Levothyroxine Supplementation in Hypothyroid Bitches During Pregnancy

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ABSTRACT

Hypothyroidism is the most common endocrine disease in dogs and has been shown to have a hereditary nature in many breeds. Previous studies have documented decreased fertility in bitches with experimentally-induced hypothyroidism, decreased viability at birth, increased periparturient mortality, and reduced birth weight in pups born to hypothyroid dogs. Hypothyroid women have an increased demand for exogenous thyroxine throughout gestation in order to maintain normal plasma concentrations of thyroid hormones and produce neuropsychologically normal children. This study was performed to determine if pregnancy causes a similar need for increased levothyroxine dosages in dogs to maintain a euthyroid state. Serum was harvested from blood collected from six bitches with experimentally-induced hypothyroidism that were receiving standard thyroid hormone replacement therapy and from four euthyroid control bitches. Thyroid function tests performed on these samples included total thyroxine (T4), free T4 (FT4), thyroid stimulating hormone (TSH), and 3,5,3'-triiodinine (T3). Thyroid concentrations were measured from ovluation through the end of pregnancy. All bitches whelped normal litters. Euthyroid bitches had no significant alterations in their hormone concentrations throughout pregnancy. None of the supplemented hypothyroid bitches had clinical signs of hypothyroidism throughout the study. Serum concentrations of T4 and FT4 were elevated at multiple sample points during gestation. The results from this study indicate that standard levothyroxine supplementation is adequate to maintain a euthyroid

state during pregnancy in experimentally-induced hypothyroid dogs. In addition, there is no evidence that canine thyroid profiles in euthyroid dogs are altered during gestation.

Dedication

This work is dedicated to my loving husband. Without his unwavering support and understanding I would not be the person that I am today. He provided consistent mentoring and laughter throughout my residency and masters. Life makes unexpected twists and turns and it was my fortune to earn the love and devotion that I experience from him every day.

In addition, I wish to dedicate this thesis to Dr. Purswell. Her jovial attitude throughout my residency was truly remarkable and will be missed. I hope that she finds as much fun in her retired years as I have had with her as a student. I am proud to say that I was trained by such a well-respected Theriogenologist and hope to be half the clinician that she is one day.

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Attributions

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Dr. Purswell is a Professor of Theriogenology in the Large Animal Clinical Sciences department at the Virginia-Maryland Regional College of Veterinary Medicine. She is the chair of this committee and was active in the procuring funding, design, planning and implementing this project. She also contributed to the writing and revisions of the thesis and manuscript for publication.

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Chapter 1: Literature Review

The Thyroid

The thyroid is a bilobed endocrine structure that resides on the anterior portion of the trachea from the third to the seventh ring. The two lobes, varying in size from one to three centimeters by one-half to one centimeter, are connected via an isthmus, although not all dogs possess this structure[39]. In humans the thyroid is the first endocrine structure to form during embryogenesis [70]. The thyroid enlage arises from the endoderm and is viewed as a proliferation of tissue at the aortic arch [70]. This tissue grows ventrally as the fetus develops. Thyroid hormones are first detectable at about eleven to thirteen weeks of gestation, although the fetal thyroid is not self-sustaining until twenty weeks and relies on maternal thyroid hormones for normal development [60,70,82]. The functional unit within the thyroid is typically described as a cystic structure lined by follicular cells or thyrocytes. The vast majority of the remaining structure is filled with colloid [39,70]. Thyroglobulin (Tg) is an iodinated glycoprotein that resides within the colloid portion of the thyroid and is a major synthesis and storage apparatus for thyroid hormone and its precursors in the tissue [14,70].

Iodine is an elemental requirement for synthesis of thyroid hormones, and dietary iodine deficiency causes hypothyroidism. Iodine is rapidly absorbed through the stomach and duodenum and transported through the plasma in an inorganic form to the tissues of the body and excreted through the kidney [14,46]. Canine plasma contains 5-10µg/dL of iodine, which is twenty times the concentration of human plasma [14]. Iodine undergoes active transport via the sodium-iodine symporter across the thyroid follicular cell basement membrane and can be

concentrated up-to two-hundred times that of the plasma concentration in the dog [14,70]. Once iodine has entered the cell it moves down a concentration gradient into the follicular lumen, is oxidized by thyroid peroxidase (TPO) and ultimately is bound to tyrosine residues of Tg, forming monoidotyrosine (MIT) and diiodotyrosine (DIT) [14,39]. Thyroid hormones, thyroxine (T4) and 3,5,3' triiodothyronine (T3) are then formed by coupling of two iodotyrosine moieties on Tg. Iodine status of the diet and/or plasma drives which hormone is produced in higher amounts. T4 is favored when iodine intake is sufficient, but in deficient states, production of T3 in the thyroid gland is preferred [14].

Regulation of thyroid hormone secretion is simply described as a negative feedback process controlled by plasma concentrations of T4 and T3 and their interactions with the anterior pituitary gland and hypothalamus [23,39]. The hypothalamus produces thyrotropin releasing hormone (TRH) which stimulates secretion of thyrotropin (TSH) from the anterior pituitary gland. Thyrotropin is a glycoprotein with α and β subunits. Although the β subunit is unique to canine TSH, the α subunit is identical to many other glycoproteins that originate in the pituitary, of which lutenizing hormone (LH) and follicle stimulating hormone (FSH) are of importance in the reproductively active canid [14].Thyrotropin in turn binds to specific cell surface receptors and through adenylate cyclase causes the thyroid to increase iodine uptake, synthesis and secretion of thyroid hormones. As a result the thyroid gland hypertrophies. As the plasma T4 and T3 concentrations increase, production is down regulated of both TSH and TRH from the pituitary and hypothalamus. [39,53].

Thyroid hormone secretion is a complex process involving pinocytosis of Tg containing colloid within the follicular cells [14,39,70]. Lysosomal proteolytic enzymes break down the Tg

and form both MIT/DIT and T4/T3. The thyroid can deiodinate T4 to form either T3 or 3,3',5'-triiodothyronine (reverse T3, rT3), an inactive form of thyroid hormone. The T4:T3 ratio of product secretion in the canine is reported to be 4:1, although the storage ratio is 12:1 [14]. The canine thyroid can produce twice the thyroid hormone that humans synthesize.

Once secreted into circulation the majority of thyroid hormones are bound to various plasma binding proteins. The lipophilic nature of T4 and T3 limits the concentrations in the plasma that are unbound to carrier proteins. In humans the main proteins are thyroid-binding globulin (TBG), transthyretin (TBPA or thyroxine-binding prealbumin) and albumin [70]. In dogs, T4 is primarily bound to TBG, but transthyretin, albumin and plasma lipoproteins account for substantial binding as well. The concentration of TBG in the dog is about fifteen percent that present in humans and canine binding proteins have weaker affinities to bind thyroid hormones when compared to humans [14,23]. Protein binding increases the plasma hormone concentration, prolonging its half-life and assisting with hormone delivery to specific tissues [70]. The weaker affinity and lower concentration of TBG results in lower plasma total T4 (T4) concentration and a higher fraction of T4 that is unbound or "free" [14,23]. The half-life of thyroid hormones is much shorter in dogs than in humans, due to the lower TBG concentration, which accounts for the higher replacement therapy dosages in canine hypothyroidism. The "free hormone hypothesis" states that it is the free hormone that is available to leave the circulation and enter cells [14,48]. Therefore, plasma fT4 is in equilibrium with tissues and appears to be the most appropriate measure of thyroid function in dogs. This equilibrium is mediated by the carrier proteins as they are the reservoir of hormone and control their entrance into the tissues. Disease states or exogenous drug administration could affect this equilibrium. The majority of T4 is in

the plasma with a portion within the cells of the liver, kidney, and interstitial fluid. The majority of T3 is present in the cells of the muscles and skin [14]. T4 rapidly enters the cells of the liver and kidney where it is converted to T3, but the majority of thyroid hormone that is in the extracellular compartments of the body is T4 and the intracellular portions are T3.

Metabolism of thyroid hormones is a complex process mediated by enzymes that either activate or deactivate the hormones. Up to sixty percent of T3 in the circulation of the dog is formed from extrathyroidal deiodination of T4 [14,23]. Three types of deiodinases, specifically 5'-deiodinase enzymes, are present in the tissues and provide the mechanism for T3 production. This process is mediated by cleavage of I from either the outer, phenolic ring of T4 or the inner, tyrosil ring of T4 both resulting in the formation of T3 or rT3, respectively. Type I-deiodinase, a selenoenzyme, can be found in its highest activity in liver, kidney, muscle and the thyroid gland. It has an absolute requirement for selenium for maximal action and works through both inner and outer ring deiodination. Activity of type 1-deiodinase primarily results in generation of T3 in the circulation. [14,70]. Type II-deiodinase is found in the central nervous system (CNS), placenta, pituitary gland, brown fat, and skin. It converts T4 into T3 via the outer ring mechanism for intracellular use [14,70]. There are conflicting views on the location of activity of type II deiondinase and if it contributes any T3 to plasma concentrations [7]. Finally, type III is found in the placenta, CNS, fetal liver, and human skin. This enzyme is an deactivator of thyroid hormones and works via the inner ring to produce the inactive hormone, reverse T3 (rT3) [14]. All three types of deiodinases will selectively deiodinate the inner or outer rings of T3 and rT3 to form T2 for further metabolism [7].

The Thyroid in Pregnancy

Pregnancy induces a number of changes that have substantial effects on thyroid function. Very little is known about thyroid function in pregnant bitches and the following discussion is based on human and experimental animal literature. There are four major factors affecting thyroid economy that are altered due to carrying a conceptus. First, there is an overall increase in TBG due to estrogen stimulation from the pregnancy hormone, human chorionic gonadotropin (hCG). Secondly, hCG has some thyrotropic activity and contributes to higher fT4 levels and the decline in TSH serum levels. Thirdly, placental deiodinases enhance the metabolism of thyroid hormones and alter their availability in both tissues and serum. Lastly, iodine is required in increased levels during pregnancy not only due to uptake by the fetal unit, but increased renal clearance of the element. Each of these factors will be discussed at length and, although not specifically elucidated in dogs, shed light on the complex process of thyroid regulation during pregnancy.

TBG has a high affinity for thyroid hormone, although in a normal state only two thirds of T4 is carried by the protein [26]. During pregnancy TBG increases in concentration and is responsible for up to three quarters of the T4 binding in circulation [64]. Pre-pregnancy levels of TBG are 15-16mg/L but increase to 30-40 mg/L just a few weeks into gestation [26]. This increase in TBG is attributed to estrogen stimulation and increased production of the proteins by the liver. In addition, TBG half-life is prolonged during pregnancy because of increased sialation of the protein [30-32]. Some authors propose that the higher proportion of T4 bound to the TBG also contributes to the increased level of TBG by increasing the stabilization of the molecule [26]. As a result of more thyroid hormone binding to the increased TBG, the serum total T4 and

T3 concentrations increase during the first half of pregnancy. Thyroid hormone secretion increases in response to the decreased free hormone that occurs when protein binding is increased. This is a transient change in hormone level during the first trimester and most normal pregnant women will maintain their fT4 and fT3 levels within the non-pregnant reference range for the duration of pregnancy [6,9].

In the first trimester there is a decrease in the plasma TSH concentration. This decrease has a direct correlation with the serum hCG concentration. For example, rising levels of hCG results in an overall increase in fT4 down-regulating TSH production and plasma concentration. Euthyroid women respond to hCG stimulation by down regulating thyroid hormone production and clinical tests would be unable to detect the transient shift in thyroid dynamics. The question remains as to why there is this affect from hCG stimulation. There is homology between the β subunit of hCG and TSH as well as the LH, hCG, and TSH receptors [25,26]. This similarity between structures explains why hCG can bind to the TSH receptor and stimulate a response from the follicular cells. This has been shown by women with lower levels of TSH with high levels of hCG and β-hCG subunits resulting in a twenty percent increase in the fT4 level during the first trimester [28].

Deiondinases are major contributors to metabolism of thyroid hormones in the body. Type II and III deiodinases are present in the human placenta and work through outer ring conversion of T4 to T3, although only type II deiodinases has been identified in canine placentas to date [72]. Activity of type II increases when plasma T4 decreases, resulting in an increased T3. Type III converts T4 to rT3 and is highly active in the placenta during pregnancy, contributing to the low T3 and high rT3 levels found in a fetus [26]. Overall, effects of placental

deiodinase activity on the mother include increased T3 and rT3 levels and decreased T4 levels in circulation. Again, although these deiodinases have increased activity during pregnancy due to their presence in the placental unit, increased synthesis and secretion of thyroid hormones compensates for any deficiency created by increased metabolism.

Finally, the presence of iodine, in sufficient quantities, for production of the thyroid hormones is essential. Generally a non-pregnant woman should consume about 100-150µg/day. During pregnancy this need increases to about 200µg/day. The need for increased intake is due to increased renal iodide clearance throughout gestation as well as fetal uptake after week ten of gestation. The average glomerular filtration clearance rate for iodide is 30ml/min and in a human consuming 150µg of iodine the thyroid will clear about 17ml/min [26]. Due to pregnancy the overall glomerular filtration rate increases, pulling iodine out in more significant quantities. In addition the thyroid increases its uptake of iodine to 60ml/min [34,59]. In the second half of gestation the fetus starts consuming more iodine to make their own thyroid hormones so the renal excretion of iodine decreases by 10-15µg/ day [27]. If the pregnant women is consuming enough dietary iodine this route of deficient thyroid production should be nullified.

Dogs have about fifteen percent the amount of TBG that humans possess and canine TBG has a weaker affinity for thyroid hormone, resulting in dogs having a lower serum T4 concentration and higher FT4 fraction when compared with humans [14,23]. In one study where T4 was measured at 30-35 days post coitum in the pregnant group or 22-44 days after the onset of estrus in the diestrus group, it was found that bitches under the influence of progesterone, either during pregnancy or in diestrus, had elevated levels of T4 before and after TSH stimulation [63]. In the bitch no known pregnancy hormone, similar to hCG, exists. In the

absence of a hormone equivalent to hCG, the thyrotropic activity would be absent. Therefore, alteration in thyroid function in pregnant dogs may be different than that of humans. Furthermore, commercial diets should provide adequate amounts of dietary iodine to support pregnancy.

Thyroid Function Tests

Testing for hypothyroidism requires a few essential tests for diagnosis of disease. Serum concentrations of T4, fT4, and TSH are the most useful for determining thyroid function in dogs. T4 is the measure of thyroid hormone, bound and free, within the plasma can be used as a screening test suspect animals. A low T4 in combination with clinical signs may warrant a therapy trial or further tests [23,52]. The reported specificity and sensitivity of a low serum T4 level are 89 and 82 percent, respectively [20,40].

At this time fT4 is the best single laboratory test for the diagnosis of hypothyroidism. The measurement only accounts for the unbound fraction of thyroid hormone and a low equilibrium dialysis measurement has a reported specificity of 93 to 94 percent and sensitivity of 80 to 98 percent [20,23,40,58]. Equilibrium dialysis compensates for the lower affinity of TBG present in dogs and may provide more accurate levels of FT4 needed to distinguish hypothyroid dogs with a low T4 and those animals with NTI [23].

TSH is the last commonly used test for diagnosis of hypothyroidism. Although in human medicine TSH levels are highly accurate in screening for disease, TSH is not as useful in dogs. Multiple studies have shown that about one fourth of hypothyroid dogs possess a normal TSH concentration [20,23,52,58,62], so although development of a canine specific immunoassay has improved our testing abilities, it has not been as helpful as one would expect.

Total T3 (T3) can be used, but is generally normal in most hypothyroid dogs due to the failing thyroid gland producing larger amounts of T3 compared to T4. That in combination with the conversion of T4 to T3 in circulation will account for normal serum T3 concentrations [52]. The only possible exception to this rule is in sighthounds, where serum concentrations of T4 and fT4 in euthyroid dogs are consistently lower than those of other breeds. Because of this, it is sometimes suggested that T3 can be used to diagnose disease in these breeds [23]. In addition the accuracy of this test is reported to be 55 percent in non-sighthounds. [20,23,40].

As in most diagnostic tests, outside factors can contribute to false results. One such factor is non-thyroidal illness (NTI). Clinical signs and a through history can be combined with thyroid hormone tests to rule out any confounding factors related to non-thyroid disease. In the event that there is an acute disease process it is best to delay any hormone tests until the disease has been resolved [23]. Specifically, T4 and T3 will decrease with NTI while TSH and rT3 may increase [53]. Much investigation has been made regarding the mechanisms for the hormone changes in NTI. Evidence exists for a variety of mechanisms, including alterations in binding of T4 and T3 to plasma binding proteins, reduced transport of thyroid hormones into cells, deiodinase activity, and decreased TSH or TRH secretion [52,53]. In humans with mild illness generally only T3 will decrease due to reduced activity of type one deiodinases [44]. In severe illness T4 may decrease. TSH usually remains normal but certain drugs, mainly glucocorticoids and dopamine, will cause a decrease in serum levels [71]. Fasting can affect serum TSH through an increase in cortisol. This is explained by euthyroid patients' TSH secretion is released in a pulse-like fashion and is inhibited by cortisol during normal diurnal rhythms [44]. NTI results in the suppression of TSH and TRH secretion [44]. T4 will decrease in NTI due to the reduction of TBG, albumin, and

transthyretin. This mechanism would only affect the bound portion of thyroid hormone. Lastly, cytokines, interleukin-1 and 6, tumor necrosis factor-α, and interferon-γ, will be elevated during NTI and have an inhibitory effect on synthesis and secretion of TSH, T3, and TBG [47,81]. All of these effects of NTI on thyroid hormones is transitory and the hormone concentrations will normalize upon resolution of disease.

Secondly, breed has a substantial influence on serum thyroid hormone concentrations. Sighthounds and some Alaskan sled dogs have lower concentrations of T4 and FT4 in a euthyroid state [23,52]. Lastly, it has been well documented that exogenous drug administration can cause alterations in the thyroid status of the patient. Specifically glcocorticosteroids, sulfonamides, anticonvulsants, and some non-steroidal anti-inflammatories have been implicated [13,15,23,52,54,57,65,80].

Canine Hypothyroidism

Hypothyroidism is present in 0.2 to 0.8 percent of the population and is diagnosed in dogs with an average age of seven years. There are conflicting studies as to the risk associated with breed, gender or reproductive status [66]. Primary hypothyroidism in dogs occurs when the thyroid gland is destroyed by one of two pathologic processes; lymphocytic thyroiditis (autoimmune thyroiditis or LT) or idiopathic follicular atrophy (IFA). Each occurs in approximately fifty percent of cases [31] and breed predilections for hypothyroidism have been well documented. LT is characterized by a diffuse infiltration of inflammatory cells within the thyroid gland, specifically lymphocytes, plasma cells, and macrophages [30,31]. Histopathologic lesions include lymphoid nodules and destruction of the follicles within the gland. Final stages of this form involve replacement of normal tissue with fibrous connective tissue. Antithyroglobulin

antibodies (TgAAs) can be found in most dogs affected by this form of the disease. Currently the presence of TgAAs in any animal may be the earliest evidence of thyroid destruction [23]. At this time this disease is considered heritable [31].

IFA is unique in the fact that there is also loss of thyroid parenchyma, but the tissue is replaced with adipose tissue [30]. There is no associated inflammation with this form of the disease. Some propose that IFA may be the end result of LT, but these claims have not been proven. Secondary and tertiary hypothyroidism is rare in the dog.

Clinical signs of hypothyroidism in dogs can be very ambiguous and at times confounding. General signs include a weight gain (lower metabolic rate), dermatologic changes including dry hair coat, seborrhea, alopecia, recurrent pyoderma and hyperpigmentation as well as neurologic abnormalities, cardiovascular changes and reproductive aberrations [51,52,66]. Changes in metabolic rate can manifest as lethargy, dullness, and weight gain [66]. The dermatologic conditions are most common, being reported in sixty to eighty percent of all cases [21,51,52]. Neurologic signs consistent with peripheral neuropathy, include proprioceptive deficits and hyporeflexia. Muscle atrophy is also commonly seen [52]. CNS signs are also sometimes noted in hypothyroid dogs, with cerebellar and central vestibular abnormalities predominating.

Hypothyroid Reproductive Manifestations

Adverse effects on reproduction due to hypothyroidism have been studied in multiple species. It is known that irregular estrous cycles associated with hypothyroidism exist in not only humans, 23.4 percent in one study, but rats, ewes and Syrian hamsters [43,49,76]. In humans and rats, it is thought that the irregularity of ovulation and estrous cycles is related to increased

prolactin levels leading to a decreased response of the ovaries to LH [4,19,35,73]. This pathway has led to complete ovulation failure in some patients [29]. In morphological studies on hypothyroid rat ovaries various authors have reported ovarian atrophy [12], smaller corpra leutea, and a decreased number of graafian follicles when compared to euthyroid controls [4]. Thyroid hormones, specifically T3 and T4, are found in human follicles and thyroid receptors are found on granulosa cell surfaces demonstrating a direct influence of thyroid hormones on fertility [77]. After conception, hypothyroid mothers are at an increased risk of abortion and miscarriage [17,36,43,61]. Hallengren et al reported fetal loss in 29% of hypothyroid pregnancies and only 6% in thyroid treated pregnancies. Hypothyroid mothers are also at risk for preterm delivery, lower birth weights of their offspring, as well as one report of increased admission to the neonatal intensive care unit [8,10,16,17,24,61]. Finally, neural development is impaired in children and rats born to overtly hypothyroid mothers as well as subclinical or inadequately supplemented hypothyroid mothers. Rats took longer in a water maze test when compared to controls and the cohort of rats that were born to treated hypothyroid mothers had similar times in the maze as the euthyroid group [78]. In humans there have been numerous studies looking at the intelligent quotient (IQ), Bayley Psychomotor Development Index (PDI), and other cognitive batteries on children born to hypothyroid mothers. Overall, children born to hypothyroid mothers scored lower on their PDI [60] and IQ tests [33] than age matched and factor controlled children.

There has been little work done on the reproductive effects of hypothyroidism in dogs. Historically it was thought that reproductive manifestations of the disease included prolonged interestrous intervals, silent estrus periods, non-cyclicity, abortions, small litters, infertility, and weak or stillborn offspring [38,66]. A kennel of Borzoi bitches with confirmed lymphocytic

thyroiditis and hypothyroidism were found to have multiple aberrations in reproduction including infertility, abortion, and stillbirths [37]. One study by Segalini failed to show a correlation between hypothyroid hormones (T4 and TSH) and infertility in large breed dogs [67]. One study looked at prolactin levels in euthyroid and hypothyroid bitches and it was found that the hypothyroid bitches had higher prolactin levels which might inhibit reproductive success [42]. More recently one study has shown that hypothyroid bitches can conceive but a prolonged course of disease results in lower pregnancy rate than euthyroid bitches. In the first round of breeding, twenty weeks after induction of hypothyroidism, all nine hypothyroid and euthyroid bitches were confirmed pregnant and carried to term. However, a second breeding in the same dogs, after fifty-six weeks of hypothyroidism, resulted in term pregnancies in only four of eight hypothyroid bitches compared with six of six euthyroid bitches. In the second breeding both sets of bitches whelp similar sized litters, but puppies have lower birth weights, an increased risk of periparturient death, and the hypothyroid bitches have more difficulty during whelping as indicated by strength and length of contractions in active labor [55,56]. The same group initiated thyroid replacement therapy on the hypothyroid dogs and found that the periparturient mortality and smaller puppy sizes were reversed with standard supplementation [56].

Levothyroxine Supplementation

Standard treatment for hypothyroidism in dogs is hormone replacement therapy with sodium levothyroxine. Tablet and liquid formulations are currently available [45,48,50]. The bioavailability of the oral formulations has been reported from ten to fifty percent [45,50]. The half-life of levothyroxine is approximately 16 hours, but a biologic effect is thought to be present for much longer [48,50]. Peak concentrations are achieved around 4 hours after oral

administration, but there is considerable variability between individual dogs with regard to absorption after oral administration and in other pharmacokinetic parameters [48]. Currently, the recommended dosage is 20µg/kg per os, once daily. There has been a range of dosages reported from 0.5mg/m^2 to as high as 44µg/kg either once daily or twice daily with varying results[22,32,48,50]. Due to individual variation, close monitoring of each dog is necessary. Monitoring hormone replacement therapy includes clinical response, serum T4 concentration, and possibly serum TSH concentration with serum samples taken approximately four to six hours after dosing levothyroxine. Hormone concentrations can be measured as early as two weeks after the start of therapy, but clinical response is best evaluated after 6-8 weeks of treatment. Maintenance of T4 above 35nmol/L and TSH within the normal reference range (<0.6ng/ml) is effective in resolving clinical signs of hypothyroidism [22]. Some patients will take up to three months for dermatologic manifestations, weight gain, and dull mentation to resolve completely. Furthermore if the clinical signs are not resolving dosages must be changed and more frequent monitoring should be initiated [22]. Currently there have been no specific studies looking at thyroid hormone supplementation during pregnancy in the dog.

The treatment of choice for human hypothyroid patients is oral levothyroxine. Dosages are based on body weight and are initiated at 1.6µg/kg/d [11]. Once therapy has been started, serum TSH is measured six to eight weeks after commencement or when dosages are changed [11]. Once the patient's condition has stabilized, as evidenced by no clinical signs, symptoms, and normalization of TSH values, measurement of serum TSH is performed every 12 to 18 months [11,75].

Alterations in thyroid economy present during pregnancy complicate thyroid hormone replacement therapy. During pregnancy euthyroid women will require an average of 20-40% increase in their thyroid production to maintain a euthyroid state and prevent detrimental effects on their offspring [68,82]. One to two percent of women of child bearing age are on levothyroxine supplementation during pregnancy [3,68]. Hypothyroid women require dosage increases that vary depending on the severity of hypothyroidism and how well controlled their TSH levels were prior to conception. For example, in two reports subclinical hypothyroid women required a greater increase in their dosage, mean increase of 70%, than overtly hypothyroid women who required on average a 40-50% dose increase [1,74]. On average, most women will require an overall increase of 25-50% of their pre-conception dosing regimen [2,3,5]. At this time it is recommended that previously diagnosed hypothyroid women increase their supplementation dose by 30% or two tablets a week [3,5,41,74,82]. Women that are screened and found to be sub-clinically hypothyroid the dosing should be started at 100-150µg/d or 2-2.4µg/kg/d. Monitoring is carried out through serial serum TSH levels and it is important to stress the use of trimester-specific ranges. One such study published ranges for the pregnant woman [18] and have been used by multiple studies and clinics to date. The Endocrine Society and American Thyroid Association state that TSH levels for the first trimester remain below 2.5mIU/L and less than 3mIU/L for the remainder of pregnancy to be considered normal [69]. Once there is a change in dosing or therapy is initiated, serum TSH values should be evaluated every 4 weeks during the first half of pregnancy and once or twice at 26 to 32 weeks of gestation [2,41,82].

Chapter 2: Manuscript

Introduction

Hypothyroidism is the most common endocrine disease in dogs and has been shown to be hereditary in many breeds [31]. Detrimental effects of hypothyroidism on reproduction have been documented in bitches with experimentally-induced disease, including decreased viability at birth, increased periparturient mortality, and reduced birth weight in pups born to hypothyroid dogs [55]. All reproductive abnormalities that were a consequence of hypothyroidism were reversed by thyroid hormone replacement therapy with levothyroxine [56]. Similar effects on reproduction have been well documented in hypothyroid women.

In women, pregnancy increases the demand for thyroid hormones through a variety of mechanisms. Pregnant women have increased thyroxine-binding globulin (TBG), the most important binding protein for thyroxine. Because circulating T4 is 99.9% protein bound in the plasma, increased protein binding creates a substantial need for supplemental thyroxine. In addition, increased metabolism of thyroid hormones occurs through placental deiodinases, thus increasing the demand for thyroid hormone synthesis and secretion [24-26]. Finally, human chorionic gonadotropin (hCG) acts as a thyrotropic hormone, stimulating release of T_3 and T_4 exerting negative feedback on the pituitary and lowering TSH levels from weeks 8-14 of gestation [24-26,28]. If the hypothalamic-pituitary-thyroid axis is normal, increased thyroid hormone production and secretion can compensate for the increased needs induced by pregnancy. However, if the only source of thyroid hormone is through administration of exogenous levothyroxine, the dosage must be increased to meet the demand. Therefore, dosage

adjustment of levothyroxine based on careful monitoring of thyroid function tests is necessary in pregnant women with hypothyroidism. The increase in levothyroxine dosage necessary to maintain women in a euthyroid state during pregnancy typically ranges from 25% - 50% [2,61,82]. Specific normal ranges of thyroid function tests have been developed for each trimester of pregnancy, and levothyroxine supplementation is altered to adhere to these ranges.

Little is known about the influence of pregnancy on thyroid function in dogs. In the only study the authors' are aware of evaluating thyroid function in pregnant bitches, the serum T4 concentration was higher in pregnant and diestrus bitches before and after TSH stimulation at 22 to 44 days after the onset of estrus or breeding in a single 24 hour sampling period [63]. There is no known hCG pregnancy hormone equivalent in the dog. In addition TBG binding and concentrations are different in the dog [13,23]. Therefore, the changes seen in thyroid hormones in pregnant women may not occur in pregnant bitches. In addition, the authors are not aware of studies evaluating levothyroxine supplementation in hypothyroid dogs during pregnancy. The purpose of the present study is to determine the effects of pregnancy on thyroid function tests in the euthyroid dog and to compare the results with those in pregnant levothyroxine-supplemented hypothyroid dogs at the same stages of pregnancy. It is hypothesized that the euthyroid bitches would have no change in their hormone profiles and that the levothyroxine treated levothyroxine treated dogs would decreased T4, T3, FT4 and increased TSH concentrations despite supplementation.

Materials and Methods

Animals

Twelve multiparous purpose bred mongrels were used in the study. Bitches ranged in age from five to six years (59-73 months) old and the euthyroid controls weighed 10.8 ± 1.18kg and the hypothyroid treated bitches weighed 10.3 ± 0.90kg. Hypothyroidism was induced in 6 randomly selected bitches by intravenous administration of 1 mCi/kg of ^{131}I, while the remaining 6 dogs acted as euthyroid controls [56]. Hypothyroidism was confirmed during anestrus 9, 38 to 45, and 76 to 82 weeks after ^{131}I by finding serum T_4 concentrations < 10 nmol/L before and 4 h after administration of 50 μg human recombinant TSH[b]. Unfortunately two of the euthyroid dogs were withdrawn from the study. One bitch failed to conceive and another bitch developed glaucoma that was unresponsive to treatment and was humanely euthanized.

Breeding and Levothyroxine Supplementation

As a part of a prior study dogs in both groups were bred twice at a median of 20 and 55 weeks after ^{131}I administration to evaluate the effects of hypothyroidism on reproduction [56]. Before the third breeding, levothyroxine supplementation was initiated in the hypothyroid bitches. The initial dosage of levothyroxine was 0.016 ± 0.002 mg/kg PO once daily with food at approximately 8 am. In order to ensure all bitches received adequate treatment prior to breeding, serum concentrations of T4 and TSH were measured monthly on samples obtained 4 hours after levothyroxine administration. The dosage was adjusted to maintain the serum T4 concentration >35 nmol/L and the serum TSH concentration in the reference range (0.03-0.6

ng/ml). No dosage change was made within 3 months before or at any time after breeding. The breeding reported in this study occurred a median of 137 (132-158; 139.6 + 9.4) weeks after radioiodine and 37 weeks after initiation of levothyroxine supplementation.

Sample collection and analysis

Blood samples were collected from all dogs (4 hours after levothyroxine administration in the levothyroxine treated group) on a weekly basis from the onset of proestrus (vulvar swelling, sero-sanguenous discharge) to seven weeks post-whelping. Ovulation was estimated using a commercial progesterone elisa (Target®, Biometalics, Inc., Princeton, NJ) and subsequently confirmed by measurement of serum progesterone using a validated radioimmunoassay (Coat-a-count Progesterone, Siemens Healthcar Diagnostics, Los angeles, CA). The blood was allowed to clot at room temperature for 1 hour and serum was collected after centrifugation at 5,000 x G and stored at -80°C until assay. Serum T4, fT4, T3, and TSH were measured in duplicate on all samples from ovulation to 63 days post ovulation. Serum T4 and T3 were measured using validated radioimmunoassays and TSH using a validated radioimmunometric assay in the investigators' laboratory [54,79]. Serum fT4 was measured using a validated modified equilibrium dialysis assay at Michigan State University [15].

Statistical Analysis

All data was analyzed with a mixed-model repeated measures ANOVA using SAS version 9.2. Statistical significance was set to $p < 0.05$. TSH data was log transformed. For each group, hormone concentrations at each time point were compared to ovulation (day 0). In addition, hormone values were compared between the levothyroxine treated and euthyroid control

groups at each time point. A third comparison was made between the change in ovulation value and each time point between the two groups was designated as the delta measurement.

Results

Hypothyroid bitches were administered levothyroxine (Soloxine, Virbac Corp, Fort Worth, TX) at a final dosage of 0.0199 $\pm$ 0.006 mg/kg PO once daily for 37 $\pm$ 14 weeks prior to breeding. The mean serum T4 and TSH concentrations of bitches receiving levothyroxine supplementation during the 3 months immediately prior to breeding were 47.4 $\pm$ 12.8 nmol/L and 0.17 $\pm$ 0.23 ng/ml, respectively. All dogs had resolution of all clinical signs of hypothyroidism including alopecia, seborrhea, weight gain, activity, and alertness. Body weight decreased in the levothyroxine treated group from 12.8 $\pm$ 1.44 kg prior to initiating levothyroxine supplementation to 10.3 $\pm$ 0.90 kg ($P < 0.0001$) immediately prior to the final breeding, while the weight in the control group did not differ during the same time period (10.6 $\pm$ 1.30 and 10.8 $\pm$ 1.18 kg, respectively). All levothyroxine treated bitches conceived and whelped healthy puppies [56].

No significant differences in TSH, T3, T4 and FT4 was noted in any measured hormone during pregnancy compared with that found prior to breeding in the euthyroid control group (Figures 1-8). All puppies whelped in both groups survived and were considered healthy at the time of adoption.

Thyroid stimulating hormone concentrations during pregnancy did not differ from those prior to breeding within the levothyroxine treated group. When the control and levothyroxine treated groups were compared at each time prior to and during pregnancy, there was no

difference in absolute concentrations or change from the pre-breeding concentration at any time. (Figure 1, 2).

No significant difference in total triiodothyronine (T3) concentration was detected between groups or deltas, (Figures 3 and 4 respectively). However, within the levothyroxine treated group, mean T3 concentration was significantly lower ($p = 0.019$) at day 56 compared to ovulation (Figure 3).

Total thyroxine (T4) concentrations within the levothyroxine treated group were significantly higher at both day 42 ($p= 0.0039$) and day 63 ($p=0.0025$) when compared to ovulation (Figure 5). Mean serum T4 concentrations were significantly higher in the levothyroxine treated group compared to the euthyroid group at days 42 and 63 ($p= 0.0176$ and $p=0.0008$) (Figure 5). Mean serum T4 deltas were significantly different between groups at the same sampling points, day 42 ($p= 0.0261$) and day 63 ($p= 0.0015$) (Figure 6).

Within the levothyroxine treated group, the mean FT4 concentrations at day 42 and day 63 were significantly higher than ovulation ($p= 0.0118$ and $p= 0.0494$) (Figure 7). The mean serum FT4 concentration was higher in the levothyroxine treated group compared with control concentrations at day 42 ($p= 0.0141$) (Figure 7). The mean FT4 concentrations between the two groups approached statistical significance for day 63 ($p= 0.0568$) (Figure 7). The mean delta FT4 in the levothyroxine treated group was significantly higher than the euthyroid group at days 35 ($p= 0.0318$), 42 ($p= 0.0004$), and 63 ($p= 0.0027$) (Figure 8).

Discussion

The present study demonstrated that euthyroid bitches do not exhibit significant alterations in serum TSH, T3, T4, or FT4 concentrations during pregnancy. The lack of

significant alteration in thyroid function tests measured in the euthyroid group suggest that either the physiologic changes in thyroid economy that occur in pregnancy are minor or bitches with an intact HPT axis are able to compensate for physiologic changes that occur during pregnancy. It is also worth noting that there is a lack of evidence, in the alteration of TSH concentrations, for thyroid stimulation through a pregnancy specific hormone, such as hCG, in pregnant dogs [25,26]. Furthermore, dogs possess lower concentrations of TBG in circulation when compared with humans. There were no alterations in the T4 concentrations of the euthyroid dogs consistent with minimal change in TBG during pregnancy when compared with humans. This affect was not observed in our euthyroid bitches at any stage in pregnancy although it is a direct contradiction of what was found previously by Reimers et al. In their study with five euthyroid pregnant bitches, they found that T4 was significantly elevated compared to dogs of any other reproductive states when samples before and after TSH stimulation were taken. Women have up-regulation of type III deiodinases resulting in a lower T3 concentrations during pregnancy [26]. No changes were detected in serum T3 concentrations in pregnant bitches of the present study or that of Reimers et al [63]. This study was limited in the fact that only one time point during pregnancy as evaluated and only five dogs of during pregnancy were represented.

In women, hCG's thyrotropic activity plays a major role in altering thyroid hormones during pregnancy. In the dog there is no such equivalent hormone, and its absence is reflectedin the concentrations of TSH, T4, and FT4 not being altered during pregnancy in our euthyroid bitches.

Total thyroxine and free thyroxine (T4 and FT4) concentrations were significantly higher in the levothyroxine treated group compared with the euthyroid group in mid to late gestation.

The parallel increase in these two hormones and the lack of change in the fT4 fraction is consistent with no change in binding of T4 to plasma transport proteins (Figure 11). There may be a relative increase in hormone concentration due to less deiodination and relative increase in the hormone pool in circulation. All of the levothyroxine treated bitches received the same levothyroxine dose throughout gestation and this would contribute to a higher concentration of thyroid hormone if there was a decrease in the conversion of T4 to T3. The metabolism of oral levothyroxine supplementation by individual dogs is highly variable [48]. Therefore, alterations in the metabolism of the administered dose of levothyroxine could contribute to a higher thyroid hormone concentration. This conclusion is supported by the variability in T4 and FT4 concentrations in the levothyroxine treated group. Bioavailability of levothyroxine is reduced when given with a meal and any changes in feeding practices, stress causing the dogs to not finish their morning meal, or a change in feeding time could contribute to an increased level of absorbed levothyroxine and increased T4 concentration.

Elevated TSH concentrations were not detected in any of the levothyroxine treated or euthyroid bitches. The few times that alterations of T4 and FT4 were detected in the levothyroxine treated group, hormone concentrations were increased, which would activate the negative feedback loop in hypothyroid bitches, suppressing TSH release. Therefore, the fixed dose of levothyroxine was not responsive to changes in the HPT axis. In the case of the levothyroxine treated bitches, the results from the present study indicate that the thyroid supplementation protocol was adequate for the physiologic needs of pregnancy and resulted in a lack of elevation in TSH concentrations during gestation. More importantly there were no significant differences in the TSH levels between groups. If there was a need for increased

thyroid hormone supplementation in the levothyroxine treated group, an increase in TSH would have been expected due to the lack of adequate thyroid gland response.

Concentrations of T3 remained consistent in the euthyroid group throughout the sampling period, but in the levothyroxine treated group there was a decrease in T3 compared to ovulation at day 56. The overall concentrations for T3 decreased throughout gestation in both groups, although no significance was found. A potential cause for this trend of a decrease in T3 concentration is altered deiodinase activity. A decrease in type 1 or type 2 deiodinase could result in less conversion of T4 to T3, or an increase in type 3 deiodinase could result in increased production of rT3 and metabolism of T3 to T2. Both circumstances would result in decreased T3 concentrations and is seen in pregnant women [26]. To our knowledge, only type II deiodinase has been detected in canine placenta, although studies are limited [72].The use of thyroid hormone ratios, specifically T3/T4 and T3/FT4, elucidate the action of deiodinases in vivo. In the present study, the both ratios trend downwards indicating a decrease in deiodinase activity (Figures 9 and 10). In comparison, there is very little change between the two groups, suggesting that, although the difference is statistically significant, the biological significance is questionable. Compensatory mechanisms for the thyroid to produce more T3 are disrupted in our cohort of levothyroxine treated bitches, therefore reliance on conversion of T4 to T3 in tissues is absolute.

One potential confounding factor in the present study is that the statistical power is low, due to the numbers of dogs used in this experiment: 4 bitches in the control euthyroid group and 6 in the supplemented levothyroxine treated group. Large intra-group variability within the levothyroxine treated group, reflected in the standard error of the mean, was present for T4 and

FT4 concentrations at time points that coincided with statistically significant differences between groups in other analytes.

Further studies investigating the physiology of thyroid metabolism during pregnancy in bitches are warranted to further elucidate the mechanisms for aberrations seen in hypothyroid pregnancies. A study with increased numbers of bitches would also strengthen our confidence in standard supplementation protocols during gestation.

In conclusion, the results from this study indicate that there were no significant alterations in thyroid hormone profiles in euthyroid bitches during pregnancy. Therefore, at present there is no evidence to implement further testing of pregnant bitches to screen for subclinical hypothyroidism or overt hypothyroidism, unless clinical signs are present. In addition, hypothyroid bitches undergoing thyroid hormone replacement therapy do have alterations in their thyroid hormone profiles during pregnancy. But this is characterized by an increase in T4 and FT4 at specific time points, indicating that standard supplementation protocols are adequate for maintaining a relative euthyroid state during gestation. The increase in T4 and FT4 concentrations late in pregnancy in the levothyroxine treated dogs was mild and likely of little clinical consequence given the lack of change in TSH, T3, and bitches having normal parturitions.

Chapter 3: Conclusion

In the present study it was determined that pregnant bitches do not exhibit the same fluctuations in thyroid hormone profiles that are documented in pregnant women. There is no evidence of TBG stimulation from estrogen in dogs. Furthermore, since canids do not possess a hormone similar to hCG, homology between the β subunits of hCG and TSH does not play a stimulatory role in dogs. Placental deiodinases are present in the canine placenta, but only type II deiodinase has been documented thus far in the literature. If type III deiodinases are present in the placenta of pregnant bitches, they do not play a major role in contributing to lower thyroid hormone concentrations. A larger scale experiment in bitches with natural disease would strengthen the conclusions from this study. Further studies are warranted to fully characterize the mechanisms of thyroid hormone production, transport, and metabolism in pregnant dogs. Studies involving thyroid function tests should be explored as well. Lastly, due to the thyroid hormone profiles, lack of clinical signs of hypothyroidism, and resolution of all previously reported effects of hypothyroidism on pregnant bitches and their offspring, we can conclude that standard supplementation protocols are adequate for maintenance of a euthyroid state during gestation.

Figures

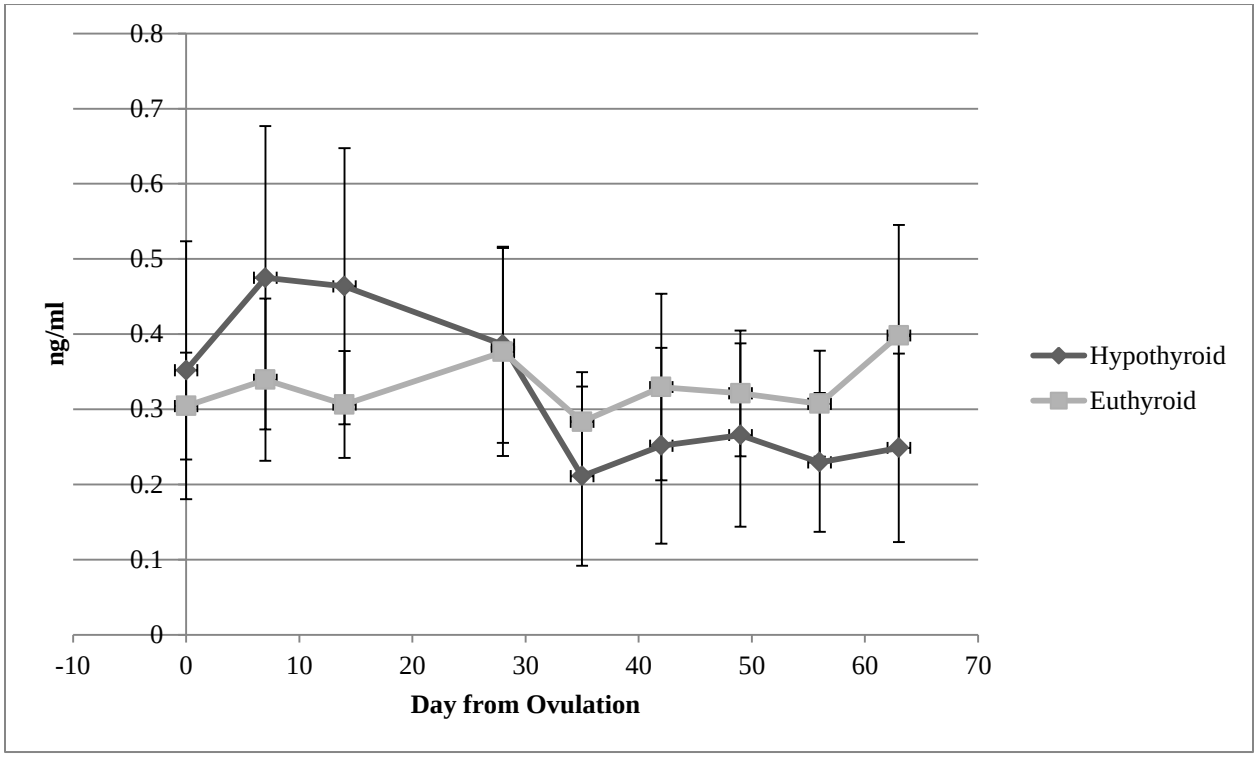

Figure 1: TSH concentration during pregnancy measured using a radioimmunometric assay. Data are presented as mean TSH concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference from ovulation, day 0, is denoted by * ($p < 0.05$). Significant difference between groups is denoted by ° ($p < 0.05$).

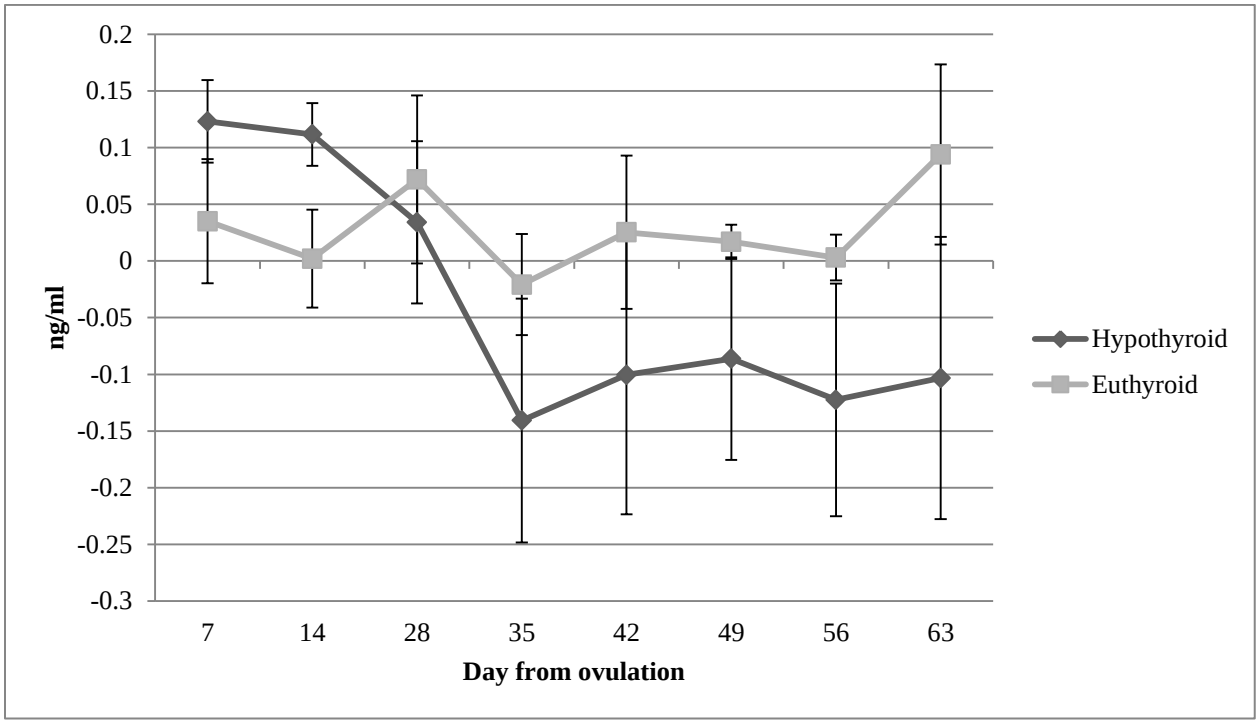

Figure 2: TSH concentration change from ovulation, day 0, between the euthyroid and levothyroxine treated groups during pregnancy. Data are presented as mean TSH concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference is denoted by * ($p < 0.05$).

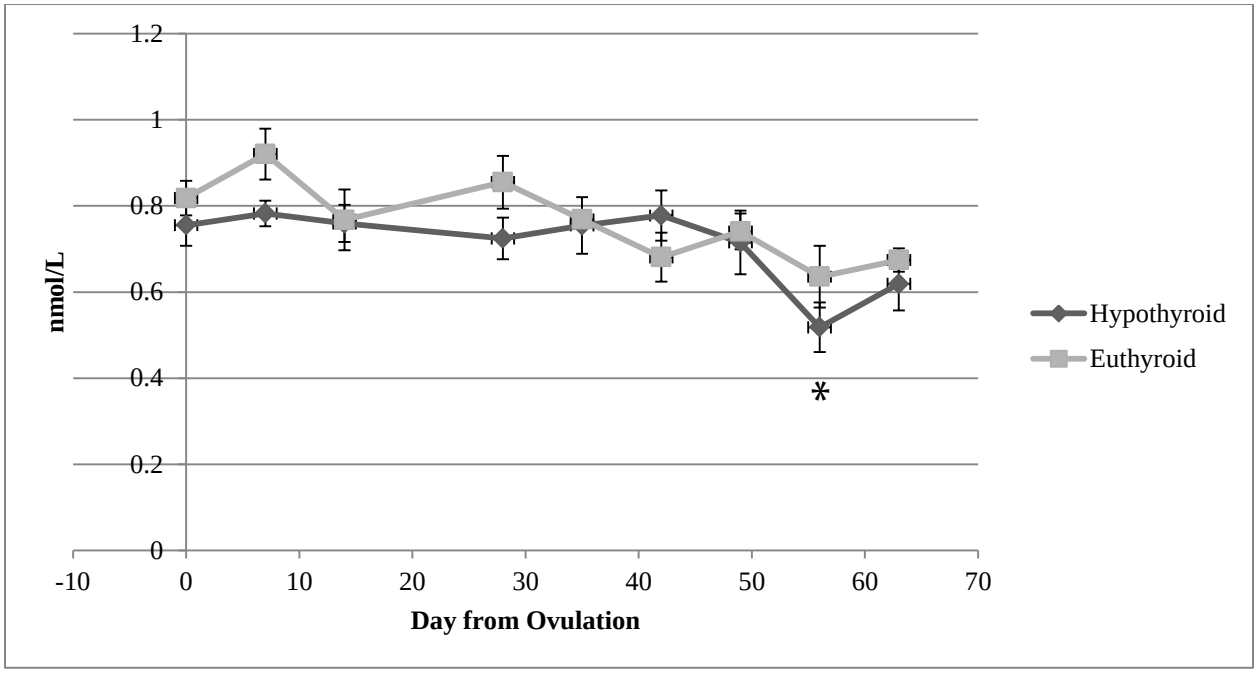

Figure 3: T3 concentration during pregnancy measured using a radioimmunoassay. Data are presented as mean T3 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference from ovulation, day 0, is denoted by * ($p < 0.05$). Significant difference between groups is denoted by ° ($p < 0.05$).

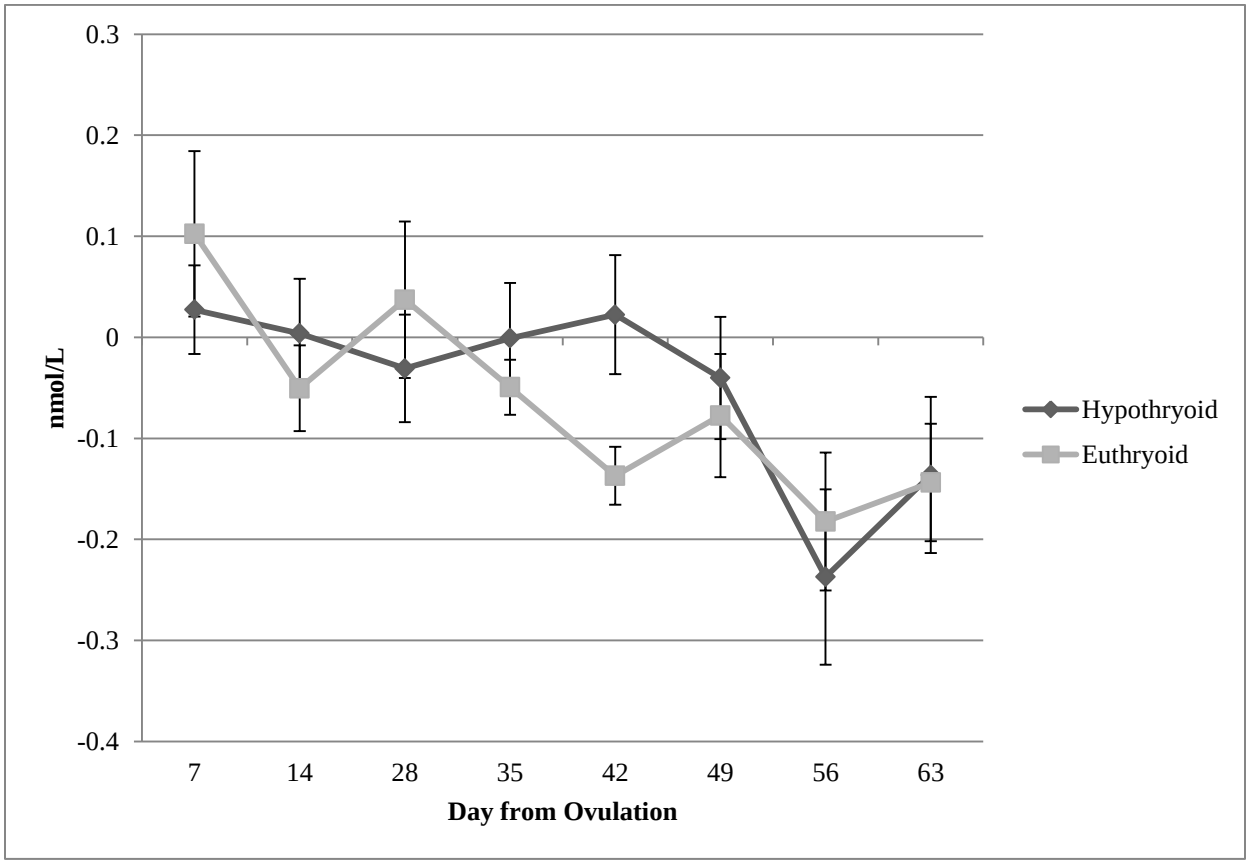

Figure 4: T3 concentration change from ovulation, day 0, between the euthyroid and levothyroxine treated groups during pregnancy. Data are presented as mean T3 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference is denoted by * ($p < 0.05$).

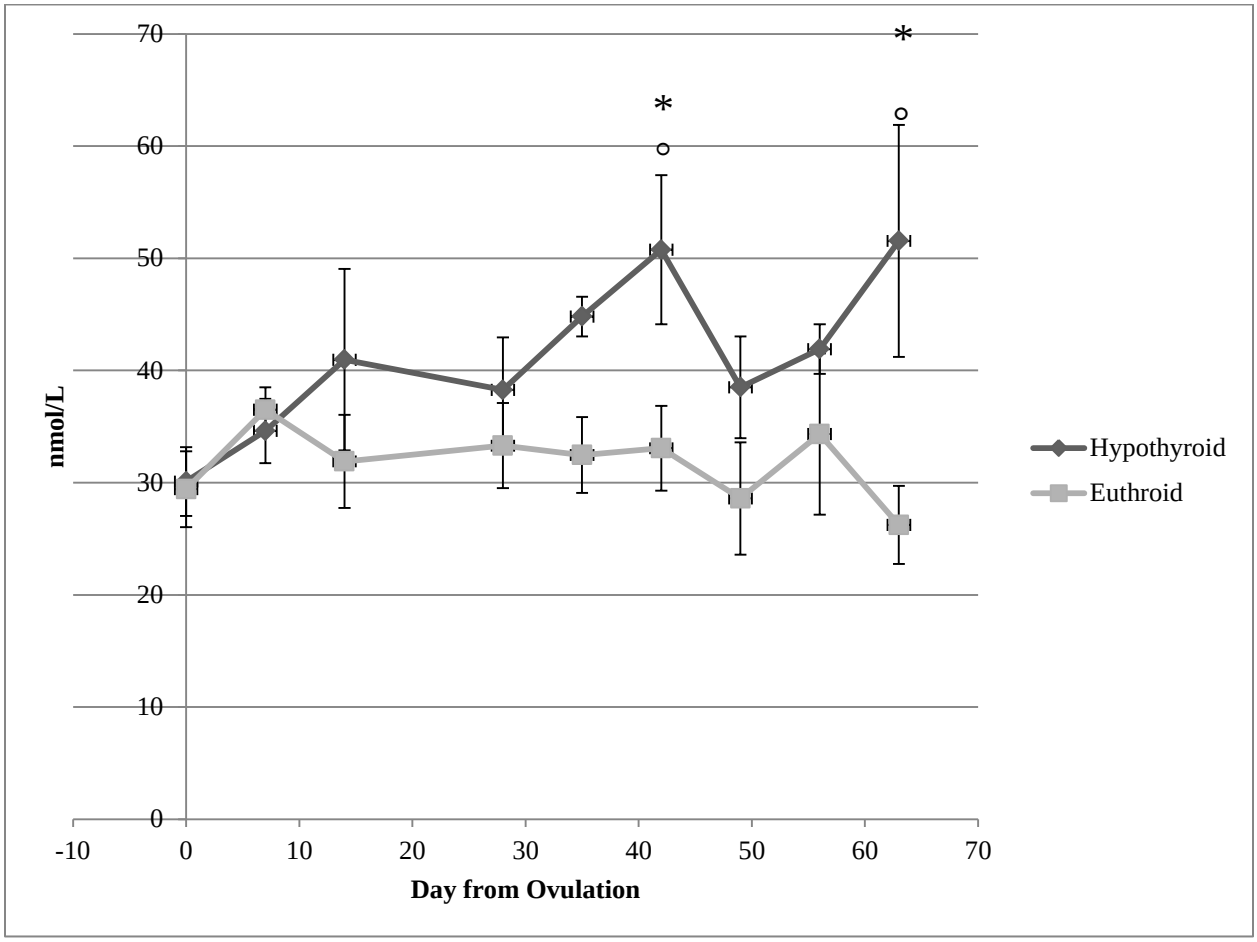

Figure 5: T4 concentration during pregnancy measured using a radioimmunoassay. Data are presented as mean T4 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference from ovulation, day 0, is denoted by * ($p < 0.05$). Significant difference between groups is denoted by ° ($p < 0.05$).

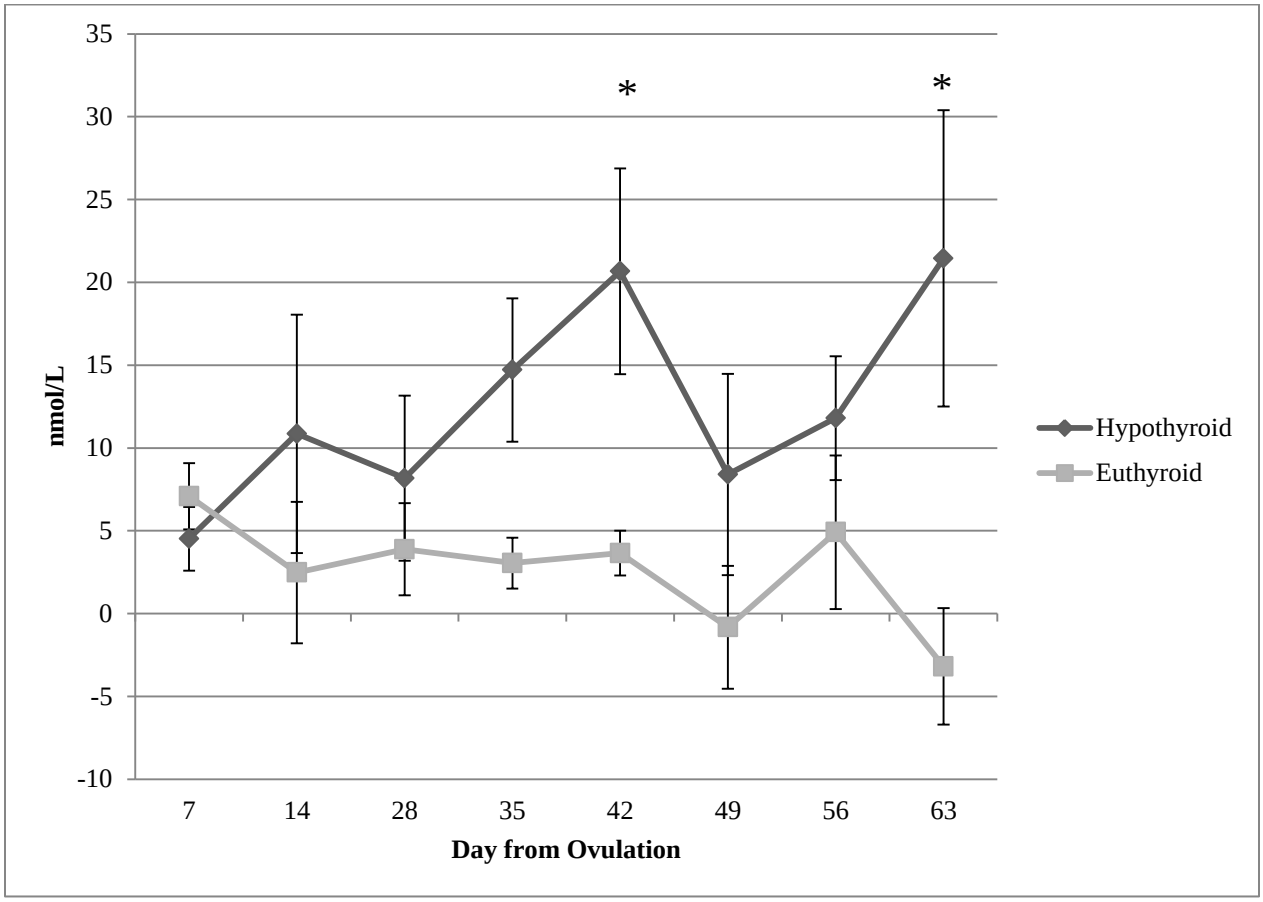

Figure 6: T4 concentration change from ovulation, day 0, between the euthyroid and levothyroxine treated groups during pregnancy. Data are presented as mean T4 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference is denoted by * ($p < 0.05$).

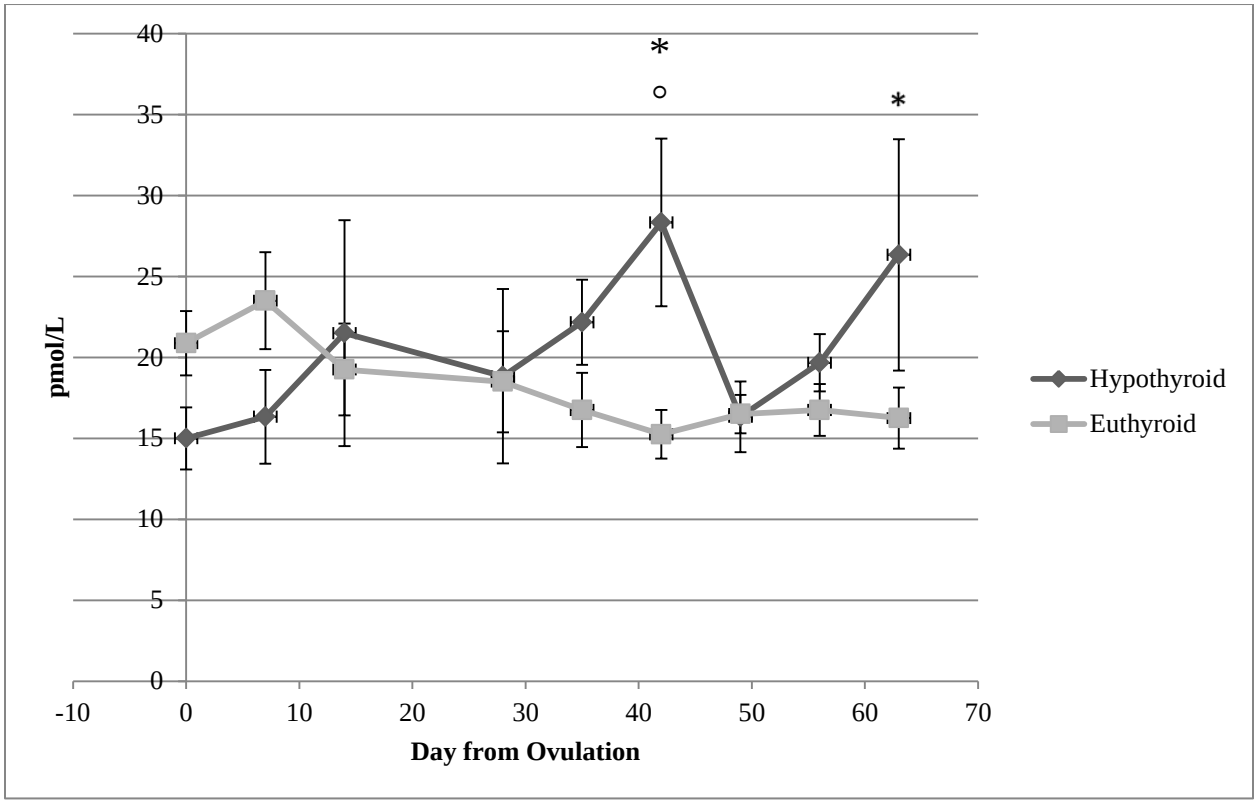

Figure 7: FT4 concentration during pregnancy measured using a modified equilibrium dialysis assay. Data are presented as mean FT4 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group).Significant difference from ovulation, day 0, is denoted by * ($p < 0.05$). Significant difference between groups is denoted by ° ($p < 0.05$).

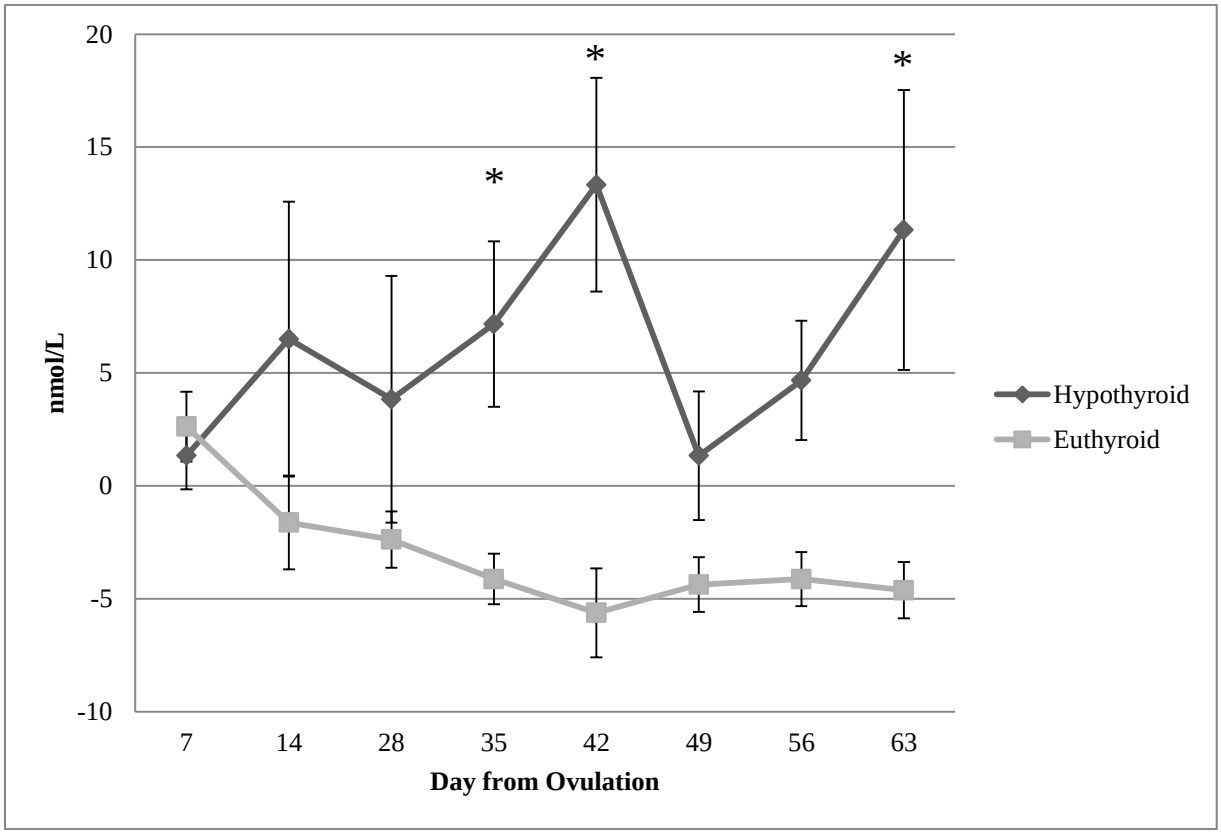

Figure 8: FT4 concentration change from ovulation, day 0, between the euthyroid and levothyroxine treated groups during pregnancy. Data are presented as mean FT4 concentration ± standard error of the mean (n=6 for the levothyroxine treated group, n=4 for the euthyroid group). Significant difference is denoted by * ($p < 0.05$).

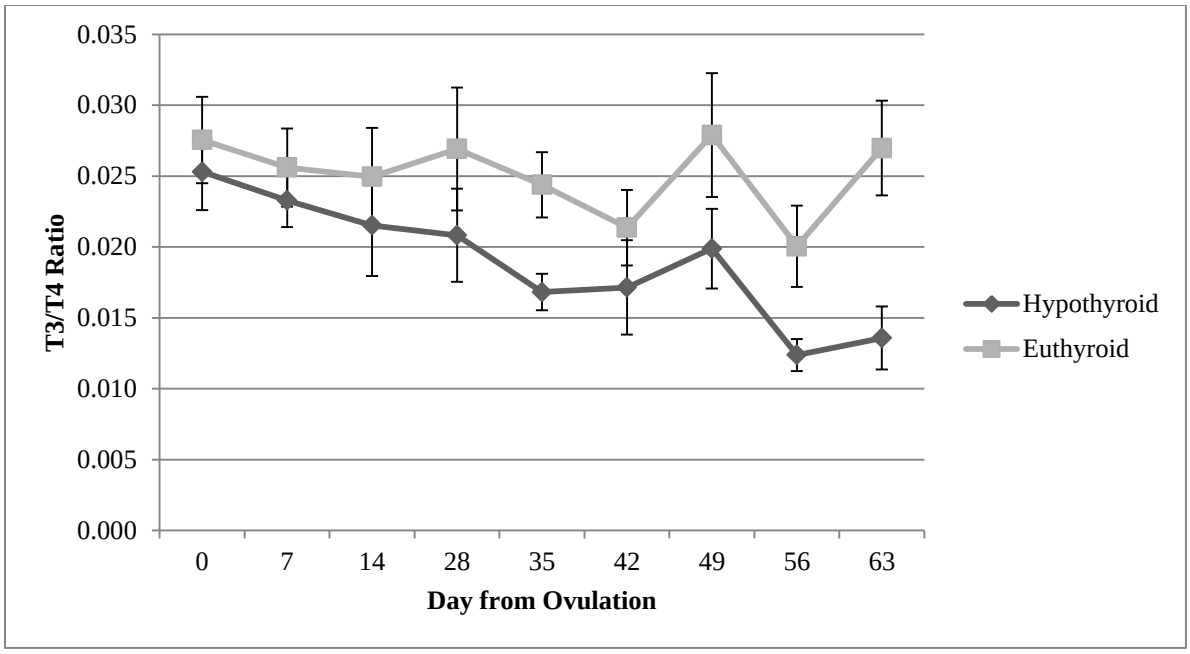

Figure 9: T3/T4 ratio as determined by dividing the average T3 concentration by the average T4 concentration in both the levothyroxine treated and euthyroid groups. This ratio is an indication of T3 conversion from T4.

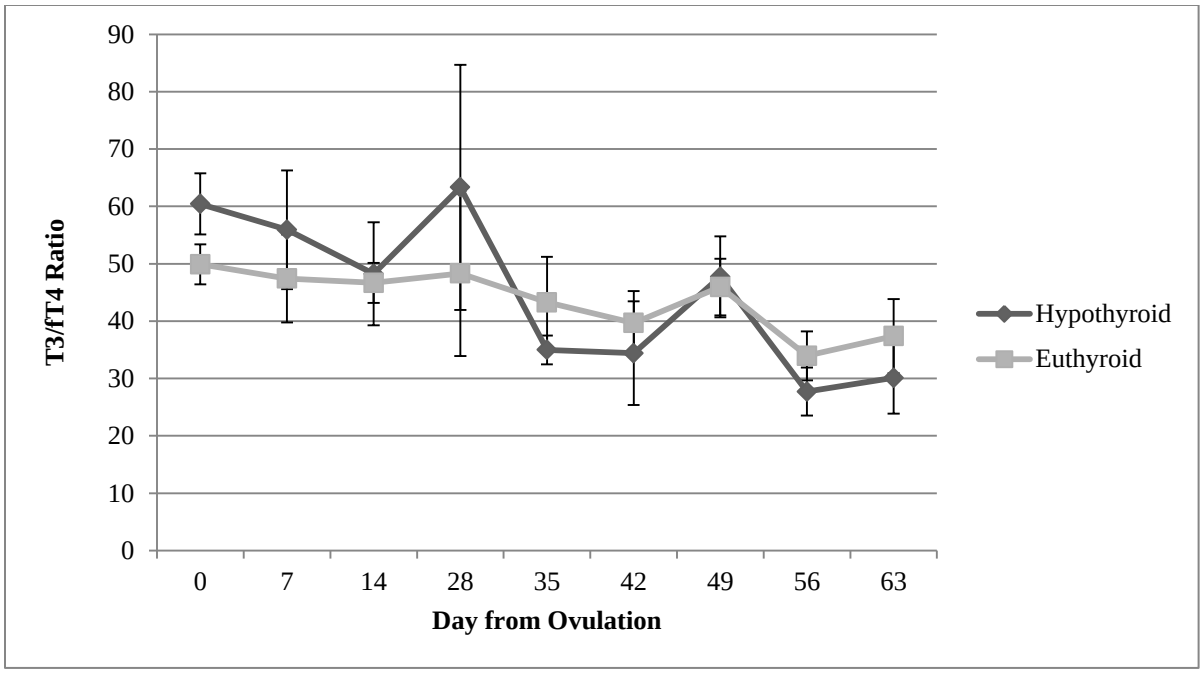

Figure 10: T3/fT4 ratio determined by dividing the average T3 concentration by the average fT4 concentration in both the levothyroxine treated and euthyroid groups. This ratio is an indication of conversion of T3 from T4.

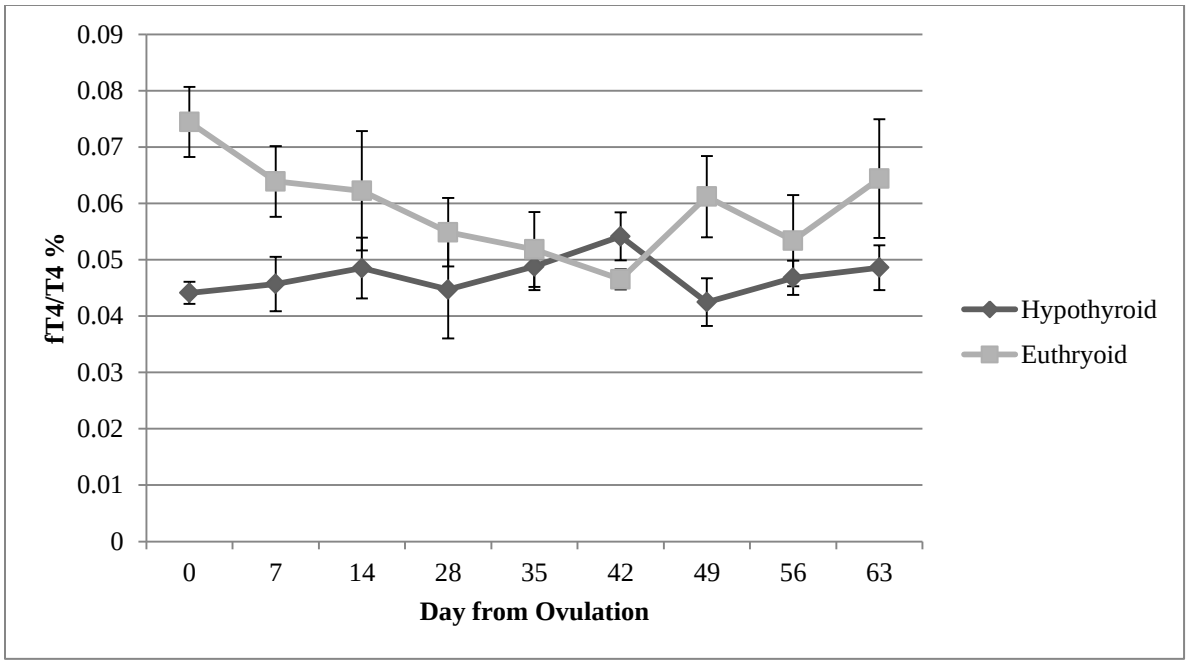

Figure 11: fT4/T4 % as determined by dividing the average fT4 concentration by the average T4 concentration, the dividing the product by 10. This is an indicator of protein binding.

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