically work for other species. Measurement of equine TT_4, FT_4, and TT_3 by RIA has been validated previously (Sojka et al., 1993). It would be expected that EIA would produce reliable measurements of thyroid hormones in horses because of

the similarities between RIA and EIA procedures and between thyroid hormone structure across species. However, differences in thyroid hormone affinities to antibodies, buffer characteristics, or interfering biomolecules make assay validation necessary to confidently use measured values.

Assay validation often evaluates the accuracy, precision, and specificity of the new method. Accuracy is the extent to which the measurement of the hormone in a sample agrees with the exact amount that is present (Reimers et al., 1981). Accuracy may be evaluated by measuring the recovery after the addition of known amounts of hormone. Accuracy may also be evaluated by comparison of measurements of the assay to be validated with the measurements of an assay that is known to be accurate, or the "gold standard". Precision is the variability in measurements of the same sample. Intra-assay variability measures the precision within one run of an assay, whereas inter-assay variability measures the precision of an assay over a time period. Specificity is the extent of freedom from interference by substances other than the one intended to be measured (Reimers et al., 1981). Specificity may be evaluated by determining the percent recoveries after serial dilutions of a sample and by measuring cross-reactivity of each antibody with substances chemically similar to the hormone intended to be measured. Biologic specificity can be evaluated by measuring hormone concentrations after a biologically activated stimulation of hormone levels.

The objective of this study was to validate and evaluate novel methods of assessing thyroid function in horses. First, the measurement of TT_4, FT_4, TT_3, and FT_3 in equine plasma using EIA was validated. Second, a TRH response test protocol was developed, evaluated, and compared to previous TRH response test procedures.

Materials and Methods

EIA validation

Specificity. Enzyme immunoassays for TT_4, TT_3, FT_4, and FT_3 (Active®, Diagnostic Systems Laboratories, Inc., Webster, TX) were validated for specificity, accuracy, and precision. Specificity was determined qualitatively by measuring the biological change in thyroid hormone concentrations after TRH administration.

Specificity was measured quantitatively as percent recovery after dilution of plasma sample with zero standard to make 1:1, 3:4, 1:2, and 1:4 dilutions.

Accuracy. Accuracy was determined by linear regression of concentrations measured by EIA and RIA for 28 plasma samples varying from low to high concentrations. Radioimmunoassay kits for TT_4, FT_4, and TT_3 (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) were previously validated for horses (Sojka et al., 1993). FT_3 RIA kits (Clinical Assays GammaCoat, DiaSorin, Stillwater, MN) have been used successfully for the measurement of FT_3 in horses (Nachreiner, personal communication) and were validated for biologic specificity, accuracy, and precision within this study. Accuracy after spiking was determined by adding 50 and 100 μL of plasma with medium and high concentrations to 100 μL of a low concentration plasma sample, and replicated by adding standards to plasma.

Precision. Precision was determined by evaluating inter- and intra-assay variability. Inter-assay variability was evaluated by measuring 28 samples on two separate days and averaging inter-assay coefficients of variation (CV). Intra-assay variability was evaluated using 4 replicate measurements within one run for low, medium, and high concentration samples.

Free T3 RIA validation

Validation of TT_4, FT_4, and TT_3 RIA kits for equine plasma had been performed previously (Sojka et al., 1993). However, since FT_3 RIA kits had not been validated for equine plasma, they were validated within this study. Intra-assay variability was determined by four repeated measurements of high, medium, and low concentration samples within one assay. Accuracy after spiking was determined by adding 100 and 200 μL of high concentration standard or plasma to 200 μL of low concentration plasma. Biologic specificity was determined by measuring the change in FT_3 concentration after TRH administration. Dilutional parallelism was evaluated by comparison of percent recoveries between FT_3 in equine plasma serially diluted with zero standard, and a high concentration RIA standard serially diluted with zero standard.

TRH response test

TRH response tests were performed on 13 mature Arabian and Arabian cross geldings (1024.6 ± 25.3 kg BW, 12.3 ± 0.9 y) between May and September 2004. Horses were housed in stalls and offered water and timothy/alfalfa hay, but feed was withheld during the day of TRH response tests. A stock solution of TRH (Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 1.0 mg TRH/mL and stored at -20°C until the day of use. One mg TRH was injected intravenously between 0700 and 0900. A 24 h trial was run on two horses to determine the time periods necessary for T_4 and T_3 to peak. Blood samples were taken via jugular catheter immediately before and 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h after TRH administration. Total T_4 and FT_4 concentrations peaked at 5 h and TT_3 and FT_3 concentrations peaked 2 to 3 h after TRH administration (Figure 1). For subsequent response tests, blood samples were collected via venipuncture into sodium heparin Vacutainer tubes inimmediately before TRH injection, 2.5, 5, and 24 h after TRH injection. Eleven horses received the 1.0 mg TRH dose and two control horses received 1 mL saline solution for each of three response tests.

Blood analysis

Blood samples were centrifuged at 3000 g for 10 min at 4°C and plasma was transferred to 2 mL polypropylene vials (Sarstedt, Newton, NC) and stored at -20°C until analysis. Plasma samples from the 24 h trial TRH response test (28 samples total) were analyzed for TT_4, FT_4, TT_3, and FT_3 using both EIA (Active®, Diagnostic Systems Laboratories, Inc., Webster, TX) and RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Plasma samples from the subsequent three TRH response tests were analyzed for TT_4, FT_4, and TT_3 via RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) and for FT_3 via EIA (Active®, Diagnostic Systems Laboratories, Inc., Webster, TX). All samples were measured in duplicate.

Statistical analysis

Recoveries for dilutions and additions were calculated as (observed concentration ÷ expected concentration) * 100%. Comparison of RIA and EIA methods using the 24 h trial TRH response test data were made by robust linear regression, calculation of the

coefficient of determination (r^2) and the P-value for the null hypothesis that the slope of the best fit line equals one (Intercooled Stata 9.0; StataCorp LP, College Station, TX). Comparisons of EIA and RIA measurements were also analyzed by Bland-Altman plots of the difference in EIA and RIA values (EIA – RIA) plotted against the mean value ([EIA + RIA] ÷ 2) (Bland & Altman, 1999). Measurements during the three subsequent TRH response tests included baseline concentration; peak concentration; area under the curve (AUC); the change in thyroid hormone concentration (ΔTH) for TT_4 (ΔTT_4), FT_4 (ΔFT_4), TT_3 (ΔTT_3), and FT_3 (ΔFT_3); and percent of baseline (POB). The magnitude of the hormone response was represented by the AUC of the increments above baseline concentrations plotted against time and calculated by trapezoidal approximation. The ΔTH was calculated as the difference between the peak concentration (at 2.5 h for TT_3 and FT_3, and 5 h for TT_4 and FT_4) and baseline concentration. The POB was calculated as (peak concentration / baseline concentration) * 100%. The ability of one measurement to predict another measurement was evaluated with robust linear regression and calculation of the coefficient of determination (r^2), 95% confidence intervals, and the P-value for the null hypothesis that the slope of the best fit line equals zero (Intercooled Stata 9.0; StataCorp LP, College Station, TX). Data was graphed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

Results

EIA validation

Specificity. Concentrations increased after TRH administration ($P < 0.01$), peaking at 200, 171, 199, and 225 POB for TT_4, FT_4, TT_3, and FT_3, respectively. Recovery after dilution ranged from 68% to 88% of predicted concentrations for all thyroid hormones (Table 1).

Accuracy. Measurements of RIA and EIA concentrations were systematically different ($P < 0.001$), but correlated for TT_4 ($r^2 = 0.85$), FT_4 ($r^2 = 0.90$), TT_3 ($r^2 = 0.98$), and FT_3 ($r^2 = 0.94$) (Figure 2). Bland-Altman plots of the data revealed that EIA measurements had positive biases of the mean differences ± 2 SD for TT_4 (3.16 ± 1.50 μg/dL), FT_4 (0.78 ± 0.23 ng/dL), TT_3 (0.74 ± 0.31 ng/mL), and FT_3 (4.49 ± 2.78 pg/mL) (Figure 3). When a low concentration plasma sample was spiked with a higher

concentration plasma sample, recovery ranged from 93.3% to 114% after spiking with a medium concentration sample and 82.2% to 106% after spiking with a high concentration sample (Table 2). When a low concentration plasma sample was spiked with a higher concentration standard, recovery ranged from 91.5% to 116% after spiking with a medium concentration standard and 92.4% to 115% after spiking with a high concentration standard (Table 3).

Precision. Inter- and intra-assay CV varied between hormones, ranging from 0.77% to 17% and 3.5% to 33% for intra- and inter-assay CV, respectively (Table 4).

Free T3 RIA validation

Concentrations increased after TRH administration ($P < 0.01$), peaking at 189% to 650% of baseline. Recovery ranged from 70% to 94% when plasma was diluted with zero standard and 72% to 87% when a high concentration standard was diluted with zero standard. Recoveries ranged from 96% to 103% when plasma was spiked with a high concentration plasma sample and 107% to 111% when plasma was spiked with a high concentration standard. Intra-assay CV was 7.1%, 1.2%, and 2.3% for low, medium, and high concentrations, respectively.

TRH response tests

Means ± SEM for TT_4, FT_4, TT_3, and FT_3 for baseline measurements were 0.86 ± 0.04 µg/dL, 0.19 ± 0.02 ng/dL, 0.66 ± 0.03 ng/mL, 5.5 ± 0.6 pg/mL; for AUC were 18.6 ± 0.9 h·µg/dL, 5.35 ± 0.40 h·ng/dL, 11.4 ± 1.1 h·ng/mL, 34.5 ± 3.7 h·pg/mL; and for ΔTH were 1.26 ± 0.05 µg/dL, 0.36 ± 0.03 ng/dL, 1.55 ± 0.16 ng/mL, 4.85 ± 0.52 pg/mL; respectively. The AUC and ΔTH were correlated for TT_4 ($r^2 = 0.92$), FT_4 ($r^2 = 0.96$), TT_3 ($r^2 = 0.80$) and FT_3 ($r^2 = 0.58$) (Figure 4). The magnitude of hormone response, measured as either AUC or ΔTH, was not correlated to baseline measurements ($r^2 < 0.21$) (Figures 5 & 6). The AUC and POB were correlated for TT_3 ($r^2 = 0.82$), FT_3 ($r^2 = 0.51$), TT_4 ($r^2 = 0.31$) and FT_4 ($r^2 = 0.27$) (Figure 7). Peak concentrations were correlated to AUC for TT_4 ($r^2 = 0.59$), FT_4 ($r^2 = 0.82$), and TT_3 ($r^2 = 0.71$), but not FT_3 ($r^2 = 0.17$) (Figure 8). Baseline concentrations were negatively correlated to POB for TT_4 ($r^2 = -0.36$), FT_4 ($r^2 = -0.53$), TT_3 ($r^2 = -0.22$), and FT_3 ($r^2 = -0.23$) (Figure 9).

Discussion

EIA validation

Specificity. Qualitative specificity was established for all thyroid hormones by measuring elevation in hormone concentrations after *in vivo* TRH administration. POB for TT_4 were within and TT_3 were below the 160 – 367 % and 220 – 321 % ranges of POB, respectively, as seen in previous studies (Beech and Garcia, 1985; Chen and Li, 1986; Harris et al., 1992; Lothrop and Nolan, 1986; Sommardahl et al., 2005). Percent recoveries of EIA measurements after dilution of plasma with zero standard were consistently low, indicating a lack of specificity (Reimers et al., 1981) (Table 1).

Accuracy. For all thyroid hormones, measurements by EIA were different than those for RIA (Figure 2). Therefore, when thyroid hormone concentrations are assessed and compared to reference values, the method of analysis should be taken into consideration. EIA measurements were consistently higher, which may result inappropriate placement of values determined by EIA within a normal reference range that has been determined by RIA.

Bland-Altman plots were used to better characterize the differences between RIA and EIA measurements (Bland & Altman, 1999). Bland-Altman plots of the data reveal a positive bias in the EIA measurements because the bias line, the mean difference in measurements (EIA – RIA), is greater than zero (Figure 3). For all thyroid hormones, both boundaries of the 95% limits of agreement, 2 SD above or below the bias line, are positive. This indicates that well over 95% of EIA measurements would be greater than RIA measurements. Data points on the Bland-Altman plots reveal a positive slope to the data resulting from proportional error. There is a greater difference between the two methods at higher concentrations; indicating that accuracy, as determined by RIA values, is lower at higher concentrations.

Differences in measurements between the two methods may be a result of differences in antibody affinities for labeled or endogenous hormones, differences in buffer composition, or differences in the influence of interfering biomolecules. Regardless of the source of inaccuracy, implementation of correction factors by creating calibration curves would make measurements comparable (Solter & Farner, 2000).

When RIA values are plotted against EIA values, the equation of the best fit line may be used for calibration (Figure 10). Replacing "x" with the measured EIA value would give the predicted RIA value and would be able to be compared to other RIA values. However, EIA values do not need to be comparable to RIA values when relative changes or differences from a control group are being measured.

Recovery rates of addition of plasma or standard to plasma samples were 100 ± 20% of expected values (Tables 2 & 3). Therefore, although EIA measurements may be different than RIA values, EIA is still accurate for the measurement of thyroid hormones in equine plasma. This is possible because although RIA may be the "gold standard" for total concentrations of thyroid hormones, its ability to measure the true concentrations is unknown. Enzyme immunoassays measure free thyroid hormone concentrations by an analog method with an enzyme-T_4 conjugate that has no measurable binding to serum proteins, especially thyroid binding globulin and albumin. For free thyroid hormone concentrations, equilibrium dialysis assays are often referred to as the "gold standard" because they minimize disturbances of the bound to unbound equilibrium (Christofides et al., 1999). These disturbances may become most apparent during states of altered serum T_4 binding capacity, such as pregnancy or illness, at which time an assay involving equilibrium dialysis may be a more reliable method. The present study used RIA for comparison, since it is often used to measure equine thyroid hormone concentrations. However comparison with an equilibrium dialysis method may have been a more reliable assessment of true accuracy.

Precision. Inter-assay CV was high for low and medium concentrations of TT_3 (> 20%, Table 4). Slight variations in the measurements of standards may cause differences in standard curves that could contribute to differences between runs. The assay may also be very sensitive to variation brought about by human error, including slight differences in incubation times or pipetting error. While inter-assay CVs were higher for TT_4 and TT_3, intra-assay CVs were higher for FT_4 and FT_3, although not greater than 20%. Free hormone concentrations may have been more influenced by interfering biomolecules or buffer characteristics than total hormone concentrations. Slight changes in these variables may disturb the equilibrium between free and bound hormone fractions, consequently adding to variability of measurements. Care should be taken while

performing the assay to minimize slight variations in incubation times, incubation temperatures, or reagent amounts.

TRH response tests

Basal thyroid hormone concentrations may change depending on a number of environmental and physiological variables, including age, gender, season, time of day, exercise, nutrition, and illness (Greenspan, 2004). For this reason, taking a basal thyroid hormone concentration at a single time period is not a reliable indication of thyroid function. Also, because of this high variability it is difficult to determine a reference range and classify a measurement as "normal".

Thyroid function tests may be less affected by extrathyroidal factors, giving a more accurate representation of thyroid function than basal measurements of thyroid hormones. However, thyroid function may be altered temporarily by factors such as nonthyroidal illness, oral levothyroxine supplementation, or season, resulting in changes in response test results (Panciera, 2001; Perera et al., 1985; Sommardahl et al., 2005)

The results of the 24 h trial TRH response test allowed for the creation of a TRH response test protocol specific to our group of mature Arabian geldings. Peak concentrations fell within the ranges established in earlier studies of 4 to 6 h for T_4 and 2 to 4 h for T_3 (Breuhaus, 2002; Chen & Li, 1986; Harris et al., 1992; Lothrop & Nolan, 1986).

The magnitude of hormone response was approximated by AUC. This measurement was chosen because it best represents the absolute change in circulating thyroid hormone concentrations during the response and recovery periods (Annett et al., 2005). It is affected by both secretion and uptake over a period of time, and measures the total amount of hormone that was available to induce physiological responses. However, constraints of labor and cost allowed only four measurements to be taken for the calculation of AUC. Infrequent sampling may cause unwanted changes in measurements when the shape of the curve changes. Therefore, the assumption that the shape of the curve does not change was made in order to evaluate differences in AUC.

The validity of using four measurements for the calculation of AUC was assessed by comparison of AUC calculated with 4 values (0, 3.0, 5.0, and 24 h) or calculated with

all 14 values of sample measurements taken during the 24 h trial TRH response tests. Mean AUC values did not differ between method of calculation for TT_4 ($P = 0.55$), FT_4 ($P = 0.59$), TT_3 ($P = 0.90$), and FT_3 ($P = 0.78$). These results support the acceptability of calculating AUC with four measurements, as long as the shape of the curves are not different. Mean values for both 4-sample and 14-sample calculations of AUC during the 24 h trial TRH test were overlaid on the regression of AUC and ΔTH (Figure 4) in order to assess the validity of using two horses to determine time periods for peak thyroid hormone concentrations (Figure 11). The inclusion of the 24 h trial measurements within the 95% prediction intervals would indicate similarity in thyroidal responses between the two horses used in the 24 h trial and the horses used for subsequent TRH response tests. Most data points for TT_4, TT_3, and FT_3, but not FT_4, were within the 95% prediction intervals (Figure 11).

By using AUC as the most accurate method of approximating magnitude of hormone response, the accuracy of using other measurements to predict the magnitude of hormone response was evaluated. Linear regression of AUC and ΔTH revealed that calculating ΔTH is an acceptable method of predicting the magnitude of hormone response (Figure 4). In contrast, baseline concentrations are not a good predictor of the magnitude of hormone response. Even when $P < 0.05$, viewing the linear regression graph reveals that there is no correlation to have any predictive value of the baseline measurements because of the wide range of the prediction intervals (Figures 5 & 6). The absence of a correlation between baseline measurements and the magnitude of hormone response indicates that baseline measurements do not accurately assess the capability of the pituitary-thyroid axis to respond to a stimulus. In addition to thyroid gland production and secretion, nonthyroidal factors such as deiodination, serum binding proteins, vascular and cellular transfer, and catabolism regulate circulating thyroid hormone concentrations (Kaptein et al., 1994). Therefore, low circulating concentrations of thyroid hormones do not reflect an inability of the thyroid gland to produce and secrete them. Using a TRH response test for the assessment of thyroid function helps make a distinction between actual dysfunction of the thyroid gland and compensation to a stressor through peripheral regulation of circulating thyroid hormone concentrations.

POB has been used as a measure of thyroid response (Chen & Li, 1986; Harris, 1992). The results of the current study show that POB does not accurately predict the magnitude of hormone response for TT_4 and FT_4, but may be acceptable for TT_3 and FT_3. POB may not predict hormone response as well as ΔTH because it is calculated relative to baseline concentrations. Having baseline concentration in the denominator of the response calculation creates a negative correlation between hormone response, as measured by POB, and baseline concentrations (Figure 9). This implies that a horse with high baseline concentrations would need a greater absolute increase in hormone concentrations to obtain a relative hormone response comparable to a horse with lower baseline concentrations. The differences in predictability of T_4 POB and T_3 POB may be because T_3 response is more independent of baseline concentrations than T_4 response (Figure 5). This is reflected in the lower correlation coefficient of FT_3 and slight negative correlation of TT_3 of AUC with baseline when compared to TT_4 and FT_4 (Figure 5). It is also reflected in the lower correlations of POB with baseline of TT_3 and FT_3 when compared to TT_4 and FT_4 (Figure 9).

Although not as strong as the predictive value of ΔTH, peak concentrations of thyroid hormones are correlated to the magnitude of hormone response (Figure 8). Previous studies have evaluated results of TRH response tests by comparing differences in hormone concentrations at each sampling time period of experimental groups with a control group (Beech & Garcia, 1985; Breuhaus, 2002).

After visual inspection of data points of individual horses, it was observed that for some individuals there were considerable differences in their responses to TRH depending on the hormone measured. Correlations were greater between comparisons of TT_4 AUC and FT_4 AUC ($r = 0.79$, $P < 0.001$) or TT_3 AUC and FT_3 AUC ($r = 0.86$, $P < 0.001$) than they were between comparisons of T_3 AUC and T_4 AUC, either free or total ($r \sim 0.65$, $P < 0.001$). Individual horses may preferentially release either T_4 or T_3 in response to a large stimulus (Oliver & Held, 1985). Therefore, measuring both T_4 and T_3 during a TRH response test will provide a more complete assessment of thyroid function.

Reference ranges were not calculated from the results of this study because our group of mature Arabian geldings is not representative of a general horse population. Usefulness of various measurements in predicting pituitary-thyroid axis responsiveness

has been determined, and their application to a general population may be accomplished when reference ranges are made for populations of horses in various physiological states. Measuring AUC during TRH response tests in hyperthyroid, euthyroid, and hypothyroid horses would allow for the creation of reference ranges and further characterize ranges of error that are acceptable for determining the predictability of ΔTH, POB, peak concentrations, and baseline concentrations.

It is necessary that the use of basal thyroid hormone concentrations for the assessment of thyroid status is replaced by an improved, unified method of assessing thyroid status in order to properly evaluate equine health. The results of the current study have depicted approaches of improving the assessment of thyroid status in horses, including the use of EIA and more critical methods of evaluating the results of TRH response tests.

Implications

Measuring thyroid hormone concentrations in equine plasma using EIA is a viable method; however results cannot be compared to RIA measurements. The use of TRH response tests to assess thyroid function should include the calculation of AUC or ΔTH, which measure absolute changes in hormone concentrations, for a more critical and accurate evaluation than relative changes in hormone concentrations.

Table 1. Recoveries (%) for EIA after dilution of equine plasma with zero standard

	Dilution		
Hormone	3:4	1:2	1:4
TT_4	85.8	77.8	75.8
FT_4	70.3	68.4	88.8
TT_3	82.3	74.9	72.2
FT_3	84.7	84.1	88.4

Table 2. Recoveries (%) for EIA after plasma addition to low concentration plasma

		Concentration of added plasma	
Hormone	Amount added	Medium	High
TT_4	50 µL	104	106
	100 µL	97.8	106
FT_4	50 µL	95.5	93.1
	100 µL	94.9	86.8
TT_3	50 µL	110	105
	100 µL	114	99.5
FT_3	50 µL	93.3	91.9
	100 µL	103	82.2

Table 3. Recoveries (%) for EIA after standard hormone addition to plasma

		Concentration of added standard	
Hormone	Amount added	Medium	High
TT_4	50 µL	115	101
	100 µL	111	100
FT_4	50 µL	91.5	101
	100 µL	96.8	106
TT_3	50 µL	114	115
	100 µL	114	113
FT_3	50 µL	104	110
	100 µL	101	92.4

Table 4. Coefficients of variation (CV, %) for thyroid hormone concentrations measured by EIA

		Concentration		
		Low	Medium	High
TT_4	Intra-assay CV	4.1	9.7	0.77
	Inter-assay CV	20	13	10
FT_4	Intra-assay CV	16	4.1	4.9
	Inter-assay CV	4.6	4.9	3.5
TT_3	Intra-assay CV	8.0	7.4	3.9
	Inter-assay CV	33	29	16
FT_3	Intra-assay CV	17	9.0	9.3
	Inter-assay CV	7.2	8.2	9.5

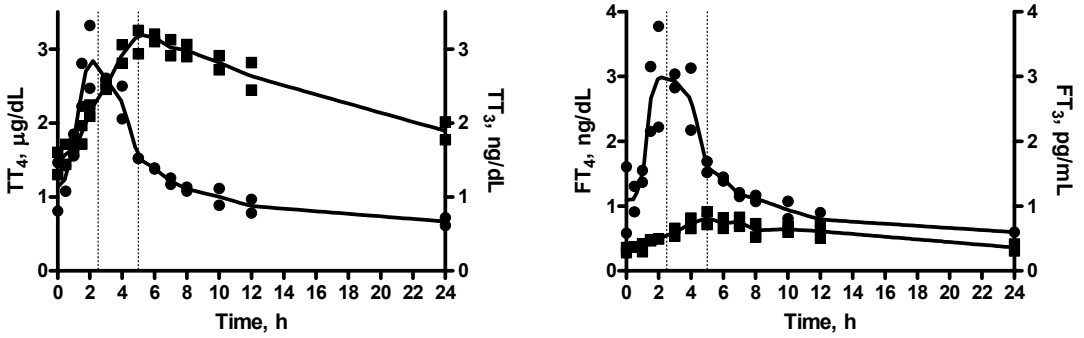

Figure 1. Thyroid hormone concentrations during the 24 h trial TRH response test (n=2). Concentrations were measured using RIA. Solid circles represent individual measurements of TT_3 or FT_3, and solid squares represent individual measurements of TT_4 or FT_4. The solid line represents a spline curve fit of the average values at each time point. The broken lines indicate the time points that were chosen to measure peak concentrations in TT_3 and FT_3 (2.5 h) or TT_4 and FT_4 (5 h) for subsequent TRH response tests.

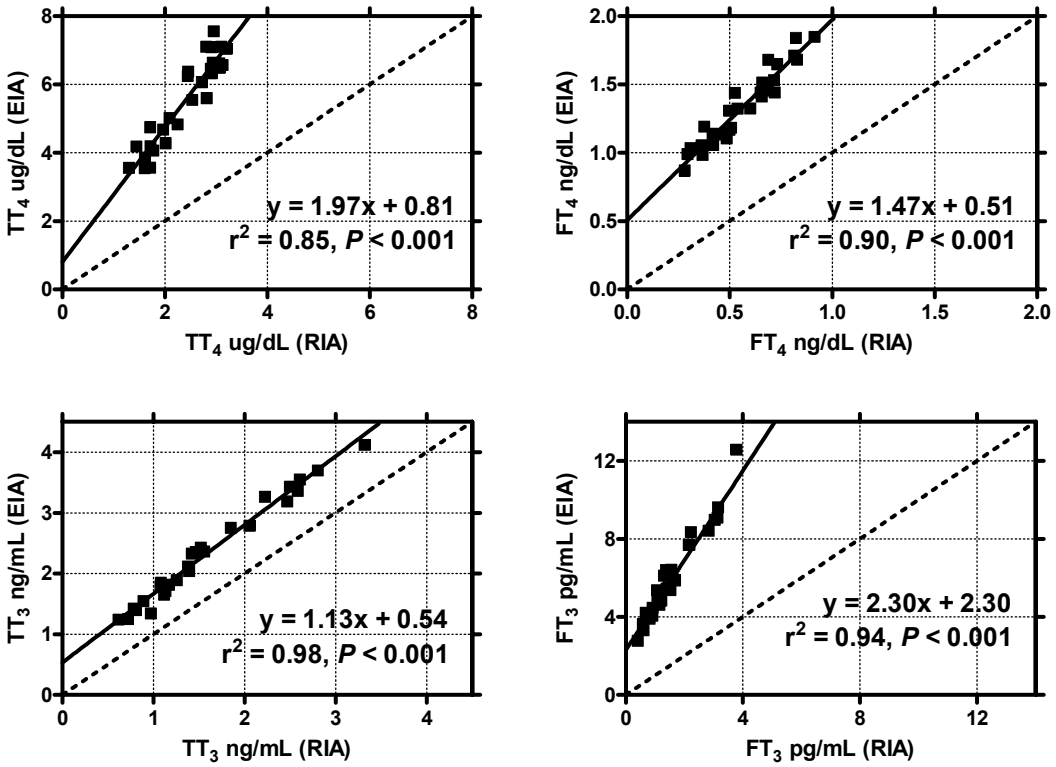

Figure 2. Comparison thyroid hormone concentrations measured by EIA (y axes) and RIA (x axes) using linear regression. The solid line represents the best-fit line and the broken line represents the line of identity (x = y).

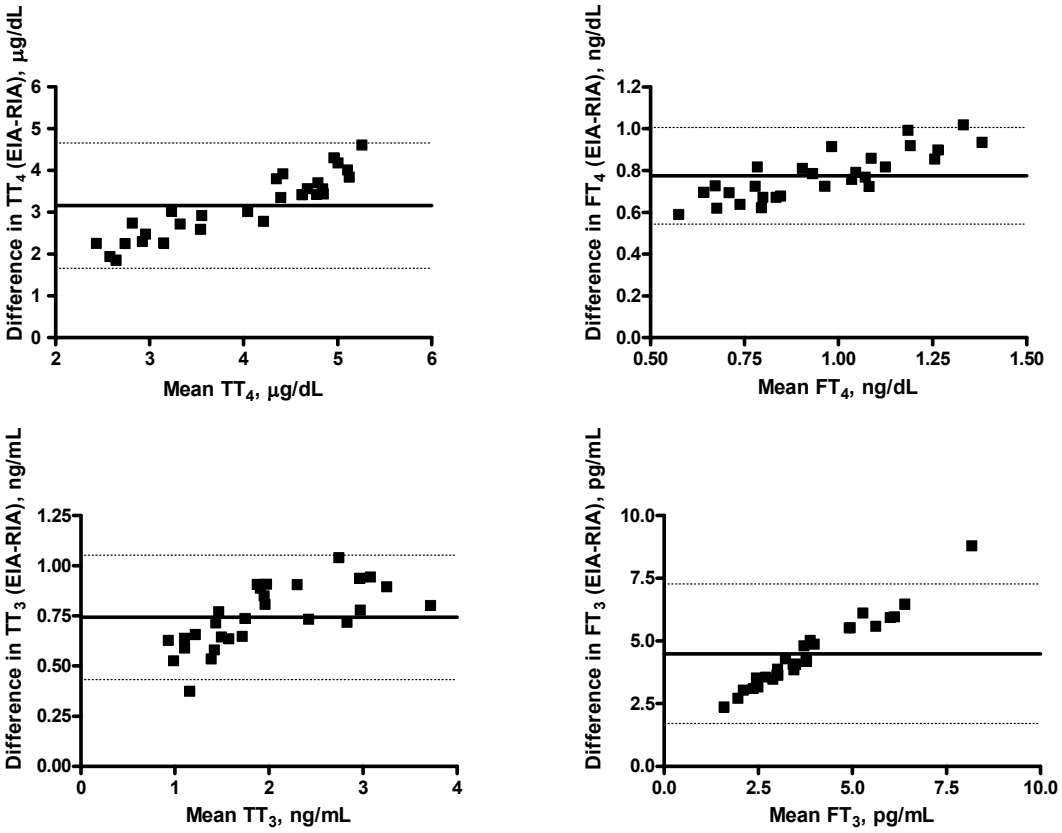

Figure 3. Bland-Altman plots of the difference in EIA and RIA measurements against mean measurement for thyroid hormone data. The solid line represents the mean difference in the two methods (bias line), the broken lines represent two standard deviations above or below the mean difference (95% limits of agreement).

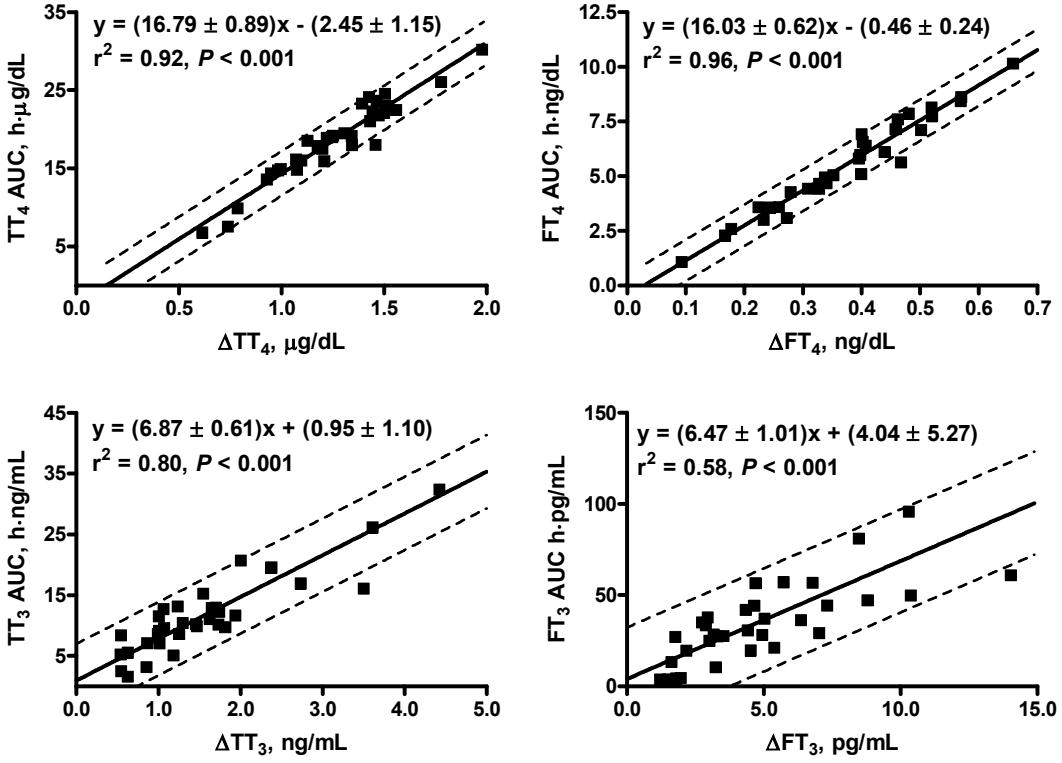

Figure 4. Comparison of the magnitude of hormone response measured as AUC and ΔTH using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

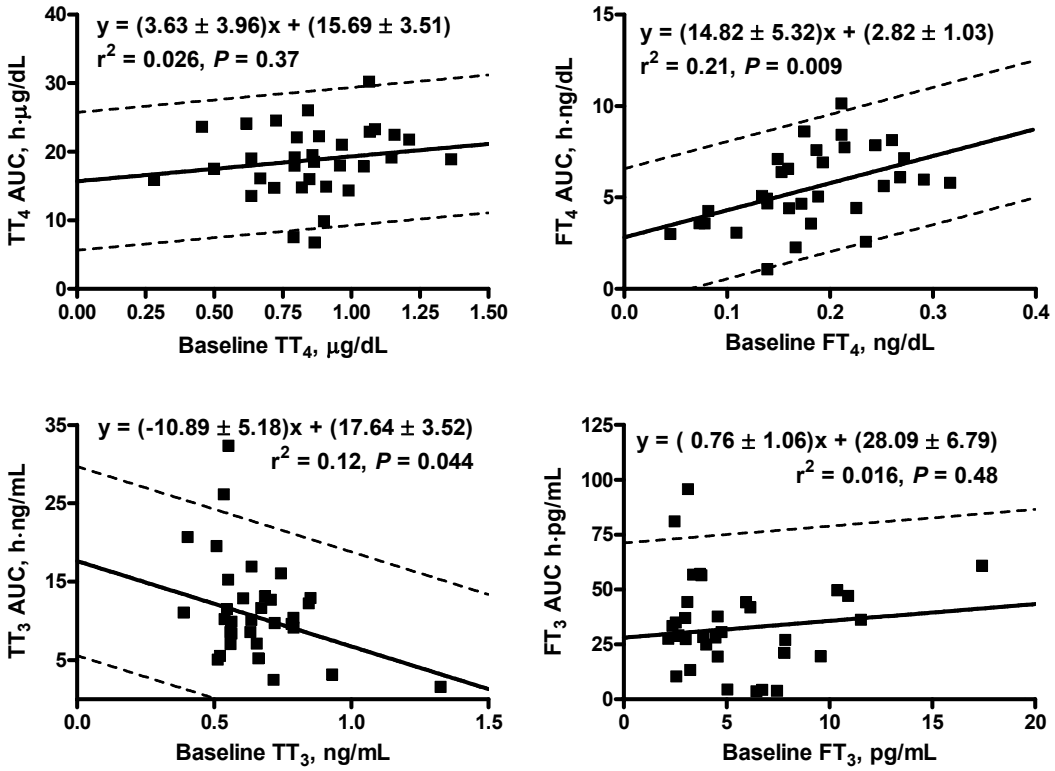

Figure 5. Comparison of the magnitude of hormone response measured by AUC and the baseline concentrations of thyroid hormones using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

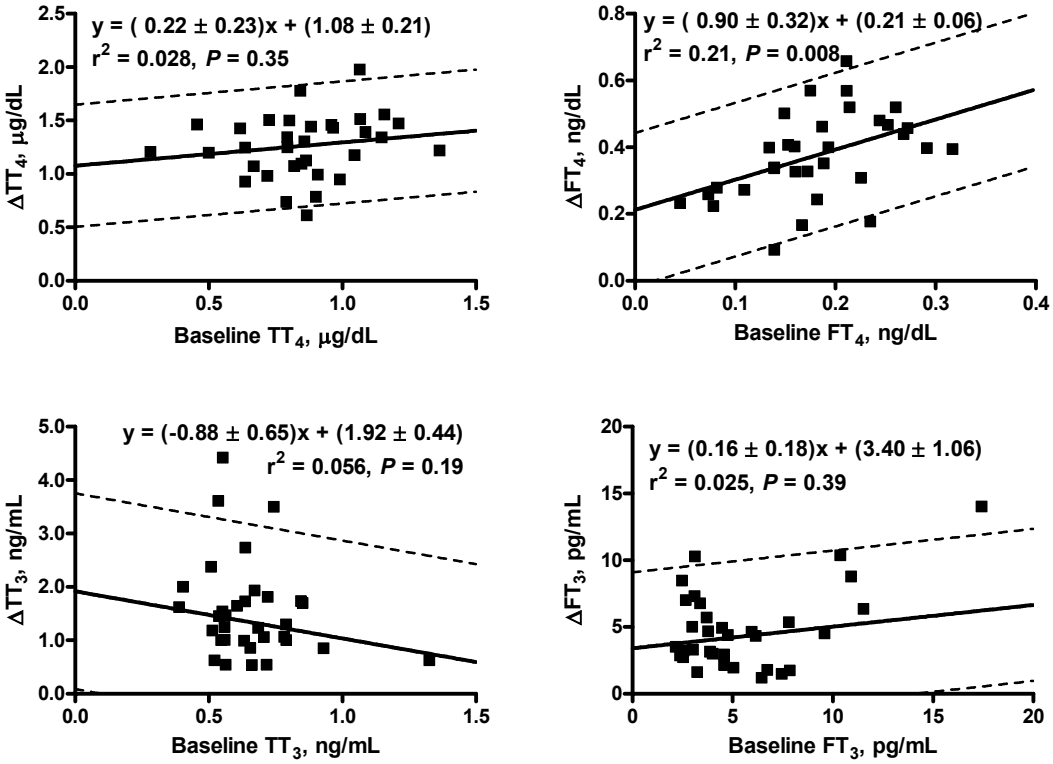

Figure 6. Comparison of the magnitude of hormone response measured by ΔTH and the baseline concentrations of thyroid hormones using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

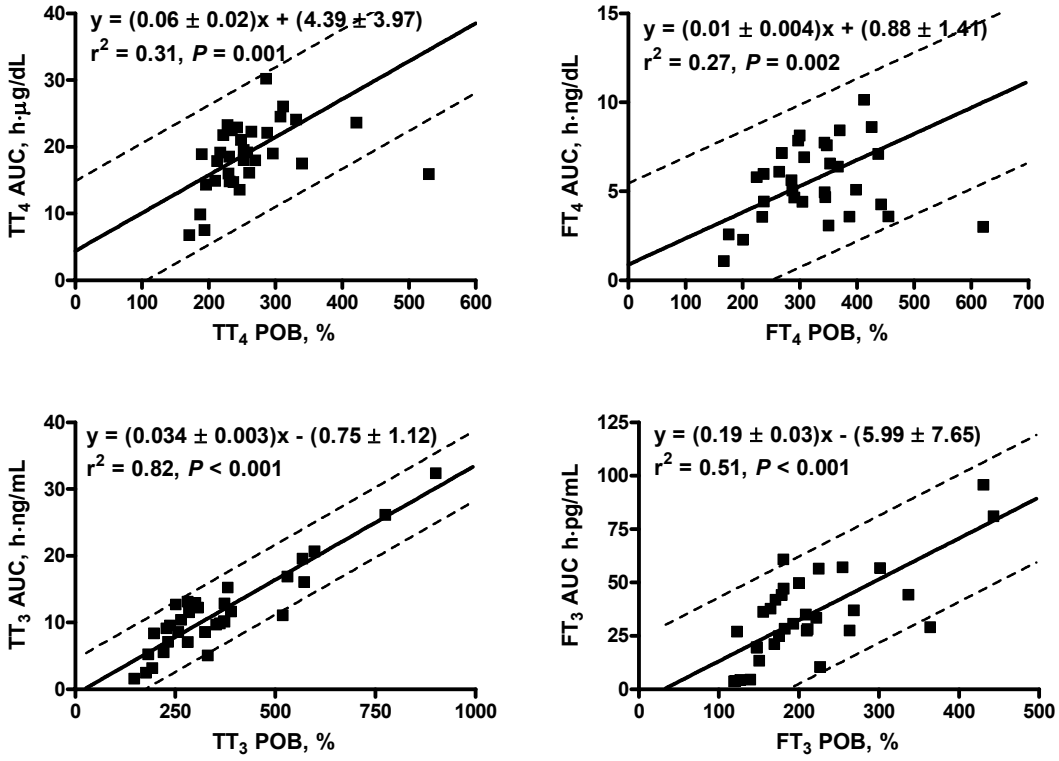

Figure 7. Comparison of the magnitude of hormone response measured by AUC and the percent of baseline (POB) of thyroid hormones using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

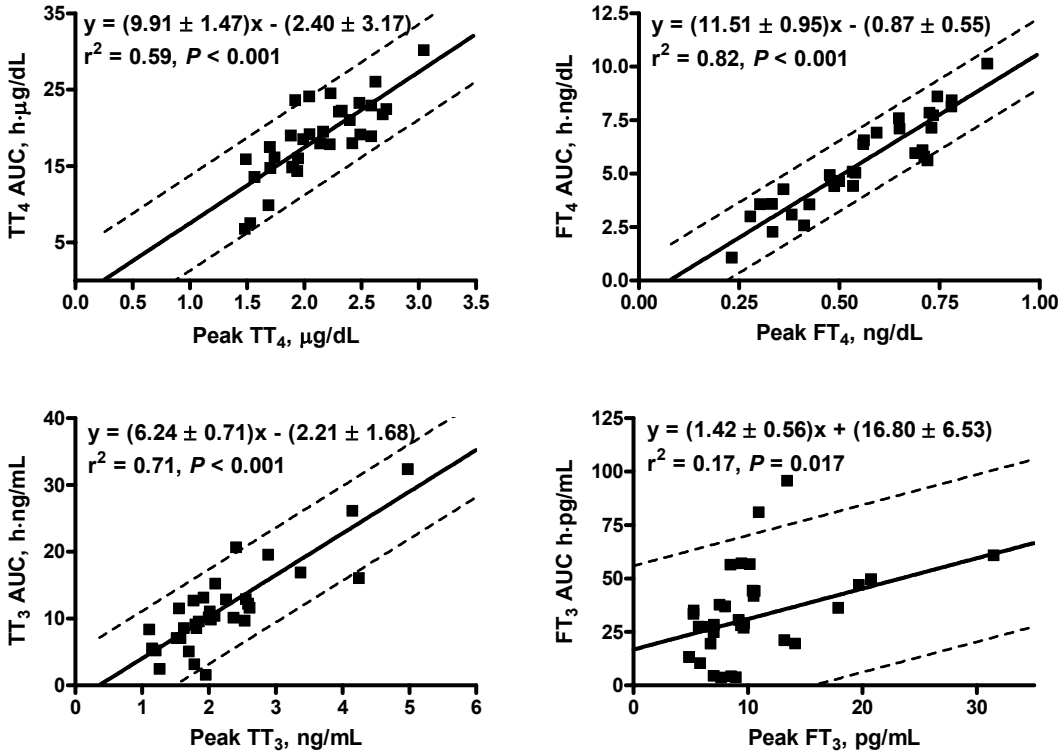

Figure 8. Comparison of the magnitude of hormone response measured by AUC and the peak concentration of thyroid hormones using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

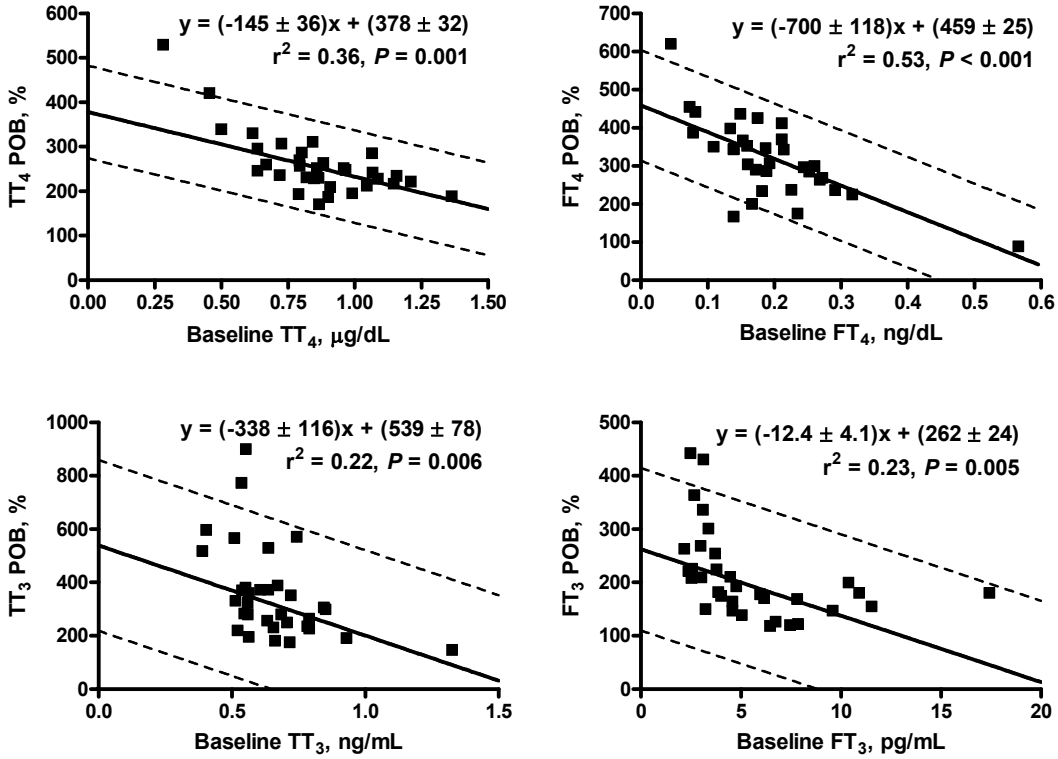

Figure 9. Comparison of percent of baseline (POB, %) and baseline concentrations of thyroid hormones using linear regression. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval.

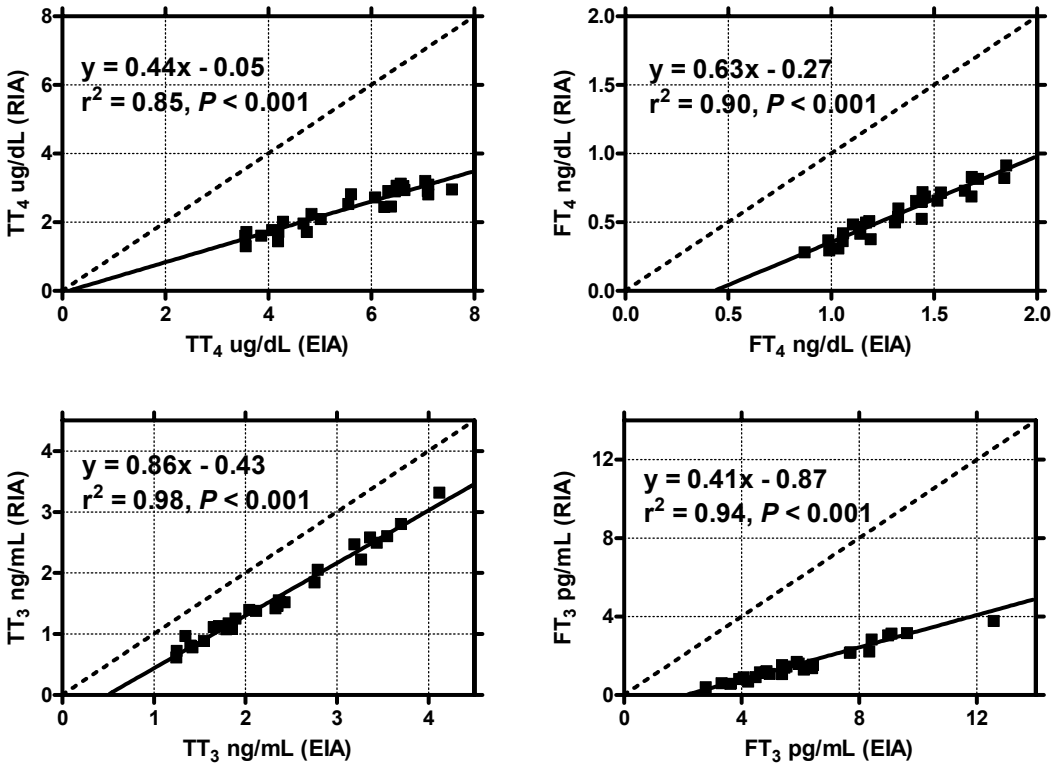

Figure 10. Calibration curves of thyroid hormone concentrations measured by RIA (y axes) and EIA (x axes) using linear regression. The solid line represents the best-fit line and the broken line represents the line of identity (x = y).

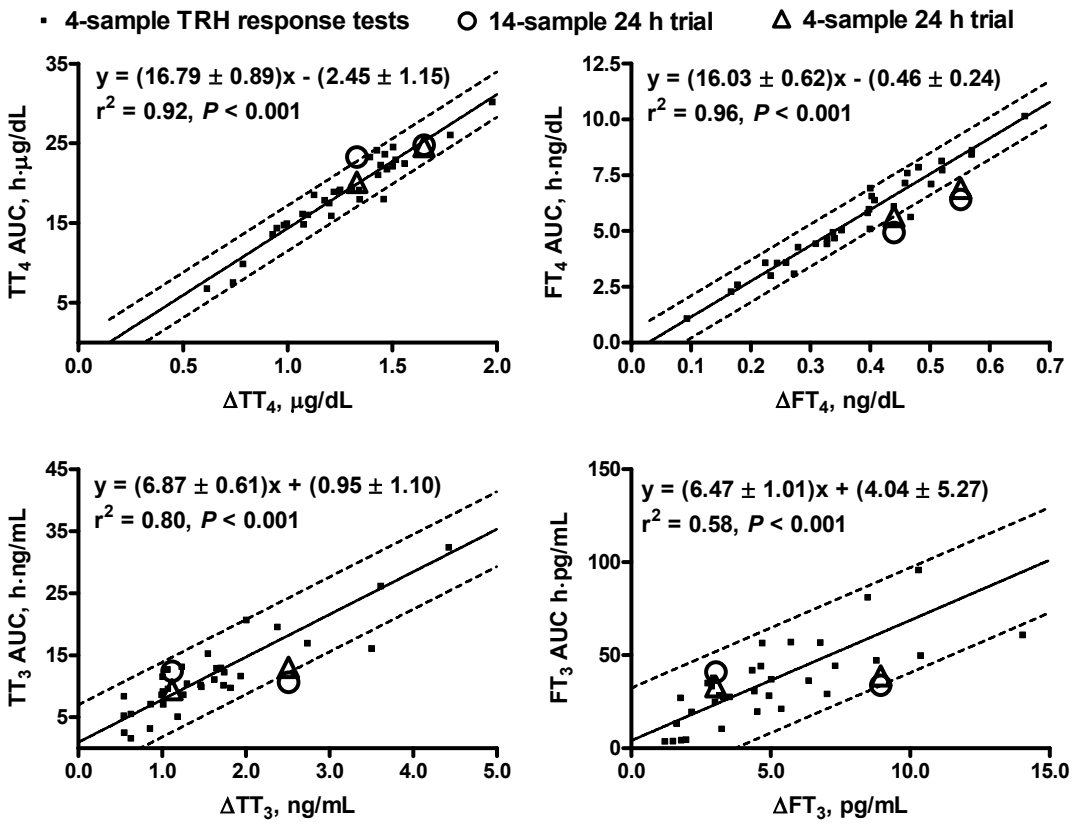

Figure 11. Comparison of the magnitude of hormone response measured as AUC and ΔTH using linear regression overlaid with measurements of the 24 h trial TRH response test. Small squares represent values from the 4-sample TRH response tests, large circles represent values from using all 14 measurements to calculated AUC during the 24 h trial TRH response tests, and large triangles represent values from using only 4 measurements to calculate AUC during the 24 h trial TRH response tests. The solid line represents the best-fit line and the broken lines represent the 95% prediction interval of the data from the 4-sample TRH response tests.

CHAPTER IV

Metabolic changes during athletic conditioning and acute exercise in Arabians fed different sources of dietary energy

ABSTRACT: Thirteen mature Arabian and Arabian cross geldings were kept on mixed grass/clover pasture. They were divided into two groups and offered feeds high in either sugar and starch (SS; n = 6) or fat and fiber (FF; n = 7). The feeds were similar in digestible energy and protein content, but differed in nonstructural carbohydrate, acid and neutral detergent fiber, and fat content. Horses began a 15 wk training period after approximately 5 mo of pasture rest. Athletic conditioning consisted of two weekly sessions of walking and trotting on an automatic walker for 1 h and one weekly session on a high speed treadmill, which progressively increased in level of difficulty as training advanced. At the end of the training period, horses performed an exercise test on the treadmill consisting of three galloping bouts 25, 20, and 15 min in duration at 6, 7, and 8 m/s, respectively. Blood samples were collected the first day of each week between 0700 and 0900 during the training period and before, during, and after the exercise test. Thyrotropin releasing hormone (TRH) response tests were performed before and after the training period and the morning after each horse completed the exercise test. During the training period, increases in total and free thyroxine (TT_4 and FT_4; respectively) occurred until week 4 of training, then continually decreased until returning to pre-training concentrations ($P < 0.001$). During weeks 3 and 4 of training, a rise in nonesterified fatty acid concentration occurred simultaneously with a decrease in triglyceride concentration ($P < 0.001$), indicating an increase in lipolysis. Concurrently, there was an increase in insulin sensitivity and decrease in insulin secretory response ($P < 0.001$). During the exercise test, TT_4, FT_4 and total triiodothyronine (TT_3) progressively increased above pre-exercise levels (0.94 ± 0.10 µg/dL; 0.19 ± 0.02 ng/dL; 0.76 ± 0.05 ng/mL; respectively), peaked after the 8 m/s gallop (1.46 ± 0.10 µg/dL; 0.30 ± 0.02 ng/dL; 1.08 ± 0.05 ng/mL; respectively), and decreased below pre-exercise levels by 24 h (0.85 ±0.10 µg/dL; 0.13 ± 0.02 ng/dL; 0.63 ± 0.05 ng/mL; respectively) ($P < 0.05$). Total T_3 and free triiodothyronine (FT_3) measurements during exercise were higher for SS fed horses than FF fed horses ($P < 0.05$). Responses of TT_4 and FT_4 to TRH stimulation decreased after

exercise ($P < 0.05$), whereas responses of FT_4 and TT_3 were higher in SS fed horses during a TRH response test after training ($P < 0.05$). Analysis of thyroid function was therefore dependent on the time that measurements were taken in relation to completion of exercise or level of training. Analysis varied depending on which thyroid hormones were measured and whether they were measured with respect to a stimulus. TT_4 and FT_4 were more affected by training and exercise than by dietary composition, whereas TT_3 and FT_3 were influenced by the level of carbohydrates in the diet. Changes in thyroid status may have facilitated improved insulin sensitivity or enhanced lipid metabolism which would spare glucose utilization.

Key Words: Thyroid, Horse, Exercise, Athletic conditioning, Thyrotropin releasing hormone

Introduction

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), play an important role in carbohydrate and lipid metabolism, protein synthesis, and energy production; and therefore are a crucial link between nutrition and metabolism. Exercise exerts additional stressors on the body over daily maintenance; therefore proper thyroid hormone action is especially critical during these times of increased metabolic rate.

Previous studies evaluating the effects athletic conditioning on thyroid status in horses have produced conflicting results. Results of increases, decreases, or no changes in thyroid hormone concentrations in response to training have been reported (Irvine, 1967; Suwannachot et al., 2000; Takagi et al., 1974). Similarly, there are no conclusive studies describing changes in thyroid hormones during exercise in horses, although increases in T_4 and T_3 have been observed after exercise (Garcia et al., 1986). Changes in thyroid hormone concentrations could be a result of changes in their production and/or metabolism during training and exercise (Harris et al., 1992; Irvine, 1967).

The importance of thyroid hormone action during exercise is made apparent by considering the physiological effects of hypothyroidism. Thyroidectomy in horses lowers resting heart rates, respiratory rates, and cardiac output (Frank et al., 2002). Exercise intolerance has been reported in thyroidectomized horses, along with reduced distances to fatigue and lower maximal oxygen consumption, maximal heart rate, and maximal velocity during a treadmill exercise test. The cardiovascular system of the thyroidectomized horse is less affected by β-adrenergic stimulation, indicating a decrease in number or function of their receptors.

Thyroid hormones are involved in basically all aspects of energy metabolism by inducing the transcription of genes of enzymes necessary for protein, carbohydrate, and lipid metabolism (Clement et al., 2002). For this reason, dietary nutrient composition may affect thyroid hormone status. Insulin and glucose stimulate T_4 deiodination to T_3, producing effects when the carbohydrate portion of the diet is altered (Gavin et al., 1987 & 1988). An influence of dietary composition on thyroid status may affect a horse's ability to make metabolic changes necessary during exercise or athletic conditioning.

The objective of this study was to determine the effects of athletic conditioning and a single exercise session on thyroid function in mature Arabian geldings and

determine if dietary energy source influences thyroid responses. It was expected that thyroid function would increase in response to both chronic and acute exercise, and that horses fed higher levels of nonstructural carbohydrates would have higher baseline concentrations of T_3.

Materials and Methods

Horses and diets

Thirteen mature Arabian and Arabian cross geldings (1024.6 ± 25.3 kg BW, 12.3 ± 0.9 y) from the Virginia Tech Middleburg Agricultural Research and Extension Center were used in this study, occurring between May and September 2004. Horses were kept on mixed grass/clover pasture. They were divided into two groups and offered feeds high in either sugar and starch (SS; n = 6) or fat and fiber (FF; n = 7) (Table 1). Horses were group fed twice daily to meet approximately one-third of their DE requirements with either the SS or FF pasture supplement (NRC, 1989). The feeds were similar in digestible energy and protein content, but differed in nonstructural carbohydrate, acid and neutral detergent fiber and fat content.

Training

Horses began a 15 wk training period after approximately five mo of pasture rest. Athletic conditioning consisted of two weekly sessions of walking and trotting on an automatic horse exerciser and one weekly session on a high speed treadmill. Training on the automatic horse exerciser progressed from 30 min walking and 10 min trotting during wk 1, to 20 min walking and 40 min trotting during wk 15. Training on the treadmill progressed from 20 min walking and 25 min trotting at 3.5 to 4.0 m/s during wk 1 to 10 min walking, 15 min trotting at 4.0 m/s, and 10 min cantering at 8.0 m/s during wk 15. Basal venous blood samples were collected in sodium heparin tubes the first day of each week of training between 0700 and 0900, after approximately 16 h on pasture without access to feed concentrates.

Exercise test

At the end of the training period, horses performed an exercise test on the treadmill consisting of three galloping bouts of 25, 20, and 15 min in duration at 6, 7, and 8 m/s, respectively (Figure 1). Catheters were placed in the jugular vein between 0700 and 0800, approximately 60 min before the start of exercise. Venous blood samples were collected at -60, 0, 10, 35, 65, 75, 95, 129, 140, 155, 185, 250, 310, and 1440 min with respect to the start of exercise.

TRH response tests

Thyrotropin releasing hormone (TRH) response tests were performed on untrained horses before athletic conditioning (TRH-U), on trained horses after the 15 week training period (TRH-T), and on exercised horses the morning after the treadmill exercise test (TRH-E). A stock solution of TRH (Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 1.0 mg TRH/mL and stored at -20°C until the day of use. Horses were housed in stalls and offered water and timothy/alfalfa hay, but feed concentrates were withheld during the day of TRH response tests. One mg TRH was injected intravenously between 0700 and 0900. Eleven horses received the TRH dose and two control horses received 1 mL saline solution. Blood samples were collected in sodium heparin tubes immediately before and 2.5, 5.0, and 24 h after TRH injection.

Blood analysis

Blood samples were centrifuged at 3000 g for 10 min at 4°C and plasma was transferred to 2 mL polypropylene vials (Sarstedt, Newton, NC) and stored at -20°C until analysis. All plasma samples were analyzed for total T_4 (TT_4), free T_4 (FT_4), and total T_3 (TT_3) using radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) and for plasma free T_3 (FT_3) using enzyme immunoassay (Active®, Diagnostic Systems Laboratories, Inc., Webster, TX). Cortisol was measured in weekly and TRH response test samples via radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA). Insulin was measured in the exercise test and weekly samples via radioimmunoassay (Coat-A-Count Insulin, Diagnostic Products Corporation, Los Angeles, CA). Cholesterol (CHOL), glucose,

nonesterified fatty acids (NEFA), and triglycerides (TG) were measured in weekly samples enzymatically using commercial kits (Beckman Instruments, CX5 Chemistry Analyzer Cholesterol 467825, Glucose 442640, Triglyceride 445850).

Statistical analysis

Data are summarized as least square means ± SEM. Main effects were evaluated using a mixed model ANOVA with repeated measures. Differences of hormone and metabolite concentrations between SS and FF groups were compared as simple effects by slicing significant interactions (SAS Version 9.1; SAS Institute Inc., Cary, NC). The magnitude of the hormone response was represented by the area under the curve (AUC) of the increments above baseline concentrations plotted against time and calculated by trapezoidal approximation. Differences in AUC between TRH tests and feeds were compared as simple effects by slicing significant interactions. Data was graphed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

During training, minimal model parameters of insulin sensitivity (SI) and acute insulin response to glucose (AIRg) were predicted by the use of proxies developed by equivalence testing with the results of minimal models performed on 46 horses (Treiber et al., in press). Insulin sensitivity and AIRg, or beta cell responsiveness, were estimated by the reciprocal inverse square of basal insulin (RISQI) and the modified insulin-to-glucose ratio (MIRG), respectively. Weekly basal insulin concentrations were used to calculate RISQI (insulin concentration$^{-0.5}$) and basal glucose and insulin were used to calculate MIRG ([800 – 0.30 (insulin concentration – 50)^2]/[glucose concentration – 30]).

Ratios of thyroid hormone concentrations were used for inferences about peripheral thyroid hormone metabolism. Deiodinase activity was estimated by FT_3:FT_4 and TT_3:TT_4 ratios. The relative affinity of thyroid hormone binding to serum carrier proteins was estimated by the free hormone fractions, or FT_3:TT_3 and FT_4:TT_4 ratios.

Relationships between parameters were assessed from Spearman's Correlation Coefficients (r) and P-values determined from data of the weekly training samples.

Results

Training

Body weight did not differ between wk 0 (1025 ± 25 kg) and wk 15 (1044 ± 28 kg) ($P = 0.61$). Weekly measurements of thyroid hormones during the training period were similar between SS and FF groups for TT_4 (1.29 ± 0.03 and 1.26 ± 0.03, respectively; $P = 0.46$), FT_4 (0.19 ± 0.01 and 0.17 ± 0.01, respectively; $P = 0.052$), and TT_3 (0.66 ± 0.02, $P = 0.89$), and a difference between SS (5.17 ± 0.45) and FF (3.69 ± 0.41) fed horses for FT_3 ($P = 0.016$). TT_4 progressively increased above pre-training levels (0.89 ± 0.08 µg/dL) until wk 4 of training (1.77 ± 0.08 µg/dL) ($P < 0.001$), then continually decreased until returning to pre-training levels by wk 12 (Figure 2). FT_4 also increased above pre-training levels (0.16 ± 0.01 ng/dL) until wk 4 (0.21 ± 0.01 ng/dL) ($P < 0.001$), then returned to pre-training levels by wk 5. TT_3 and FT_3 did not show this systematic variation from pre-training concentrations (0.64 ± 0.04 ng/mL; 4.78 ± 1.04 pg/mL; respectively) (Figure 2).

Results for the thyroid hormone ratios, FT_4:TT_4, FT_3:TT_3, FT_3:FT_4, and TT_3:TT_4, did not differ between SS and FF fed horses ($P > 0.20$). There was an effect of week of training on all thyroid hormone ratios ($P < 0.001$) (Figure 3).

There was an effect of week of training on the nonthyroidal blood parameters NEFA ($P < 0.001$), TG ($P < 0.001$), glucose ($P = 0.007$), and cortisol ($P = 0.002$) (Figure 4). There was an effect of feed on TG ($P < 0.001$), glucose ($P = 0.002$), cortisol ($P = 0.001$), and insulin ($P < 0.001$) (Figure 5).

There was an effect of week on both RISQI and MIRG ($P < 0.001$, $P = 0.003$; respectively) (Figure 6). RISQI increased above untrained values during wk 1, 3, and 4 ($P < 0.05$). There was an effect of feed on RISQI ($P < 0.001$), with FF fed horses having higher values than SS fed horses. MIRG decreased below untrained values during wk 3 ($P < 0.05$). There was an effect of feed on MIRG ($P < 0.001$), with SS fed horses having higher values than FF fed horses.

Correlations of nonthyroidal blood parameters with thyroid hormone parameters are summarized in Table 2.

Exercise test

During the exercise test TT_4, FT_4, and TT_3 progressively increased above pre-exercise levels (0.94 ± 0.10 µg/dL; 0.19 ± 0.02 ng/dL; 0.76 ± 0.05 ng/mL; respectively), peaking after the 8 m/s gallop (1.46 ± 0.10 µg/dL; 0.30 ± 0.02 ng/dL; 1.08 ± 0.05 ng/mL; respectively), and decreased to or below pre-exercise levels by 24 h (0.85 ±0.10 µg/dL; 0.13 ± 0.02 ng/dL; 0.63 ± 0.05 ng/mL; respectively) (Figure 7). FT_3 had a large inter-horse variability and did not increase above the pre-exercise concentration (5.42 ± 1.05 pg/mL) ($P > 0.05$) (Figure 7). Measurements during exercise were similar between SS and FF groups for TT_4 (1.18 ± 0.04 and 1.25 ± 0.04, respectively; $P = 0.23$) and FT_4 (0.25 ± 0.01, $P = 0.72$). There was an effect of feed on TT_3 and FT_3 measurements during exercise, with SS fed horses having higher values than FF fed horses for both TT_3 (0.92 ± 0.02 and 0.78 ± 0.02, respectively; $P < 0.001$) and FT_3 (6.52 ± 0.42 and 4.33 ± 0.39, respectively; $P = 0.001$) (Figure 8). Insulin concentrations were higher in SS fed horses than FF fed horses during exercise ($P < 0.001$) (Figure 8).

There was an effect of time on FT_3:FT_4 ($P = 0.040$) and FT_4:TT_4 ($P = 0.024$) ratios, but not TT_3:TT_4 ($P = 0.38$) and FT_3:TT_3 ($P = 0.93$) ratios (Figure 9). There was an effect of feed on TT_3:TT_4 ($P < 0.001$), FT_3:FT_4 ($P = 0.012$), and FT_3:TT_3 ($P = 0.048$) ratios, but not FT_4:TT_4 ($P = 0.12$) ratio.

TRH response tests

Thyroid hormone concentrations increased above baseline concentrations during all TRH response tests (Figure 10). The changes in TT_4 and FT_4 concentrations during a TRH response test, as represented by AUC, were greater after training (21.6 ± 1.5 h·µg/dL; 6.87 ± 0.58 h·ng/dL; respectively) than after exercise (15.8 ± 1.5 h·µg/dL; 3.92 ± 0.58 h·ng/dL; respectively) ($P < 0.010$), but neither responses were different than pre-training responses (18.5 ± 1.5 h·µg/dL; 5.46 ± 0.58 h·ng/dL; respectively) ($P > 0.05$) (Figure 11). The changes in TT_3 and FT_3 concentrations during a TRH response test did not change from untrained levels (13.1 ± 1.9 h·ng/mL; 41.1 ± 6.3 h·pg/mL; respectively) in response to training (11.2 ± 1.9 h·ng/mL; 36.9 ± 6.3 h·pg/mL; respectively) ($P > 0.20$) or exercise (10.4 ± 1.9 h·ng/mL; 27.2 ± 6.3 h·pg/mL; respectively) ($P > 0.10$). When

compared to the FF group, horses fed SS had greater AUC for FT_4 ($P = 0.047$) and TT_3 ($P = 0.046$) during TRH-T and a trend for FT_3 during TRH-T ($P = 0.068$) (Figure 11).

There was no effect of feed on cortisol concentrations during TRH response tests ($P = 0.39$). Cortisol concentrations 5 h after TRH administration were below baseline concentrations during TRH-U ($P < 0.001$), TRH-T ($P = 0.045$), and TRH-E ($P = 0.005$) (Figure 12). The only difference between tests within a single time period was between the 0 h sample during TRH-U and all other measurements ($P < 0.001$).

Discussion

Training

Pre-training measurements of TT_4 and TT_3 were similar to, FT_4 lower than, and FT_3 greater than previously measured concentrations for normal adult horses (Anderson et al., 1988; Bayly et al., 1996; Breuhaus, 2002; Messer et al., 1995b).

The results of the present study indicate that thyroid status, as measured by basal thyroid hormone concentrations, is dependent on the time since initiation of training and on which thyroid hormone was measured. The only responses of thyroid hormones to training were increases in TT_4 and FT_4 during the early stages of training. It has been suggested that T_4 could be used to monitor training reserves in racehorses, after finding that T_4 concentrations increased during the latter stages of training (Takagi et al., 1974). Conversely, decreases in protein-bound iodine (representative of thyroid hormones) were measured in horses during training, and no changes in thyroid hormones were measured in ponies during mild exercise training (Irvine, 1967; Suwannachot et al., 2000).

Although TT_4 and FT_4 concentrations increased until wk 4 of training, they then decreased until reaching concentrations not different from baseline by wk 12 and wk 5, respectively. Physiological explanations may be that thyroid hormone action only facilitates initial changes necessary during the early stages of training; alternatively a new equilibrium may have formed among the different body compartments, allowing peripheral regulatory mechanisms to return circulating thyroid hormone concentrations to basal levels. Environmental explanations for the changes in TT_4 and FT_4 include the possibility of seasonal influences. Seasonal increases in thyroid hormone concentrations have been observed in horses being acclimated to colder temperatures (McBride et al.,

1985). However, the duration of our study was from May to September, before colder temperatures were experienced, and there was no effect of temperature on thyroid hormone concentrations ($P > 0.05$). Conversely, another study found peaks in T_4 concentration during July and February in non-pregnant mares (Flisinska-Bojanowska et al., 1991). The peak in T_4 during wk 2 to 4 of training corresponds to the first half of June, approximately one month prior to the peak observed by Flisinska-Bojanowska et al. (1991).

The decreases in free hormone ratios and T_3 to T_4 ratios after the initiation of training indicate increased production, degradation, and serum carrier protein concentration or affinity, along with decreased relative deiodinase activity and cellular uptake (Figure 3). These results are in agreement with previous findings that athletic conditioning in horses increases T_4 secretion rate and metabolism and excretion of thyroid hormones with training (Irvine, 1967).

Ratios of thyroid hormone concentrations were used for inferences about peripheral thyroid hormone metabolism. Circulating thyroid hormone concentrations are influenced by both production and secretion from the thyroid gland and by peripheral regulatory mechanisms, including transfer between circulation and tissue sites, production of T_3 from T_4 deiodination, hormone binding to serum carrier proteins, and degradation rates. Activities of these peripheral regulatory mechanisms affect thyroid hormone ratios, including free hormone fractions and T_3 to T_4 ratios. The complexity of their interactions do not allow for their direct analysis through the use of thyroid hormone ratios, although inferences may be made to form a better understanding of thyroid status. Deiodinase activity and cellular uptake are expected to positively influence, and degradation rates negatively influence FT_3:FT_4 and TT_3:TT_4 ratios (Bianco et al., 2002; Peeters et al., 2005; Reyns et al., 2002). Degradation rates, the relative affinity of thyroid hormone binding to serum carrier proteins, and the concentration of serum carrier proteins is expected to vary inversely with the free hormone fractions, or FT_3:TT_3 and FT_4:TT_4 ratios (Irvine & Evans, 1975). The influence of negative feedback to the hypothalamus and pituitary would regulate production and secretion from the thyroid gland to be negatively associated with free hormone fraction and T_3 to T_4 ratios (Kaptein et al., 1994; Larsen et al., 1979).

The changes in thyroid hormone parameters during the early weeks of training may facilitate metabolic changes during the initiation of training. These metabolic changes are observed as changes in other physiological parameters, including TG, NEFA, RISQI, and MIRG. Correlations were made between nonthyroidal blood parameters and thyroid hormone parameters to better characterize their relationships in order to help explain mechanisms of causation for the changes seen during the early weeks of training.

During wk 3 and 4, a rise in NEFA concentrations occurs simultaneously with a decrease in TG concentrations (Figure 3), indicating an increase in lipolysis. NEFA was positively correlated to FT_4 ($r = 0.20$) and TT_3 ($r = 0.30$), and TG was negatively correlated to TT_4 ($r = -0.43$) and FT_4 ($r = 0.27$), supporting a role of thyroid hormones in stimulating lipolysis, perhaps through the induction of lipase activity. Decreases in hepatic lipase activity and NEFA concentrations and increases in TG concentrations have been found in thyroidectomized horses (Frank et al., 1999; Frank et al., 2004).

During wk 3, there is an increase in RISQI and decrease in MIRG (Figure 5), indicating increased insulin sensitivity and decreased pancreatic beta cell response, respectively. Correlations of thyroid hormone parameters with insulin, RISQI, and MIRG (Table 2) support mechanisms of a T_4-induced increase in insulin sensitivity and an insulin-stimulated increase in T_4 deiodination (Frank et al., 2005; Gavin et al., 1987).

An increase in metabolic rate by increased lipolysis and insulin sensitivity facilitate the lipid and carbohydrate metabolism needed to fulfill the additional energy requirements of regular exercise. The concurrent increase in TT_4 and FT_4, through intracellular conversion to T_3, may have provided the cellular signaling necessary for these metabolic changes to occur.

Exercise test

Increases in TT_4, FT_4, and TT_3 were seen as exercise progressed and into the recovery period (Figure 7). Among their many roles during exercise, thyroid hormones initiate the transcription of genes necessary for cardiac function, glucose transport, neuromuscular excitability, and nutrient metabolism. Thyroid hormones have a pronounced effect on the cardiovascular system, including increasing vasodilation, cardiac contractility, and cardiac output (Danzi & Klein, 2004). Thyroid hormone

actions may contribute to the mechanism by which insulin sensitivity increases in exercising horses. Insulin sensitivity is elevated by an increase in glucose transporter abundance in rats supplemented with thyroid hormone or exercised (Ploug et al., 1990; Romero et al., 2000; Weinstein et al., 1994). Insulin sensitivity is also elevated in thyroid hormone supplemented and exercised horses, although the mechanism of this change is unknown (Frank et al., 2005; Treiber et al., 2005). Thyroid hormones are also important in maintaining neuromuscular excitability through transcription of ion transporter genes. They induce transcription of ion transporters that tightly regulated calcium, sodium, and potassium concentration gradients in order to facilitate muscle contraction (Clausen, 1998; Hudecova et al., 2004).

Thyroid hormones increase resting metabolic rate and mechanical energy efficiency by directly activating uncoupling protein (UCP) genes through interaction of thyroid receptors with the proximal promoter regions (Solanes et al., 2005). UCP increases energy metabolism by uncoupling respiration from ATP production by diminishing the proton gradient across the inner mitochondrial membrane. Acute exercise up regulates UCP, whereas endurance training down regulates UCP (Schrauwen et al., 2003). This is in agreement with exercise-induced increases in metabolic rate and training-induced increases in mechanical energy efficiency. Concentrations of TT_4, FT_4, and TT_3 during the exercise test correlate well with expected expression of UCP, increasing during and up to a few hours after exercise and decreasing by 24 h after exercise, providing a possible functional role of the changes in thyroid hormone concentrations. During exercise the increase in thyroid hormone concentrations may induce changes in metabolic rate by uncoupling respiration, in addition to increasing lipid and carbohydrate catabolism.

Conversely, training-induced increases in TT_4 and FT_4 in this study are not in agreement with expected increases in mechanical energy efficiency. Therefore, either the circulating concentration of thyroid hormones was not representative of their bioactivity, or changes in UCP expression did not produce changes in mechanical energy efficiency.

TT_3 and FT_3 were greater in SS fed horses than FF fed horses during exercise, perhaps as a result of increased insulin concentrations in the SS fed horses (Figure 8). Incubation of cultured rat hepatocytes with insulin or glucose increased T_3 neogenesis,

indicating a stimulatory effect on hepatic T_4 5'-deiodinase activity (Gavin et al., 1987). The influence of insulin on deiodinase activity may be the reason why T_3 neogenesis is increased by the carbohydrate rather than the protein portion of the diet (Gavin et al., 1988). Insulin stimulated T_3 neogenesis may be a physiological mechanism to increase insulin sensitivity. T_3 is the more physiologically active form and increases insulin sensitivity by increasing glucose transporter abundance (Romero et al., 2000; Weinstein et al., 1994). However, if there was an increase in deiodinase activity with SS feeding it was not reflected in the T_3 to T_4 ratios, since there was no effect of feed on these parameters.

There was an effect of exercise on decreasing thyroid hormone ratios (Figure 9). Interpretation of these ratios would suggest increased production, degradation, serum carrier protein concentration or affinity and decreased deiodinase activity during exercise.

TRH response tests

During the TRH response tests, TT_4 AUC and FT_4 AUC were lower during TRH-E when compared to TRH-T, indicating a decreased ability of the pituitary-thyroid axis to respond the morning after an exhaustive exercise session. This may be caused by decreased sensitivity of the pituitary from overstimulation by thyroid hormone negative feedback during exercise, when thyroid hormone concentrations are increased. Alternatively, the thyroid gland may be depleted and have less hormone to secrete in response to stimulation.

Unlike TT_4 and FT_4, alterations in TT_3 and FT_3 concentrations during the TRH response tests were not changed after the exercise test. The peak increase in T_3 occurred before the peak increase in T_4, perhaps because of preferential release of T_3 after a large stimulation of the thyroid gland by TSH (Oliver & Held, 1985). This preferential release may not be affected by physiological changes during exercise.

In a previous study, horses in training had a significantly greater increase in T_4 4 h after TRH administration when compared to horses out of work, representing an increased sensitivity of the pituitary-thyroid axis to a stimulus (Harris et al., 1992). Similar responses were not seen in this study, since responses during TRH-T were not significantly different than TRH-U. However, results may have been different if TRH

response tests had been performed during wk 4, when there were significant changes in thyroid hormone concentrations and nonthyroidal blood parameters. Therefore, performing only two TRH response tests, one before and one after training, does not exclude the possibility that, in general, training could have an effect on thyroid function test results.

Responses to thyroid stimulation tests may demonstrate seasonal variability; however this variability may be species specific and has not been studied in horses. In lactating cows, greater responses are seen in spring and summer than in winter (Perera et al., 1985). Similarly, in reindeer the smallest response was during winter; however the greatest response was in autumn (Timisjarvi et al., 1994). Conversely, the greatest responses were seen during winter in humans (Harrop et al., 1985). Since there was no control group that was not undergoing training, it is unknown whether there would have been changes in response test results during 15 wk in untrained horses.

Total T_3 and FT_3 responses were greater in SS fed horses than FF fed horses during TRH-T. This response is similar to the greater increases in TT_3 and FT_3 during exercise in SS fed horses. There may not only be insulin-stimulated increases in peripheral deiodinase activity in SS fed horses, as part of the mechanism for differences during exercise, but there may also be insulin-stimulated increases in deiodinase activity within the thyroid gland. This would create a larger supply of T_3 within the thyroid gland of SS fed horses and cause greater secretion of T_3 in response to a stimulus, including both exercise and TRH response tests. Similar results were seen in a previous study where horses with obesity-associated laminitis and hyperinsulinemia had similar TT_4 and FT_4 responses and greater TT_3 and FT_3 responses to TRH response tests than horses with chronic lameness due to musculoskeletal disorders (Graves et al., 2002).

For this reason, it may be suspected that dietary adaptation to high levels of nonstructural carbohydrates would be beneficial during exercise, in order to supply the body with more physiologically active thyroid hormone, T_3. However, the undesirable effects of elevated insulin concentrations and decreased insulin sensitivity may outweigh the benefits of greater T_3 concentrations. Also, increased concentrations of T_3 may increase UCP expression, causing decreased metabolic efficiency and increased heat production (Solanes et al., 2005).

Although TRH response tests are often used to evaluate thyroid gland function, they may also be used to evaluate pituitary gland function and diagnose pituitary adenomas. Baseline cortisol concentrations are lower in horses suspected of having a pituitary adenoma and increase after TRH administration, returning to baseline within 24 h. This response is different than what is seen in normal horses, where cortisol concentrations decrease or increase then decrease after TRH administration, decreasing below baseline concentrations between 5 and 24 h (Beech & Garcia, 1985; Eiler et al., 1997). More distinct analysis may be performed when used in conjunction with a dexamethasone suppression test, where increases in cortisol are only seen in horses with pituitary adenomas (Eiler et al., 1997). The results of the present study support the observation of decreased cortisol concentrations 5 h after TRH administration in healthy horses (Figure 12). The abnormally high baseline concentrations of cortisol during TRH-U may be in part due to stress brought about by the novelty of working with the horses again and bringing them into stalls.

In conclusion, the results of this study indicate that the thyroid gland increased thyroid hormone output during exercise. After exercise ceased, thyroid hormone levels dropped below pre-exercise levels, and the ability of the thyroid gland to respond to stimulation decreased. During athletic conditioning, the thyroid gland responded to the increased demand for thyroid hormones and increased its output, mainly in the form of T_4. Analysis of thyroid function is therefore dependent on the time that measurements are taken in relation to completion of exercise or level of training. Analysis may also vary depending on which thyroid hormones are measured and whether they are measured with respect to a stimulus. TT_4 and FT_4 were more affected by training and exercise than by dietary composition, whereas TT_3 and FT_3 were influenced by the level of carbohydrates in the diet.

Implications

Thyroid status assessment should be planned at a time when variables such as exercise will have minimal influence on results, and interpretation of results should take into consideration the effects of training and dietary composition on thyroid status.

Table 1. Nutrient composition of pasture, sugar and starch (SS) feed, and fat and fiber (FF) feed. Analysis was performed by the Dairy One Forage Laboratory (Ithaca, NY). Data are summarized on a DM basis as mean ± SEM.

Nutrient	SS (n = 18)	FF (n = 24)	Pasture (n = 9)
DM, %	88.7 ± 0.3[a]	92.1 ± 0.3[b]	21.56 ± 0.98
DE, Mcal/kg	3.09 ± 0.03	2.99 ± 0.03	2.45 ± 0.04
CP, %	14.49 ± 0.31	15.04 ± 0.26	22.18 ± 1.44
ADF, %	10.63 ± 0.60[a]	28.31 ± 0.50[b]	30.62 ± 0.69
NDF, %	19.28 ± 0.68[a]	44.63 ± 0.56[b]	56.24 ± 1.24
Fat, %	3.61 ± 0.24[a]	14.51 ± 0.20[b]	3.78 ± 0.24
NSC, %	49.26 ± 1.35[a]	12.02 ± 1.13[b]	8.36 ± 0.61
Ash, %	7.09 ± 0.26[a]	9.05 ± 0.22[b]	10.47 ± 0.42
Ca, %	1.27 ± 0.07[a]	1.64 ± 0.06[b]	0.61 ± 0.03
P, %	0.72 ± 0.02	0.71 ± 0.02	0.41 ± 0.02
Mg, %	0.27 ± 0.02	0.22 ± 0.01	0.22 ± 0.01
K, %	1.16 ± 0.03[a]	1.35 ± 0.03[b]	3.37 ± 0.11
Na, %	0.22 ± 0.01	0.21 ± 0.01	0.005 ± 0.001
Cl, %	0.51 ± 0.02[c]	0.59 ± 0.02[d]	1.01 ± 0.05
Fe, ppm	418 ± 24[a]	533 ± 20[b]	175 ± 30
Zn, ppm	134 ± 7	133 ± 6	17.2 ± 0.8
Cu, ppm	51.3 ± 3.2	51.2 ± 2.6	5.67 ± 0.37
Mn, ppm	77.4 ± 4.4	88.7 ± 3.7	44.2 ± 2.35
Mo, ppm	1.16 ± 0.09	1.15 ± 0.07	0.722 ± 0.100

[a, b]Nutrient means of the SS and FF feeds differ ($P < 0.001$)
[c, d]Nutrient means of the SS and FF feeds differ ($P < 0.01$)

Table 2. Analysis of correlations of blood plasma parameters

Comparisons	Spearman's correlation coefficient	P-value
Compared with NEFA		
FT_4	0.20	0.013
TT_3	0.30	< 0.001
Compared with TG		
TT_4	-0.43	< 0.001
FT_4	-0.27	0.001
Compared with insulin		
TT_4	-0.26	0.001
FT_4	-0.24	0.003
TT_3:TT_4	0.34	< 0.001
FT_3:FT_4	0.27	0.001
Compared with RISQI		
TT_4	0.26	0.001
FT_4	0.24	0.003
TT_3:TT_4	-0.34	< 0.001
FT_3:FT_4	-0.27	0.001
Compared with MIRG		
TT_4	-0.22	0.005
FT_4	-0.22	0.006
TT_3:TT_4	0.31	< 0.001
FT_3:FT_4	0.26	0.001

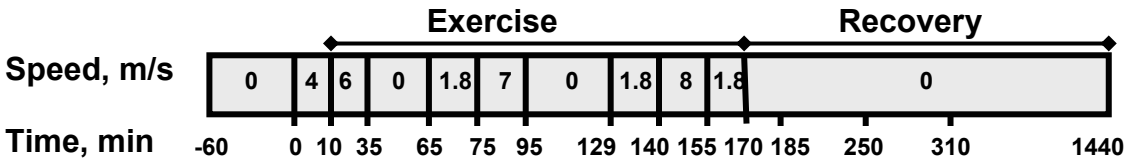

Figure 1. Time intervals and speeds during the treadmill exercise test.

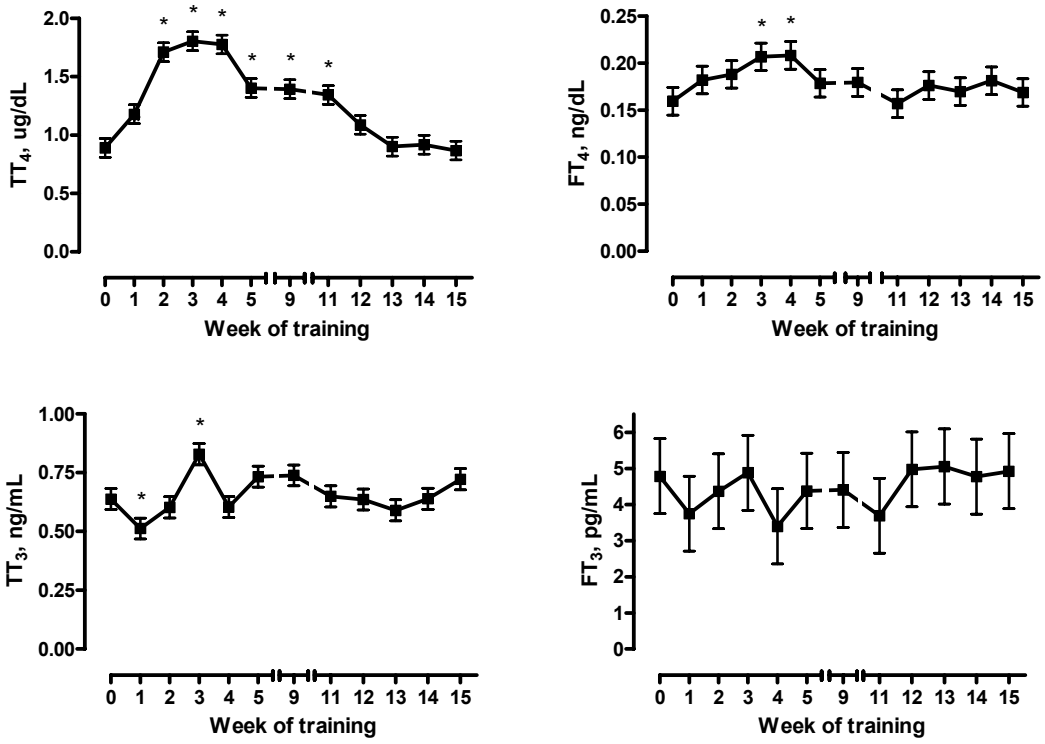

Figure 2. Basal thyroid hormone concentrations during the 15 week training period. Asterisk indicates significant difference from untrained concentrations at week 0 ($P <$ 0.05).

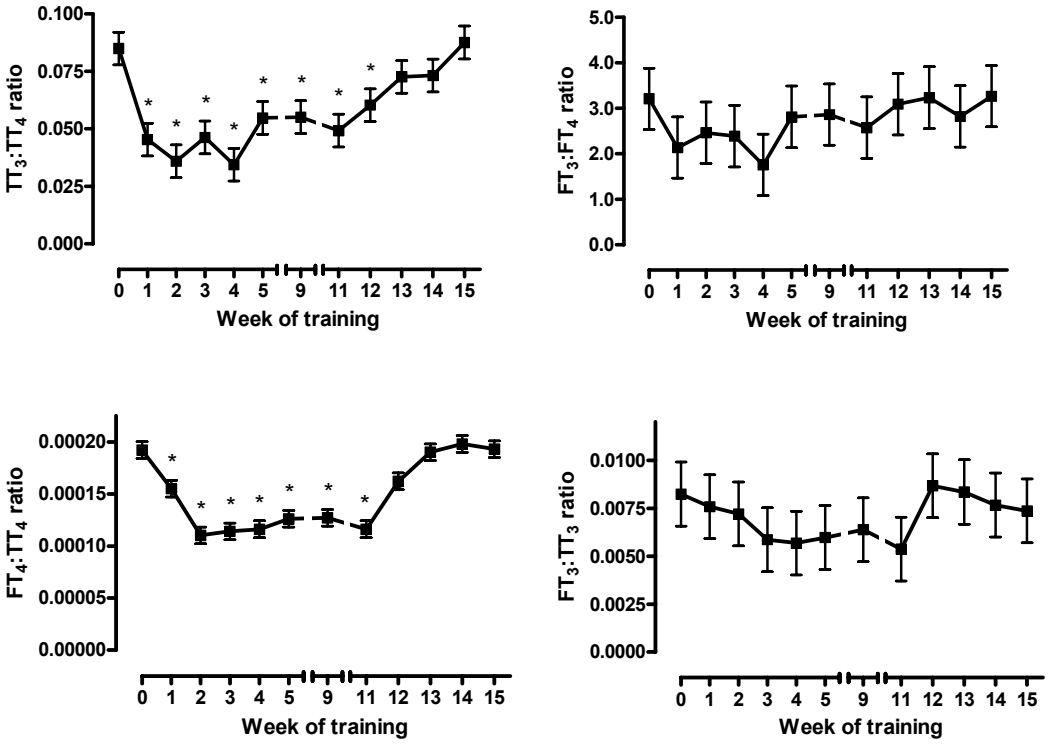

Figure 3. Ratios of basal thyroid hormone concentrations during the 15 week training period. Asterisk indicates significant difference from untrained concentrations at week 0 ($P < 0.05$).

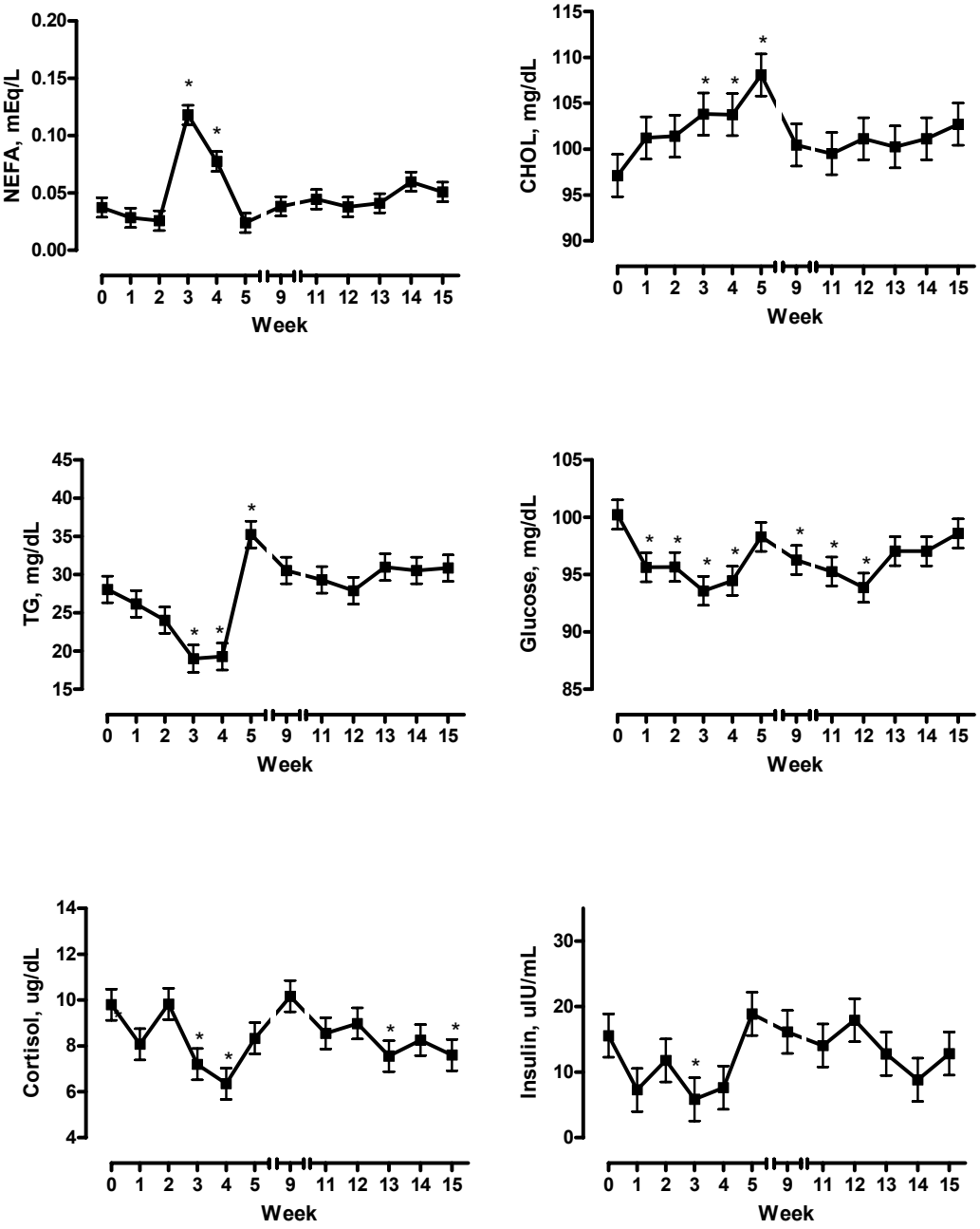

Figure 4. Basal concentrations of NEFA, CHOL, TG, glucose, cortisol, and insulin during the 15 week training period. Asterisk indicates significant difference from untrained concentrations at week 0 ($P < 0.05$).

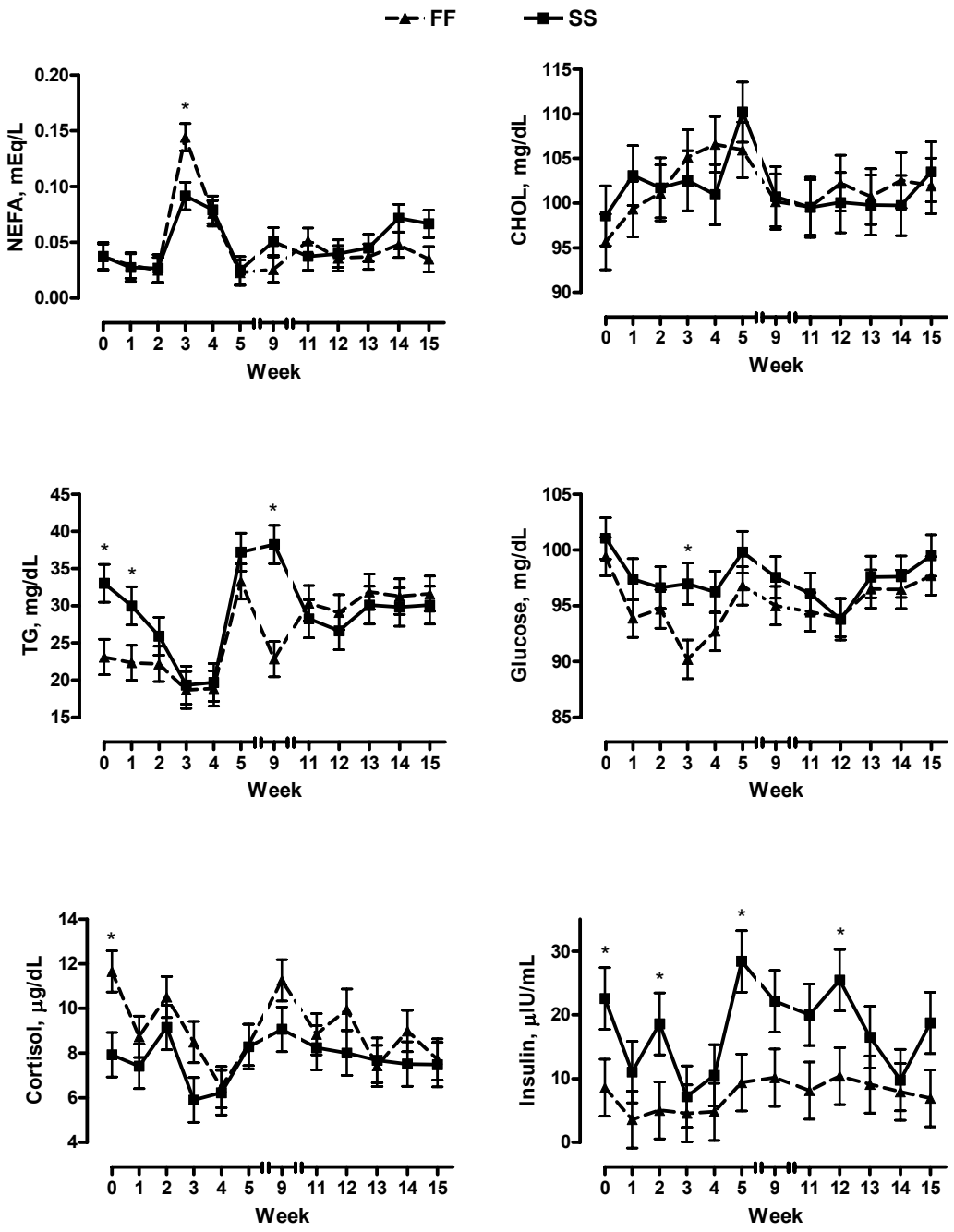

Figure 5. Basal concentrations of NEFA, CHOL, TG, glucose, cortisol, and insulin for SS and FF fed horses during the 15 week training period. Asterisk indicates significant difference between feed groups within a week ($P < 0.05$).

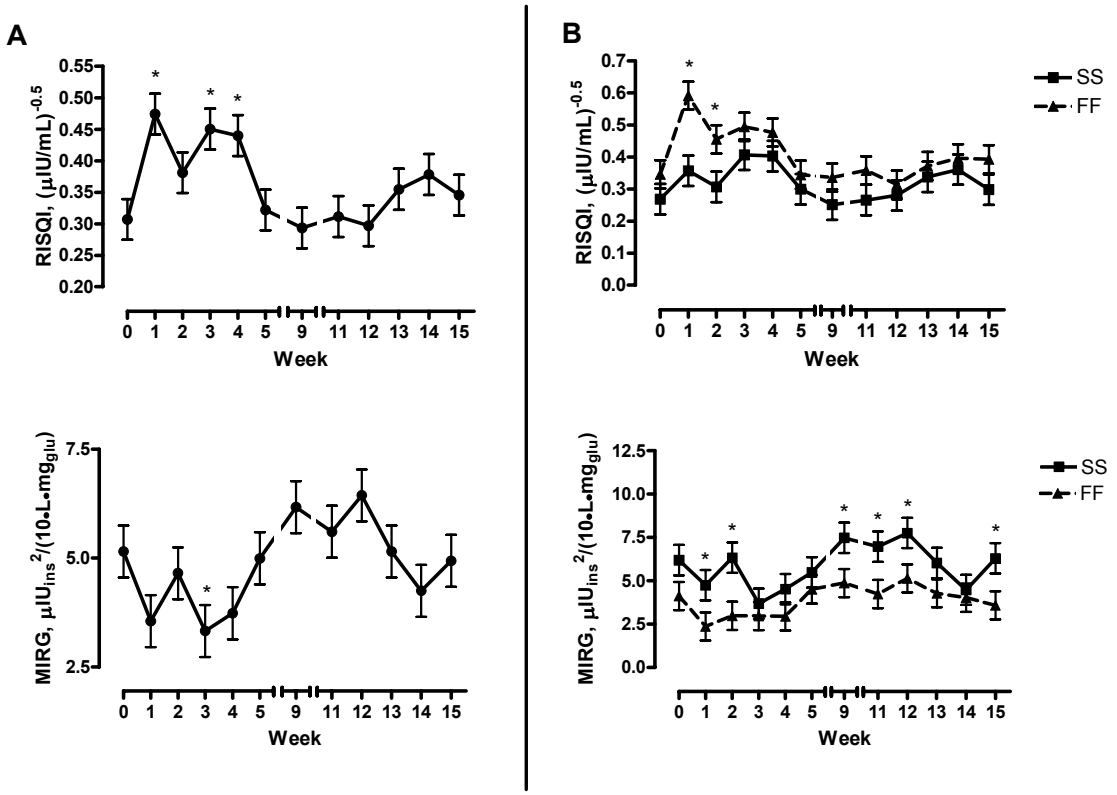

Figure 6. Mean ± SEM values for proxies of insulin sensitivity (RISQI) and beta cell responsiveness (MIRG) for all horses (A) and for SS and FF fed horses (B) during the 15 week training period. In A, asterisks indicate significant difference from untrained concentrations at week 0 ($P < 0.05$). In B, asterisks indicate significant difference between feed groups within a week ($P < 0.05$).

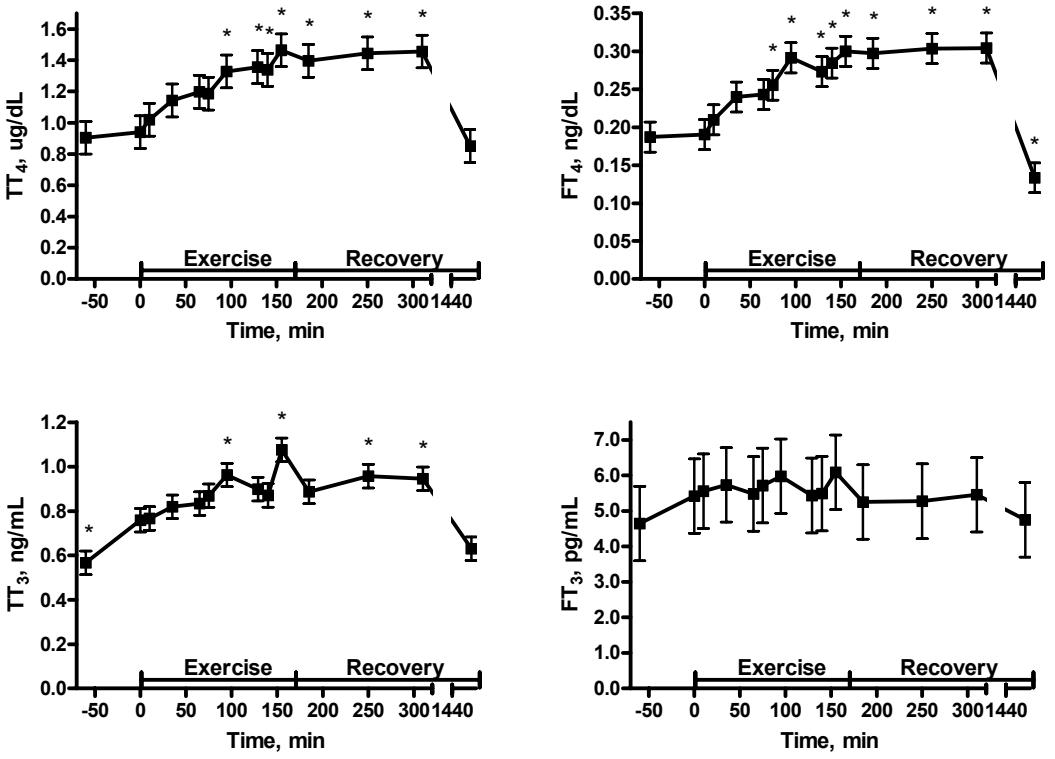

Figure 7. Thyroid hormone concentrations during the exercise test. Asterisk indicates significant difference from the concentration immediately before the start of exercise at 0 min ($P < 0.05$).

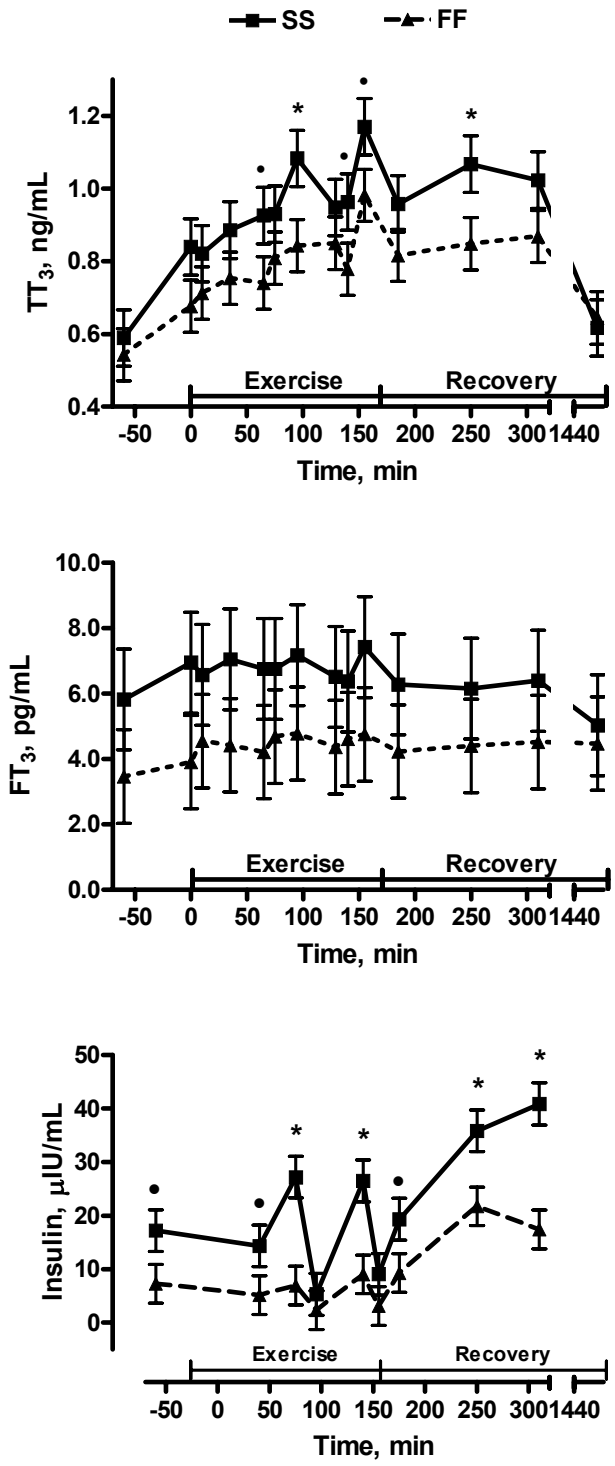

Figure 8. TT_3, FT_3, and insulin concentrations during the exercise test in SS and FF fed horses. Asterisk indicates significant difference between SS and FF groups ($P < 0.05$); solid circle indicates a trend for a difference between SS and FF groups ($P < 0.10$).

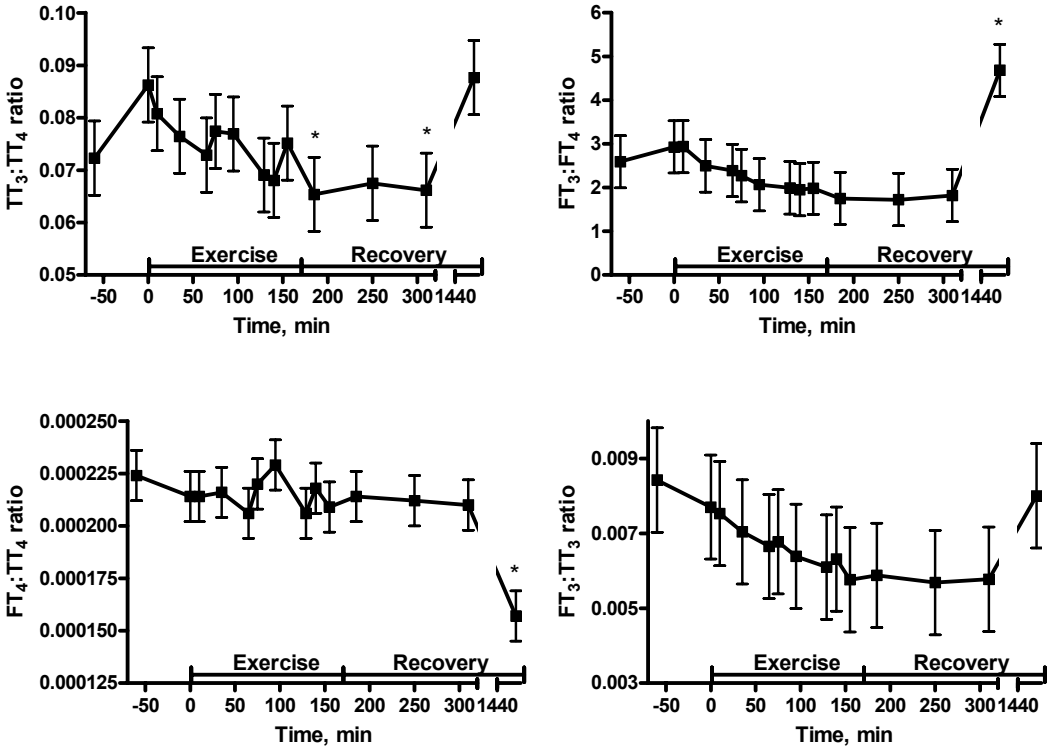

Figure 9. Ratios of thyroid hormone concentrations during the exercise test. Asterisk indicates significant difference from the concentration immediately before the start of exercise at 0 min ($P < 0.05$).

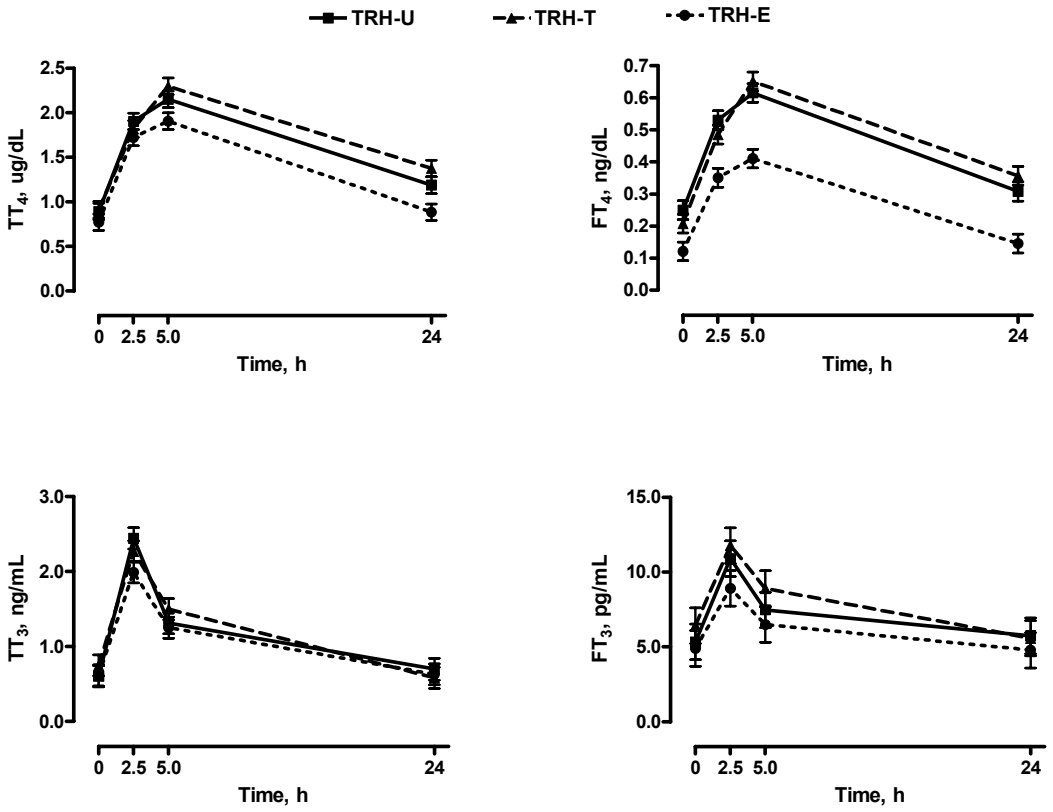

Figure 10. Thyroid hormone concentrations immediately before (0 h) and 2.5, 5.0, and 24 h after intravenous administration of 1 mg TRH in untrained (TRH-U), trained (TRH-T), and exercised (TRH-E) horses (n = 11).

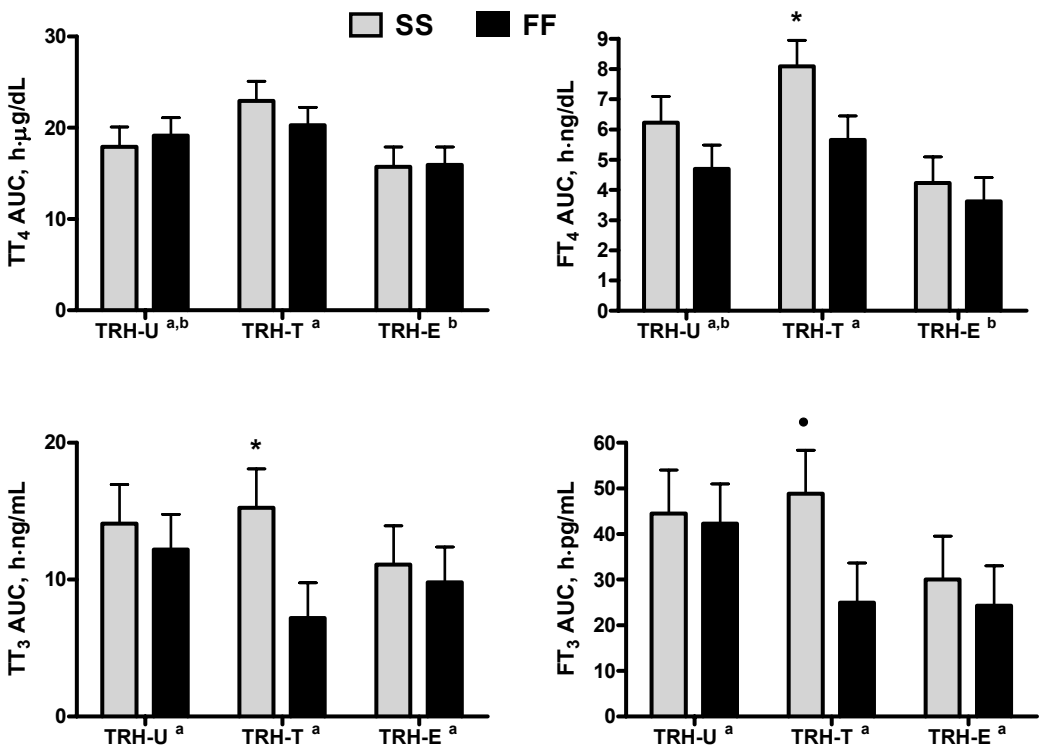

Figure 11. Magnitude of thyroid hormone response, represented as the area under the curve (AUC) of TT_4, FT_4, TT_3, and FT_3 for TRH response tests on horses before athletic conditioning (TRH-U), after a 15 wk training period (TRH-T), and the morning after an exhaustive exercise test (TRH-E). Letters indicate significant differences between TRH response tests ($P < 0.05$), Asterisks indicate differences between SS and FF fed horses within a test ($P < 0.05$), Solid circles indicate trends for differences between SS and FF fed horses within a test ($P < 0.10$).

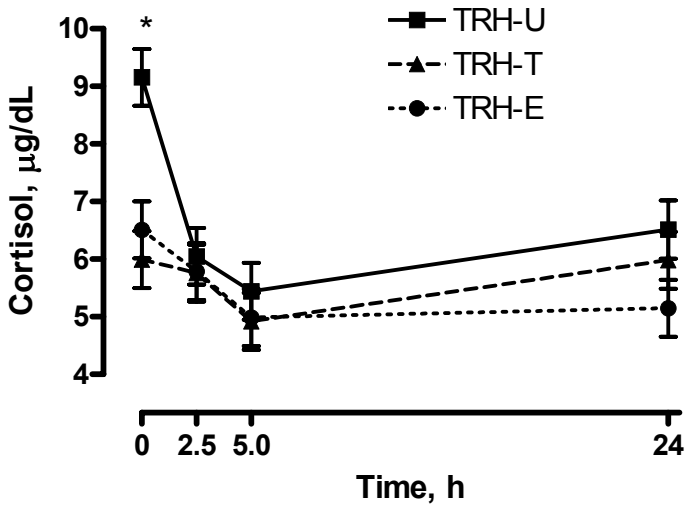

Figure 12. Cortisol concentrations during TRH response tests. Asterisk indicates a difference between TRH-U and all other TRH response tests within a time period.

CHAPTER V

Circulating thyroid hormone concentrations in ponies with pasture-associated laminitis

ABSTRACT: Total and free thyroxine (TT_4 and FT_4, respectively) and triiodothyronine (TT_3 and FT_3, respectively) were measured in 126 Dartmoor, Welsh or crossbred ponies in March and May 2004. Ponies were maintained on a single farm under similar pasture grazing conditions. Ponies were categorized as either previously laminitic (PL) or never laminitic (NL) according to their histories reported by the farm managers, and evaluated for current laminitis (CL) based on digital pulse, increased coronary band temperature, and signs of discomfort or pain. During both March and May, TT_4 and FT_4 were greater in NL than PL ponies ($P < 0.05$). In May, TT_4 and FT_4 concentrations in CL ponies were similar to both NL and PL ponies ($P > 0.10$). There was no effect of history of laminitis on TT_3 or FT_3 concentrations. TT_3 decreased between March and May ($P = 0.044$), whereas FT_3 increased ($P < 0.001$). These results indicate that basal thyroid hormone concentrations are not useful as a predictor or hormonal characteristic of pasture-associated laminitis. However, changes in thyroid hormone parameters may be influenced by seasonal or environmental changes and associated with a pony's physiological state, including its insulin sensitivity and pregnancy.

Key Words: Pony, Thyroid hormone, Laminitis, Insulin sensitivity

Introduction

Laminitis is a painful and disabling disease in which there is separation of the secondary epidermal and dermal lamellae of the hoof (Pollitt, 1996). Horses and ponies affected by laminitis often experience a decreased quality of life and usefulness, which may eventually lead to a loss of life itself. The cause of laminitis is incompletely understood, and multiple theories on pathogenic mechanisms have been proposed (Bailey et al., 2004). In reality, the pathogenesis of laminitis probably has multiple causal mechanisms with multiple component causes within each mechanism, which creates the difficulty in studying this disease. Recent surveys in the United States have found that 13% of horse owners/operations have reported problems with laminitis in their horses over the previous 12 mo, and that laminitis was ranked first in need of research by equine practitioners (AAEP, 2003; USDA, 2000). The prevalence of laminitis in the equine industry and the lack of a proper understanding of its pathophysiology have created this need for research on laminitis.

The role of thyroid function in the pathogenesis of laminitis is not understood, with the results of experimental studies conflicting with clinical observations and practice. However, the importance of thyroid hormone action in energy metabolism makes it a key area of interest when studying metabolic disorders involving insulin resistance, such as laminitis.

Hypothyroidism has been proposed as a predisposing factor for laminitis (Colles & Jeffcott, 1977), and suggestions of low thyroid hormone levels in horses affected with laminitis have been made (Johnson, 2002). However, the results of experimental studies do not always support these propositions. Experimental thyroidectomy in adult horses does not induce laminitis or obesity (Lowe et al., 1974; Vischer et al., 1999; Frank et al., 1999, 2003a, 2003b, 2004). Studies on laminitic horses indicate that thyroid gland dysfunction does not play a role in the development of laminitis, and that circulating thyroid hormone concentrations may not be depressed in laminitic horses (Graves et al., 2002; Hood et al., 1987).

The association of thyroid dysfunction with laminitis has led to the use of oral thyroid hormone supplementation for the management of laminitis. However, controlled

studies have not been performed to determine whether thyroid hormone supplementation is beneficial during acute episodes of laminitis.

The objective of this study was to determine if differences exist in basal thyroid hormone concentrations between ponies that have never had laminitis (NL), those that have previously had laminitis (PL), and those that developed laminitis during the sampling period (CL). Any differences may be useful as indicators of a predisposition to laminitis and increase the understanding of peripheral changes that occur in thyroid hormones in response to laminitis.

Materials and Methods

One hundred twenty six Dartmoor, Welsh, or crossbred ponies (36 pregnant mares, 51 non-pregnant mares, 21 geldings, and 18 stallions) that were housed on the same farm in Northern Virginia were studied in March and May 2004. Ponies were kept on mixed grass/clover pastures. Grass species comprised of mostly cool season grasses, including tall fescue. Age (10.0 ± 0.7 y; range 3 – 32 y), BW (320 ± 5 kg; range 196 – 490 kg), and body condition score (BCS) on a scale from 1 to 9 (6.2 ± 0.1; range 3.5 – 8.0) were recorded (Henneke et al., 1983). Ponies were categorized as either previously laminitic (PL; n = 54) or never laminitic (NL; n = 72) according to their histories reported by the farm managers, and evaluated for current laminitis (CL; n = 13, May only) based on digital pulse, increased coronary band temperature, and signs of discomfort or pain.

Basal venous blood samples were collected in sodium heparin tubes between 0800 and 1000. Blood samples were centrifuged at 3000 g for 10 min at 4°C and plasma was transferred to 2 mL polypropylene vials (Sarstedt, Newton, NC) and stored at -20°C until analysis. Plasma samples were analyzed for total and free thyroxine (TT_4, FT_4), and total triiodothyronine (TT_3) via radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) and for free triiodothyronine (FT_3) via enzyme immunoassay (Active, Diagnostic Systems Laboratories, Inc., Webster, TX). Insulin and cortisol were measured using RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). Triglycerides (TG), and total bilirubin (TBIL) were measured enzymatically using commercial kits (Beckman Instruments, CX5 Chemistry Analyzer, Triglyceride 445850).

Thyroid hormone ratios of TT_3:TT_4, FT_3:FT_4, FT_4:TT_4, and FT_3:TT_3 were calculated by converting both measurements to the same unit, then dividing the first by the second measurement. The minimal model parameter of insulin sensitivity (SI) was predicted by the use of a proxy developed by equivalence testing with the results of minimal models performed on 46 horses (Treiber et al., in press). Insulin sensitivity was estimated by the reciprocal inverse square of basal insulin (RISQI), calculated by using basal insulin concentrations (insulin concentration$^{-0.5}$).

Pasture samples were collected in March and May, and analyzed for nonstructural carbohydrate (NSC), sugar (water-soluble carbohydrate), and starch contents (Dairy One Forage Laboratory, Ithaca, NY).

Data are summarized as least squares means ± SEM. Main effects of month and history of laminitis were evaluated using a mixed model ANOVA. Means were compared as simple effects by slicing significant interactions. Relationships between parameters were assessed from Spearman's Correlation Coefficients (r) and P-values determined from the data obtained during both months (SAS Version 9.1; SAS Institute Inc., Cary, NC). Data was graphed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

Results

There was no effect of age or sex on thyroid hormone parameters. There were no clinical cases of laminitis found in March. All ponies that developed laminitis in May (CL) had previous histories of laminitis and were categorized into the PL group in March.

During both March and May, TT_4 was greater in NL than PL ponies ($P = 0.018$) (Figure 1). TT_4 concentrations were lower in May than in March ($P < 0.001$). During May, CL ponies had similar concentrations of TT_4 to NL and PL ponies ($P = 0.82$, 0.18). During both March and May, FT_4 was greater in NL than PL ponies ($P = 0.020$, < 0.001). FT_4 concentrations were lower in May than in March ($P < 0.001$). During May, CL ponies had similar concentrations of FT_4 to NL ponies ($P = 0.72$) and PL ponies ($P = 0.062$). There was no effect of history of laminitis on TT_3 concentrations during March ($P = 0.44$) or May ($P = 0.42$). TT_3 decreased between March and May in PL ponies ($P = 0.026$), but not NL ponies ($P = 0.63$). There was no effect of history of laminitis on FT_3

concentrations during March ($P = 0.27$) or May ($P = 0.17$). TT_3 increased between March and May ($P < 0.001$).

There was an effect of pregnancy on TT_4 ($P = 0.021$) and FT_4 ($P = 0.001$), but not TT_3 ($P = 0.12$) and FT_3 ($P = 0.97$) concentrations. Lower TT_4 and FT_4 concentrations were observed in pregnant (1.05 ± 0.04 μg/dL and 0.22 ± 0.01 ng/dL, respectively) than non-pregnant (1.27 ± 0.03 μg/dL and 0.31 ± 0.01 ng/dL, respectively) ponies. A greater proportion of ponies were pregnant in the PL group (30/54 = 56%) than the NL group (6/72 = 8%) or CL group (3/13 = 23%; May only). When pregnant ponies were excluded from the analysis, there was no difference in TT_4 and FT_4 concentrations between NL and PL ponies during March ($P = 0.75$, 0.99; respectively) (Figure 2). Although there was no overall effect of history of laminitis on TT_4, FT_4, TT_3, or FT_3 concentrations; PL ponies had lower TT_4 ($P = 0.028$) and FT_4 ($P = 0.030$) concentrations than NL ponies during March. In non-pregnant ponies, there was an effect of month on TT_4 ($P < 0.001$) and FT_4 ($P < 0.001$), but not TT_3 ($P = 0.52$) and FT_3 ($P = 0.017$) concentrations.

For thyroid hormone ratios, there was an effect of month on the FT_4:TT_4, FT_3:TT_3, and FT_3:FT_4 ratios ($P < 0.001$); there was an effect of history of laminitis on the TT_3:TT_4 ($P = 0.014$), FT_3:FT_4 ($P = 0.001$), and FT_4:TT_4 ($P < 0.001$) ratios; and a month by history of laminitis effect on FT_3:FT_4 ($P = 0.001$) and FT_4:TT_4 ($P = 0.002$) ratios (Figure 3).

Insulin concentrations were higher in May (30.2 ± 4.3 μIU/mL) than in March (18.1 ± 2.0 μIU/mL) ($P = 0.034$). During March, insulin concentrations were higher in PL ponies (25.1 ± 4.4 μIU/mL) than NL ponies (12.8 ± 3.8 μIU/mL) ($P = 0.036$). During May, insulin concentrations were higher in PL ponies (35.7 ± 5.2 μIU/mL) than NL ponies (16.9 ± 4.2 μIU/mL) ($P = 0.005$), and were higher in CL ponies (86.5 ± 9.0 μIU/mL) than both PL ponies and NL ponies ($P < 0.001$).

RISQI values were similar in March (0.30 ± 0.01 $[mIU/mL]^{-0.5}$) and May (0.31 ± 0.03 $[mIU/mL]^{-0.5}$) ($P = 0.31$). During March, RISQI values were higher in PL ponies than NL ponies ($P = 0.008$). During May, RISQI values were higher in CL ponies (0.20 ± 0.05 $[mIU/mL]^{-0.5}$) than NL ponies (0.33 ± 0.02 $[mIU/mL]^{-0.5}$) ($P = 0.013$), and had a trend for higher values than PL ponies (0.30 ± 0.03 $[mIU/mL]^{-0.5}$) ($P = 0.076$).

Total bilirubin concentrations were higher in March (1.94 ± 0.08 mg/dL) than in May (1.01 ± 0.03 mg/dL) ($P < 0.001$). During March, TBIL concentrations were higher in PL ponies (2.36 ± 0.09 mg/dL) than NL ponies (1.63 ± 0.08 mg/dL) ($P < 0.001$), however concentrations were not different between groups in May ($P = 0.98$).

BCS was higher in May (6.5 ± 0.1) than in March (6.2 ± 0.1) ($P < 0.001$). During March, BCS was higher in PL ponies (6.4 ± 0.1) than NL ponies (6.0 ± 0.1) ($P = 0.004$), however there were no differences in BCS between groups in May ($P = 0.41$).

Nonstructural carbohydrate content of pastures increased from March (5.10 ± 1.00 % DM) to May (9.70 ± 1.20 % DM) ($P = 0.039$). The NSC component of starch increased from March (4.20 ± 1.00 % DM) to May (7.80 ± 0.61 % DM) ($P = 0.040$), whereas sugar was not different in March (0.87 ± 0.12 % DM) and May (1.80 ± 0.55 % DM) ($P = 0.16$).

Correlations of thyroid hormone parameters with insulin, RISQI, TBIL, and BCS are summarized in Table 1.

Discussion

In all groups of ponies during both months, mean concentrations of TT_4 and TT_3 were similar to, FT_4 lower than, and FT_3 greater than previously measured concentrations for normal adult horses (Anderson et al., 1988; Bayly et al., 1996; Breuhaus, 2002; Messer et al., 1995b).

Although PL ponies had lower TT_4 and FT_4 concentrations in March, this difference could not be used as a predictor for the development of laminitis. When CL ponies from May were retrospectively separated into a group in March, TT_4 concentrations were similar to both NL and PL ponies and FT_4 concentrations were similar to NL ponies (Figure 4). By taking into account that at both time periods TT_4 and FT_4 concentrations in CL are not different than NL, and that concentrations in PL are lower than NL, the decreased TT_4 and FT_4 in PL may be an indication of a response or compensation to laminitis and may facilitate the metabolic changes necessary to cope with the disease. These metabolic changes may be the animal's means of recovering from laminitis and avoiding it during subsequent years.

The ponies that were analyzed spanned a variety of physiological states, including pregnancy. Pregnant ponies had lower TT_4 and FT_4 concentrations than non-pregnant ponies. In humans, pregnancy is known to affect thyroid status by increasing total thyroid hormone concentrations through an increase in binding protein concentration (Ain et al., 1987). However, in horses, lower concentrations of TT_4 and TT_3 have been observed in pregnant horses when compared to non-pregnant horses (Cebulj-Kadunc et al., 2003). In a study measuring equine thyroid hormone concentrations across a wide range of physiological states, TT_3 and FT_3 were similar across all groups, whereas TT_4 and FT_4 differed between physiological states (Anderson et al., 1988). Pregnant ponies had the lowest FT_4 concentrations and the lowest FT_4:TT_4 ratios of all the physiological states.

Excluding pregnant pony data from TT_4 and FT_4 analyses eliminated differences between NL and PL groups in March (Figure 2). This further illustrates that thyroid hormone concentrations cannot predict a risk for laminitis. However, since TT_4 and FT_4 concentrations were still lower in PL ponies in May, decreasing thyroid hormone concentrations may facilitate metabolic changes necessary to avoid laminitis during periods of increased risk or changes in environmental conditions.

The similarity of thyroid hormone concentrations between NL and CL ponies supports the results of previous studies reporting that thyroid gland dysfunction does not play a role in the development of laminitis, and circulating thyroid hormone concentrations may not be depressed in laminitic horses. One such study found that although serum levels of T_4 and T_3 were depressed in horses with carbohydrate-induced acute laminitis, horses with chronic laminitis had elevated T_3 concentrations and similar T_4 concentrations with respect to control values (Hood et al., 1987). Thyroid function, as determined by TSH stimulation tests, was similar for control horses and those affected with chronic laminitis. In another study, horses with obesity-associated laminitis and hyperinsulinemia had similar TT_4 and FT_4 responses and greater TT_3 and FT_3 responses to TRH response tests as horses with chronic lameness due to musculoskeletal disorders (Graves et al., 2002). Baseline thyroid hormone concentrations were similar between the two experimental groups in that study.

The increases in T_3 to T_4 ratios in PL when compared to NL ponies indicate increased deiodinase activity and cellular uptake, and decreased production and degradation. Ratios of thyroid hormone concentrations were used for inferences about peripheral thyroid hormone metabolism. Circulating thyroid hormone concentrations are influenced by both production and secretion from the thyroid gland and by peripheral regulatory mechanisms, including transfer between circulation and tissue sites, production of T_3 from T_4 deiodination, hormone binding to serum carrier proteins, and degradation rates (Kaptein et al., 1994). Activities of these peripheral regulatory mechanisms affect thyroid hormone ratios, including free hormone fractions and T_3 to T_4 ratios. The complexity of their interactions do not allow for their direct analysis through the use of thyroid hormone ratios, although inferences may be made to form a better understanding of thyroid status. Selenodeiodinase activity of T_4 deiodination to T_3 and cellular uptake are expected to positively influence, and degradation rates negatively influence FT_3:FT_4 and TT_3:TT_4 ratios (Bianco et al., 2002; Peeters et al., 2005; Reyns et al., 2002). Degradation rates, the relative affinity of thyroid hormone binding to serum carrier proteins, and the concentration of serum carrier proteins is expected to vary inversely with the free hormone fractions, or FT_3:TT_3 and FT_4:TT_4 ratios (Irvine & Evans, 1975). The influence of negative feedback to the hypothalamus and pituitary would regulate production and secretion from the thyroid gland to be negatively associated with free hormone fraction and T_3 to T_4 ratios (Kaptein et al., 1994; Larsen et al., 1979).

In humans, illness may cause abnormalities in thyroid hormone metabolism, resulting in the development of nonthyroidal illness (NTI). These subjects have normal thyroid gland function, but abnormal concentrations of thyroid hormones from decreases in T_4 deiodination to T_3 (Peeters et al., 2003). These changes in peripheral deiodinase activity result in decreased plasma T_3, whereas T_4 may be increased in mild illness and normal or decreased during critical illness. The greater T_3 to T_4 ratios in PL ponies and similar ratios in CL ponies when compared to NL ponies do not indicate that laminitis has similar effects as NTI on thyroid hormone metabolism.

The decreases in TT_4 and FT_4 and increases in FT_3 and FT_3:FT_4 ratio from March to May indicate increased deiodinase activity. Interestingly, TT_3 also decreased between

months, causing a large increase in FT_3:TT_3 ratio, opposite of the decrease in FT_4:TT_4 ratio, and causing the TT_3:TT_4 ratio to remain unchanged. This inverse relationship between TT_3 and FT_3 from March to May could be caused by changes in serum carrier protein affinity or interfering biomolecules *in vivo* or *in vitro*. *In vivo* effects may be caused by nonesterified fatty acids, which compete for binding sites on serum carrier proteins, consequently increasing the free hormone fraction (Liewendahl et al., 1992). *In vitro* effects could be caused by the assay methods used to measure hormone concentrations. When measuring free hormone concentrations with an analog method, such as radioimmunoassay or enzyme immunoassay, the equilibrium between the free and bound state may become disturbed during the assay. The use of an equilibrium dialysis assay minimizes disturbances of the bound to unbound equilibrium (Christofides et al., 1999). These disturbances may become most apparent during states of altered serum T_4 binding capacity, such as pregnancy or illness (perhaps including laminitis), at which time an assay involving equilibrium dialysis may be a more reliable method. Alterations in the binding capacity may have occurred between March and May because of physiological changes brought about by changes in the environment.

Environmental factors influencing the changes in thyroid hormone parameters between March and May could include seasonal effects or changes in pasture composition and intake. Seasonal increases in thyroid hormone concentrations have been observed in horses being acclimated to colder temperatures (McBride et al., 1985). The duration of our study was from March to May, as temperatures were beginning to rise, which may have caused the opposite response by decreasing TT_4, FT_4, and TT_3. Increases in pasture starch levels were seen between March and May ($P = 0.040$), facilitating an increase in plasma insulin concentrations ($P = 0.034$). Increased insulin concentrations could result in an insulin-stimulated increase in T_4 deiodination to T_3 by an increase in T_4 deiodinase activity (Gavin et al., 1987). In addition to nutrient composition of the pastures affecting thyroid status, an increase in palatability may cause increased pasture intake during May, although this variable was not measured. Overfeeding, or overnutrition, in humans results in increased T_3 levels while T_4 concentrations remain constant (Danforth et al., 1979; Utiger, 1982). These results are

seen during overnutrition, regardless if the component of the diet overfed was carbohydrate, fat, or protein.

Correlations between thyroid hormone parameters and nonthyroidal blood parameters were used to determine relationships between thyroid status and metabolic state. Correlations of thyroid hormone parameters with insulin and RISQI support mechanisms of a T_4-induced increase in insulin sensitivity and an insulin-stimulated increase in T_4 deiodination (Frank et al., 2005; Gavin et al., 1987). Thyroid hormones facilitate the regulation of plasma glucose concentrations by stimulating glucose transporters, GLUT1 and GLUT4, expression in skeletal muscle and adipose tissue, increasing insulin stimulated glucose transport (Romero et al., 2000; Shimizu et al., 2002; Weinstein et al., 1994). These activities of thyroid hormones may be part of the mechanism for oral levothyroxine supplementation increasing insulin sensitivity and accelerating insulin disposal in resting horses (Frank et al., 2005). Thyroid hormones may facilitate the changes in carbohydrate metabolism seen in laminitis, a state associated with insulin resistance (Coffman & Colles, 1983). Facilitation of these physiological changes may be especially critical in the hoof, since chronic laminitis is characterized by loss of GLUT1 and GLUT4 in laminar keratinocytes (Mobasheri et al., 2004). Also, *in vitro* studies of horse hoof explants indicate that the integrity of the explants was dependent on consumption of glucose (Pass et al., 1998).

Insulin stimulated T_3 neogenesis may be part of the compensation for insulin resistance by providing more physiologically active hormone to induce glucose transporter expression. Insulin stimulated deiodination could partly explain the differences seen between months and between PL and NL ponies, however it does not explain the results seen in CL ponies. CL ponies had the lowest insulin sensitivity; therefore they would be expected to have higher T_3 concentrations and higher T_3 to T_4 ratios. Possible explanations of why there are similar concentrations of thyroid hormones in NL ponies and CL ponies may include that laminitis mediated changes in thyroid hormone concentrations occur over a longer period of time and are not apparent in CL ponies yet, or because metabolism of T_3 occurs at a rate fast enough to counterbalance increased T_3 neogenesis to reduce negative feedback.

Total bilirubin was positively correlated with FT_4 (r = 0.25) and negatively correlated with FT_3 (r = -0.25) and FT_3:FT_4 ratio (r = -0.39), indicating a decrease in deiodination, perhaps as a result of decreased T_4 transport into cells. The transport of T_4 into cells is rate limiting for subsequent thyroid hormone metabolism, including intracellular T_4 deiodination to T_3 (Hennemann et al., 1986). High concentrations of bilirubin and NEFA inhibited T_4 transport into cells and subsequent deiodination in cultured rat hepatocytes, and is predicted as the mechanism for reduced plasma T_3 concentrations in critically ill patients (Lim et al., 1993). Also, bilirubin is negatively correlated to liver deiodinase activity in critically ill patients (Peeters et al., 2003). Elevated TBIL concentrations were observed in PL ponies in March, providing a possible link between laminitic history and TT_4 and FT_4 concentrations; however this is not supported in May, when TBIL concentrations were not different between groups.

The assumption that hypothyroidism is related to obesity-associated laminitis is often made (Johnson, 2002), perhaps because hypothyroidism has been suggested to be a predisposing factor for obesity through a decrease in basal metabolic rate. However, thyroid hormone concentrations and their metabolism are normal in obese humans (Roti et al., 2000) and experimental thyroidectomy in adult horses does not induce laminitis or obesity (Lowe et al., 1974; Vischer et al., 1999; Frank et al., 1999, 2003a, 2003b, 2004). The results of this study indicate that the incidence of obesity may be associated with previous occurrences of laminitis; however it was not associated as a cause of current laminitis. Obesity may be an indication of a risk factor for laminitis, since BCS was negatively correlated to RISQI (r = -0.36), and CL ponies had lower RISQI values than NL ponies ($P = 0.013$). Total T_4, FT_4, and the T_3 to T_4 ratios also had correlations with RISQI (Table 1), indicating an interrelationship between BCS, thyroid status, and insulin sensitivity.

The association between thyroid status and insulin sensitivity may be explained physiologically as a cyclic relationship. Insulin resistance creates increases in basal insulin concentrations. Elevated insulin then stimulates deiodinase activity, causing increased T_3 to T_4 ratios as insulin sensitivity decreases (Gavin et al., 1987). However, thyroid hormones are known to increase insulin sensitivity through increasing glucose transporter expression (Romero et al., 2000). Therefore, an increase in deiodinase

activity with insulin resistance may be a physiological mechanism to counterbalance insulin resistance by providing more physiologically active hormone to induce glucose transporter expression. In summary, increased T_4 concentrations may cause increased insulin sensitivity, whereas decreased insulin sensitivity may cause increased T_3 to T_4 ratios. These internal physiological changes occurring with insulin sensitivity and thyroid status create changes in nutrient metabolism and body composition, which are then manifested externally as changes in BCS.

Oral thyroid hormone supplementation is commonly used in the management of chronic laminitis; however controlled studies have not been performed to determine whether thyroid hormone supplementation is beneficial during acute episodes of laminitis. Beneficial effects of thyroid hormone supplementation may result from weight loss caused by creating a state of iatrogenic hyperthyroidism. Levothyroxine supplementation has been shown to cause weight reduction in horses, perhaps through decreasing energy efficiency and enhancing lipolysis (Frank et al., 2005). While the use of thyroid hormone supplementation has been suggested as an acceptable treatment for obesity (Krotkiewski, 2000), human medicine has largely shifted away from this approach because of the side effects of inappropriate supplementation. Tachycardia, increased heart rate, palpitations, atrial arrhythmia, widened pulse pressure, increased systolic and decreased diastolic pressures, dyspnea, exercise intolerance, and in severe cases congestive heart failure may occur from high circulating thyroid hormone concentrations (Danzi & Klein, 2004). Another effect of improper supplementation is a decrease in thyroid gland capabilities because of increased negative feedback to the hypothalamus and pituitary, which becomes a problem when thyroid hormone treatment is stopped. Oral levothyroxine supplementation decreases the concentrations of TT_4, TT_3, FT_3, and TSH in response to TRH injection (Sommardahl et al., 2005).

After considering the influence of thyroid hormone action on insulin sensitivity, a more plausible mechanism than a decrease in weight for therapeutic effects of exogenous thyroid hormones is their influence to increase insulin sensitivity. Another possible mechanism for beneficial effects of supplementation during laminitis may be that thyroid hormones stimulate vasodilation by increasing β-adrenergic receptor number and sensitivity, which may increase circulation to the laminitic hoof (Breuhaus, 2003). More

research is needed to determine whether thyroid hormone supplementation decreases the number or severity of laminitic episodes, and to determine any harmful physiological effects that may be caused by using thyroid hormone supplements in euthyroid horses.

Implications

Basal thyroid hormone concentrations are not useful as a predictor or symptom of pasture-associated laminitis. Decreases in TT_4 or FT_4 may occur in response to laminitis; however these decreases are not associated with the causation of laminitis.

Table 1. Correlations of physiological parameters

Comparisons	Spearman's correlation coefficient	P-value
Compared with insulin		
TT_4	-0.31	< 0.001
FT_4	-0.24	< 0.001
TT_3:TT_4	0.30	< 0.001
FT_3:FT_4	0.27	< 0.001
Compared with RISQI		
TT_4	0.32	< 0.001
FT_4	0.25	< 0.001
TT_3:TT_4	-0.30	< 0.001
FT_3:FT_4	-0.27	< 0.001
Compared with TBIL		
FT_4	0.25	< 0.001
FT_3	-0.25	< 0.001
FT_3:FT_4	-0.39	< 0.001
Compared with BCS		
TT_4	-0.21	0.001
FT_4	-0.23	0.001
FT_3:FT_4	0.26	< 0.001
TT_3:TT_4	0.27	< 0.001
RISQI	-0.36	< 0.001
Insulin	0.37	< 0.001

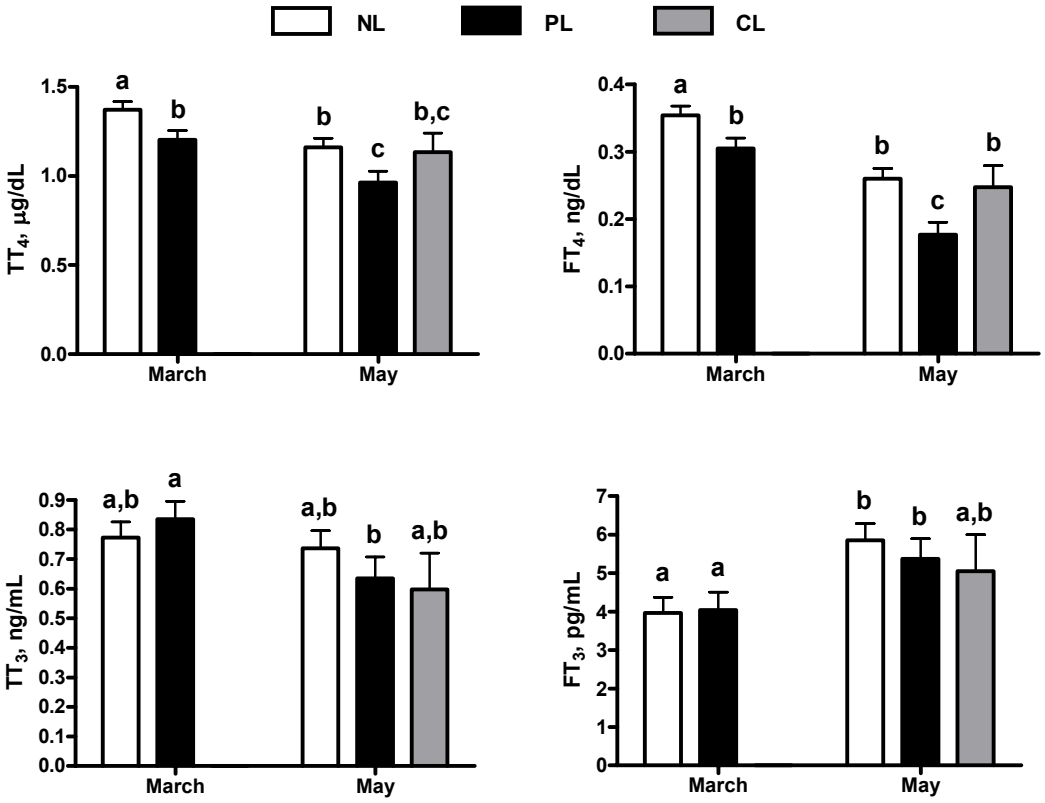

Figure 1. Basal thyroid hormone concentrations in March and May in ponies that have never had laminitis (NL), those that previously had laminitis (PL), and those that developed laminitis in May (CL). Letters denote differences between means ($P < 0.05$).

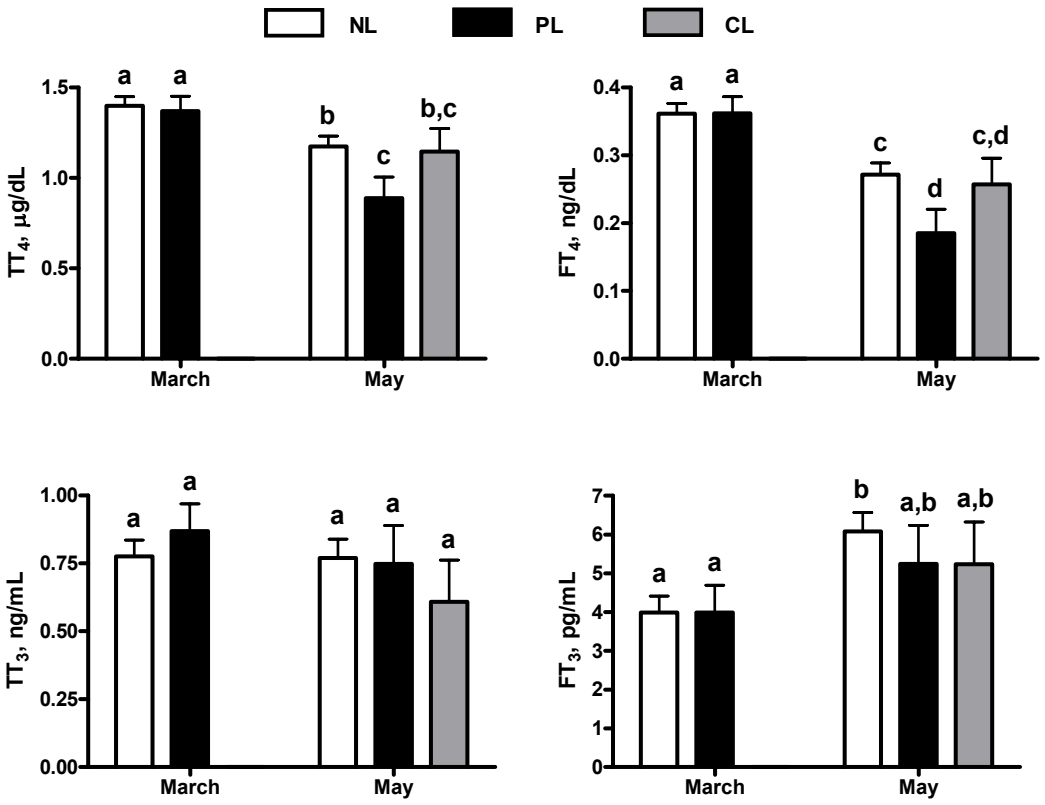

Figure 2. Basal thyroid hormone concentrations in non-pregnant ponies during March and May in ponies that have never had laminitis (NL), those that previously had laminitis (PL), and those that developed laminitis in May (CL). Letters denote differences between means ($P < 0.05$).

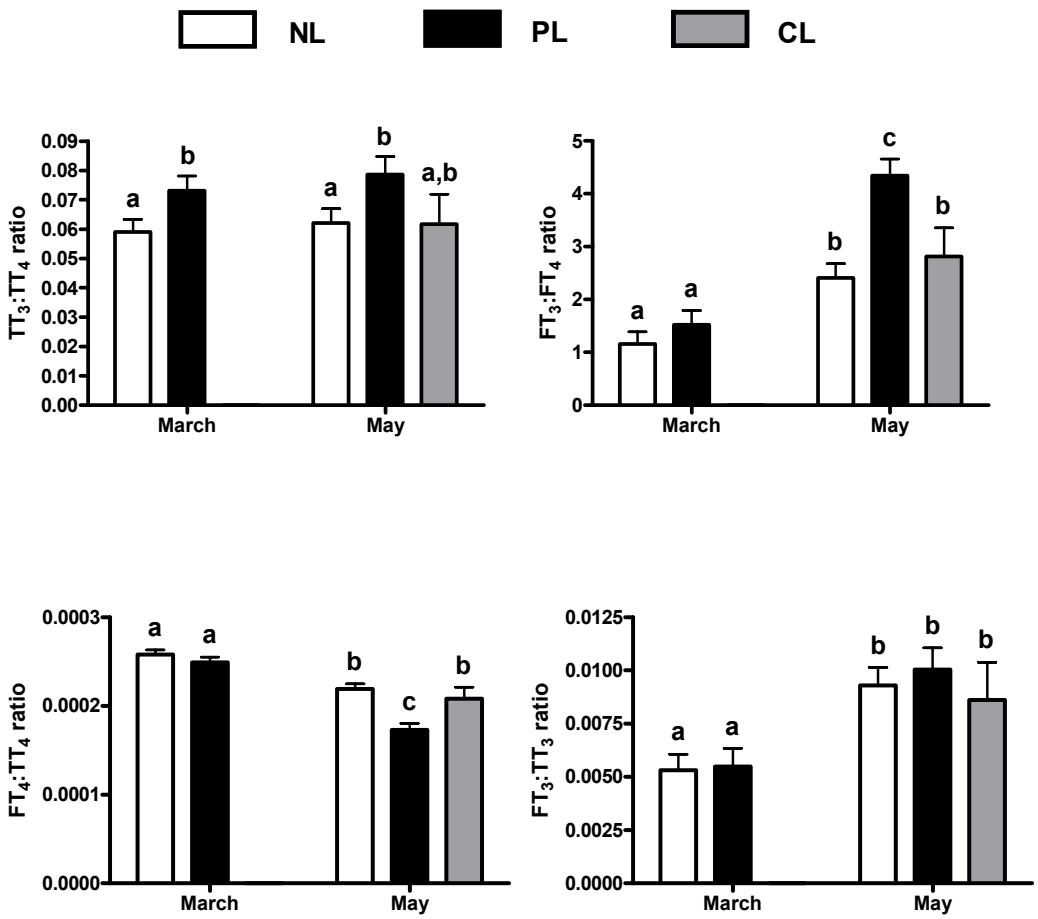

Figure 3. Ratios of thyroid hormone concentrations in March and May in ponies that have never had laminitis (NL), those that previously had laminitis (PL), and those that developed laminitis in May (CL). Letters denote differences between means ($P < 0.05$).

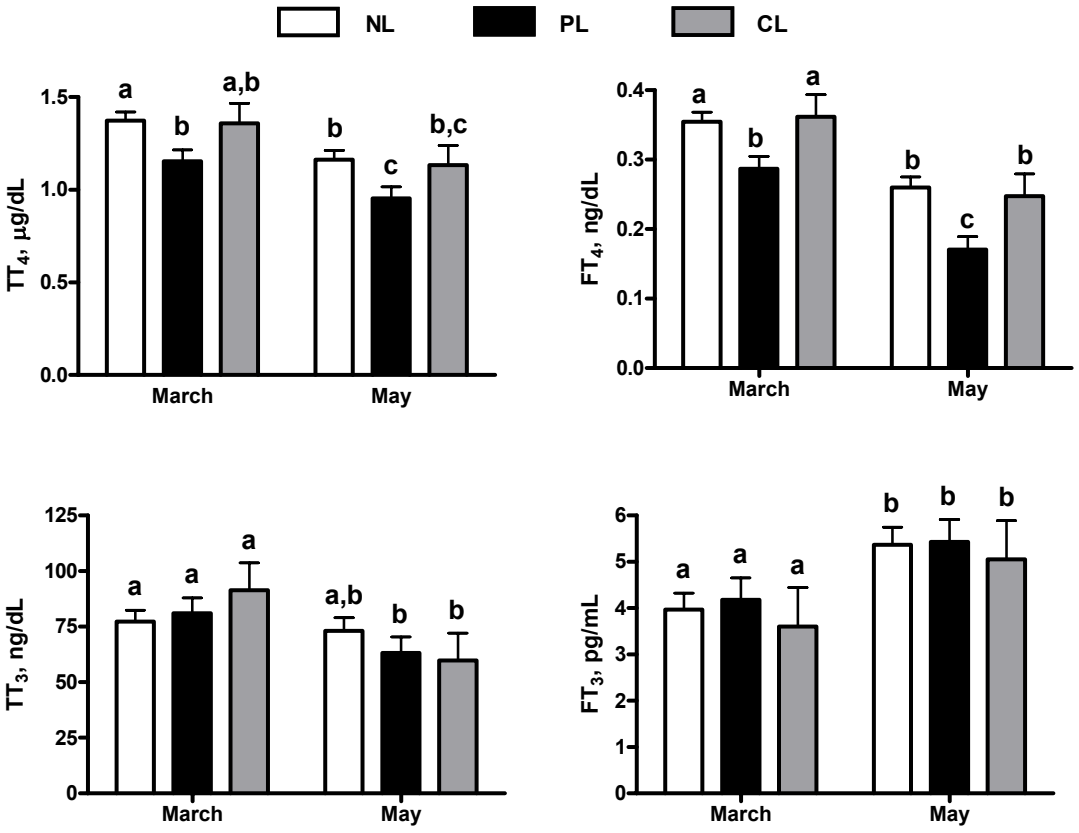

Figure 4. Basal thyroid hormone concentrations in March and May in ponies that have never had laminitis (NL), those that previously had laminitis (PL), and those that developed laminitis in May (CL), with CL ponies separated into a group in March. Ponies that developed laminitis in May were retrospectively placed into the CL group in March, even though symptoms did not appear until May. Letters denote differences between means ($P < 0.05$).

CHAPTER VI

General Summary

The objectives of the present studies were to evaluate and develop methods of assessing thyroid status in horses, assess effects of exercise and feed composition on thyroid status in mature geldings, and assess thyroid status in ponies with pasture-associated laminitis.

The results of the first study indicated that measuring thyroid hormone concentrations in equine plasma using an enzyme immunoassay is a viable method; however results cannot be compared to radioimmunoassay measurements unless values are converted by calibration. The use of TRH response tests to assess thyroid function should include the calculation of the area under the curve or the change in thyroid hormone concentrations, which measure absolute changes in hormone concentrations, making a more critical and accurate evaluation than relative changes in hormone concentrations.

The results of the second study indicated that the assessment of thyroid status should be planned at a time when variables such as exercise will have minimal influence on results, and interpretation of results should take into consideration the effects of training and dietary composition on thyroid status. Horses in training may have elevated T_4 concentrations, whereas horses fed high levels of nonstructural carbohydrates may have greater T_3 responses to a TRH response test.

The results of the third study indicated that basal thyroid hormone concentrations are not useful as a predictor or hormonal characteristic of pasture-associated laminitis. Decreases in total or free T_4 may occur in response to laminitis; however these decreases are not associated with the causation of laminitis. Thyroid hormone concentrations may be associated with metabolic changes or risk factors for laminitis, including insulin sensitivity.

The failure of baseline concentrations to predict thyroid gland activity, the relationships between thyroid hormone parameters and other metabolic parameters, and the failure to detect thyroid dysfunction as a cause for a metabolic disorder indicate that peripheral metabolism of thyroid hormones may be unrelated to thyroid gland activity,

but may be important for causing changes in metabolism. More research needs to be performed focusing on factors that influence peripheral metabolism of thyroid hormones and may interfere with their bioactivity. Also, more research is needed on the role of thyroid hormone action during metabolic disorders, such as laminitis, before the use of exogenous thyroid hormones for their treatment is justified.

CHAPTER VII

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