Effects of levothyroxine administration and withdrawal on the hypothalamic-pituitary-thyroid axis in euthyroid dogs

Vincent A. Ziglioli

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David L. Panciera

William Edward Monroe

Gregory C. Troy

Katie M. Boes

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ABSTRACT

Background: Because of the vague clinical signs and limitations of thyroid function tests, misdiagnosis of hypothyroidism in dogs is common and leads to inappropriate treatment with levothyroxine. Chronic supplementation can suppress the hypothalamic-pituitary-thyroid axis (HPTA) and make it difficult to assess thyroid function following withdrawal of levothyroxine.
Objectives: To determine if the HPTA is suppressed following levothyroxine administration in euthyroid dogs and the time required for resolution of any suppression.
Animals: Twenty-eight healthy euthyroid dogs
Methods: A prospective randomized study administering levothyroxine to euthyroid dogs with levothyroxine, for either 8 weeks (group 1) or 16 weeks (group 2). Serum concentrations of total thyroxine (T_4), free thyroxine (fT_4) by equilibrium dialysis, thyrotropin (TSH), and 3,5,3'-triiodothyronine (T_3) were measured every 4 weeks during supplementation and for 16 weeks after levothyroxine was discontinued.
Results: Mean serum T_4 and fT_4 were significantly higher and TSH was lower in all dogs during levothyroxine administration compared to baseline. Mean serum concentrations of T_4 and fT_4 in both groups and TSH in group 1, beginning 1 week after levothyroxine was discontinued, were significantly different compared to values during levothyroxine administration but not compared to baseline values.
Conclusions and Clinical Importance: Suppression of the HPTA occurred during levothyroxine supplementation and mean serum T_4, fT_4 and TSH concentrations were not significantly different compared to baseline 1 week after discontinuation in both groups. Assessing thyroid function tests 1 week after cessation of levothyroxine will likely provide an accurate assessment of thyroid function in euthyroid dogs.

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LIST OF ABBREVIATIONS

HPTA	hypothalamic-pituitary-thyroid axis
fT_4	free thyroxine
LT4	levothyroxine
SE	standard error
SD	standard deviation
T_3	3,5,3'-triiodothyronine
T_4	total thyroxine
TRH	thyrotropin releasing hormone
TSH	thyrotropin; thyroid stimulating hormone

CHAPTER 1: LITERATURE REVIEW

A. Hypothyroidism

Hypothyroidism is a common endocrinopathy of medium to large breed dogs, primarily affecting middle to older aged animals. Hypothyroidism can result from deficiency of any hormone in the hypothalamic-pituitary-thyroid axis (HPTA), including thyrotropin releasing hormone (TRH) from the hypothalamus; thyroid stimulating hormone (TSH) from the pituitary; or thyroid hormones secreted by the thyroid gland. Primary hypothyroidism, resulting from lymphocytic thyroiditis or idiopathic atrophy accounts for 95% of clinical cases.[1-3] Secondary and tertiary hypothyroidism are rare, caused by either a deficiency of TSH or TRH, respectively, and associated with pituitary tumors or trauma.[4-6] Other uncommon causes include iatrogenic or congenital diseases such as dysgenesis of the thyroid gland or enzymatic deficiency in thyroid hormone synthesis.[4-6]

Dogs affected by hypothyroidism commonly present with diverse and vague clinical signs that may mimic other diseases. In 76% of cases, dogs are reported to have weakness, lethargy and exercise intolerance.[3,7,8] Dermatologic lesions, such as dry skin, changes in hair coat quality, friction alopecia and seborrhea, are present in 60-80% of cases with symmetrical

bilateral alopecia being present 25% of the time.[3,7-9] Other clinical signs include weight gain, peripheral vestibular disease, peripheral neuropathy, cerebellar disease, myxedema stupor or coma, and lipid keratopathy.[10-14]

The biochemical changes of hypothyroidism are non-specific and can be observed with other diseases. Hypercholesterolemia, present in about 75% of hypothyroid cases, is thought to be due to decreased clearance and hepatic uptake along with increased hepatic production.[8,15,16] Approximately 30% of dogs exhibit a non-regenerative anemia with a multifactorial pathogenesis including decreased erythropoiesis resulting from decreased erythropoietin secretion, lack of thyroid hormone stimulation on early hematopoietic cells, and reduction in oxygen distribution to tissues.[8,15-19] Both hypercholesterolemia and non-regenerative anemia are observed in many other diseases such as hyperadrenocorticism, diabetes mellitus, and glomerular diseases.[20,21] Because of the non-specific clinical and biochemical findings in hypothyroid dogs, the diagnosis must not be based solely on clinical suspicion; rather it should be confirmed by appropriate thyroid function tests.

B. Hypothalamic-pituitary-thyroid axis

To properly interpret thyroid function tests, one must have a thorough understanding of the canine HPTA. The HTPA is an intricate and complex hormonal feedback loop. Iodine is an essential element in the synthesis of thyroid hormones and is acquired by dietary absorption of iodide, which is bound to plasma proteins and transported to the thyroid gland. Iodide is actively transported into the thyroid gland via the sodium/iodide symporter, which is found on the basolateral surface of the thyroid follicular cell.[22] This symporter is stimulated by TSH from the anterior pituitary gland.[22,23] After entering the cell, iodide is oxidized to iodine via thyroid peroxidase. This allows the iodination of tyrosine residues on thyroglobulin, a process of organification. The number of iodine molecules incorporated on the thyroglobulin tyrosine residue determines whether it is stored in the colloid as monoiodotyrosine (MIT) or di-iodotyrosine (DIT).[24] The coupling of two DIT molecules forms thyroxine (T_4) where the coupling of one MIT and one DIT forms triiodothyronine (T_3).

Differences between humans and canines exist with respect to iodide requirement and circulating concentrations. The iodine requirement in dogs is approximately 9-11 times higher than in people, because more circulating iodine is excreted rather than reused by the thyroid gland.[24] Dogs also have a plasma iodide concentration that is 12.6 times higher than humans, likely

due to decreased fractional rate of iodide loss from the plasma and increased intake.[24,25]

Thyroglobulin is an iodinated glycoprotein that serves as a synthesis and storage site for thyroid hormones and their precursors. When secretion occurs, thyroglobulin enters the follicular cell through micro and macrocytosis, which involves lysosome fusion with a colloid droplet to form a phagolysosome. TSH stimulates endocytosis of thyroglobulin. Once inside the follicular cell, thyroglobulin is hydrolyzed and releases T_4 and, to a lesser extent T_3, in a 4: 1 ratio. Both hormones are secreted into the blood stream, also regulated by TSH, and the liberated MIT and DIT are deiodinated, thus allowing iodide to be reused.[3,24,26]

The vast majority of thyroid hormones in circulation are bound to plasma proteins.[27] Thyroid hormones bind to thyroxine binding globulin (TBG), albumin, transthyretin and high and low density lipoproteins.[27-31] TBG is the most important binding protein in dogs due to a large plasma concentration and its high binding capacity. Transthyretin is found in lower concentrations and has a higher affinity but lower binding capacity compared with TBP. Additionally, TBG, and to a lesser extent albumin, bind both T_4 and T_3. About 60% of circulating T_4 is bound to TBG[28], 17% bound to transthyretin with about 12% and 11% bound to albumin and lipoprotein respectively. A

small proportion, about 0.1%, of unbound T_4, called free thyroxine (fT_4) in circulation represents the physiologically active portion of thyroid hormone available to tissues. The free fraction is lower and the concentration of bound T_4 is higher in people than dogs because of higher serum protein binding in humans.[25,28]

Peripheral metabolism of thyroid hormones differs among species, but most of the work to elucidate the metabolism of thyroid hormones has been performed in species other than the dog. Thyroid hormones are metabolized by deiodination, conjugated to sulfate and glucuronide, or by having their ether bond cleaved. The main hormone secreted by the thyroid gland is T_4, which is considered a prohormone.[24,27,32-34] T_4 is converted to the more active form, T_3, via deiodination of the outer ring by type 1 and type 2 deidoinases.[35] About 40-60% of T_3 is produced in peripheral tissue by deiodination. Since T_4 is 3-5 times less potent than T_3, T_3 is responsible for most of the effects on the target organs.[36] T_3 exerts its effects by binding to alpha and beta thyroid hormone receptors within the nucleus and can enhance or suppress transcription.[37]

The distribution of deiodinases helps regulate thyroid hormone homeostasis by maintaining consistent levels within a given tissue. This is

performed by activation or deactivation of the thyroid hormones, modulating excretion of thyroid hormones, and contributing to the negative feedback inhibition of the HPTA. Type 1 deiodinase is localized in the plasma membrane of the liver and kidney. It is relatively inefficient at catalyzing the reaction of T_4 to T_3 and contributes primarily to circulating T_3 concentration.[35,37,38] In contrast, type 2 deiodinase, located within the cell including in the nucleus, is efficient at catalyzing T_4 to T_3 and therefore is responsible for controlling the intracellular T_3 concentration.[37] Type 3 deiodinase produces an inactive product of both T_4 and T_3 by catalyzing the deiodination of the inner phenolic ring.[36,37] Type 1 deiodinase, also has the capacity to catalyze the inner phenolic ring of T_4 to produce the inactive rT_3.[35,39]

In the dog, type 1 deiodinase primarily is located in the thyroid, liver and kidney, with similar deiodinase activity between them.[38,40,41] Type 2 deiodinase is located in the cochlea, skeleton, brown fat, pituitary and hypothalamus.[42,43] Type 2 deiodinase is integral in control of TRH and TSH secretion as it contributes to the generation of intracellular T_3 in the hypothalamus and pituitary gland. Type 3 deiodinase is found in limited tissues such as the skin, brain, and uterus, while its distribution is more widespread in fetal tissues, limiting the exposure of the fetus to thyroid

hormones.[38,44] Other thyroid hormone metabolic pathways include glucuronidation and sulfation.[31,45 46] In the dog, about 55% of T_4 and 30% of T_3 is excreted in the bile where urinary excretion appears to have little contribution to the overall hormone elimination.

Before thyroid hormones can exert their effects on target organs, they enter the cell through carrier-mediated active transport, once thought to be a passive process.[47-49] Thyroid hormone transporters include organic anion transporting polypeptides (OATPs), amino acid transports, and the monocarboxylate transporters MCT8 and MCT10.[50-54] T_4 and T_3 bind to the thyroid hormone receptors on the plasma membranes, enter the cell, and bind to their respective intracellular receptors. Active transport, requiring both ATP and Na, are involved in transporting thyroid hormones across the plasma membrane.[55] The distribution of the thyroid hormone transporters is necessary for maintenance of tissue thyroid hormone concentrations and hence normal development in utero. This is evidenced by a well-characterized syndrome in people known as Allan-Herndon-Dudley syndrome, which leads to severe psychomotor retardation in affected patients.[56,57] This syndrome is the result of mutations in the MCT8 gene, reducing thyroid hormone transport into cells ultimately depriving neural

tissue of T_3, which is essential for the normal development of the nervous system.[53]

Thyroid hormone secretion is regulated by negative feedback in the HPTA. Free thyroid hormone, mainly T_3 but also T_4, is inhibitory to the hypothalamus and pituitary. However, intracellular T_3 derived from the activity of type 2 deioindinase on T4, seems to be more important in activating the T_3 nuclear receptor beta, compared to circulating T_3.[58] This in turn inhibits TRH and TSH secretion from the hypothalamus and pituitary thyrotropes, respectively.[59,60] Iodine itself also appears to be inhibitory, with high concentrations of iodine inhibiting thyroid peroxidase mRNA and protein synthesis, a phenomenon commonly known as the Wolff Chiakoff effect.[61] These cellular mechanisms are in place to avoid states of excessive or low circulating thyroid hormones, which can result in detrimental consequences in the affected target organs.

C. Thyroid function tests in Dogs

Thyroid function testing involves measuring various hormones in the serum, such as T_4, fT_4, T_3, free triiodothyronine (fT_3) and TSH. Thyroid function tests should be performed in cases where there is a high degree of

clinical suspicion for hypothyroidism since numerous factors affect their accuracy. Limitations exist with each test, since concurrent diseases, medications or other factors including biologic variation or innate characteristics such as age and breed can affect circulating thyroid hormone levels. As such, thyroid function tests should not be used as screening tests for asymptomatic patients, but rather be carefully chosen to minimize misdiagnoses and subsequent inappropriate supplementation for canine hypothyroidism.

The most commonly used laboratory test for diagnosis of hypothyroidism in dogs is the serum T_4 concentration due to its availability and low cost. It has a high sensitivity, with a reported range of 89% to approaching 100% and as such, a serum T_4 concentration within the reference interval can exclude hypothyroidism in the majority of cases.[8,62] However, with only a fair specificity of 75-82%, finding a serum T_4 concentration below the reference range may result in an inappropriate diagnosis of hypothyroidism 18-25% of the time.[8,62]

There are notable limitations with using low serum T_4 concentration as a definitive diagnosis for hypothyroidism. Biological variation of up to 17.3% in the circulating serum T_4 concentration reduces accuracy of the test.[63] Random fluctuations are more common in euthyroid dogs with atopic

dermatitis compared to healthy euthyroid dogs but the study failed to identify a circadian rhythm of thyroid hormone secretion in a large population of dogs.[64] However, conflicting data from a smaller number of healthy dogs found lower serum T_4 levels in the morning (8am) compared to mid-day (between 11am and 2pm) values.[65,66] Lastly, there are significant effects of non-thyroidal illnesses and administration of certain drugs on circulating T_4, with up to 30% of euthyroid dogs with non-thyroidal illnesses having a serum T_4 concentrations below the reference interval.[67,68]

Increased serum TSH concentrations are expected in hypothyroid dogs due to the lack of negative feedback of thyroid hormones, making it an ideal thyroid function test. However, 24-37% of hypothyroid dogs have serum TSH concentrations within the reference interval.[8,62,69,70] Additionally, there are marked daily fluctuations in TSH concentrations ranging from 38% to 59%.[71,72] If measured alone, the sensitivity of endogenous TSH serum concentration is approximately 63-76%.[8,62,69,70] The specificity is higher (81-93%),[8,62,67] and if combined with T_4 or fT_4, specificity approaches 98%.[67,73]

FT_4 accounts for less than 1% of circulating T_4 and represents the unbound, biologically active form found in serum. Since it is not bound to plasma proteins, it is able to leave the circulation to enter cells, bind to

thyroid hormone receptors and produce the biological effects within that cell. Theoretically, serum fT4 concentration should be a better reflection of thyroid function compared to T_4, but its low serum concentration makes it difficult to measure. FT_4 is the most sensitive and specific single test for diagnosing canine hypothyroidism when measured using equilibrium dialysis.[62,74,75] Radioimmunoassay (RIA) or chemiluminescent immunoassay (CLI) are less reliable and have no distinct advantage over total T_4 measurement.[75,76] Equilibrium dialysis (ED) uses a semi-permeable membrane that allows passage of small fT_4 molecules but not transport proteins or circulating thyroid autoantibodies. A limitation of fT_4 in the diagnosis of canine hypothyroidism is the biologic variation is about 24.3% due to a circadian pattern and is suggested it be measured between 11-2pm since levels tend to be lower in the morning.[63,66]

Measurement of serum T_3 is not currently recommended for the diagnosis of canine hypothyroidism. Although it is the most potent thyroid hormone at the cellular level, about 40-60% of T3 is produced in extrathyroidal tissues.[27] In addition, since most T_3 is located intracellularly, serum T_3 concentration does not reflect thyroid function.[3] It has a wide reported range of sensitivity and specificity of 10-52% and 45-92%, respectively with poor accuracy of 47-55%.[62,64] This is understandable since significant daily fluctuations in T_3

exist and no significant difference in concentrations are reported between hypothyroid, euthyroid and dogs with non-thyroidal illnesses.[62,64,77]

Other tests used in the diagnosis of canine hypothyroidism are the TSH and TRH stimulation tests, in which the responsiveness of the thyroid and pituitary glands are evaluated. The TSH stimulation test uses exogenous TSH, which is not species specific, and helps differentiate non-thyroidal illnesses and hypothyroidism in most cases.[62,73,78,79] This test is currently considered the gold standard for the diagnosis of canine hypothyroidism but recently, thyroid scintigraphy has been suggested to be superior at differentiating hypothyroidism from euthyroid sick dogs.[80] The TRH stimulation test provides little advantage over measuring basal serum thyroid hormone concentrations. This test is difficult to interpret since exogenous TRH administration causes relatively small changes in serum T_4 in normal dogs.[73] Although measuring the change in TSH response after administering TRH can identify hypothyroid dogs with an accuracy of 90%, it has little advantage over measuring baseline TSH and total or fT_4.[73,81] However, it can differentiate secondary and tertiary forms of hypothyroidism from primary disease.[73] Because secondary and tertiary diseases are rare, the TRH response test is rarely used.

Ten to 30% of hypothyroid dogs with circulating thyroglobulin autoantibodies also have circulating antibodies to T_4 and T_3.[68,82,83] The presence of these antibodies render invalid serum T_4 by all assays and fT_4 results determined by CLI or RIA. Circulating autoantibodies to T_4 and T_3 cause a false elevation of the hormone measurement in most assays, but fT_4 is not altered if measured using an equilibrium dialysis assay.[83,84] Autoantibodies to T_4 or T_3 are present in 2% of dogs with clinical signs of hypothyroidism and 15% of dogs diagnosed with hypothyroidism.[85,86]

Aside from factors affecting hormone measurements, there are endogenous and exogenous variables that can affect test results. Older dogs tend to have serum T_4 concentrations 21-40% lower than young dogs,[87-89] while serum TSH concentration increases with age. Serum T_3 is minimally affected by age.[88,90] The exact cause for changes in hormone concentrations as a dog ages has yet to be determined but may be due to the propensity for concurrent illnesses, subclinical thyroid disease, or change in responsiveness of the thyroid gland to TSH.

Breed specific differences in reference intervals for T_4 and fT_4 also exist. Most notably sighthounds have lower serum T_4 and fT_4 concentrations than other breeds.[91] For example, serum T_4 and fT_4 are below general population reference intervals in 91% and 21% of Greyhounds respectively.[91] Other

breeds documented to have serum thyroid hormone concentrations below general population reference intervals include Whippets, Salukis, Basenjis, Wolfhounds, English Setters, Golden Retrievers, Samoyeds, and Keeshond among others.[91-93] Ideally, breed specific reference intervals should be used when evaluating thyroid function tests for more accurate assessment. Additionally, it is recommended to evaluate circulating TSH levels concurrently when suspecting hypothyroidism.

Non-thyroidal illnesses can influence thyroid function tests and result in misdiagnosis of hypothyroidism. Serum T_4 is usually decreased to a larger extent than fT_4, and TSH is increased in 3-8% of dogs with non-thyroidal illness.[67,68] The effects of non-thyroidal illness on thyroid hormone concentration reflects the severity rather than specific disease.[67] Therefore, dogs with a more severe disease may have more pronounced thyroid hormone suppression than dogs with mild disease.

In addition, dogs with non-thyroidal illnesses may be receiving medications that affect thyroid function tests. Well-known effects of glucocorticoids, sulfonamides, tricyclic antidepressants, and aspirin have been documented with varying underlying mechanisms, where most other NSAIDs such as etodolac, deracoxib, ketoprofen, carprofen and meloxicam cause no significant change in serum thyroid hormone levels.[94-106]

Phenobarbital tends to decrease both T_4 and fT_4 by increasing the metabolism and excretion of T_4 and has little to no effect on circulating TSH initially, while prolonged use tends to cause an elevation in the TSH.[100,101,107] Glucocorticoids decrease TSH secretion, decrease binding of T_4 to carrier proteins, alter the clearance and metabolism of the hormone and decrease conversion of T_4 to T_3 at peripheral sites, ultimately decreasing circulating T_4 concentrations. The degree of thyroid hormone suppression differs based on the dose, the route of administration, and the duration of treatment.[102-105] Lastly, sulfonamides can impair thyroid hormone synthesis by inhibiting TPO, which is responsible for oxidation of iodide and iodination of tyrosine residues on thyroglobulin, leading to a decrease in plasma T_4 and a resultant increase in TSH.[108-110]

D. Hypothalamic-pituitary-thyroid axis during and after thyroid hormone supplementation in dogs and people

The normal HTPA is altered by thyroid hormone supplementation. Because of its potential effects on diagnosis of hypothyroidism, it is important to understand how thyroid hormone administration and subsequent withdrawal alters thyroid function tests. Most studies evaluating

the dynamics of thyroid hormone supplementation on the HPT axis have been in people. Very little information is available in dogs.

There are a number of situations where thyroid hormone supplementation is used intentionally in euthyroid humans. In children with complicated congenital heart defects corrected surgically, T_3 concentrations fall post-operatively, so treatment with T_3 is given, which improves cardiovascular hemodynamics and renal perfusion.[111,112] In addition, people with congestive heart failure may exhibit changes in gene expression similar to hypothyroid individuals and may benefit from thyroid hormone supplementation, since cardiac output would increase and systemic vascular resistance would be reduced.[113,114] However, supplementation with levothyroxine in dogs with congestive heart failure did not affect survival compared to placebo.[115] Differentiated thyroid carcinoma contains TSH receptors and as such, TSH stimulates growth of the carcinoma. [116] Supplementation with thyroid hormone to suppress TSH is used in humans to inhibit growth of thyroid carcinoma. In a study over 30 years, 25% fewer patients that had undergone thyroidectomy for primary neoplasia had recurrence of carcinoma while on thyroxine therapy than unsupplemented individuals.[117] In addition, supplementation with thyroxine in humans with nontoxic goiter may reduce goiter volume by decreasing serum TSH

concentration, since TSH is the main stimulator of thyroid tissue growth.[118,119]

The HPTA is controlled by feedback inhibition at all levels. At the cellular level in the hypothalamus, different isoforms of the thyroid receptor (TR), alpha and beta, are responsible for the increased or decreased gene expression of TRH respectively. More specifically, when T_3 binds to the TRbeta1 or 2 isoform, the expression of TRH is decreased.[59,120] This inhibition occurs rapidly, with suppression of the TRH gene within 5 hours of exogenous thyroid hormone administration.[121] In addition to circulating T_3 suppressing TRH secretion, circulating T_4 is converted to T_3 in the hypothalamus by deiodinase type 2.[36] In this manner, T_4 acts as a regulatory signal to the hypothalamus in states of high or low circulating thyroid hormones.[122]

Similarly, in the pituitary, thyroid hormones also have a direct effect on TSH secretion. Not only does the circulating level of T_3 affect TSH in the same manner as in the hypothalamus, but also studies support the importance of T_4 conversion to T_3 in the regulation of TSH at the pituitary level.[123] Euthyroid individuals were given iopodate, a contrast agent that blocks the conversion of T_4 to T_3, prior to infusion of either T_4 or T_3. The individuals given both T_4 and iopodate had comparable levels of TSH to

untreated euthyroid individuals. However, administration of iopodate and T_3 suppressed TSH levels supporting the greater importance of T_3 inhibition on the pituitary.[124] Plasma TSH is regulated by TRH secretion by the hypothalamus. There are TRH G protein-coupled receptors in the pituitary thyrotropes, with calcium acting as a second messenger.[125,126] Activation of TRH receptors increases TSH secretion. After secretion into the hypothalamic-hypophyseal portal system, TRH can be degraded by a cell surface peptidase known as TRH degrading ectoenzyme, which is up regulated by T_4.[127-129]

In addition to thyroid hormones acting directly to influence TRH and TSH expression, TSH influences its own secretion and that of TRH via binding to TSH receptors in the hypothalamus and pituitary. [130,131] This ultra short loop control involves TSH inhibiting the subsequent secretion of TSH in the pituitary.[130] A similar mechanism may influence TRH secretion by the hypothalamus via TSH receptors, but the physiologic mechanism is not clearly understood.[131] Other substances that control TSH secretion include dopamine, somatostatin and cytokines such as IL-1β and IL6, which inhibit TSH secretion, and alpha-adrenergic agonists and opioids, which stimulate TSH secretion.[132-135]

The suppressive effects of exogenous thyroid hormone and subsequent recovery of the HPT axis after discontinuation is documented in humans. The pattern of recovery after withdrawal of thyroid hormone is variable and is affected by factors that include duration of treatment, serum T_4 and T_3 concentration during supplementation, type and dose of supplementation used and other factors such as age and concurrent disease.[136-139] Understanding the pattern of recovery is pivotal in patients inappropriately supplemented with thyroid hormone replacement since knowing the ideal time to assess thyroid function after cessation of treatment is important for proper interpretation.

In general, levothyroxine supplementation causes suppression of TSH, which results in decreased thyroid hormone synthesis and secretion. The continual suppression of TSH secretion results in atrophy of the thyrotropes and the low TSH causes thyroid gland atrophy. When supplementation is discontinued, an initial drop in serum thyroid hormones is followed by an increase in serum TSH concentrations that precedes a rise in T_4 and T_3. The physiologic mechanisms of how supplementation affects the HPTA and recovery of the axis after withdrawal of treatment are discussed below.

The hypothalamus is affected by exogenously administered thyroid hormones, which affects sites such as the pituitary and ultimately the thyroid

glands. Both T_4 and T_3 suppress TRH production at the transcriptional level within the hypothalamic paraventricular nucleus.[140,141] TRH plays a critical role in the HPTA and with TRH deficiency there is a decrease in TSH biosynthesis, which causes decreases in thyroid hormone production.[142]

Administration of exogenous thyroid hormones suppresses TSH secretion within 1 hour of administration, with no difference observed between euthyroid and hypothyroid individuals.[143] However, the dose and half-life of the thyroid hormone administered influences the time at which TSH escapes suppression.[143] In one study[144], the higher the dose of T_3 administered, the longer it took for TSH to normalize after discontinuation of supplementation. Also, with T_4 having a longer half-life compared with T_3, the time TSH escaped suppression was greater than 100hr vs 40hr respectively. [143-145] The initial TSH suppression is likely due to inhibition of secretion of preformed TSH. This finding is supported by the exaggerated response to TRH-mediated TSH secretion within the first 1 to 2 days of thyroid hormone replacement.[146] The subsequent suppression is due to a decrease in biosynthesis of TSH and reduction of pituitary TRH receptors[147] resulting in blunted TRH-mediated TSH secretion after chronic T_4 hormone supplementation.[148] If thyroid hormone supplementation is continued

indefinitely, constitutive TSH secretion maintains low plasma TSH concentrations.[143]

Chronic thyroid hormone supplementation may also influence both intrathyroidal and extrathyroidal thyroid hormone metabolism. In general, high circulating thyroxine levels down regulate D2 activity and low circulating thyroxine levels up regulate D2 activity in extrathyroidal tissue.[149] In the thyroid gland, TSH up regulates D1 and causes increased T3 production.[150,151] In theory, a low TSH concentration results in the negative feedback of exogenous T4 which down regulates D1 activity within the thyroid. In thyroidectomized people supplemented with thyroxine, there is an increase in D3 activity, which increases T4 and T3 clearance and rT3 production and a decrease in D1 and D2 activity, which reduces T3 production. The ultimate goal is prevention of harmful effects of excessive circulating thyroid hormones.[152]

Abrupt cessation of thyroid hormone supplementation in euthyroid humans results in an initial drop in the serum T_4 and T_3 concentrations with an eventual rise to pretreatment concentrations. Serum T_4 and T_3 concentrations can decrease below reference intervals 1 to 3 weeks after withdrawal of levothyroxine and remain low for several weeks or longer.[137,139] Serum concentrations of T_4 and T_3 can increase to within

respective reference intervals as soon as 3 weeks after stopping treatment but more prolonged suppression is typical.[137,139,153] However, individual variation exists with some individuals experiencing a decrease of serum T_4 while T_3 concentrations remain within the reference interval.[137,139] The age of the patient as well as the dose and duration of supplementation may influence the variability in serum T_4 and T_3 concentrations after discontinuing thyroid hormone supplementation.[137,139,153]

The pattern of recovery of TSH after discontinuation of thyroid hormone supplementation in euthyroid individuals provides insight into recovery of the HPTA. Serum TSH begins to rise from very low concentrations within the first 2 weeks after withdrawal, but inappropriately low compared to serum T_4 and T_3 concentrations.[137-139] For at least 6 weeks after withdrawal of levothyroxine treatment, TSH concentrations in euthyroid individuals may remain below the elevated levels found in hypothyroid individuals.[137,139] During this time, a transient period of unresponsiveness or inappropriately reduced TSH responsiveness to exogenous TRH administration occurs despite subnormal serum T_4 and T_3 concentrations. This finding may persist for several weeks or longer after thyroid hormone withdrawal and can be observed whether the etiology of TSH suppression results from exogenous or endogenous thyroid

hormones.[137,139,153-155] The reason for the inappropriate and prolonged decrease in TSH and response to TRH administration is thyrotroph atrophy and decreased bioactivity of TSH.[156-159]

Little information is available regarding the effect of levothyroxine administration on the HPTA in euthyroid dogs. However, similarities exist between the dog and human in recovery of the thyroid hormone profile and histologic changes during and after thyroid hormone supplementation. In 10 euthyroid dogs supplemented with levothyroxine twice a day at 0.5mg/m^2, suppression of the T_4 response to TSH was identified at 4 weeks and to TRH stimulation at 6 weeks. This finding persisted throughout the 8 weeks of supplementation.[160] In contrast, 5 euthyroid dogs supplemented with 0.4mg twice per day of levothyroxine had no documented suppression during a 5-week supplementation period.[161] The reason for this difference may be that the dose was not high enough to suppress the HPTA. This study did not state the time of sampling after administration nor the mg/kg dose. Measurement of circulating TSH concentration could have aided in evaluating the level and degree of thyroid hormone suppression. In people, there is a characteristic decrease in TSH once supplementation is started and the level of circulating T_4 has an effect on the degree of suppression.[143,144]

Once supplementation of levothyroxine was discontinued in the study by Panciera, et al, thyroid hormone response to TRH returned to normal in all remaining six dogs. However, two of the dogs had subnormal T_4 response to TSH at the conclusion of the study, supporting the variability in the pattern of recovery as observed in people. Unfortunately, the pattern of recovery for TSH was not addressed because an assay for canine TSH was not available. In people, it appears that serum concentrations of T4 and T3 return to normal prior to the TSH normalizing and it is hypothesized that this observation will occur in dogs.[137-139]

Histologic evaluation of euthyroid dogs supplemented with levothyroxine helps support the physiological effects that occur in the HTPA.[162] During supplementation, dogs experience atrophy of thyrotropes demonstrating negative feedback inhibition within the HPTA. Circulating thyroid hormones decrease TRH and TSH synthesis by decreasing mRNA synthesis or transcription and cause a reduction of the TRH receptors on the pituitary gland.[130,131,143] Atrophy of the thyroid gland was also found including decreased epithelial volume density, height, and increased colloid volume density. This is likely caused by the decreased trophic effects of circulating TSH due to the suppressive effects of exogenous thyroid hormone supplementation.[158] After discontinuation of thyroid hormone

supplementation, serum thyroid hormones normalized with histological changes in the thyrotropes supportive of increased TSH secretion.[159,162] The histologic changes seen in the pituitary indicate that even after normalization of serum thyroid hormones there is still some degree of thyroid atrophy or subnormal responsiveness of the thyroid gland to TSH, and thus the HTPA has not completely recovered. Therefore, care must be taken in evaluating and diagnosing hypothyroidism, as thyroid hormone supplementation in euthyroid dogs or people can result in significant and variable changes to the HTPA, making the time at which the circulating serum thyroid hormones normalize unpredictable.

E. Pharmacodynamics and pharmacokinetics of thyroid hormone supplementation in dogs

Thyroid hormone supplementation is available in numerous formulations ranging from synthetic hormone to naturally occurring extracts. In veterinary medicine, the synthetic forms of thyroid hormone supplementation are used almost exclusively, with the tablet formulation being the most common in the United States. Dosages for dogs are higher than those used in humans due to a shorter circulating half-life observed in

dogs. The average half-life in people is approximately 1 week compared to the average of 7-15 hours in dogs, is due to a higher fecal excretion. In addition, there is a lower oral bioavailability in dogs compared to humans.[25,27] The oral bioavailability of 10-50% compared to 80% in people contributes to the higher dose necessary in dogs.[25,163]

The pharmacokinetics of oral synthetic thyroxine supplementation are similar between euthyroid and hypothyroid dogs, with innate variability amongst individuals.[164-166] The half-life ranges from 7-15 hours and is affected by dose, with higher doses causing a shorter half-life by increasing the metabolism or excretion.[164,165,167,168] The time to maximum serum T_4 concentration is approximately 4 hours, with a range from 1.5 to 6 hours after administration.[164,165,167,168] The bioavailability between individuals and more importantly between formulations varies greatly.[165] The oral liquid formulation of levothyroxine, Leventa®, has a bioavailability twice that of a tablet formulation of levothyroxine.[165] However, a different liquid formulation studied in Australia has a comparable bioavailability to the tablet formulation, with the liquid form having a 10% higher bioavailability.[168] Bioavailability is increased by fasting compared to administration with food, ultimately causing a shorter Tmax (mean of 2.5 hours compared to 5 hours), increased Cmax (mean 76 nmol/L compared to

42nmol/L) and a shorter half life (11.4 hours compared to 14.1 hours).[165] However, if given with food and the appropriate control is achieved, dietary changes have negligible effects on the pharmacokinetic parameters Tmax and Cmax, in dogs.[169]

Once daily administration of levothyroxine is adequate to resolve clinical signs of hypothyroidism in most dogs.[167] Also, it appears that steady state can be reached on the first day of treatment with once daily dosing of Leventa® or twice daily dosing of Soloxine®, as the pharmacokinetic parameters were similar on the first day and day 14 of treatment in euthyroid dogs.[165] Many practitioners advocate for twice daily dosing due to the optimal physiologic control of T_4 serum concentrations by evidence of pharmacokinetic studies using once verse twice daily dosing at different dosages. Higher peak and lower trough levels occur with once a day dosing compared to the same dose divided twice daily. Although there is more fluctuation of the serum T_4 concentration with once daily dosing, serum T_4 remains above the lower reference interval for 12-24 hours.[164] Once daily dosing maintained the T_4 serum concentration above the reference interval longer than the same dose divided twice daily.[164] It reported that the liquid formulation, Leventa®, at once daily dosing maintains a T_4 above the lower limit of the reference interval for at least 24 hours.[165] Although serum T_4

reaches a higher Cmax and there are more fluctuation of serum T_4, once daily administration is adequate for most dogs for the following reasons.[85,164-166,170] T_4 is highly protein bound which serves as a reservoir for thyroid hormone concentrations.[167] Thyroid hormones exert their physiologic effects by binding to nuclear receptors that cause transcription and subsequent translation, likely having a prolonged duration of action persisting beyond the measured serum hormone concentrations.[164,168] Lastly, treatment once a day may be associated with increase compliance and ultimately better overall control of hypothyroid patients.[167]

F. Monitoring treatment of hypothyroidism and side effects of hormone supplementation in dogs and people

Monitoring serum T_4 concentration and clinical response is recommended following thyroid hormone supplementation therapy in dogs.[167] In people, TSH is typically used to monitor thyroid hormone therapy and is shown to be more sensitive than fT_3 and fT_4 allowing subtle dose adjustments.[171] It appears that a persistently elevated TSH in hypothyroid dogs treated with levothyroxine is associated with inadequate supplementation and persistent clinical signs.[167] Response to treatment is

generally seen within the first two weeks and involves improvement in the metabolic signs such as lethargy and mental dullness followed by weight loss.[167] Dermatologic abnormalities generally take several weeks to months to resolve.[7,167]

As mentioned before, hypothyroidism presents a diagnostic challenge and inappropriate supplementation of euthyroid dogs may have long term deleterious effects. In a broader sense, thyroid hormones play a crucial role in differentiation, growth and metabolism.[172] Therefore, inappropriate supplementation can result in a wide variety of physiological effects that may cause side effects.

Thyrotoxicosis can be caused by exogenous thyroid hormone supplementation. The effects are specific to the target tissues such as the bone, heart, and blood and can cause a variety of unwarranted consequences. People who experience long-term over supplementation have decreases in bone density with an increase risk of fractures.[173-175] Older individuals that are over-supplemented with levothyroxine have an odds ratio of 1.88 (of death) compared to others that were previously treated.[174] Patients receiving levothyroxine supplementation experienced an increase risk of dysrhythmias and cardiovascular morbidity and mortality.[174] Lastly, euthyroid people and people with subclinical thyrotoxicosis also experience hemostatic risks that

appear to be dose dependent. Levothyroxine supplementation increases levels of vWF, factor VIII, FIX, and FX and inhibits fibrinolysis thereby increasing the risk of venous thrombosis.[176,177] Therefore, inappropriate thyroid hormone supplementation can result in untoward consequences in euthyroid patients.

G. Conclusion and research justification

Dogs suspected of hypothyroidism are often administered levothyroxine without a definitive diagnosis. This occurs because measurement of serum total thyroxine (T_4) concentration, a commonly utilized thyroid function test, has limited specificity and is influenced by drugs and concurrent illnesses.[8,62,67,68,178] When confirming a diagnosis of hypothyroidism in a dog receiving levothyroxine, it is necessary to withdraw treatment prior to thyroid function testing. Because levothyroxine administration suppresses the hypothalamic-pituitary-thyroid axis (HPTA), thyroid function tests are altered after cessation of therapy.[160]

This proposed study will investigate the time for recovery of the HPTA after it is suppressed by levothyroxine administration in euthyroid dogs. We anticipate identifying a minimum length of time after

discontinuing chronic levothyroxine treatment that will allow accurate evaluation of thyroid function tests to distinguish iatrogenic thyroid atrophy from hypothyroidism. Our first hypothesis is that levothyroxine administration will suppress the HPTA in euthyroid dogs, with the degree of suppression coinciding with the length of treatment. A second hypothesis is that the duration of suppression after levothyroxine withdrawal will be the same regardless of whether dogs are treated for 8 or 16 weeks, and that the HPTA will recover within 8 weeks.

CHAPTER 2: EFFECTS OF LEVOTHYROXINE ADMINISTRATION AND WITHDRAWAL ON THE HYPOTHALAMIC-PITUITARY-THYROID AXIS IN EUTHYROID DOGS

A. Introduction

Dogs suspected of hypothyroidism are sometimes administered levothyroxine without a definitive diagnosis. This can occur because measurement of serum total thyroxine (T_4) concentration, a commonly utilized thyroid function test, has limited specificity and is influenced by drugs and concurrent illnesses.[62,67,68,74,178] When evaluating a diagnosis of hypothyroidism in a dog receiving levothyroxine, it is necessary to withdraw treatment prior to thyroid function testing. Because levothyroxine administration suppresses the hypothalamic-pituitary-thyroid axis (HPTA), thyroid function tests may be altered after cessation of therapy.[160]

Thyroid hormone replacement therapy in euthyroid patients suppresses hypothalamic and pituitary function by negative feedback of thyroid hormones on thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (thyrotropin; TSH).[137,139,158,159] Chronic suppression of the HPTA will result in pituitary thyrotrope atrophy and subsequently, thyroid gland atrophy and impaired secretion of thyroid hormones.[137,153,159]

In humans, withdrawal of therapy after prolonged treatment can result in serum thyroid hormones and TSH concentrations below their respective reference ranges, with the length and degree of suppression influenced by the type, dose and duration of replacement therapy.[136-139] Additionally, thyroid function tests can be affected for months after long-term thyroid hormone administration. During recovery from suppression, serum TSH concentration increases prior to thyroid hormones and can result in hormone levels similar to those found in primary hypothyroidism. Studies evaluating the effects of levothyroxine administration on the HPTA in euthyroid dogs are conflicting.[160,161] Levothyroxine administration to healthy dogs for 5 weeks did not suppress T_4 response to TRH administration in one study[161], while another study documented complete suppression of TRH induced T_4 secretion after 6 weeks of treatment. More importantly, suppression of the thyroid hormone response to TSH persisted 4 weeks after withdrawal of levothyroxine treatment.[160]

The suppressive effects of thyroid hormone therapy and subsequent recovery of the HPTA presents a diagnostic challenge when evaluating thyroid function. The influence of dosage, duration of treatment, and effects on serum TSH concentrations of levothyroxine administration have not been studied in the euthyroid dog. We evaluated the degree and duration of

suppression of the HPTA that occurs after chronic administration to euthyroid dogs in order to identify when thyroid function tests accurately document euthyroidism after withdrawing supplementation. We hypothesized that levothyroxine administration would suppress the HPTA in euthyroid dogs and that the HPTA would recover within 8 weeks in all dogs, regardless of the duration of treatment.

B. Methods and Materials

Dogs

This study was approved by the Institutional Animal Care and Use Committee at Virginia-Maryland College of Veterinary Medicine and by the Veterinary Teaching Hospital Board. This was a prospective, randomized study performed at the Virginia-Maryland College of Veterinary Medicine between July 2014 and May 2015. Dogs enrolled in this study were recruited from faculty, staff, and students. Inclusion criteria included dogs 1-7 years of age with body weight greater than 5 kg that were documented to be healthy based on results of history, physical examination, complete blood count, serum biochemistry, urinalysis, and serum concentrations of T_4 and TSH. Dogs were excluded from the study if they were a sighthound breed, had been diagnosed previously with hypothyroidism or another chronic

disease, or received medication known to affect thyroid function (glucocorticoids, phenobarbital, sulfonamides, and tricyclic antidepressants) within 2 months of enrollment in the study. Dogs with a serum T_4 concentration below the reference interval or a TSH concentration above the reference interval were excluded.

Treatment and sampling

Twenty-eight dogs enrolled in the study were randomly assigned to one of two equal groups using number generated randomization.[a] Dogs in group 1 received levothyroxine for 8 weeks (denoted as week 1-8 of the supplementation period), and those in group 2 received levothyroxine for 16 weeks (denoted as week 1-16 of the supplementation period). Levothyroxine (20-26 μg/kg rounded to the nearest 0.1 mg) was dispensed to the owner to be administered orally every 24 hours 30 minutes prior to a meal.

Prior to administration of levothyroxine, 2 blood samples for measurement of serum concentrations of T_4, free thyroxine (fT_4), 3,5,3'-triiodothyronine (T_3), and TSH were obtained 1 week apart, with the mean concentration of each analyte serving as the pretreatment baseline value for each dog. One week after initiating treatment, a blood sample was obtained

4-6 hours after levothyroxine administration for measurement of serum T_4. If the serum T_4 concentration was outside the target therapeutic range (40-70 nmol/L), the levothyroxine dose was increased or decreased by 25% or to the nearest tablet size. The serum T_4 concentration was re-evaluated one week later, and dogs not reaching the target therapeutic range were excluded from the study. Week 1 of the supplementation period was designated as the time the target therapeutic range was reached. Dogs were evaluated every 4 weeks during the supplementation period by standardized history and physical examinations, and a blood sample was obtained 4-6 hours after levothyroxine administration for measurement of serum concentrations of T_4, fT_4, T_3, and TSH. At each evaluation, prescribed levothyroxine tablets were counted to determine owner compliance. After completion of levothyroxine administration (denoted as week 1-16 of the withdrawal period), dogs were evaluated at 1 and 4 weeks and then every 4 weeks for a total of 16 weeks after cessation of treatment by standardized history and physical examination and measurement of serum T_4, fT_4, T_3, and TSH concentrations. Any dog that developed an illness or required administration of a drug other than routine anti-parasite prophylaxis were reviewed by two of the investigators to determine if it should be excluded from the study. At each sample collection, 8-10 ml of blood was obtained by jugular or cephalic

venipuncture, allowed to clot for 30-60 minutes at room temperature and then centrifuged at 2500xG for 15 minutes. Harvested serum was stored at -70 C until analysis.

All thyroid function tests (T_4, fT_4, T_3, and TSH concentrations) used for statistical analysis were measured at Michigan State University, Diagnostic Center for Population and Animal Health.

Hormone measurements

Serum concentrations of T_4 were measured with a commercially available radioimmunoassy kit[c]. The volume of samples and reagents were used as per the manufacturer's protocol but the incubation period was extended to two hours in a 37C water bath. The analytical sensitivity, estimated as the mean concentration of T_4 at 90% specific binding (10 assays), was 3.4 nmol/L (range 3.1-4.0 nmol/L). Aliquots of canine serum with T_4 concentrations of 8 and 85 nmol/L were mixed in volume combinations of 1:1, 1:2, 2:1, and 4:1 to assess parallelism. The results from assay of the mixtures showed respective % observed/expected recovery rates of 90%, 96%, 86%, and 90%. When aliquots of a canine serum sample were mixed with an added stock of 26, 64, 129, and 193 nmol/L T_4, the respective % recovery rates were 86%, 89%, 99%, and 101%. Assay repeatability was

determined from three pools of canine serum with mean concentrations of T_4 of 12, 26, and 85 nmol/L. The respective intraassay % coefficients of variation (CV) were 8.5%, 9.5%, and 8.1% for 10 replicates. In 10 assay runs, the respective interassay %CV for each pool were 19.6%, 13.0%, and 9.5%.

Serum concentrations of TSH were measured with a solid-phase chemiluminescent immunometric assay[d]. The manufacturer reports an analytical sensitivity to 0.01 ng/ml. Two canine serum samples with TSH concentrations of 0.10 and 2.91 ng/ml were mixed in respective volume combinations of 1:1, 1:2, 3:1, and 5:1 to assess parallelism. The results from assay of the various mixtures showed % observed/expected recovery rates of 106%, 105%, 110%, and 102%, respectively. Assay repeatability was assessed with four pools of canine serum with mean concentrations of 0.12, 0.43, 1.45, and 3.70 ng/ml. The respective intraassay % coefficients of variation for ten replicates of each pool were 3.4%, 2.2%, 2.4%, and 2.3%. Five replicates for each pool were run on three consecutive days and the respective interassay % CV for the daily means of each pool were 1.0%, 1.0%, 1.1%, and 1.7%.

Serum concentrations of fT_4 were measured in dialysate with a commercially available kit including dialysis cells and a sensitive T_4

radioimmunoassay[e] that has previously been described by the laboratory.[103] Serum concentrations of T_3 were measured with an in-house charcoal-separation radioimmunoassay where the procedures.[179] and use in canine serum[160] have been previously described.

Statistical analysis

The minimum number of dogs in the study was determined to be 8 per group based on a power analysis, setting the level of significance at 0.05 and power at 0.8, assuming data distribution similar to that previously described.[160]

Normal probability plots showed that serum concentrations of T_4, T_3, fT_4, and TSH as well as changes in the hormones from baseline values (an average of 2 measurements) followed a Gaussian distribution. Subsequently, data were summarized as means ± standard deviation. Effect of time on each outcome within each group was assessed using mixed model ANOVA followed by Tukey's procedure for multiple comparisons. The linear model specified sample week as a fixed effect and dog identification as the random effect. Correlation among residuals was modeled by specifying the AR(1) covariance matrix. For change from baseline and for individual hormonal concentrations during treatment (weeks 0, 1, 4, and 8) the treatment groups

were compared using mixed model ANOVA. The linear model specified group, time, and the interaction between group and time as fixed effects while dog identification within group constituted the random effect. Correlation among residuals was modeled by specifying the AR(1) covariance matrix. To specifically compare the groups at time point as appropriate, the slicediff option of proc glimmix was applied to the interaction between group and time. Results are expressed as means ± standard deviation and were considered significant at $P < 0.05$. All analyses were performed using SAS version 9.4.[b]

C. Results

Of 37 dogs evaluated, nine were excluded. Three dogs were excluded initially for having a serum T_4 or TSH concentration outside the reference interval, three were excluded after two weeks on supplementation because the T_4 concentration did not reach the target therapeutic range, and two were excluded due to failed compliance. Additionally, one dog was excluded due to an elevated serum T_4 (>100nmol/L) and concurrent weight loss documented at week 4 of supplementation.

Twenty-eight dogs completed the study period; 14 were castrated males and 14 were spayed females. Breeds included mixed (n=14) Labrador Retriever (n=2), Staffordshire Terrier (n=2), German Shorthair Pointer (n=2), and 1 each of the following: Siberian Husky, Catahoula Leopard Dog, Golden Retriever, Bull Mastiff, Rottweiler, French Bulldog, Australian Cattle Dog, and German Shepherd. The mean ± SD age was 4.5 ± 1.9 years and weight was 25.04 ± 9.8 kg. There were no significant differences in age or weight between the two groups. No clinically relevant abnormalities were identified on physical exam or routine laboratory testing.

The mean initial levothyroxine dose in all dogs was 0.024 ± 0.002 mg/kg every 24 hours. There was a significant difference (P <0.05) in the mean initial levothyroxine dose between group 1 (0.023 ± 0.002 mg/kg) and group 2 (0.025 ± 0.001 mg/kg). The levothyroxine dose was increased in 3 dogs and decreased in 2 dogs based on failure to reach the target therapeutic range during week 1. The mean final levothyroxine dose in all dogs was 0.024 ± 0.003 mg/kg and was not significantly different from the mean initial dose of levothyroxine in all dogs. There was a significant difference (P<0.05) in mean final dose of levothyroxine between group 1 (0.023 ± 0.002 mg/kg) and group 2 (0.026 ± 0.002 mg/kg).

Compliance of levothyroxine administration was 100% in 15 (54%)

dogs while the remaining 13 (46%) dogs missed an average of 2 doses over their respective treatment periods. Five dogs in group 1 and eight in group 2 had less than perfect compliance.

Supplementation Period

Mean serum T_4 and fT_4 concentrations (Figure 1 and 2) in both groups during the supplementation period were higher than baseline ($P<0.0001$; table 2). Mean serum TSH concentrations in group 1 were lower during the supplementation period compared to baseline ($P<0.0002$; table 2). Mean serum TSH concentrations in group 2 were not different during supplementation compared to baseline ($P>0.3$; table 2). When both groups were combined (n=28; Figure 3), the mean TSH concentration was lower during the supplementation period at weeks 1, 4 and 8 compared to baseline ($P<0.0001$; table 4). The mean serum T_3 concentration was lower compared to baseline at week 4 in group 2 ($P=0.0495$), but was not different between any other time periods ($P>0.7$; table 2).

At week 4, T_4, fT_4, and T_3 concentrations were higher in group 1 compared to group 2 ($P=0.009$, 0.02, and 0.01, respectively). There were no significant differences between groups at any other time during the

supplementation period. No dog that completed the study had clinical signs or physical examination abnormalities consistent with hyperthyroidism.

Withdrawal Period

There was no difference in mean serum T_4 or fT_4 concentrations (Figures 1 and 2) in either group during the withdrawal period compared to baseline ($P>0.9$; table 3). Mean serum T_4 and fT_4 concentrations in both groups were lower during the withdrawal period compared to the supplementation period ($P<0.0001$; table 3). The mean serum T_3 concentrations in both groups were not different between any time periods ($P>0.1$; table 3). Mean serum TSH concentrations in both groups were not different during the withdrawal period compared to baseline ($P>0.3$; table 3). The mean serum TSH concentration in group 1 was lower at all times during the supplementation period compared to the withdrawal period, except week 1 ($P<0.01$ and $P>0.06$ respectively). The mean serum TSH concentration in group 2 was higher at week 4 of the withdrawal period compared to weeks 4, 8, 12 and 16 of the supplementation period ($P<0.03$).

The serum TSH concentration was higher in group 2 than in group 1 at week 4 of the withdrawal period ($P=0.02$). There were no significant differences between groups at any other time during the withdrawal period.

The serum T_4 concentration was below the reference interval in one dog in group 2 at week 12 of the withdrawal period, but the serum concentrations of TSH, fT_4 and T_3 were within the reference interval. One dog in group 2 had elevated serum TSH concentrations throughout the withdrawal period, but normal serum T_3, T_4 and fT_4 concentrations. One dog in group 1 had a serum T_4 and fT_4 concentration that was above the reference interval at week 8 of the withdrawal period, but had a normal serum TSH concentration. At no point during the withdrawal period did a study subject show clinical signs of hypothyroidism or have low serum T_4 and fT_4 with an elevated serum TSH concentration.

D. Discussion

Results of this study demonstrate that TSH secretion is suppressed during levothyroxine administration to euthyroid dogs, but the effect does not persist after discontinuation of treatment. The anticipated suppression of serum T_4, fT_4, T_3 and TSH concentrations after up to 16 weeks of treatment with levothyroxine was not present. Therefore, thyroid function can be accurately investigated as early as one week after cessation of levothyroxine supplementation of similar duration.

Levothyroxine administration suppresses pituitary thyrotrope function and may result in atrophy as well as suppressed secretion of TSH. As a consequence of prolonged reduction in plasma TSH, thyroid gland atrophy can occur.[180-182] Dogs in the present study showed little evidence of residual effects of the negative feedback of exogenous levothyroxine on the HPTA. Only one dog had a sustained effect attributable to levothyroxine treatment, with elevation of serum TSH concentrations up to 16 weeks after discontinuing supplementation, consistent with thyroid gland atrophy. However, serum concentrations of T_4, fT_4 and T_3 were within their respective reference intervals despite elevation of TSH in this dog. Additionally, one dog exhibited a low T_4 at one time point during the withdrawal period, but the concurrent TSH concentration was normal. At no point during the withdrawal period did a study dog exhibit a low serum T_4 or fT_4 and a high TSH concentration. This emphasizes the value of measuring serum T_4 and/or fT_4 and TSH concentrations concurrently when assessing thyroid function in euthyroid dogs inappropriately supplemented with levothyroxine.

Similar to a previous study of levothyroxine administration to euthyroid dogs, suppression of the HPTA was documented during the supplementation period in both groups.[160] Although the serum TSH

concentration during supplementation was not significantly different compared to baseline in group 2 in the present study, significant suppression of serum TSH concentrations occurred when all dogs were analyzed together, indicating a type II error. Suppression of TSH was quite marked in group 2 dogs, with all having a decrease in the TSH concentration by more than the 38% that is attributable to biological variation of the hormone.[183] While dogs in the present study exhibited normalization of the HPTA one week after discontinuation of levothyroxine, the HPTA was suppressed for at least 4 weeks after cessation of supplementation in a previous study.[160] The assessment of HTPA function using dynamic thyroid testing (TRH and TSH stimulation tests) may account for the difference since endogenous TSH concentrations may be less sensitive than tests of thyroid reserve. In addition, levothyroxine was administered at approximately twice the daily dosage in the previous study compared with the present one.

Elevated serum TSH concentration was noted 4 weeks after withdrawal of levothyroxine in dogs treated for 16 weeks compared with those supplemented for 8 weeks. This is likely the result of thyroid gland atrophy as found in another study[162] caused by suppression of TSH during more prolonged levothyroxine treatment. Because serum thyroid hormone concentrations were not suppressed after ceasing treatment, it is likely the

elevated serum TSH concentration stimulated secretion of T_4 and T_3 from the thyroid gland sufficient to maintain normal function. This phenomenon has previously been shown histologically by an increase in pituitary thyrotropes number and size with concurrent high activity of the thyroid gland after withdrawal of levothyroxine.[162] Therefore, levothyroxine administration for longer than 16 weeks may affect thyroid function tests to a greater degree.

The investigators chose to administer levothyroxine once daily based on the resolution of clinical abnormalities of hypothyroidism in dogs supplemented in a similar manner.[167,184] Although once daily dosing results in more fluctuation in serum T_4 concentrations compared to twice daily dosing, the duration of action of T_4 is longer than its plasma half-life.[85,164,166,170] In previous studies of the HPTA, dogs were administered levothyroxine twice daily, making comparisons difficult.[160,161]

Humans can have marked suppression of the HTPA after thyroid hormone supplementation, with more protracted treatment associated with prolonged recovery that may require many months.[139,153] It may be inappropriate to extrapolate findings in humans to dogs since the half-life of T_4 in humans is substantially longer than in dogs, which would cause a greater degree of both thyrotrope and thyroid gland atrophy. Additionally,

the magnitude of TSH suppression in hypothyroid dogs supplemented with levothyroxine is directly correlated with the T_4 serum concentration.[185] Since the degree of HTPA suppression is dependent on the dose and frequency of levothyroxine administration, this may explain the serum TSH, T_4 and fT_4 concentrations normalizing within one week after cessation of once daily administration of levothyroxine in the present study. Moreover, the findings of the present study cannot be extrapolated to dogs receiving levothyroxine supplementation at a different dose, frequency of administration or duration.

Shortcomings of the present study are that dogs acted as their own controls and using client owned animals introduced intrinsic differences in environment and husbandry. However, these effects may be more clinically applicable as it closely resembles the practice setting compared to facility owned and housed dogs. Compliance can contribute a variable that might affect hormone concentrations, particularly given the relatively short half-life of levothyroxine in the dog. To circumvent this problem, tablets were counted at every recheck appointment and the overall compliance was 98.4%. Additionally, the canine TSH assay is not sufficiently sensitive to determine when TSH is below the reference interval that would indicate excessive supplementation, which makes it difficult to assess the appropriateness of treatment. We used a wide range of acceptable serum T4

concentrations that others have considered representative of adequate treatment in this study.[167] In humans where accurate and precise measurement of serum TSH is possible, it is used as a more appropriate marker of tissue thyroid hormone concentrations.

In conclusion, suppression of the HPTA occurred during levothyroxine supplementation for 8 or 16 weeks, with mean serum T_4, fT_4 and TSH concentrations returning to the reference interval by 1 week after discontinuation in both groups. It appears that assessing thyroid function tests 1 week after cessation of once daily levothyroxine supplementation will likely provide an accurate assessment of thyroid function in euthyroid dogs.

CHAPTER 3: CONCLUSIONS AND FURTHER RESEARCH

Suppression of the HPTA occurred during levothyroxine supplementation, with no significant difference in the mean serum T_4, fT_4 and TSH concentrations compared to baseline 1 week after discontinuation in both groups. It appears that assessing thyroid function tests 1 week after cessation of levothyroxine supplementation will likely provide an accurate assessment of thyroid function in euthyroid dogs.

Further studies should focus on the difference in suppressive effects on the HPTA with once daily administration compared to twice daily administration of levothyroxine. Additionally, a longer supplementation period should also be evaluated to assess how long the HPTA would take to normalized after discontinuation of long-term (greater than 16 weeks) of administration of levothyroxine.

This study demonstrates that a euthyroid dog being supplemented with levothyroxine can be assessed with thyroid function tests 1 week after discontinuation of levothyroxine. This information will help general practitioners in situations where the original diagnosis of hypothyroidism is questioned and confirmation of euthyroidism is necessary.

FOOTNOTES

[a]Microsoft ® Excel 2011

[b]SAS version 9.4, Cary, NC, USA

[c] T_4 MAb Solid Phase Component System, MP Biomedicals, Diagnostics Division, Orangeburg, NY

[d] Immulite 2000 Canine TSH, Siemens Healthcare Diagnostics, Llanberis, Gwynedd, United Kingdom

[e] Free T_4 – by Equilibrium dialysis, Antech Diagnostics, Irvine, CA

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APPENDIX A: FIGURES

Figure 1: Mean ± standard deviation T_4, fT_4, and TSH serum concentrations during the supplementation (outlined in blue) and withdrawal period (outlined in red) in Group 1.

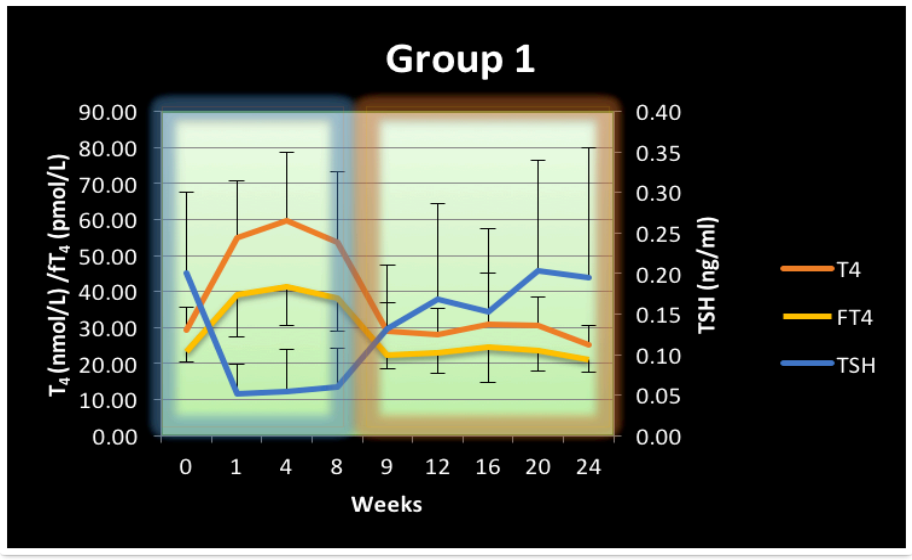


Figure 2: Mean ± standard deviation T_4, fT_4, and TSH serum concentrations during the supplementation (outlined in blue) and withdrawal period (outlined in red) in Group 2.

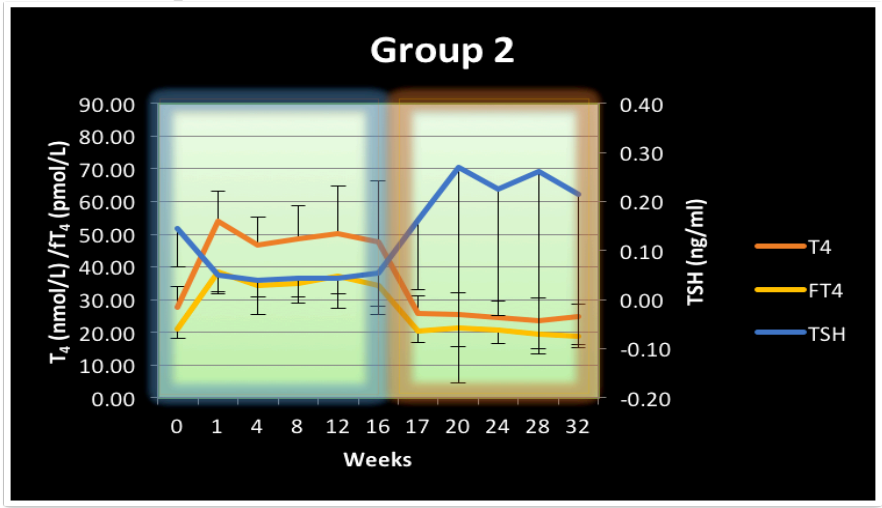
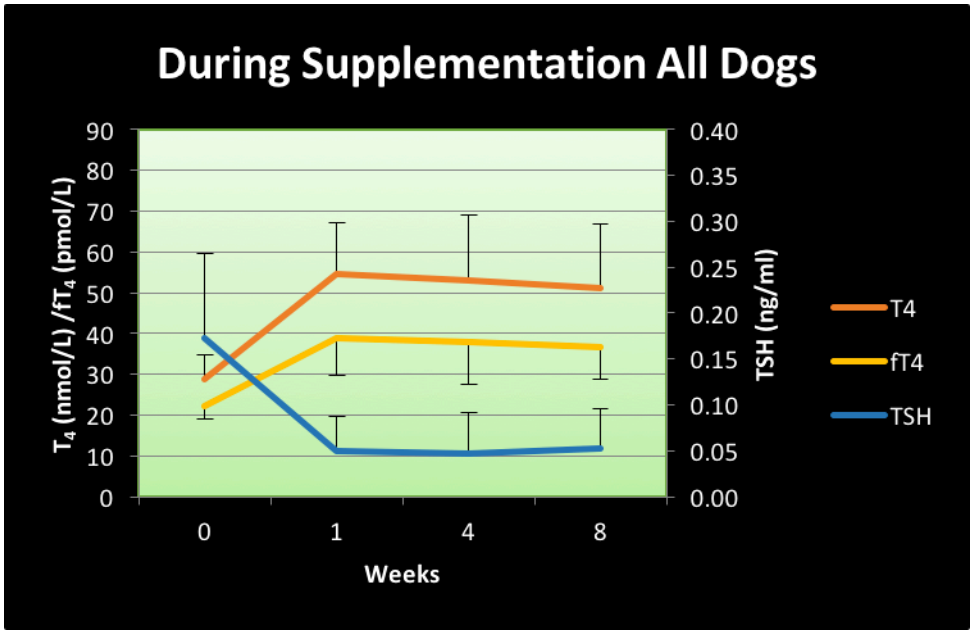


Figure 3: Mean ± standard deviation T_4, fT_4, and TSH serum concentrations during the first 8 weeks of the supplementation period for all dogs.



APPENDIX B: TABLES

Table 1: Average weight, age, initial and final levothyroxine dose and percent compliance in group 1 and 2.

	Average Weight (kg)	Average Age (years)	Average Initial dose (mg/kg)	Average final dose (mg/kg)	Compliance (%)
Group 1 (n=14)	22.01 ±7.9	4.57 ±1.9	0.023 ± 0.003	0.025 ±0.002	98.8
Group 2 (n=14)	28.06 ±10.7	4.36 ± 1.8	0.025 ± 0.001	0.026 ± 0.002	98.4
P value	0.1	0.052	0.01	0.0002	

Table 2: Significance of each hormone in group 1 and group 2 during the supplementation period. *P* values represent mean hormone concentration compared to baseline (week "0").

Sample week	T_4	SD	*P* value	T_3	SD	*P* value	fT_4	SD	*P* value	TSH	SD	*P* value
Group 1	(nmol/L)			(nmol/L)			(pmol/L)			(ng/ml)		
0	29.46	6.21		1.06	0.24		23.25	2.76		0.20	0.10	
1	54.93	15.71	<0.0001	1.04	0.28	1	39.21	11.67	<0.0001	0.05	0.04	<0.0001
4	59.71	19.05	<0.0001	1.09	0.25	1	41.29	10.82	<0.0001	0.06	0.05	<0.0001
8	53.79	19.56	<0.0001	1.07	0.26	1	38.21	9.35	<0.0001	0.06	0.05	0.0002
Group 2												
0	27.86	6.11		1.05	0.22		21.18	3.10		0.15	0.08	
1	54.00	9.31	<0.0001	1.01	0.30	0.9996	38.36	5.80	<0.0001	0.05	0.04	0.3154
4	46.57	8.58	<0.0001	0.85	0.19	0.0495	34.36	9.03	<0.0001	0.04	0.03	0.5982
8	48.50	10.20	<0.0001	0.94	0.21	0.7298	35.14	6.30	<0.0001	0.04	0.04	0.8152
12	50.29	14.37	<0.0001	0.99	0.18	0.9972	37.14	9.88	<0.0001	0.04	0.03	0.8753
16	47.71	18.51	<0.0001	0.96	0.20	0.9073	34.36	8.85	<0.0001	0.06	0.07	0.9561

Table 3: Significance of each hormone in group 1 and group 2 during the withdrawal period. *P* values represent mean hormone concentration compared to baseline.

Sample week	T_4	SD	*P* value	T_3	SD	*P* value	fT_4	SD	*P* value	TSH	SD	*P* value
Group 1	(nmol/L)			(nmol/L)			(pmol/L)			(ng/ml)		
1	29.07	7.88	1	0.99	0.23	0.9702	22.36	3.93	1	0.13	0.08	0.349
4	28.07	7.16	1	1.14	0.21	0.948	23.00	5.67	1	0.17	0.12	0.9763
8	30.93	14.23	1	1.11	0.23	0.999	24.50	9.82	0.9999	0.15	0.10	0.816
12	30.57	7.80	1	1.11	0.21	0.999	23.50	5.54	1	0.20	0.14	1
16	25.07	5.36	0.98	1.09	0.18	1	21.07	3.58	0.997	0.19	0.16	1
Group 2												
1	25.71	5.61	0.99	0.96	0.20	0.9073	20.50	3.70	1	0.16	0.14	1
4	25.43	6.65	0.99	1.01	0.19	0.9998	21.21	5.60	1	0.27	0.44	0.7905
8	24.43	5.09	0.99	1.04	0.17	1	20.64	3.99	1	0.23	0.26	0.9894
12	23.50	7.15	0.96	1.04	0.22	1	19.43	4.33	0.9993	0.26	0.37	0.8813
16	24.79	3.85	0.99	1.02	0.22	1	18.93	3.56	0.9944	0.22	0.31	0.9968

Table 4: Significance of each hormone in all dogs combined during the supplementation period. *P* values represent mean hormone concentration compared to baseline (week "0").

Sample week	T_4 (nmol/L)	SD	*P* value	T_3 (nmol/L)	SD	*P* value	fT_4 (pmol/L)	SD	*P* value	TSH (ng/ml)	SD	*P* value
0	28.66	6.10		1.06	0.23		22.21	3.07		0.17	0.09	
1	54.46	12.68	<0.0001	1.03	0.29	<0.0001	38.79	9.05	0.4722	0.05	0.04	<0.0001
4	53.14	15.97	<0.0001	0.97	0.25	<0.0001	37.82	10.39	0.07	0.05	0.04	<0.0001
8	51.14	15.54	<0.0001	1.00	0.24	<0.0001	36.68	7.98	0.2753	0.05	0.04	<0.0001

Table 5: Significance in mean serum hormone concentration between Group 1 and 2 during the supplementation period.

	T_4			T_3			fT_4			TSH		
	Mean Difference	SE	P value	Mean Difference	SE	P value	Mean Difference	SE	P value	Mean Difference	SE	P value
Week 1	0.93	4.88	0.85	0.04	0.09	0.70	0.86	3.02	0.78	0.00	0.02	0.92
Week 4	13.14	4.88	0.01	0.24	0.09	0.01	6.93	3.02	0.03	0.02	0.02	0.50
Week 8	5.29	4.88	0.28	0.14	0.09	0.15	3.07	3.02	0.31	0.02	0.02	0.46

Table 6: Significance in mean serum hormone concentration between Group 1 and 2 during the withdrawal period.

	T_4			T_3			fT_4			TSH		
	Mean Difference	SE	P value	Mean Difference	SE	P value	Mean Difference	SE	P value	Mean Difference	SE	P value
Week 1	1.75	4.20	0.68	0.02	0.08	0.79	-0.21	2.37	0.93	-0.09	0.07	0.21
Week 4	1.04	4.20	0.81	0.12	0.08	0.14	-0.29	2.37	0.90	-0.16	0.07	0.02
Week 8	4.89	4.20	0.25	0.06	0.08	0.48	1.79	2.37	0.45	-0.13	0.07	0.07
Week 12	5.46	4.20	0.20	0.06	0.08	0.48	2.00	2.37	0.40	-0.11	0.07	0.11
Week 16	-1.32	4.20	0.75	0.06	0.08	0.48	0.07	2.37	0.98	-0.08	0.07	0.27